11-17-2022 1:00 PM

Characterization of SPL4's role in drought stress and trichome development in alfalfa

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

ABSTRACT

The impacts of climate change are expected to increase the demand for crops that are resistant to drought stress. Understanding the molecular mechanisms involved in the response of plants to such stresses is thus crucial for preventing losses in crop yield. In this study, the role of alfalfa SPL4, a target of the non-coding RNA, miR156, was examined in response to drought stress and with respect to the development of trichomes. I found that transgenic alfalfa plants with RNAi-silenced *SPL4* exhibited increased trichome density under both control and drought conditions. Furthermore, in response to withholding water for 14 days, *SPL4-RNAi* plants exhibited increased root length, water content, chlorophyll content, stomatal conductance, and increased water potential in leaves when compared to wild-type plants. RT-qPCR revealed that *SPL4-RNAi* plants displayed altered expression levels of genes involved in drought tolerance (*SPL9*, *SPL13*), antioxidant biosynthesis (*CAT*), and trichome production (*GL1*, *GL3*). This study demonstrates that SPL4 has a role in both trichome development and in the drought stress response, making it a potential target for the improvement of alfalfa and potentially other crops.

Keywords

Alfalfa, Trichome, Drought, Gene Regulation, miR156, SPL4, Abiotic Stress Tolerance

SUMMARY FOR LAY AUDIENCE

To maintain food security as the human population increases, more efficient crop production methods must be developed. This need is exacerbated by global climate change, which causes agricultural areas to suffer extreme weather patterns, such as drought. Drought causes the accumulation of reactive oxygen species (ROS), which can interfere with biological processes and limit plant growth. This is of special interest to Canada, as large parts of the prairies are predicted to be subject to even harsher droughts as climate change progresses. Silencing through miRNAs is a promising molecular tool for inducing desirable changes in plant physiology. One example of this is miR156, which through its interaction with proteins named Squamosa Promoter Binding-Like transcription factors (SPLs), acts as a regulator of both plant development and stress tolerance. In this study, the role of SPL4, a member of the SPL family, was investigated through the comparison of wild-type (WT) alfalfa plants with alfalfa plants exhibiting reduced *SPL4* expression. It was found that under drought conditions, plants with reduced *SPL4* expression were greener than WT alfalfa, and had increased root length, water content, water potential, chlorophyll content, and stomatal conductance, indicating a greater tolerance for drought conditions. Trichomes, hair-like structures present on most plant leaves and partly responsible for maintaining water content, were found in greater densities on plants with reduced *SPL4* expression under both drought and stress-free conditions. The *SPL9* and *SPL13* genes involved in negatively regulating drought stress tolerance, were found to have lowered expression, while *CATALASE* (*CAT*), a gene involved in removing ROS, and *GLABROUS 1* (*GL1*) and *GLABROUS 3* (*GL3*) genes involved in trichome development were found to have increased expression in *SPL4* silenced alfalfa. These results suggest that *SPL4* is a possible target for molecular manipulation in order to improve drought tolerance in alfalfa.

STATEMENT OF CO-AUTHORSHIP

The following people contributed to the publication of work undertaken as part of the thesis:

M.Sc. Candidate - Matei Dan-Dobre, University of Western Ontario: The M.Sc. candidate contributed to defining the scientific inquiry and the methods used to solve it. The M.Sc. candidate wrote the entire draft version of the thesis, and revised it according to co-author #1 and #2's comments. The M.Sc. candidate determined the methodology used together with co-author #1, and the candidate implemented all experiments and interpreted all data collected alongside advice from co-author #1.

Co-author #1 – Dr. Abdelali Hannoufa, Supervisor: Co-author #1 defined the overall inquiry together with the M.Sc. candidate, and provided guidance on experiments chosen and the interpretation of the data collected. They assisted in reviewing of the thesis and proposed refinements to the draft proposal made by the candidate.

Co-author #2 – Dr. Jim Karagiannis, Co-supervisor: Co-author #2 provided assistance in interpreting data collected by the conducted experiments, and assisted in reviewing of the thesis and proposed refinements to the draft proposal made by the candidate.

Co-author #3 – Ruimin Gao: Co-author #3 generated the construct for the RNAi-induced silencing of the SPL4 gene while at the Hannoufa lab. His work was unpublished, and pertains to the construct used for the transformation of alfalfa used for the methods described in Chapter 2.

Co-author #4 – Mimmie Lu: Co-author #4 generated the transgenic alfalfa plants by performing alfalfa transformation using the construct generated by co-author #3 as part of their work at AAFC. Her work was unpublished, and pertains to the construct used for the transformation of alfalfa used for the methods described in Chapter 2.

ACKNOWLEDGMENTS

I would like to acknowledge everyone who assisted me in my research and with putting together my thesis. Thank you to Dr. Abdelali Hannoufa and Dr. Jim Karagiannis for their guidance and experience, providing direction to my research as it progressed and helping me write and organize that research into this thesis. Thank you to the members of my advisory committee, Dr. Sheila Macfie and Dr. Rima Menassa, who listened to the updates on my work's progress, providing me with their insights and encouragement. Thank you to Lisa Amyot, who gave me my initial training when I first came to AAFC, and Ling Chen, who helped me through the seemingly endless troubleshooting required to complete my experiments when they inevitably ran into problems. I would like to thank Alex Molnar for his help with graphics. Thank you to my friends for their support, listening to my gripes when I became frustrated and helping me relax afterwards. Thank you to my family for their interest in my work, and for their lighthearted taunts about when I would complete my thesis. And finally, a special thank you to my Mom and Dad, without whom my education would not have been possible. Your encouragement and advice were a constant that I depended on. I dedicate this thesis to you.

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LIST OF ABBREVIATIONS

AbA – Aureobasidin A

ABA – Abscisic Acid

AGO - Argonaute

ASC – Ascorbate

ASC-GSH – Ascorbate-glutathione

APX – Ascorbate Peroxidase

bHLH – Basic Helix-Loop-Helix

CAT – Catalase

CPC - Caprice

DHAR – Dehydroascorbate Reductase

DFR – Dihidroflavonol-4-reductase

DMSO - Dimethylsulfoxide

EGL3 – Enhancer of GL3

ETC1 – Enhancer of TRY and CPC1

GL1 – Glabrous 1

GL3- Glabrous 3

GR – GSH Reductase

GSH – Glutathione

HEN1 – HUA Enhancer

Leu – Leucine

LWC – Leaf Water Content

LWP – Leaf Water Potential

MDAR – Monohydroascorbate Reductase

Mha – Mega Hectare

miRNA – micro RNA

miR156 – microRNA 156

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NAT – Naturally Antisense siRNA

OD – Optical Density

PCR – Polymerase Chain Reaction

PAP1 – Production of Anthocyanin Pigment 1

piRNA – Piwi-Interacting RNA

PP2C – Protein Phosphatase 2C

RCAR1 – Regulatory Component of ABA Receptor 1

RISC – RNA-Induced Protein Complex

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RT-qPCR – Reverse Transcription quantitative PCR

SD – Synthetic Defined

siRNA – Short Interfering RNA

SnRK2 – SWF1-Related Protein Kinase 2

SPL – Squamosa Promoter Binding-Like

SOD – Superoxide Dismutase

TAS – Trans-Acting siRNA

TRY - Triptychon

TTG1 – Transparent Testa Glabra1

TT8 – Testa8

WT – Wild-Type

YDPA – Yeast Peptone Dextrose Adenine

Y1H – Yeast One-Hybrid

CHAPTER 1

1 INTRODUCTION

1.1 Crop improvement and its importance

The human population has steadily increased over the past three centuries, with the annual growth rate peaking at 2.1% in 1968 and currently standing at ~ 1.05%. Furthermore, the United Nations projects that the world's population will increase from 7.7 billion individuals in 2021 to nearly 11 billion by the end of the century (Lutz et al., 2018). To maintain food security for this ever-growing population, crop production will need to increase in order to keep pace with the projected rise in demand (Godfray et al., 2010; Tilman et al., 2011). To accomplish this, strategies based on both extensification (an increase in the amount of land used) and intensification (the improvement of agricultural output for the same acreage) will need to be utilized (Tilman et al., 2011). While both strategies will undoubtedly be needed, it should be noted that extensification results in environmental damage due to the conversion of wilderness into farmland, whereas intensification can result in improved crop yields without such negative ecological impacts (Tilman et al., 2011).

Compounding the need for increased crop efficiency are abiotic stressors, which are responsible for up to a 70% reduction in crop yield by limiting plant growth as plants divert resources to counter the effects of stress conditions (Boyer, 1982). As climate change continues to escalate, extreme weather phenomena are predicted to increase in both frequency and severity (Mukherjee, Mishra & Trenberth, 2018). Canada in particular has seen declining annual precipitation in regions that historically have had issues with severe drought, such as the Canadian prairies (Schindler & Donahue, 2006). Breeding programs have had some success in producing cultivars that can grow under water deficient conditions (Slama et al., 2013), but yield

improvements in *Medicago sativa* (alfalfa) through conventional breeding have been limited, with little increase in yield being achieved through breeding programs from the 1950s through the 1990s (Volenec et al., 2002). This is likely due to alfalfa's 1 Gb polyploid genome, tetrasomic inheritance, and allogamous reproduction (Li and Brummer, 2012). Due to these impediments, the development of molecular tools for improving alfalfa yields is of vital importance to achieving sustainable intensification.

1.2 Alfalfa as a forage crop

Legumes are the third largest family of plants and are an important source of both forage and food (Wang et al., 2015). They also have utility as 'pioneer' plants, growing in nutrient-poor soil due to their ability to form symbiotic relationships with nitrogen-fixing Rhizobium bacteria and nutrient-scavenging soil fungi (Márquez et al., 2005). Alfalfa is a forage legume crop that is widely used by the Canadian agriculture industry. It is grown on an estimated 30 Mha globally (Annicchiarico et al., 2015) and on 3.8 million hectares in Canada (mostly in the western prairie provinces) (Statistics Canada, 2016). This plant possesses a vigorous and deep root system, making it very useful to counteract soil erosion and providing it with a high water efficiency (the amount of water required to provide one crop yield) (Putnam et al., 2001). Alfalfa also provides permanent vegetation cover as it is a perennial crop, enhancing soil fertility by adding organic matter to the soil (Putnam et al., 2001).

Additionally, due to alfalfa's ability to compete with weeds in growth rate, as well as its propensity for producing alleopathic compounds, it can be used as a smother crop, inhibiting the growth of undesirable plants and thus resulting in fields requiring less herbicide for crop production (Small, 2011). Another key attribute of alfalfa is its ability to form a symbiotic relationship with Rhizobia, a nitrogen fixing bacterium that establishes itself in alfalfa root

nodules, thereby reducing the need for fertilizers in crops that are grown on fields subsequent to alfalfa (Blesh & Drinkwater, 2013; Small, 2011). Furthermore, due to its perenniality, high yield, and relative ability to withstand abiotic stress conditions, alfalfa is also groomed as a potential biofuel feed stock (Sanderson & Adler, 2008).

Improving alfalfa yields through molecular approaches is of great importance since the use of conventional breeding has had limited success and since pest and drought stress will likely increase in frequency as the climate continues to warm (Deutsch et al., 2018). While alfalfa already possesses a hardy nature and a relative resilience to heat damage and drought stress when compared to many other crops (Erice et al., 2010), additional improvements are warranted since despite alfalfa's resilience, yields are still negatively affected (Shao et al., 2009). Increasing the capacity of alfalfa to withstand drought and heat may also increase its ability to tolerate cold, as the response to this stress shares some physiological characteristics that typically confer resistance to heat and drought (e.g., smaller leaves, reduced internode length, increased pubescence, and increased biomass allocation to the root system) (Small, 2011). Taken together, these enhancements are likely to improve the economic value and the environmental sustainability of alfalfa production.

1.3 Abiotic stress response

As sessile organisms, plants are unable to remove themselves from environmental constraints, so rather they have evolved mechanisms to cope with stress. Stress conditions could be biotic (e.g., pathogen infection, herbivore predation) or abiotic (e.g., drought, heat, cold, nutrient deficiency, salinity, toxic metals) (Federoff et al., 2010). Under heat stress, cellular processes are disrupted resulting in protein misfolding and denaturation and increased membrane fluidity (Bernstam, 1978). To reduce internal temperatures in response to heat, plants increase

transpiration, thus lowering temperatures through evaporative cooling (Lin et al., 2017). Unfortunately, high temperatures and drought tend to coincide, resulting in the plant's inability to use transpiration to lower temperature (due to the reduced stomatal conductance necessary to prevent the plant from losing too much water from drought) (Zandalinas et al., 2018).

The broader plant response to drought stress involves changes in gene expression, changing rates of transpiration through stomatal closures, reducing leaf surface, the delaying of senescence, and the general focusing of plant resources on maintaining vital functions and minimizing water loss (Lamaoui et al., 2018; Sicher et al., 2012). Key to the drought response is abscisic acid (ABA), a plant hormone that increases in concentration upon drought stress, and which is vital in stomatal closure and in promoting root growth (Spollen et al., 2000; Zhang & Davies, 1989). ABA production starts in the roots upon encountering water-deficient soil, and ABA is then transported through the xylem to the shoots (Zhang & Davies, 1989). Ethylene, a hormone that is involved in controlling both plant growth and senescence, is concomitantly reduced in the plant shoots upon drought and root growth is promoted to ensure the uptake of any remaining moisture left in the soil (Spollen et al., 2000).

The regulation of the ABA response is mediated through the SNF1-Related Protein Kinase 2 (SnRK2) protein family. This is evidenced by the fact that plants with non-functional SnRK2 lack any ABA-related response to drought (Umezawa et al., 2009). Typically, SnRK2 is inactivated due to dephosphorylation by Protein Phosphatase 2C (PP2C) (Umezawa et al., 2009). When a plant requires ABA responses, PP2C is inactivated through the binding of a Regulatory Component of ABA Receptor 1 (RCAR1), preventing SnRK2 from being dephosphorylated and allowing it to function with ABA to induce responses to drought stress (Umezawa et al., 2009).

Most stress factors ultimately result in oxidative damage to plants through the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), which damage nucleic acids, proteins, cellular membranes, and both cellular and membrane-bound organelles (Kong & Lin, 2010; Costa et al., 2011; Stark, 2005). Organelles are damaged by lipid peroxidization or oxidative damage to membrane-bound proteins, and when severe enough, this damage accumulates and results in a loss of cellular function (Stark, 2005). Damage caused by ROS also results in other ROS species as by-products, such as peroxide and hydroxyl ions, which themselves are also highly reactive, and can cause a chain of further damage to nucleic acids and proteins (Møller & Wallin, 1998). Damage caused by ROS manifests physiologically, disrupting plant growth and development when mild, and resulting in cellular necrosis and/or chlorosis through destruction of chloroplasts and chlorophyll when severe (Briantais et al., 1996). This damage by ROS results from the plant's cellular defenses against oxidative damage being overwhelmed (Apel & Hirt, 2004).

One such line of defence is comprised of antioxidants, which convert ROS into more stable forms of oxygen that lack the free radicals that make ROS so damaging (Sarker & Oba, 2018). Antioxidants can function through both enzymatic and non-enzymatic mechanisms (Sarker & Oba, 2018). An example of an enzymatic antioxidant is catalase (CAT), which catalyzes the conversion of hydrogen peroxide into water (Choudhury et al., 2013). An example of non-enzymatic antioxidant function is the ascorbate-glutathione (ASC-GSH) metabolic cycle, which is composed of many dedicated reactions that detoxify hydrogen peroxide (Choudhury et al., 2013). First, hydrogen peroxide is converted to water by ascorbate peroxidase (APX) via ascorbate's (ASC) donation of an electron (Wells & Xu, 1994). The oxidized ascorbate is then regenerated by monohydroascorbate reductase (MDAR), which itself is a radical, and thus is reduced by

dehydroascorbate reductase (DHAR) through donation of an electron from GSH (Wells & Xu, 1994). The oxidized GHS is then reduced by GSH reductase (GR) using NADPH as an electron donor, resulting in regenerated ASC and GSH for further uses in the ASC-GSH cycle (Whitbread et al., 2005).

Another method plants use to remove ROS is through specialized metabolite biosynthesis (Schaefer & Rolshausen, 2006). Specialized metabolites include anthocyanins, flavonoids, carotenoids, and tocopherols, which each play roles beyond defending plants from ROS (e.g., plant development, pigmentation, protection from ultraviolet light, and signaling between plants and microorganisms) (Mathesius, 2018; Armstrong & Hearst, 1996; Mari, Bosch & Alegre, 2010). A good example of such specialized metabolite function in the plant response to biotic stresses is the role of anthocyanins in conferring resistance to insect predation (Natatsuka et al., 2007; Schaefer & Rolshausen, 2006). Anthocyanin production is tightly regulated, in part through *DFR* transcription (Gonzales et al., 2008). *DFR* is transcribed when the *DFR* transcription activation complex binds to its promoter (Gou et al., 2011). This transcription activation complex is composed of members of the MYB family of transcription factors, basic helix-loop-helix (bHLH) factors, and the WD40 repeat protein Transparent Testa Glabra1 (TTG1) (Gou et al., 2011). A member of the SPL family, SPL9, can compete with bHLH protein Testa8 (TT8) for binding to Production of Anthocyanin Pigment 1 (PAP1) to prevent the assembly of this complex and inhibit *DFR* expression, thus inhibiting the production of anthocyanins (Gou et al., 2011).

1.4 Role of microRNAs in plant development and stress response

The regulation of plant gene expression is governed by transcription factors, epigenetic factors, and distinct small RNA molecules 21 to 24 nucleotides in length, which act at the transcriptional and post-transcriptional levels (Jamalkandi and Masoudi-Nejad, 2009; Voinnet,

2009). Regulation by small RNAs occurs after the generation of double-stranded RNAs (or single stranded RNAs that are folded into a stem-loop/hairpin structure), which are recognized by RNase III-like enzymes called Dicer-Like for processing into small interfering RNAs and which are combined with RISC, culminating in the silencing of gene expression through the recognition of complementary binding sites on mRNA transcripts (Ramachandran and Chen, 2008). Different and distinct biochemical pathways can generate different classes of small RNAs. These include piwi-interacting RNAs (piRNAs, occurring only in animals), short interfering RNAs (siRNAs), trans-acting siRNAs (TAS), naturally anti-sense siRNAs (NAT), and microRNAs (miRNA) (Liu and Paroo, 2010).

To produce miRNAs, a miRNA (MIR) gene is transcribed by RNA polymerase II into a precursor pri-microRNA, stabilized and cleaved by Dicer-Like proteins into a pre-microRNA, and then further processed into a mature miRNA while in the nucleus (**Figure 1**) (Voinnet, 2009). HUA enhancer 1 (HEN1) methylates the mature miRNA at the 2'-hydroxy termini of both strands, after which one strand becomes the processed mature miRNA which is transported to the cytoplasm, and the other strand of pri-microRNA is degraded (Krol et al., 2010). At this point, Argonaute (AGO) proteins recognize miRNAs and direct them to mRNA transcripts which hold complementary sequences (Krol et al., 2010). Mature miRNAs, created after cleavage of the precursor RNA (Perron and Provost, 2008), negatively regulate gene expression through interaction with RNA-induced silencing complexes (RISC), which bind to target transcript sequences and induce cleavage of the target sequence or silencing through translation inhibition (Dugas and Bartel, 2004; Kidner and Martienssen, 2005). When perfect complementarity exists between miRNA and its mRNA target, cleavage at the complementary site occurs (Hutvagner and Zamore, 2002). When complementarity is imperfect, destabilization

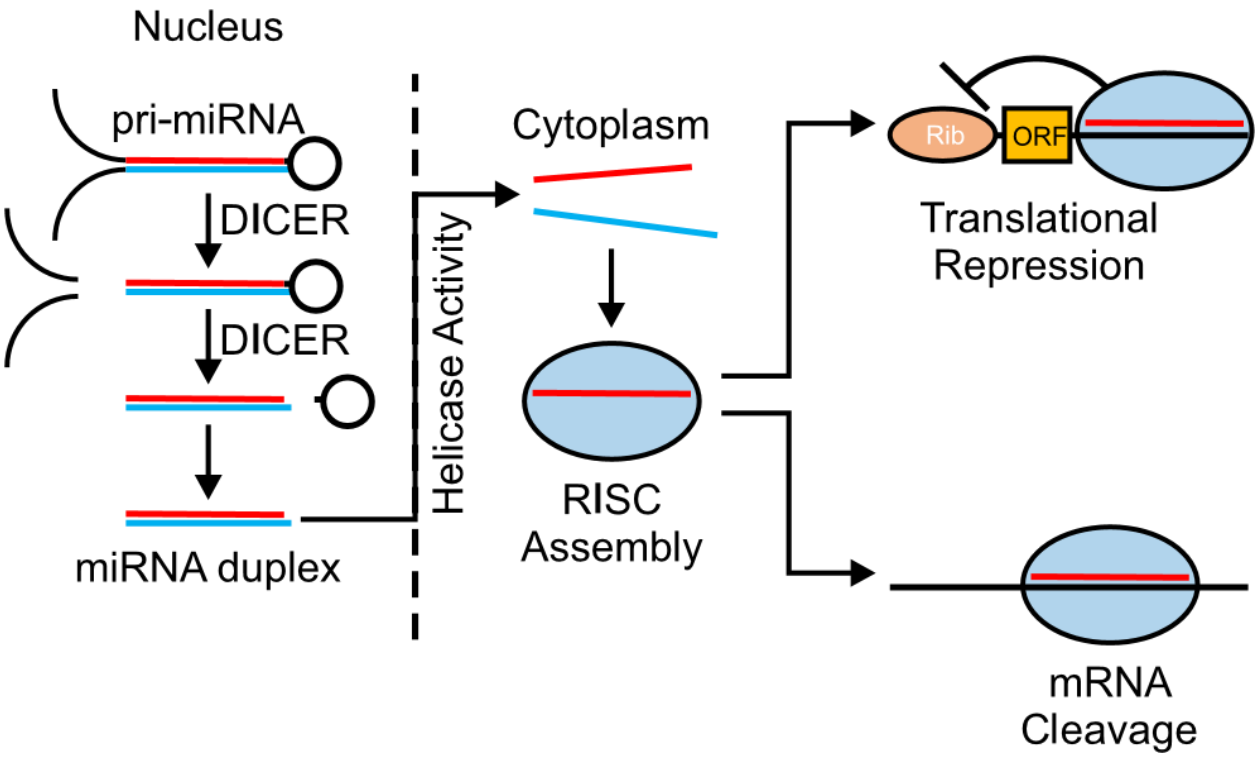

Nucleus
pri-miRNA
DICER
DICER
miRNA duplex
Helicase Activity
Cytoplasm
RISC
Assembly
Rib
ORF
Translational
Repression
mRNA
Cleavage

Figure 1: miRNA biogenesis and mode of action

Double-stranded pri-miRNA is created in the nucleus, and is then modified by DICER into short double-stranded miRNAs. These miRNAs bind to RISC protein complexes resulting in silencing of target gene mRNA expression through cleavage or translational repression**.**

of mRNA, and hence inhibition of translation, occurs through deadenylation or trimming of the 3’ poly-A tail (Wu et al., 2006).

In plants, mutations in miRNA biogenesis genes have been shown to produce acute effects on development, indicating their crucial role in this process (Xie et al., 2006). An example of miRNAs is the *miR156* family that regulate SPL transcription factors in *Arabidopsis thaliana*, and which play important roles in both shoot and root development (Baker et al., 2005). Mutations in *AthmiR156*a and *AthmiR156*b resulted in plants with more roots relative to wild-type plants (Guo et al., 2008). *miR156* is one of the most conserved miRNAs in plants, being found in mosses, monocotyledons, and dicotyledons (Arazi et al., 2005; Xie et al., 2006).

The role of *miR156* role in plant development involves the downregulation of transcription factors, including those belonging to the SPL family (Aung et al., 2015). SPL proteins are critical in regulating plant growth. For example, SPL4 regulates the duration of the vegetative state and flowering time, as well as impacting nodulation in *Lotus japonicas* and branching in *Arabidopsis thaliana*, *Lotus japonicus*, *Solanum Lycoperisium (tomato)*, *Zea mays* (maize), and many other plants (Wu and Poethig, 2006; Wang et al., 2015; Chuck et al., 2011). Arabidopsis plants that overexpress *miR156* are semi-dwarf, with altered numbers of leaves and longer vegetative phases (Wang et al., 2008; Yu et al., 2012). Overexpression of *miR156* in Arabidopsis also improves drought tolerance (Cui et al., 2014) and increases trichome numbers (Wei et al., 2012). *miR156* is crucial for establishing lateral meristems in maize (Chuck et al., 2010). In rice, *SPL14-miR156* interaction governs plant architecture, with loss of function mutants possessing a reduced number of tillers (aboveground branches on grasses) and an increased number of branches in the panicles (loose clusters of flowers) (Jiao et al., 2010; Miura et al., 2010).

In alfalfa and many other plants (e.g., Arabidopsis, Lotus, maize, and tomato) *miR156* positively regulates the response to drought, heat and salinity (Arshad et al., 2017; Visentin et al., 2020; Hanly et al., 2020; Feyissa et al., 2019; Matthews et al., 2019). As an example, *miR156* silences *SPL13* in alfalfa, resulting in an altered root architecture that improves water uptake, an increase in photosynthesis under drought conditions, and an increase in the synthesis of anthocyanins and other stress-related hormones, leading to a general enhanced resilience to drought (Feyissa et al., 2019). However, increasing levels of *miR156* excessively can result in the opposite effect, high levels of *miR156* overexpression result in drought susceptibility, with plants with the highest levels of *miR156* possessing the lowest amounts of stress-related metabolites and the fewest physiological adjustments that would be beneficial under drought conditions (Feyissa et al., 2019). This result was also shown in tomato, where *miR156*-overexpressing plants display increased ABA sensitivity and lower stomatal conductance, as well as an increase in the amount of time taken to fully reopen stomata after drought conditions have subsided (Visentin et al., 2020). Together, these findings show how *miR156* is deeply involved in the response to abiotic stress for many plants, and depending on the level of expression, can both increase or decrease a plant's ability to withstand drought stress (Feyissa et al., 2019; Visentin et al., 2020).

1.5 Role of SPLs in plant development and stress response

SPL transcription factors play important roles in plant development (Wu et al., 2009). SPL3, SPL4 and SPL5 have been shown to accelerate flowering in *Arabidopsis* (Wu & Poethig, 2006). SPLs are also involved in determining leaf size and shape by controlling the number and size of cells in the leaves of *Arabidopsis* (Usami et al., 2009). When overexpressed, *SPL9* was shown to increase leaf size, while simultaneously reducing the rate of leaf initiation, and a similar phenotype was also observed in gain of function *SPL15* mutants (Usami et al., 2009). Wu et al.

(2010) showed that SPL9 (itself regulated by *miR156*) acts as a regulator for *miR172b*, which controls the temporal coordination of the vegetative phase and floral induction in *Arabidopsis*. SPL9 positively regulates trichome development and negatively regulates anthocyanin formation in *Arabidopsis* (Yu et al., 2010). SPL3, SPL9 and SPL10 are involved in lateral root growth, and SPL10, alongside SPL11 and SPL12, regulate morphological changes such as lamina shape and trichome distribution (Yu et al., 2010; Shikata et al., 2009).

SPLs also play an important role in the plant's response to stress (e.g., in mediating the response to recurring heat stress) (Cui et al, 2014). The ability of SPLs to affect root architecture also implicates them in the plant response to drought stress, as root architecture is vital in a plant's ability to efficiently acquire water and nutrients (Osmont et al., 2007). Root architecture is also vital for plant stability due to anchorage, allowing plants to remain stable in waterlogged or desiccated ground, thus preventing plants from uprooting during times of drought or flood stress (Osmont et al., 2007). Hanley et al. (2020) showed that alfalfa plants with RNAi-silenced *SPL9* had decreased senescence and increased water content under drought, and accumulated more anthocyanins (stress response antioxidants) when compared to wild type alfalfa under both well-watered and drought conditions. In alfalfa, plants containing more than one copy of mutated *SPL8* were shown to possess an increased ability to survive under drought conditions alongside a reduced leaf size and an early flowering time, with plants containing more than two copies of mutant *SPL8* also exhibiting decreases in plant height, shoot and root biomass, and root length (Singer et al., 2022). In *Medicago truncatula, spl8* mutants had enhanced branching and biomass yield, while overexpression of *SPL8* had the opposite effect (Gou et al., 2018). In the study done by Gou et al. (2018), *SPL8* was found to affect branching by being responsible for axillary bud formation, with overexpressed *SPL8* repressing axillary bud formation thus reducing branching and by extension

overall biomass (Gou et al., 2018). In alfalfa, the downregulation and upregulation of *SPL8* resulted in similar phenotypes to *M. truncatula SPL8* mutant and overexpression lines, while also possessing an increased tolerance to salt stress (Gou et al., 2018).

RNAi-silencing of *SPL13* in alfalfa resulted in increased tolerance to flood stress, with *SPL13RNAi* plants having a much more efficient rate of photosynthesis, and sharing the up and downregulation of many differentially expressed genes with that of flood tolerant alfalfa cultivar AAC-Trueman cultivar, such as the upregulation of Flavanone 3 hydroxylase, Flavanone 3'5'hydroxylase and *DFR* to scavenge the ROS produced during flood stress (Feyissa et al., 2021). *SPL13RNAi* also reduced water loss and increased survival in alfalfa plants undergoing drought stress, with higher levels of water retention, stomatal conductance, chlorophyll concentration, and photosynthesis efficiency (Arshad et al., 2017). Later studies conducted by Feyissa et al. (2019) found that the interplay between miR156/*SPL13* and *WD40-1/DFR* was responsible for regulating the alfalfa response to drought stress, with *miR156* suppressing *SPL13* expression, resulting in an increased expression of *WD40-1* which itself is responsible for the control of *DFR*. This results in enhanced biosynthesis of flavonoids and anthocyanins, allowing *SPL13RNAi* alfalfa to better control the amount of ROS present and mitigate the adverse effects of drought stress (Feyissa et al., 2019).

1.6 Trichomes and their functions

Trichomes are anatomical features that are present on leaves, stems, and/or flowers of both monocot and dicot plants (Peter et al., 1995). The role of trichomes as a defense mechanism against insects is well documented (Levin, 1973; Stipanovic, 1983; Webster et al., 1972). This function is achieved in one of three ways: 1) trichomes may act as a physical barrier to limit an insect's contact with the plant, 2) by the production of toxic compounds which poison the insect through contact,

ingestion, and/or inhalation, or 3) by the production of an adhesive substance which impedes insect movement (David and Easwaramoorthy, 1988; Duffey, 1986). Alfalfa trichomes utilize the first method of deterring insect predation, acting as physical barriers to a variety of pests (David and Easwaramoorthy, 1988; Duffey, 1986). The effectiveness of this mechanism is dependent on the length, density, and orientation of the trichomes, as well as on the insect's size, mode of locomotion and mouthparts (Southwood, 1986). Longer and denser hairs confer a greater resistance to insect predation, providing a more formidable barrier, as well as interfering with the insect's digestion (Wellso, 1973). Alfalfa utilizes trichomes to provide resistance to pest stressors such as the alfalfa weevil (*Hypera variabilis*) and the alfalfa blotch leafminer (*Agromyza frontella*) (Danielson et al., 1987; Maclean and Byers, 1983).

Trichome density has also been linked with the water content of plant material under adverse environments, with mature foliage in drier climates possessing the greatest trichome density (Johnson, 1968). Trichomes function as laminar flow inhibitors, creating turbulent flow in the boundary layer (Schull, 1929, Schreuder et al., 2001). Turbulent boundary layers have been linked to increased exchange of CO_2 and H_2O when compared to laminar boundary layers (often one or more orders of magnitude larger) (Schreuder et al., 2001). A layer of trichomes decreases air movement next to the leaf surface, creating a water potential gradient, an area of still air through which water vapour diffuses while moving from the water-saturated leaf interior to the drier air of the environment (Wooley et al., 1964). A study conducted by Sletvold and Ågren in 2011 found trichome-producing plants were more tolerant to drought than glabrous plants. In addition, low vapour pressure deficits have been shown to reduce glandular trichome density on silver birch leaves (Lihavainen et al., 2017).

Trichome development is regulated by a network of transcription factors, including GL1 and GL3 (Gruber et al., 2006). For example, enhanced expression of *GL3* and *GL1* results in an increase in trichome density in *Brassica napus*, with GL1 having been found to increase trichome density in Arabidopsis (Gruber et al., 2006) and GL3 to dramatically (~1000-fold) enhance leaf and stem trichome coverage in *Brassica napus* (Gruber et al., 2006). Yeast two-hybrid analysis showed that GL1 and GL3 are both required for maximum trichome initiation (Payne et al., 2000). Trichome development results from MYB transcription factors GL1 and MYB23, the bHLH factors GL3 and Enhancer of GL3 (EGL3), and the WD40 repeat protein TTG1 forming a trichome promoting trimeric complex due to the binding of a MYB factor and TTG1 to a bHLH factor (Galway et al., 1994). Transcription factors Triptychon (TRY), Caprice (CPC), Enhancer of TRY and CPC1 (ETC1) and Trichomeless 1 inhibit trichome production through competition for binding to GL3 or EGL3, preventing the formation of the trimeric complex (Esch et al., 2003). This complex initiates cell differentiation into trichomes, localizes itself in the epidermis, and is able to move between cells, initiating trichome development in many epidermal cells (Bouyer et al., 2008). Since members of the SPL family have shown an inhibitory effect on MYB-bHLH-TTG1 promoter complexes (Gou et al., 2011), it is possible that SPL4 may be involved in promoting trichome development.

1.7 **Proposed Research**

The impacts of climate change are expected to increase the demand for crops resistant to drought stress (Schindler & Donahue, 2006). Understanding the molecular mechanisms that control the plant response to stress is thus crucial for preventing losses in crop yield. For *Medicago sativa* (alfalfa) this need is compounded by the inability of traditional breeding techniques to

significantly improve crop productivity (Volonec et al., 2002). Studies conducted in alfalfa have shown that genetic modifications can induce plant mechanisms that increase stress tolerance (Feyissa et al., 2019).

miR156 is a non-coding RNA which negatively regulates gene expression at the post-transcriptional level and is a potent tool for improving alfalfa yield due to reducing expression of complementary target genes (Gao et al., 2016). Previous research has shown that *miR156* regulates the stress response in alfalfa via silencing *SPL* genes (Aung et al., 2015; Feyissa et al., 2019), and that overexpression of *miR156* enhances trichome density in Arabidopsis (Wei et al., 2012). I thus decided to investigate the role of SPL4 in drought tolerance, especially since *SPL4* is one of the genes silenced by *miR156* in alfalfa (Gao et al., 2016, Feyissa et al., 2019). Furthermore, a role for SPL4 in regulating trichome development is further supported by the fact that members of the SPL family are known to be involved in the MYB-bHLH-TTG1 promoter complex (Gou et al., 2011), which is also involved in determining the cell fate of epidermal cells with respect to becoming trichomes.

Investigating physiological traits that are involved in plant tolerance to drought stress in *SPL4*-silenced alfalfa can provide insight into how the *miR156*/SPL4 regulatory module affects drought tolerance and trichome development in alfalfa. This study will investigate the role of SPL4 in *miR156*-mediated alfalfa response to drought stress by altering *SPL4* expression and evaluating the following phenotypic changes relative to WT alfalfa: plant water status, water content, fresh weight and dry weight, root and shoot height, chlorophyll content and stomatal conductance. In addition, the phenotypic effect *SPL4* silencing on trichome development in alfalfa will be evaluated through measuring trichome density in *SPL4*-silenced alfalfa plants relative to WT alfalfa. Additionally, molecular interactions of SPL4 will be investigated to determine if SPL4

directly or indirectly controls the expression of other genes that play important roles in alfalfa's response to drought and trichome development.

1.8 Hypothesis

I hypothesize that silencing *SPL4* in alfalfa will impact expression of genes involved in trichome development (e.g., *GL1* and *GL3*), drought tolerance (e.g., *SPL9* and *SPL13*), and genes involved in antioxidant biosynthesis (e.g., *SOD*, *PP2C* and *CAT*) and will significantly enhance both trichome development and density, as well as phenotypic responses to drought.

1.9 Objectives/goals

The objectives of this study are as follows:

1. To investigate the role of SPL4 in trichome development and drought response by conducting drought experiments using alfalfa plants with RNAi-silenced *SPL*4 and assessing the effects on trichome number and stress response.
2. To identify downstream genes that may be regulated by SPL4 to affect drought response and trichome development. These genes will include *GL1*, *GL3*, *SPL9*, *SPL13*, and *CAT*.

Through the execution of this work, I hope to generate insights into the functional role of SPL4 in drought tolerance as well as to provide a foundation for future research into drought tolerance in both alfalfa and other plants.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

All plant materials used in this study were derived from alfalfa (*Medicago sativa*) clone N4.4.2 (Badhan et al., 2014) and were developed in the laboratory of Dr. Abdelali Hannoufa (Agriculture and Agri-Food Canada, London, Ontario, Canada). Individual transgenic lines were propagated through vegetative cuttings, which were collected and inserted into a wet sponge growth medium (Oasis Rootcubes) and grown under a plastic dome for four weeks. After four weeks, the dome was shifted to allow slight air flow from the greenhouse (for the purpose of acclimating the plants to the greenhouse environment). Plants were then transplanted to dark soil (PRO-MIX®, Smithers-Oasis North America, Kent, OH) and grown at 21 - 23ºC in a fully automated greenhouse under a 16-hour light/8-hour dark regime with a light intensity of 380-450 W/m2 and a relative humidity of 70%. Plants were rotated on the bench periodically to minimize the effects of microclimate differences. Plants were grown for 60 days (reaching maturity) before being subjected to drought treatments.

2.2 Drought treatment

Cuttings of SPL4RNAi and wild-type (WT) alfalfa were inserted into a wet sponge growth medium (Oasis Rootcubes) and grown for four weeks. Rooted stem cuttings of SPL4RNAi and WT alfalfa were transferred to 15 cm diameter pots containing equal amounts of BX Micorrhizae (PRO-MIX®, Smithers-Oasis North America) soil, and grown for two months before the start of the experiment. For this experiment, plants that exhibited visibly reduced or enhanced growth were excluded to ensure as little variation as possible. A total of 16 biological replicates per genotype

were used and the plants were distributed in a random block design and subsequently maintained in a greenhouse under long day conditions (16h light/8h dark, 56 relative humidity, 23℃) and watered twice a week. When alfalfa plants were two months old, a 50% soil water content was established in each pot using a Fieldscout soil sensor reader (Spectrum Technologies Inc.) and then water was withheld. During the drought trial, pots were rotated randomly every day on the bench to minimize variations due to microclimate effects. Data on water content/loss, water potential, stomatal conductance, biomass weight, and chlorophyll content were collected as described in Arshad et al. (2017) after a week with no water. Another set of plants prepared for drought as above were re-watered after the stress period to assess the ability of the plants to recover from drought.

2.3 Phenotypic characterization of alfalfa SPL4 RNAi plants

A morphological analysis of alfalfa was conducted for the purpose of determining the effects of *SPL4* silencing and drought stress on plant development. Plant height was measured as the length between the tip and the neck of the tallest alfalfa shoot. Similarly, root length was measured as the length between the tip to the neck of the longest root. Root and shoot biomass were measured by collecting roots and shoots of the alfalfa separately and measuring their wet weight by weighing them on a Denver Instrument SI402 scale.

Water content was measured for the shoots and roots by weighing the shoots and roots of alfalfa both immediately after collection, and after placing them at 65℃ for five days. The difference in weight between the original measurements and the desiccated weight was then recorded. Leaf water content was measured by collecting two 0.5 g samples from alfalfa leaves on the second level, the level immediately below the terminal buds. One sample was placed in an

oven at 65℃ for five days and the other submerged in water for one day. The difference in weight between the two samples was recorded.

Water potential was measured by excising leaves immediately below the terminal buds at the petiole and placing them in the pressure chamber of a Plant Water Status Console (Hoskin Scientific, Model 3115) with the stem of the leaf extruding from the chamber. Pressure was steadily increased in the chamber, and pressure was recorded when water could be seen escaping from the stem of the leaf.

To measure stomatal conductance from WT and SPL4RNAi alfalfa, a Leaf Porometer (Decagon Devices, Inc Pullman, WA) was used as described in Arshad et al., (2017). To determine chlorophyll content, a 1000 mm^2 area of alfalfa leaves on the second level were collected from WT and SPL4RNAi plants, and chilled prior to chlorophyll extraction. The tissue sample was then placed in a homogenization tube and homogenized for 30 sec (MoBio Laboratories Inc Powerlyzer 24). The tube was then refrigerated for 2 h, and 5 mL of 80% aqueous acetone subsequently pipetted into the homogenization tubes. Samples were then homogenized for a further 30 sec. The sample extract was moved to a centrifuge tube and centrifuged for 20 min at 500 *g*. The supernatant was then decanted into a 10 mL graduated cylinder and the volume brought to 10 mL with 80% acetone. Using 80% acetone as a blank, samples were analyzed by spectroscopy (BioRad SmartSpec Plus) at 695 nm and again at 663 nm to determine chlorophyll concentration.

Trichome density was determined by counting the number of trichomes present on a section of second level leaf using a Nikon SMZ1500 stereoscopic zoom microscope at 10x magnification. Sections were kept standard by avoiding xylem bundles and by observing the abaxial side of the leaf.

2.4 Expression analysis of genes linked to trichome development and drought stress

The transcript levels of candidate genes involved in trichome development or stress response were measured to determine any differences resulting from *SPL4* silencing and drought stress. Candidate genes included *GL1* and *GL3*, which are involved in trichome development (Gruber et al., 2006), *SPL9*, *SPL13* and *PP2C*, which are involved in drought tolerance (Aung et al., 2015; Ma et al., 2009), and *DFR, SOD,* and *CAT* genes that are related to the biosynthesis of stress-alleviating antioxidants (Arshad et al., 2017). Analysis of the transcript levels of these genes was conducted using qRT-PCR as follows. Leaf tissue was collected from alfalfa flag leaves using scissors, and the tissue was frozen in liquid nitrogen and ground to a fine powder. RNA was extracted using the Qiagen RNeasy Plant Mini Kit. The extracted RNA was treated with TURBO DNase to remove any residual DNA. cDNA was then synthesized using iScript™ Reverse Transcription Supermix for qRT-PCR (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The resulting cDNA was diluted 20x in DNase free water. *Actin* and *cyclophilin* were uses as reference genes as their expression levels remain relatively unchanged across environmental conditions (Castonguay et al., 2015). Each reaction was repeated three times and consisted of a Mastermix created using a ratio of 10x BioRad SsoFast EvaGreen Supermix to 1x forward and 1x reverse primers. For every well, 6 µL of Mastermix and 4 µL of diluted cDNA was loaded. The qPCR protocol was as follows: 95ºC for 30 sec, 95ºC for 5 sec, a primer specific annealing temperature of 58ºC (for *SPL4, actin* and *cyclophilin* primers) for 15 sec for 45 cycles, with a melt curve temperature of 65ºC for 31 sec, increasing the temperature by 0.5ºC per cycle and 0.5 ºC per sec for 60 cycles. Transcript analysis was carried out using CFX Maestro™ software (Bio-Rad).

2.5 Statistical analysis

For each plant genotype, at least eight biological replicates per genotype per treatment were used for phenotypic characterization. For comparisons between two groups, t-tests with applied Bonferroni corrections were used.

CHAPTER 3

3 RESULTS

The non-coding RNA, *miR156,* regulates the response of alfalfa to various stresses via silencing genes encoding members of the SPL family of transcription factors (Stief et al., 2014, Feyissa et al., 2021). SPL4, a member of the SPL transcription factor family, is a known target of *miR156* (Gao et al., 2016), but otherwise has not been extensively studied in alfalfa and other crop species. To better understand how the *miR156*/SPL regulatory module affects drought tolerance in alfalfa, SPL4 was chosen for analysis involving RNAi-mediated gene silencing so that the phenotypic, physiological, and molecular effects of *SPL4* knockdown could be evaluated in this crop plant.

3.1 Analysis of *SPL4* expression in *SPL4RNAi* plants

To initiate the study, I first assessed the level of *SPL4* silencing in previously generated transgenic plants harboring the *SPL4RNAi* transgene. RNA was collected from eight independently generated *SPL4RNAi* genotypic lines (*SPL4RNAi-1, SPL4RNAi-3, SPL4RNAi-7, SPL4RNAi-8A, SPL4RNAi-8B, SPL4RNAi-9B, SPL4RNAi-13* and *SPL4RNAi-14)* and *SPL4* transcript levels were determined by RT-qPCR in relation to a WT control genotype. *β-actin* and *cyclophilin* were used as reference genes (Kozera & Rapacz, 2013). Based on these experiments, three *SPL4RNAi* genotypes (*SPL4RNAi-7, SPL4RNAi-9B, SPL4RNAi-14*) were chosen for further characterization due to their decreased *SPL4* transcript levels relative to WT alfalfa (*SPL4RNAi-7* exhibited ~ 50% of the *SPL4* transcript level observed in WT, whereas *SPL4RNAi-9B* and *SPL4RNAi-14* exhibited ~ 30% and ~ 25% the WT level, respectively) (**Figure 2)**.

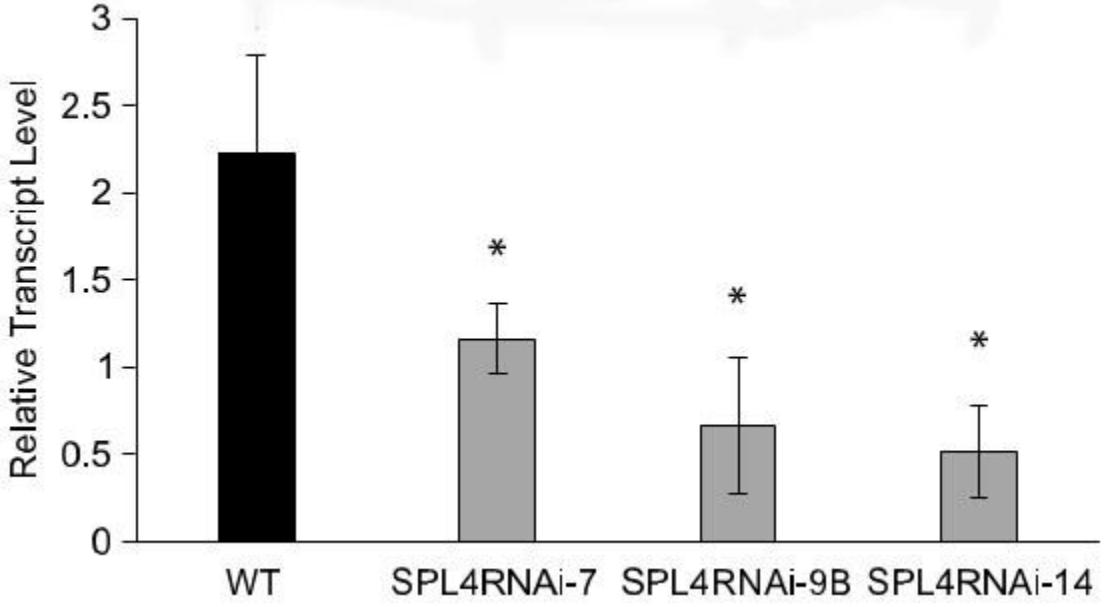

Relative Transcript Level
3
2.5
2
1.5
1
0.5
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9B
SPL4RNAi-14

Figure 2: Expression analysis of SPL4 in WT and *SPL4RNAi* alfalfa leaves using qRT-PCR. The experiment consisted of three biological and three technical replicates for each genotype. Asterisks indicate significant differences relative to the WT control using unpaired t-tests ($p<0.05$). Error bars represent the standard error. All characterized plants were at the same developmental stage.

3.2 Phenotypic effects of *SPL4* silencing under drought Stress

3.2.1 Effect of *SPL4* silencing on plant architecture under drought

To determine the effect of *SPL4* silencing on the drought response in alfalfa plants, the *SPL4RNAi-7, SPL4RNAi-9B,* and *SPL4RNAi-14* plants, as well as a WT control, were subjected to drought stress or non-drought control conditions as described in the Materials and Methods and then plant height and root length were measured. Following 14 days of drought treatment, no statistically significant differences in plant height were observed between WT and *SPL4RNAi* plants. As seen in **Figure 3A**, plant height was similar between *SPL4RNAi* plants and WT under both drought and well-watered control conditions. Previous studies have shown that the ability of alfalfa plants overexpressing *miR156* to withstand drought is due partially to elongated roots (Arshad et al., 2017). Consistent with this observation, both *SPL4RNAi-9B* and *SPL4RNAi-14* genotypes showed a ~ 30% increase in root length when compared to WT under drought (**Figure 3B**). *SPL4RNAi-7* did not show an increase in root length, suggesting that a dose dependent effect may occur with *SPL4* transcript levels. Within genotypes, WT root length was reduced ~ 30% under drought conditions, while *SPL4RNAi-7* showed no significant reduction in length. *SPL4RNAi-9* and *SPL4RNAi-14* exhibited a ~ 10% increase in root length under drought conditions compared to their unstressed counterparts (**Figure 3B**).

3.2.2 Effect of *SPL4* silencing on fresh root and shoot biomass under drought

As shoot and root biomass are affected in plants grown under drought stress, I set out to examine these parameters in *SPL4RNAi* plants under drought. After 14 days of drought treatment, no phenotypic differences were observed with respect to root or shoot biomass between WT and *SPL4RNAi* plants (**Figures 4A, 4B**). However, within genotypes, both WT and all *SPL4RNAi*

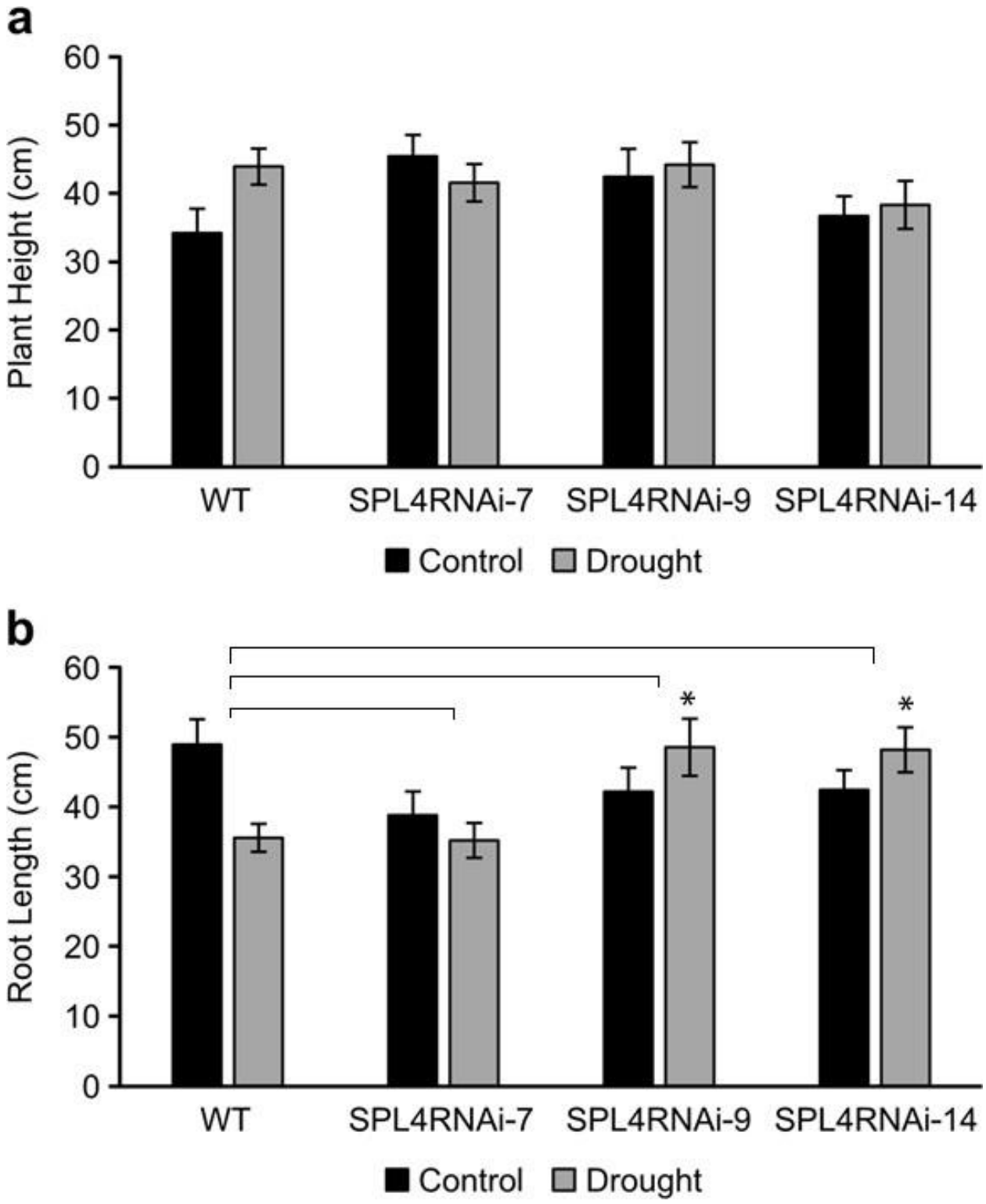

a
Plant Height (cm)
60
50
40
30
20
10
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
b
Root Length (cm)
60
50
40
30
20
10
0
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought

Figure 3: Effect of *SPL4* silencing on plant architecture under drought stress.

A) Effect of *SPL4* silencing on shoot height under drought; B) Effect of *SPL4* silencing on root length under drought. The experiment consisted of eight biological replicates of each genotype. Asterisks indicate significant differences relative to the WT control under drought conditions using unpaired t-tests ($p < 0.05$). Brackets indicate significant changes in the difference between the control and drought conditions for *SPL4RNAi* plants compared to WT using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All characterized plants were at the same developmental stage.

plants showed a significant reduction in both shoot and root biomass under drought. *SPL4RNAi* plants showed a greater decrease in shoot and root biomass, with shoot biomass decreasing ~ 50% compared to WT, where shoot biomass decreased by approximately 25% (**Figure 4A**). Root biomass decreased about 25% in WT, 30% in *SPL4RNAi-7* and *SPL4RNAi-14* and 75% in *SPL4RNAi-9B* (**Figure 4B**). Despite the larger decrease in root and shoot biomass in *SPL4RNAi* plants compared to WT under drought, SPL4RNAi plants retained an overall biomass similar to that of WT, owing to having consistently (although not always statistically significant) higher root and shoot biomass under well-watered conditions (**Figure 4A, B**).

3.3 Effect of *SPL4* silencing on water relations under drought

Total water content is the total water contained within the plant tissue and is a good indicator of tolerance or susceptibility to drought. This parameter has been used to assess drought tolerance in many plants, including Arabidopsis (van der Weele et al., 2000) and alfalfa (Matthews, 2019, Arshad et al., 2017, Feyissa et al., 2017, Zhang et al., 2018). Water potential, another indicator of the effect of drought on water movement, has been used to assess the severity of drought on plants, including alfalfa (Matthews et al., 2019, Feyissa et al., 2017, Hanly et al., 2020). Movement of water through plant tissues enables cell growth and eliminates water deficits, resulting in mitigation of the effects of drought conditions (Boyer, 1968). This parameter is normally measured based on the amount of pressure required to force water out of plant tissue under drought conditions. These two water relations parameters were used in this study to assess the effect of *SPL4* silencing on drought tolerance in alfalfa.

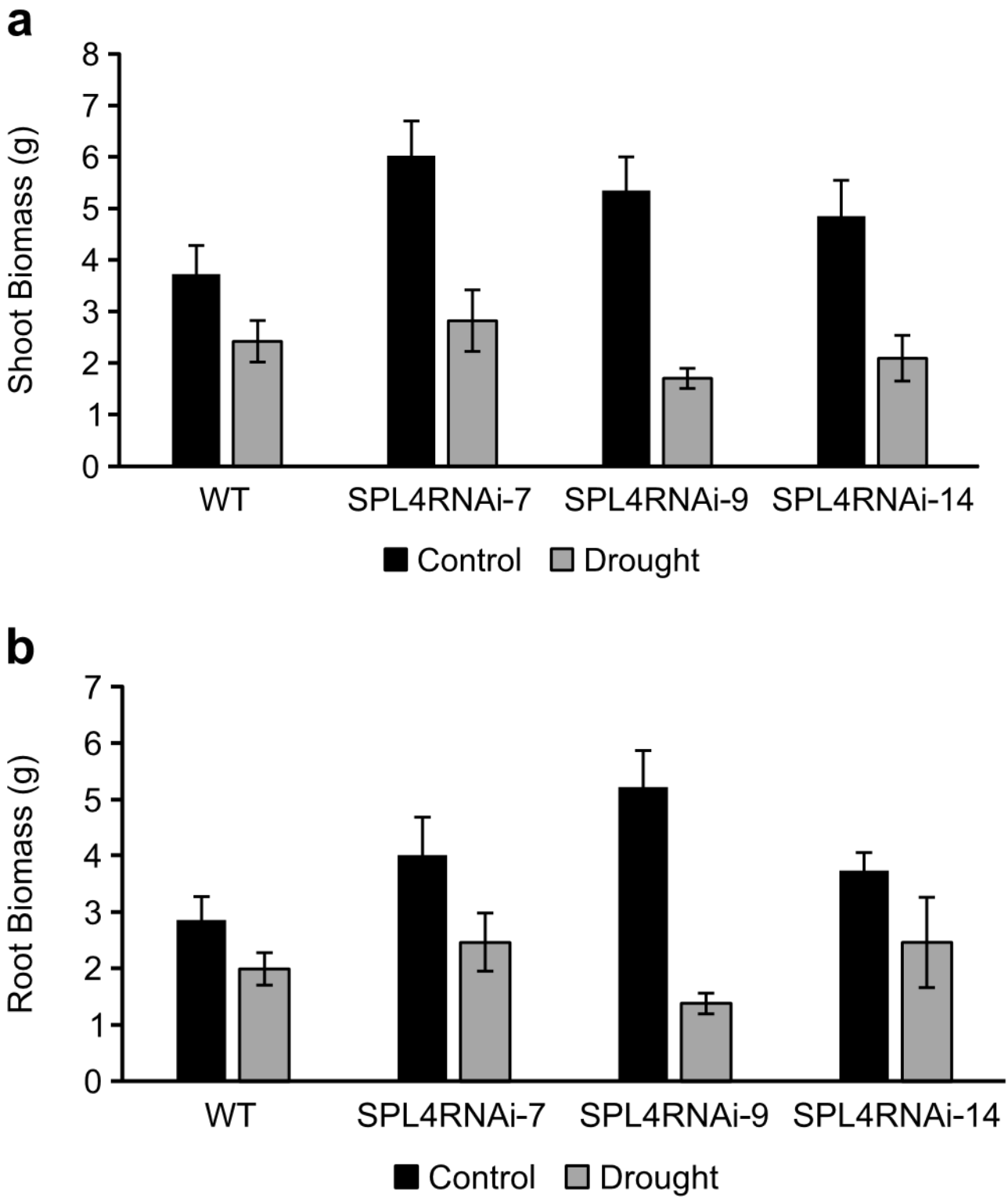

a
Shoot Biomass (g)
8
7
6
5
4
3
2
1
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
b
Root Biomass (g)
7
6
5
4
3
2
1
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought

Figure 4: Effect of *SPL4* silencing on plant biomass under drought stress.

A) Effect of *SPL*4 silencing on shoot biomass; B) Effect of *SPL4* silencing on root biomass. Shoot biomass was measured 14 days after vegetative cutting. The experiment consisted of eight biological replicates of each genotype. Error bars represent the standard error. All characterized plants were at the same developmental stage.

3.3.1 Effect on total leaf water content

To evaluate the role of SPL4 in the regulation of drought tolerance, leaf water content (LWC) was compared under well-watered and drought conditions in WT and *SPL4RNAi* plants. Under drought, there was a 60% reduction in water content in the leaves of WT alfalfa, while the greatest decrease in water content among *SPL4RNAi* plants was 30% in *SPL4RNAi-4* (**Figure 5A**). This finding is consistent with qualitative observations of plant health under drought conditions, where WT plants appeared to be more severely impacted by drought and more chlorotic than *SPL4RNAi* plants (**Figure 5B**). Under well-watered conditions, there was no statistically significant difference in LWC between WT and *SPL4RNAi* plants (**Figure 5A**). Under drought conditions, all *SPL4RNAi* genotypes showed more than 35% higher water content than WT (**Figure 5A**).

3.3.2 Effect on leaf water potential

Another indicator of plant water status, leaf water potential (LWP) (Biruk et al., 2017), was measured in WT and *SPL4RNAi* genotypes under well-watered and drought conditions. While LWP was unaffected in WT relative to *SPL4RNAi* plants under control conditions, there was a significant increase in LWP under drought stress in WT. The increase in LWP was 20% lower in the transgenic plants compared to WT, indicating a larger volume of water present in *SPL4RNAi*

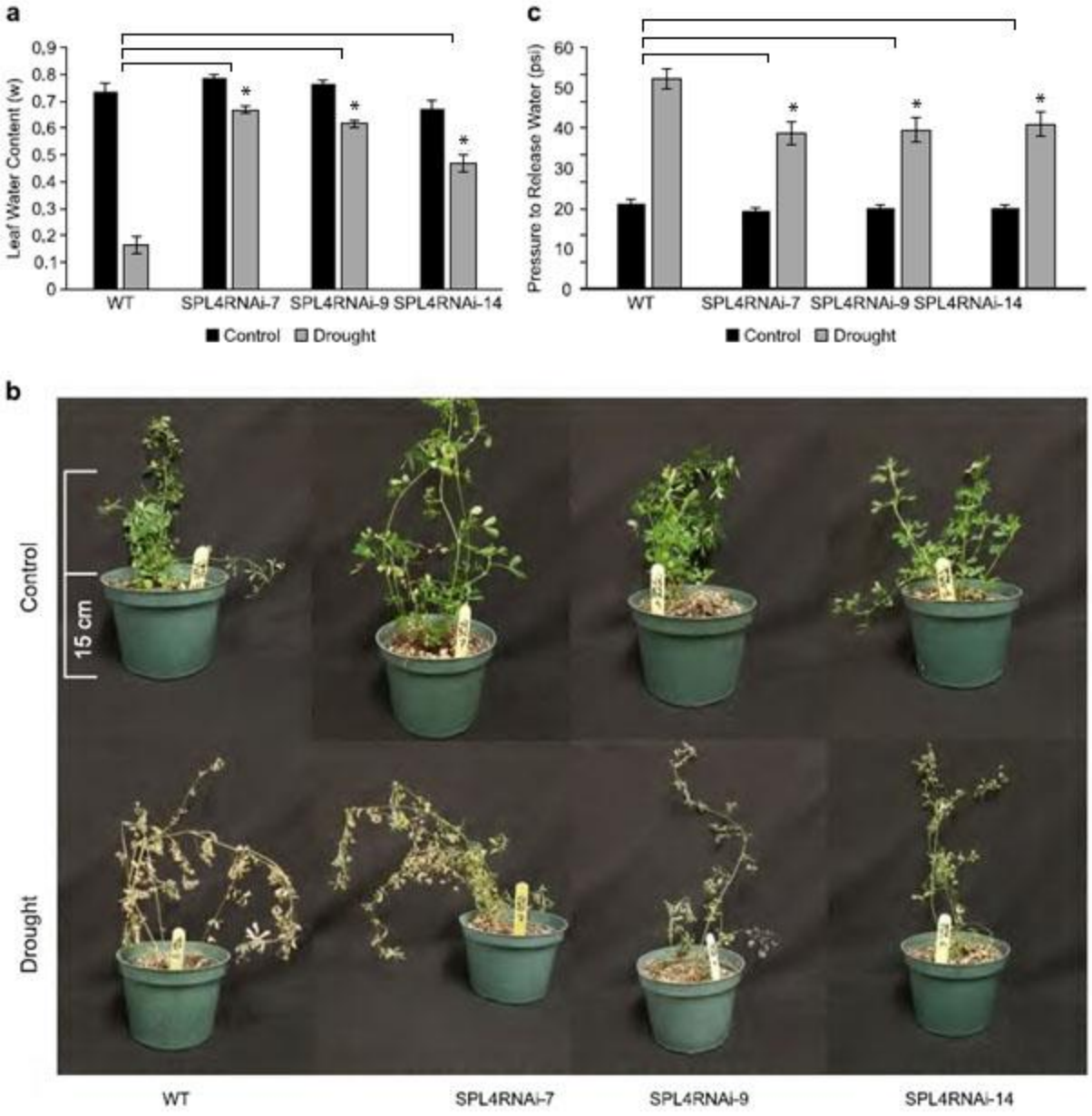

a
Leaf Water Content (w)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
c
Pressure to Release Water (psi)
60
50
40
30
20
10
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
b
Control
15 cm
Drought
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14

Figure 5: Effect of *SPL4* silencing on water relations under drought in alfalfa.

A) Leaf water content of WT and *SPL4RNAi* alfalfa plants under drought stress; B) Representative WT and *SPL4RNAi* plants under control and drought conditions; C) Leaf water potential in WT and *SPL4RNAi* alfalfa under drought. For A and C, the experiment consisted of eight biological replicates of each genotype. Asterisks indicate significant differences relative to the WT control under drought conditions using unpaired t-tests ($p < 0.05$). Brackets indicate significant changes in the difference between the control and drought conditions for *SPL4RNAi* plants compared to WT using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All characterized plants were at the same developmental age.

drought-affected leaves. Within genotype, drought resulted in a ~ 175% increase in LWP in WT, while in *SPL4RNAi* LWP was only about 100% higher (**Figure 5C**). The difference in LWP between the two sets of plants indicates an improved response of *SPL4RNAi* plants to water stress, which is consistent with phenotypic observations of alfalfa plants subjected to drought conditions (**Figure 5B**).

3.4 Effect of *SPL4* silencing on chlorophyll concentration in alfalfa under drought

The level of chlorophyll in green tissues is a parameter that can be used to evaluate the ability of plants grown under stress to conduct photosynthesis and synthesize primary metabolites. It can also be used an indicator of overall plant health (Arshad et al., 2017). To determine if SPL4 is involved in regulating chlorophyll accumulation in alfalfa, *SPL4RNAi* plants were compared with WT under well-watered and drought conditions for chlorophyll A, chlorophyll B, and total chlorophyll concentrations. Under drought stress, *SPL4RNAi-7*, *SPL4RNAi-9* and *SPL4RNAi-14* genotypes exhibited approximately 9, 10, and 11% higher chlorophyll A concentrations, respectively, compared to WT (**Figure 6A**). Under well-watered conditions, there was a significant difference between the chlorophyll A content of WT and *SPL4RNAi-14* plants, with an ~ 8% increase in chlorophyll A concentration. Within genotypes, WT alfalfa showed no significant difference in chlorophyll A concentration between well-watered and control conditions, when compared to SPL4-silenced genotypes (**Figure 6A**).

For chlorophyll B, while there was no significant difference in concentration between *SPL4RNAi* plants and WT under well-watered conditions, *SPL4RNAi* plants had an ~ 40% higher concentration under drought conditions (**Figure 6B**). Within genotypes, drought stress caused

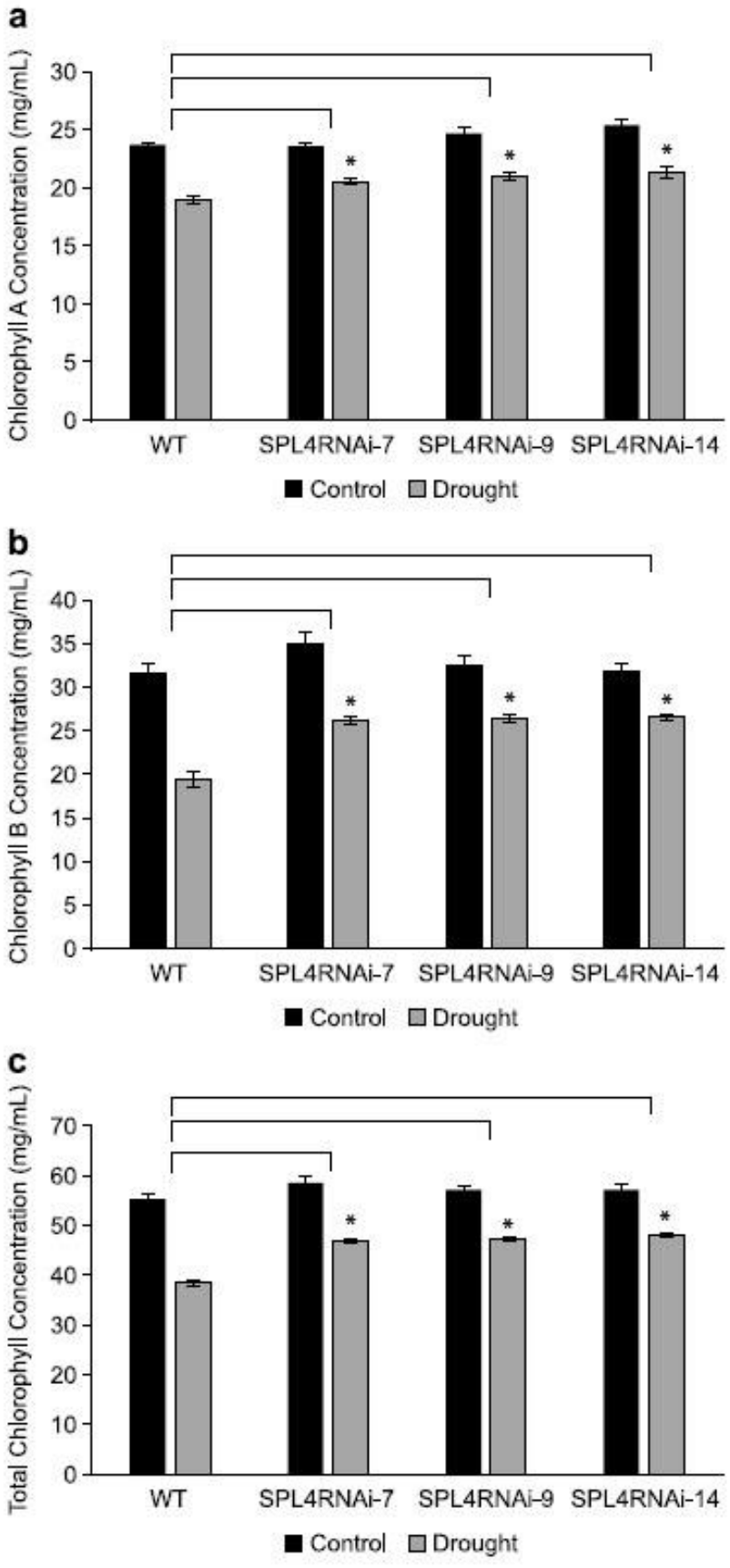

a
Chlorophyll A Concentration (mg/mL)
30
25
20
15
10
5
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
b
Chlorophyll B Concentration (mg/mL)
40
35
30
25
20
15
10
5
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought
c
Total Chlorophyll Concentration (mg/mL)
70
60
50
40
30
20
10
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought

Figure 6: Effect of *SPL4* silencing on chlorophyll concentration in alfalfa under drought.

A) Concentration of chlorophyll A; B) Concentration of chlorophyll B; C) Concentration of total chlorophyll. The experiment consisted of 10 biological replicates of each genotype Asterisks indicate significant differences relative to the WT control under drought conditions using unpaired t-tests ($p < 0.05$). Brackets indicate significant changes in the difference between the control and drought conditions for *SPL4RNAi* plants compared to WT using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All characterized plants were at the same developmental age.

a 24% decrease in chlorophyll B concentration in *SPL4RNAi-7* and a 19% decrease in *SPL4RNAi-9* and *SPL4RNAi-14*, whereas WT alfalfa had a significantly more pronounced decrease of about 44% (**Figure 6B**), indicating a higher susceptibility of WT to drought compared to *SPL4RNAi* genotypes.

Total chlorophyll content in *SPL4RNAi* genotypes was higher under drought conditions in *SPL4RNAi* genotypes when compared to WT, with up to an ~ 25% increase in total chlorophyll, while no difference was detected between WT and *SPL4RNAi* genotypes under well-watered conditions (**Figure 6C**). Within genotypes, WT alfalfa had a 33% decrease in total chlorophyll, while *SPL4RNAi-7, SPL4RNAi-9* and *SPL4RNAi-14* had about an 18% decrease under drought. These findings are consistent with qualitative observations conducted over the course of the drought treatment, and provide an explanation for the reduced yellow coloration on drought-afflicted *SPL4RNAi* plants (**Figure 6B**).

3.5 Effect of *SPL4* Silencing on Stomatal Conductance in Alfalfa under Drought

Stomatal conductance is a measurement of water vapor movement out of plant leaves and is an indicator of the effect of drought on water transfer between the plants and their surrounding environment (Arshad et al., 2017). Stomatal conductance was measured to determine the extent of stomata opening and the release of water vapor while plants were subjected to drought stress. Under well-watered conditions, there was no difference in stomatal conductance between WT and *SPL4RNAi* alfalfa. While all genotypes showed a decrease in conductance under drought, WT showed the most severe response with a decrease of 65%. In contrast, *SPL4RNAi-7* and

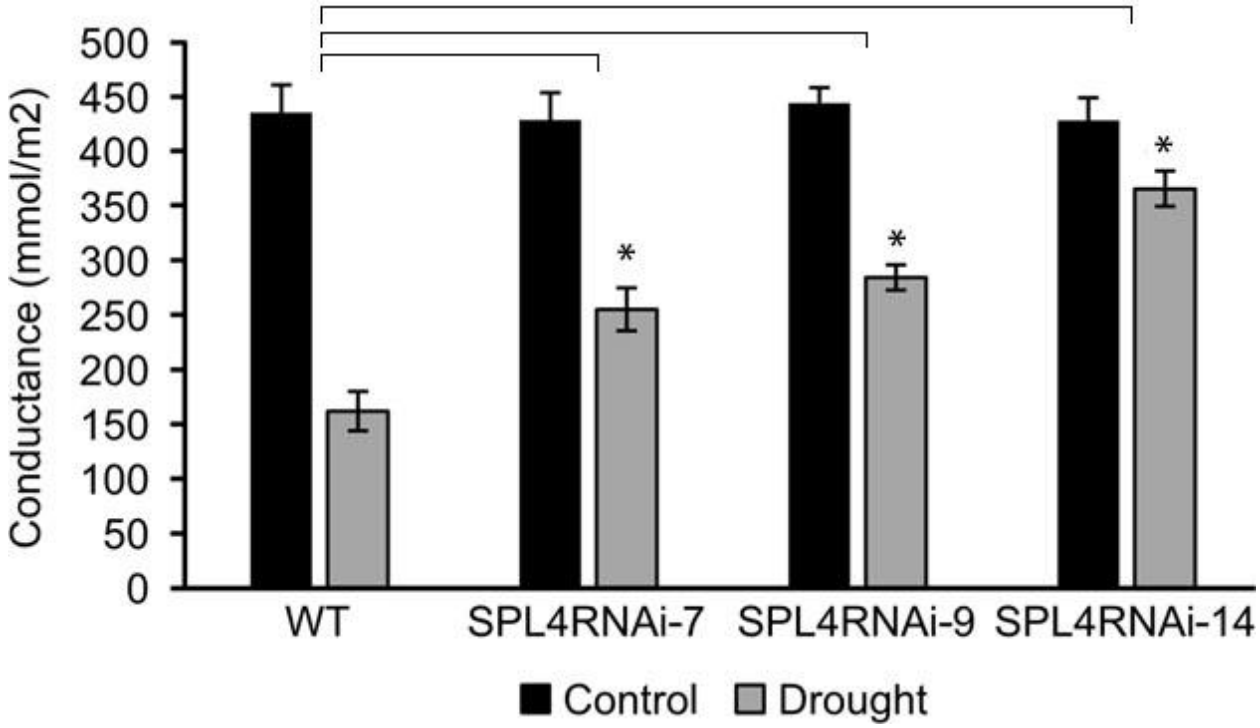

Conductance (mmol/m2)
500
450
400
350
300
250
200
150
100
50
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought

Figure 7: Stomatal conductance in alfalfa under drought stress.

The experiment consisted of 10 biological replicates of each genotype. Asterisks indicate significant differences relative to the WT control under drought conditions using unpaired t-tests ($p < 0.05$). Brackets indicate significant changes in the difference between the control and drought conditions for *SPL4RNAi* plants compared to WT using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All characterized plants were at the same developmental age.

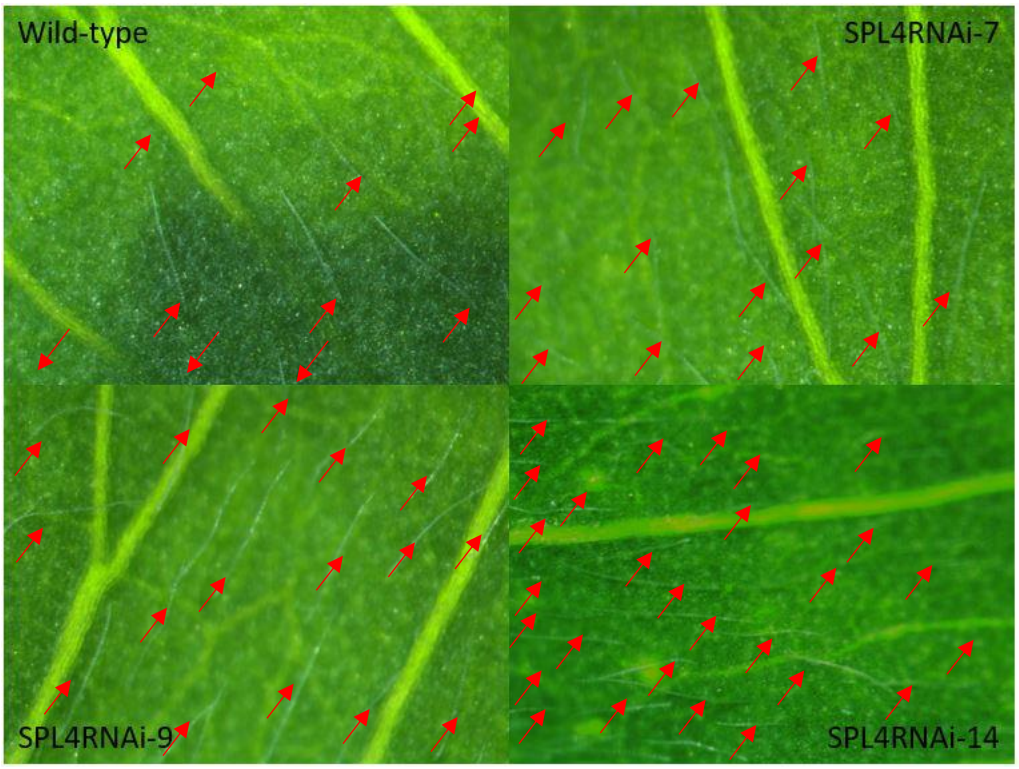
SPL4RNAi-9 plants showed decreases of only ~ 40%, and *SPL4RNAi-14* plants a decrease of only 10%. Under drought conditions, *SPL4RNAi-7, SPL4RNAi-9* and *SPL4RNAi-14* all had higher stomatal conductances (~ 66%, ~ 73%, and 133%, respectively) than WT, indicating more water transfer was occurring in *SPL4RNAi* plants compared to WT (**Figure 7**).

3.6 Effect of SPL4 silencing on trichome density

Based on preliminary qualitative observations regarding the number of trichomes present on leaves of *SPL4RNAi* plants (Hannoufa lab, unpublished), I decided to conduct a thorough quantitative analysis of trichomes on these plants relative to WT, especially under drought stress. To that end, trichomes were assessed both visually (**Figure 8A**) and quantitatively (**Figure 8B**) in *SPL4RNAi* plants relative to WT under both well-watered conditions and water scarcity (drought). This analysis revealed that, regardless of water availability, there was significantly higher (~ 50%) trichome density on leaves of *SPL4RNAi* plants (**Figure 8A**) compared to WT, indicating that SPL4 may be a negative regulator of trichome development in alfalfa.

Given the aforementioned finding, I set out to determine the effect of SPL4 on the relative expression of genes known to be involved in trichome development, namely *GL1* and *GL3* (Gruber et al., 2006). The results showed a 30% and 60% increase in transcript levels of *GL1* and *GL3*, respectively, in *SPL4RNAi* plants when compared to WT alfalfa (**Figure 9A, B**). These findings are consistent with observed differences in trichome density between WT and *SPL4RNAi* alfalfa plants (**Figure 8A**).

a


Wild-type
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14

b

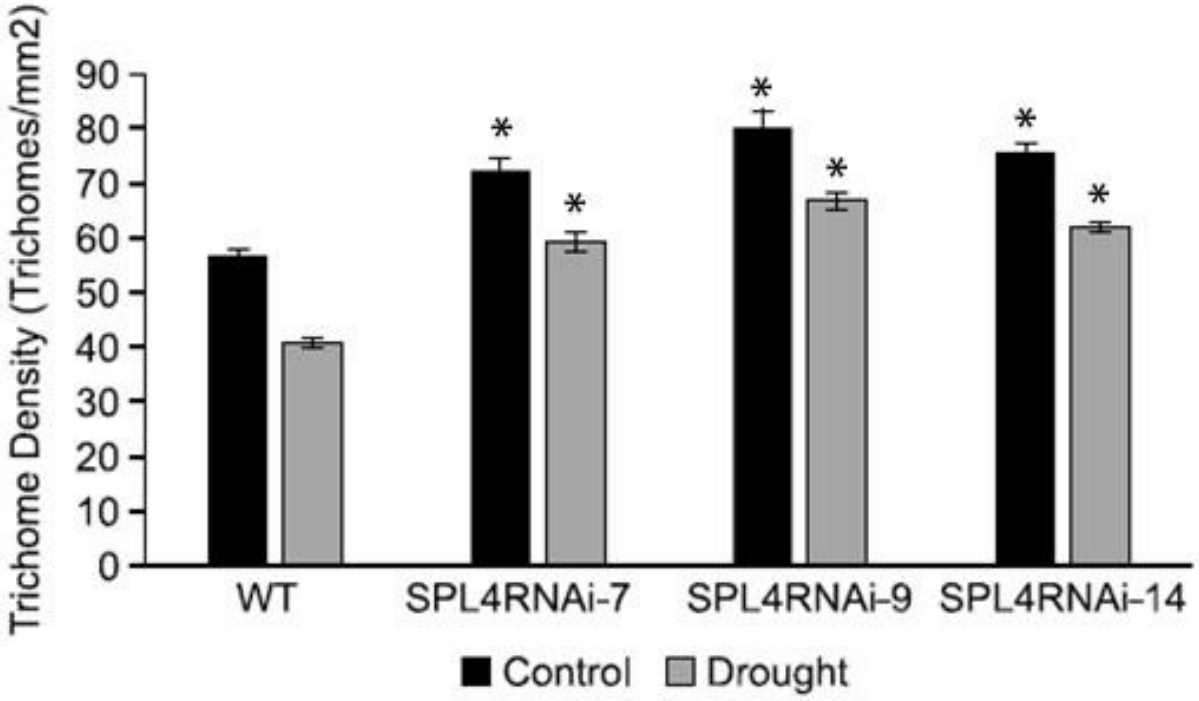

Trichome Density (Trichomes/mm2)
90
80
70
60
50
40
30
20
10
0
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
Control
Drought

Figure 8: Trichome density of *SPL4RNAi* plants.

A) Representative WT and *SPL4RNAi* trichome densities. Red arrows indicate some visible trichomes, illustrating the difference in quantity between WT and *SPL4RNAi* genotypes.

B) Effect of *SPL*4 silencing on trichome density in alfalfa under drought. The experiment consisted of eight biological replicates and three technical replicates of each genotype. Asterisks indicate significant differences relative to the WT control using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All displayed plants were at the same developmental age.

3.7 Effect drought on expression of stress-related genes in SPL4RNAi alfalfa

Having conducted experiments evaluating the phenotypic effects of *SPL4* silencing on stress response, I next determined if silencing *SPL4* gene expression had an effect on the expression of other genes involved in the plant stress response. These included *SPL9* and *SPL13*, genes involved in regulating drought stress (Aung et al., 2015; Gao et al., 2016), and *CAT*, a gene involved in antioxidant biosynthesis (Choudhury et al., 2013) (**Figure 10**). This analysis revealed that when *SPL4* is silenced, stress related genes are similarly affected. In *SPL4RNAi* alfalfa, *SPL9* transcript levels were ~ 30% lower than in WT alfalfa (**Figure 10A**), and similarly those of *SPL13* were 30% lower in *SPL4RNAi-7* and *SPL4RNAi-14* compared to WT, but *SPL4RNAi-9* did not show a significant reduction in *SPL13* transcript levels (**Figure 10B**). In *SPL4RNAi* plants, *CAT* expression was ~ 40% higher in all SPL4RNAi genotypes, suggesting that *SPL4* is involved in regulation of *CAT* expression (**Figure 10C**).

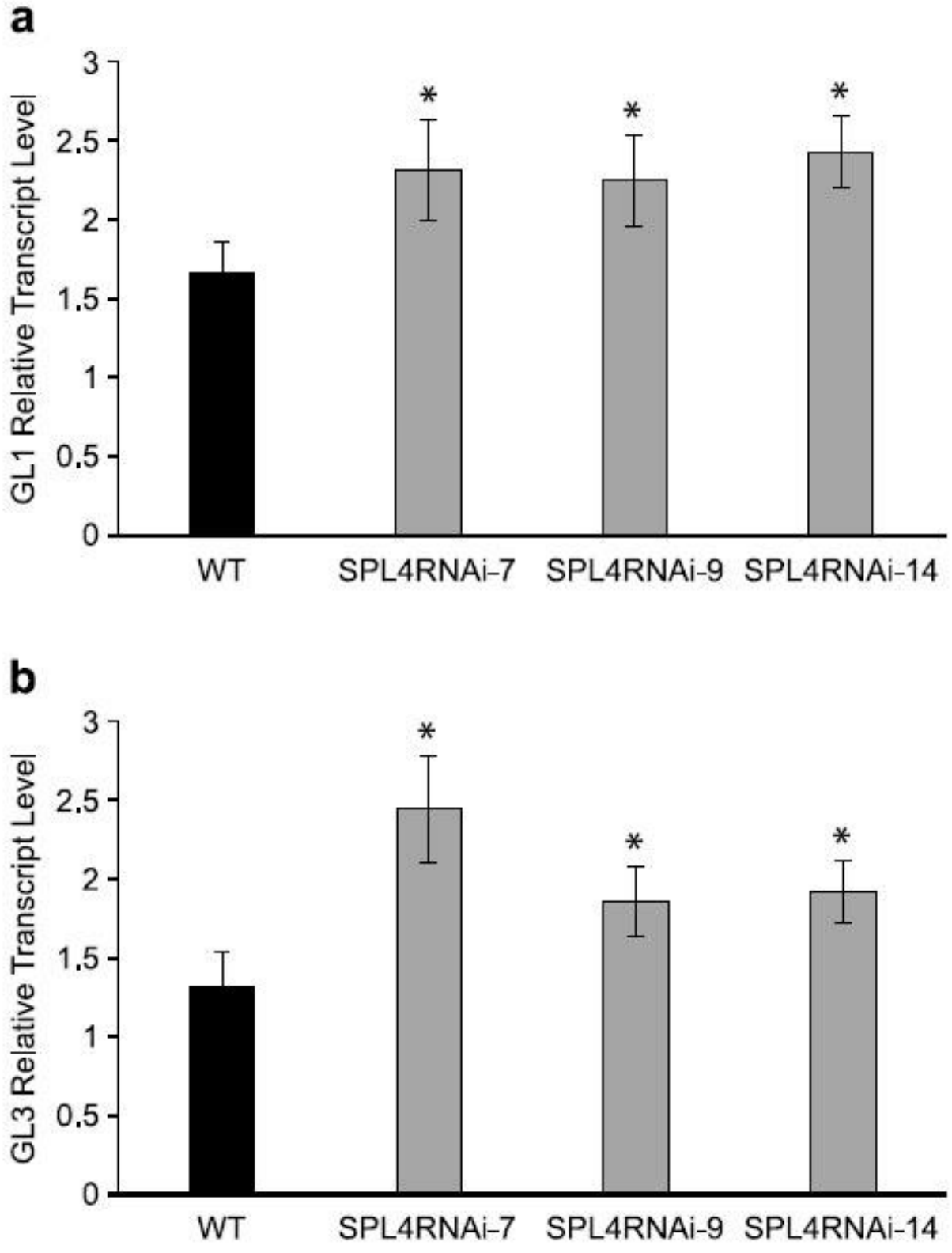

a
GL1 Relative Transcript Level
3
2.5
2
1.5
1
0.5
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
b
GL3 Relative Transcript Level
3
2.5
2
1.5
1
0.5
0
*
*
*
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14

Figure 9: Analysis of expression of *GL1* and *GL3* in *SPL4RNAi* plants.

Relative transcript level of A) *GL1* and B) *GL3*. The experiment consisted of three biological and three technical replicates of each genotype. Brackets indicate significant differences relative to the WT control using unpaired t-tests ($p < 0.05$). Error bars represent the standard error. All characterized plants were at the same developmental age.

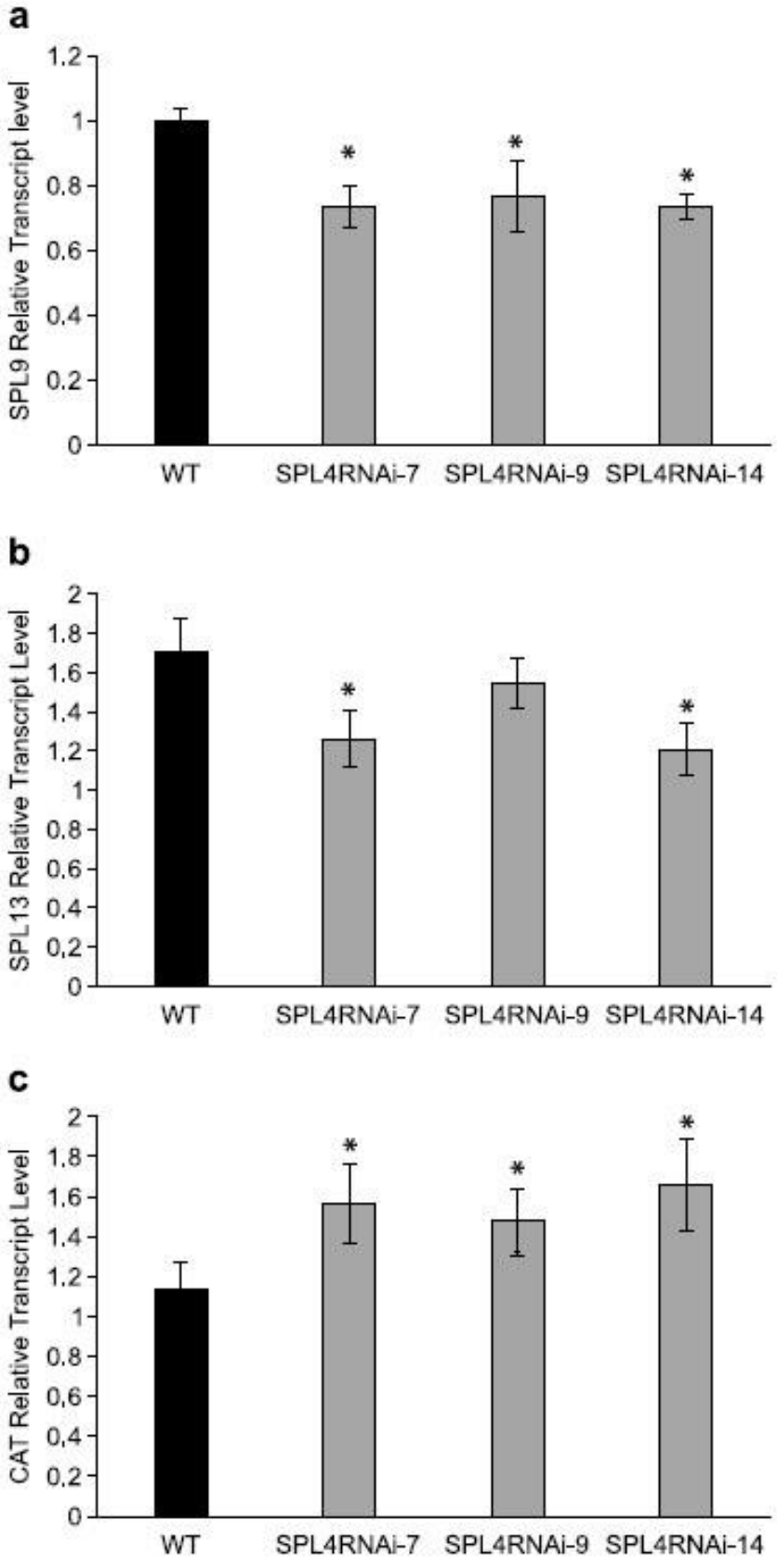

a
SPL9 Relative Transcript level
1.2
1
0.8
0.6
0.4
0.2
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
b
SPL13 Relative Transcript Level
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0,2
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14
c
CAT Relative Transcript Level
2
1.8
1.6
1.4
1.2
1
0.8
0,6
0,4
0.2
0
WT
SPL4RNAi-7
SPL4RNAi-9
SPL4RNAi-14

Figure 10: Expression analysis of stress response genes in *SPL4RNAi* alfalfa.

Analysis of relative transcript levels of A) *SPL9*, B) *SPL13*, and C) *CAT*. The experiment consisted of three biological and three technical replicates of each genotype. Asterisks indicate significant differences relative to the WT control using unpaired t-tests ($p<0.05$). Error bars represent the standard error. All characterized plants were at the same developmental age.

CHAPTER 4

4 DISCUSSION

4.1 Overview of research

miR156 regulates plant development as well as the response to a variety of plant stresses. This is mediated through its silencing of the SPL family of transcription factors, most of which are involved in regulating plant growth (Baker et al., 2005; Arshad et al., 2017). In alfalfa, *miR156* overexpression results in the silencing of *SPL* genes, a decrease in plant height, an increase in branching and root nodulation, a reduction in stem thickness, and a longer vegetative phase (Aung et al., 2015, Gao et al., 2016). In *Arabidopsis thaliana*, overexpression of *miR156* was shown to improve drought tolerance, and to result in an increase in trichome number (Cui et al., 2014; Wei et al., 2012). In maize, *miR156* is crucial for the establishment of lateral meristems (Chuck et al., 2011).

In Arabidopsis, *SPL* genes targeted by *miR156* are grouped into four clades, SPL3/SPL4/SPL5, SPL2/SPL10/SPL11, SPL9/SPL15, and SPL6/SPL13 (Guo et al., 2008). Feyissa et al. (2021) also generated a phylogenetic tree for the SPL family and divided newly discovered alfalfa SPLs into eight clades, suggesting possible functions related to known SPLs within the same clades. SPL1 was identified to be in the same clade as SPL12, known to regulate thermotolerance in *Arabidopsis* (Feyissa et al. 2021; Chao et al., 2017). SPL7, SPL8 and SPL13 were also identified, all of which were downregulated under flooding stress (Feyissa et al., 2021). SPL7 was found to be closely related to SPL2, SPL3, and SPL4 in alfalfa, as well as SPL3, SPL4, and SPL5 in *Arabidopsis*, suggesting that all of these SPLs potentially play roles in regulating response to flood stress in many plant species (Feyissa et al., 2021).

As suggested by the phylogenetic tree constructed by Feyissa et al. (2019), SPLs play roles in response to several forms of stress as well as development in both *Arabidopsis* and alfalfa, but SPLs have been identified in many other plant species. For example, in rice the *SPL14-miR156* module was shown to govern branching in tillers and panicles (Jiao et al., 2010; Miura et al., 2010). SPL4 and SPL5 accelerate flowering in *Arabidopsis* (Wu & Poethig, 2006), and overexpression of *SPL9* increased leaf size while reducing the rate of leaf initiation (Usami et al., 2009), whereas silencing of *SPL9* resulted in increased trichome density. SPL4 influences the duration of the vegetative state and flowering time of *Lotus japonicus* and also affects branching in tomato and maize (Wu and Poethig, 2006; Wang et al., 2015; Chuck et al., 2011). SPLs are also critical regulators of the stress response. Silencing of SPL13 by miR156 in alfalfa resulted in changes in root architecture, including an increase in water uptake (Feyissa et al., 2019). In this context, it is important to note the work of Osmont et al. (2007) who demonstrated that changes in root architecture are vital to the plants' ability to respond to drought stress by reducing water loss and allowing it to acquire water and nutrients from deeper in the soil (as well as to the plants' ability to remain stable in waterlogged ground during times of flood stress).

SPL9-silenced alfalfa plants showed a decrease in senescence under drought, with a higher accumulation of anthocyanins (Hanly et al., 2020). SPL9 and SPL15 were both shown to increase leaf size and reduce the rate of leaf initiation in *Arabidopsis* (Usami et al., 2009). SPL3, SPL9 and SPL10 have been identified as regulators of lateral root growth in Arabidopsis (Yu et al., 2010). In *Medicago truncatula,* SPL8 overexpression caused a reduction in shoot branching and biomass yield (Gou et al., 2018). With regards to trichome development, silencing of *SPL3, SPL9, SPL10, SPL11* and *SPL12* in *Arabidopsis* resulted in greater trichome density, but overexpression of *SPL*

genes caused the opposite effect (Yu et al., 2010). As previously shown by Feyissa et al. (2021), SPL4 is in the same clade as SPL3, indicating that it too may play a role in trichome development.

In *Arabidopsis*, a mutant with hyper-induced *miR156* expression was shown to enhance trichome development on cauline stems and leaves (Wei et al., 2012). A negative feedback interaction between *miR156* and SPL15 was identified, where increased *miR156* expression resulted in reduced *SPL15* expression and a subsequent increase in trichome density (Wei et al., 2012). SPL4, while not in the same phylogenetic clade as SPL15 (Feyissa et al., 2021), is negatively regulated by *miR156* (Gao et al., 2016), and prior preliminary observations have suggested that silencing of *SPL4* in alfalfa resulted in increased trichome density in alfalfa (Hannoufa lab, unpublished). These findings suggest that SPL4 likely plays a role in regulating trichome development in alfalfa and potentially other plants.

SnRK2, a protein kinase, regulates ABA, a plant hormone responsible for many plant responses to stress, including promoting root growth and initiating stomatal closure (Spollen et al., 2000; Zhang & Davies, 1989; Umezawa et al., 2009), aiding in the plants ability to uptake and retain water while subjected to drought stress. Feyissa et al. (2021) found that upon *miRNA156* overexpression (*miR156*OE), *SnRK2* transcripts were significantly increased, and that inactivation of SnRK2 kinases caused a decrease in *miR156* biogenesis under stress conditions. A proposed model was put forward where SnRK2 positively regulates *miR156*, which itself negatively regulates many SPLs, including SPL4 (Feyissa et al., 2021). ABA also is involved in the regulation of trichome development (Shi et al., 2018). Its close link with SnRK2, and subsequently the SnRK2 protein kinase family's regulation of *miR156*/SPL4 (Feyissa et al., 2021), indicates that SPL4 may be involved in both drought tolerance and trichome development.

After considering prior research, the role of SPL4 in drought tolerance and trichome development was investigated using the hypothesis that silencing *SPL4* would impact trichome development and drought tolerance in alfalfa. Over the course of this study, it was found that *SPL4RNAi* alfalfa conferred an improved tolerance to drought stress, exhibiting greater root lengths, as well as an increased leaf LWC, LWP, chlorophyll content and stomatal conductance relative to WT controls. Reduced *SPL4* expression was also shown to result in an increase in trichome density.

4.2 Impact of *SPL4* silencing on alfalfa morphology

miR156 functions in part by silencing *SPL4*, as *miR156*OE has been shown to cause reduced expression of *SPL4* in alfalfa (Gao et al., 2016). *miR156*OE alfalfa has also shown increased shoot and root biomass under drought conditions (Arshad et al., 2017), and so similar phenotypes were expected for *SPL4RNAi* alfalfa. In this study, however, reduced levels of *SPL4* did not result in many phenotypic changes to plant architecture. Plant height was unaffected in the three *SPL4RNAi* lines under drought conditions when compared to WT. Similarly, both shoot and root biomass were unaffected by lowered *SPL4* transcript levels under drought conditions and were also indistinguishable from WT. Since increased *miR156* expression has been shown to cause phenotypic changes in shoot length and biomass (Gao et al., 2016), *SPL4* may not be sufficient on its own to cause changes to shoot architecture, and other regulators may be required to cause a change in phenotype. Interestingly, root length was increased in *SPL4RNAi-9* and *SPL4RNAi-14*, but not in *SPL4RNAi-7*. This result suggests that SPL4 can affect root architecture without the influence of other regulators, and that a certain threshold of *SPL4* transcript level must be achieved before phenotypic effects are observed (as *SPL4RNAi-7* exhibited higher *SPL4* transcript levels

relative to *SPL4RNAi-9* and *SPL4RNAi-14).* Root length may perhaps be affected only when downregulation of *SPL4* reaches a certain level, and the higher *SPL4* transcripts of *SPL4RNAi-7* may not have crossed the threshold required to significantly affect root length. This finding is similar to that of Feyissa et al. (2019) and Hanly et al. (2020), where *SPL13* and *SPL9* both showed dose-dependent effects. In *SPL13RNAi* plants, *SPL13* mRNA transcript expression levels below a certain threshold showed significant drought tolerance compared to higher expression levels (Feyissa et al., 2019), and similarly in plants with a high level of *SPL9* silencing, it was found that drought tolerance was only affected in plants where *SPL9* expression remained below a threshold (Hanly et al., 2020).

4.3 *SPL4* impact on alfalfa water status

To confirm if the silencing of *SPL4* in alfalfa plays a role in drought response, the ability of *SPL4RNAi* plants to resist the deleterious effects of drought was evaluated. A qualitative examination appeared to show that both WT and *SPL4RNAi* plants were affected by drought, but *SPL4RNAi* plants were less chlorotic than WT under drought conditions.

First, LWC was measured in leaves of WT and *SPL4RNAi* plants exposed to drought. *SPL4RNAi* plants had significantly less reduction in LWC than WT under drought, providing evidence that *SPL4* silenced plants were more tolerant to drought stress than WT. This observation is in line with that of Arshad et al. (2017) who found that overexpressing *miR156* caused significantly higher LWC when compared to WT under drought conditions, as well as under well-watered conditions.

Second, LWP was measured in leaves of WT and *SPL4RNAi* plants under drought and well-watered conditions. Once again, there was no change in LWP under well-watered conditions, but there was a significant increase in LWP in WT when compared to *SPL4RNAi* plants, revealing that more pressure was required to force water out of WT than *SPL4RNAi* leaves under drought conditions. This finding is consistent with work showing that *SPL13RNAi* alfalfa plants had higher LWP than WT alfalfa under drought conditions (Feyissa et al., 2019). This result, combined with the increase in LWP, suggests that there was more water present in *SPL4*-silenced alfalfa plants than in WT when exposed to drought stress.

4.4 *SPL4* regulates response to drought in alfalfa

An indicator of overall plant health under stress is the level of chlorophyll in green tissues since photosynthesis is reduced under stress conditions (Schulze, 1986; Rizhsky et al., 2002). Chlorophyll A and B levels in WT and *SPL4RNAi* leaves were quantified, and total chlorophyll levels were calculated. In all cases, there was a significant increase in chlorophyll concentration in *SPL4RNAi* plants when compared to WT. This result is consistent with the observation that *SPL4RNAi* plants were greener than WT plants when subjected to drought stress, indicating that the *SPL4*-silenced plants were much healthier under drought conditions, and can endure drought stress with less difficulty than WT.

Stomatal conductance, another indicator of plant health, measures the movement of water vapor out of stomata (Levitt, 1980). When stomatal conductance was measured, no significant difference was found when comparing WT and *SPL4RNAi* alfalfa under well-watered conditions, but there was a significant increase in stomatal conductance in *SPL4RNAi* alfalfa compared to WT under drought. This result was surprising, as plants that performed better under drought conditions were expected to allow less water vapor out of their leaves, to better conserve water content. An

explanation for this phenomenon is that *SPL4RNAi* plants are more efficient at conserving water than WT plants, allowing the *SPL4*-silenced plants to keep stomata open for gaseous exchange. This result matches that found by Arshad et al. (2017) where *miR156*OE plants also had greater stomatal conductances than WT, and it was argued that since WT plants lost water at a faster rate, they desiccated quicker and as a result had lower stomatal conductances than *miR156*OE plants.

4.5 *SPL4* and its role in trichome development

Based on preliminary observations, an increased trichome density was noticed in *SPL4RNAi* when compared to WT plants (Hannoufa lab, unpublished). Therefore, I conducted a more thorough quantitative and qualitative analysis of trichomes in *SPL4RNAi* plants. A significantly higher trichome density was detected in leaves of *SPL4RNAi* when compared to WT leaves under both conditions. This result is consistent with findings in the literature, where *miR156* overexpression in *Arabidopsis* resulted in significantly increased trichome density on leaves and stems (Wei et al., 2012). This result also confirms the previous observations from the Hannoufa lab and is evidence that *SPL4* plays a role in trichome development in alfalfa.

With the intent of shedding some light on how SPL4 affects trichome development at the molecular level, the transcript levels of *GL1* and *GL3*, two genes associated with trichome development in plants (Gruber et al., 2006), were determined in *SPL4RNAi* plants. Both *GL1* and *GL3* showed significant higher transcript levels when compared to WT. This finding provides an explanation as to why *SPL4RNAi* plants have a higher trichome density when compared to WT, and suggests that SPL4 plays a role in regulating *GL1* and *GL3* expression in alfalfa.

4.6 *SPL4* and its effect on expression of stress-related genes

Expression of stress-related genes was determined in WT and *SPL4RNAi* alfalfa under control conditions. *SPL9* and *SPL13*, members of the SPL transcription factor family, are both involved in regulating response to drought stress in alfalfa (Aung et al., 2015; Gao et al., 2016). Interestingly, their transcript levels were significantly lowered in *SPL4RNAi* plants compared to WT (except for *SPL4RNAi-9*, which showed no significant difference in *SPL13* expression). This result suggests SPL4 may play a role in regulating the expression of *SPL9* and *SPL13*, both genes associated with drought tolerance, a result supported by phenotypic findings showing that SPL4 functions to regulate biological processes associated with drought stress. *CAT*, an antioxidant biosynthesis gene (Choudhury et al., 2013), also had higher transcript levels in *SPL4RNAi* plants relative to WT, suggesting that SPL4 plays a role in regulating antioxidant biosynthesis.

4.7 Conclusions

Abiotic stress causes huge losses in crop yield each year, and compounding this issue is a growing population, increasing temperatures and recurring drought worldwide (Goujon et al., 2018; Boyer et al., 1982; Mukherjee, Mishra & Trenberth, 2018). To deal with these issues, varieties of alfalfa and other important crops will need to be developed to increase production without relying on increases in land usage to overcome future environmental challenges, and feed a growing population (Godfray et al., 2010; Tilman et al., 2011). *miR156* has previously been shown to be a powerful tool in increasing alfalfa forage yield in the face of abiotic stressors (Aung et al., 2015, Arshad et al., 2017). Furthermore, its regulation of the SPL transcription factor family will need to be better understood in order to efficiently utilize it as a potential regulator of stress in crops (Feyissa et al., 2019).

SPL9 and SPL13 have previously been shown to play roles in stress response (Hanly et al., 2020; Feyissa et al., 2019), but the roles of many of the SPLs in plant development and stress tolerance are still elusive. Since *SPL4RNAi* plants exhibited increased root lengths in response to drought conditions, it can be concluded that SPL4 plays a role in root growth in alfalfa. In addition, SPL4 affects alfalfa's ability to retain water under drought stress, as *SPL4RNAi* plants exhibited an increased LWC and decreased LWP under drought conditions. *miR156*OE plants had similar traits according to Arshad et al. (2017) and Feyissa et al. (2019), which confirms that SPL4 plays a role in regulating water retention in alfalfa under drought stress. This is further supported by *SPL4RNAi* plants possessing increased chlorophyll concentrations and stomatal conductance when compared to WT alfalfa. Expression levels of genes known to be involved in drought stress, *SPL9* and *SPL13*, and *CAT*, a known antioxidant biosynthesis gene, were found to be altered when *SPL4* is silenced. This also supports the conclusion that SPL4 regulates how alfalfa responds to drought stress. The finding that *SPL4RNAi-7* did not show increased root length leads to the conclusion that in some cases the effect of SPL4 may be dose-dependent. Finally, prior observations were confirmed when SPL4 was shown to increase trichome density and cause increased expression of *GL1* and *GL3* genes in alfalfa, regardless of whether or not alfalfa plants were subject to drought stress.

CHAPTER 5

5 FUTURE DIRECTIONS

While this study focused on drought stress to the exclusion of other abiotic stressors, it should be noted that *miR156* affects alfalfa's responses to other stressors, including flooding, salinity and heat (Feyissa et al., 2021; Arshad et al., 2017; Matthews et al., 2019). Since SPL4 is regulated by *miR156* (Gao et al., 2016), it may also play a role in response to other stress factors. Additionally, further exploration into the mechanism by which SPL4 regulates drought stress responses such as water retention should be examined further. Similarly, this study focused on whether SPL4 affected trichome development in alfalfa, having shown that it affects trichome density and the expression of *GL1* and *GL3*. *miR156* has been previously shown to affect trichome distribution in *Arabidopsis thaliana* (Yu et al., 2010; Wei et al., 2012). Having confirmed that SPL4 affects trichomes, and with prior research showing a link between *miR156* and trichome development, further research into other members of the SPL family and their involvement in trichome development and regulation of trichome genes is warranted. Potential candidates include other SPLs from the same clade as SPL4 as identified by Feyissa et al. (2021), namely SPL2, SLP3, and SPL7.

In addition, a Y1H assay should be conducted to definitevely determine whether SPL4 regulates *GL3* directly. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) could also be used to identify all genes SPL4 directly regulates through DNA binding. ChIP-seq aggregates large amounts of DNA, amplifying specific DNA-protein complexes using an antibody specific to a protein of interest (Johnson et al., 2007). Parallel DNA sequencing then allows precise identification of global binding sites for the protein of interest (Johnson et al., 2007). Finally, a study could be conducted to determine if SPL4 regulates drought tolerance and/or trichome density

in organisms other than alfalfa. *miR156*/SPL has been shown to affect plant development and stress tolerances in model organisms such as *Arabidopsis* and *Lotus japonicus* (Wei et al., 2012; Wu & Poethig, 2006), as well as other crops such as tomato, canola, rice and maize (Wang et al., 2015; Jiao et al., 2010; Weit et al., 2012; Chuck et al., 2011), and it is possible that SPL4 will play a similar role in these organisms as it does 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
MsSPL4-F	TTGCACCTGCCGTACACATTGCA	qPCR SPL4 Transcript Level and Y1H Prey Protein Synthesis
MsSPL4-R	AACTGCAATGTGTACGGCAGGTG	qPCR SPL4 Transcript Level and Y1H Prey Protein Synthesis
SPL4RNAi-F	CACCATGGAGACAAGAAGGTCAGAGGGA	SPL4RNAi Construct Cloning
SPL4RNAi-R	GCTTTGGCATGATACTCACAGAC	SPL4RNAi Construct Cloning
GL1-F	TGCTTTAATTAAGACTCCCATC	qPCR GL1 Transcript level
GL1-R	AATTTACACTTGTTTTGGAC	qPCR GL1 Transcript Level
GL3-F	GTATTGGAAGATCTCAGTCTCAT	qPCR GL3 Transcript Level
GL3-R	CCTTACTATTTGAAGGAACCATT	qPCR GL3 Transcript Level
SPL9-F	TTATTCTTTTTCAAGTCCATTTT	qPCR SPL9 Transcript Level
SPL9-R	AATTAACAACACTAGTCTCT	qPCR SPL9 Transcript Level
SPL13-F	TATGAATGACTATGATAGTAAGT	qPCR SPL13 Transcript Level
SPL13-R	CTTACCTTGGTAATTGGACAAAA	qPCR SPL13 Transcript Level
CAT-F	AGGGTAACTTTGACCTTGTTGGA	qPCR CAT Transcript Level
CAT-R	TAAATTACCTCTTCATCCCTGTG	qPCR CAT Transcript Level

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