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Characterizing the Role of the miR156-SPL Network in Heat Stress Response in Medicago sativa

Craig Matthews, *The University of Western Ontario*

Supervisor: Hannoufa, Abdelali, *The University of Western Ontario*
Co-Supervisor: Macfie, Sheila, *The University of Western Ontario*
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Abstract

Heat stress negatively impacts plant development by disrupting regular plant functions, including molecular, physiological and anatomical processes, reducing crop production. Alfalfa (*Medicago sativa*) is an important forage crop and developing abiotic stress resistant cultivars would help mitigate crop losses. Members of the *miR156* family regulate *SPL* genes, impacting plant growth and development and are involved in stress response. Here, alfalfa with *miR156* overexpression (*miR156OE*) and *SPL13* RNAi knockdown (*SPL13i*) showed increased tolerance to heat stress (40°C) while *SPL9* RNAi alfalfa did not. Heat-stressed *miR156OE* and *SPL13i* plants had increased antioxidant levels, including anthocyanins. Additionally, genes associated with *miR156* involved in hormone and antioxidant biosynthesis were differentially regulated under heat stress in transgenic alfalfa. These results demonstrate that *miR156* contributes to heat stress tolerance in alfalfa at least partially by silencing *SPL13* and suggest *SPL13* could be useful for improving abiotic stress tolerance in alfalfa and potentially other crops.

Keywords

miR156, *SPL* genes, abiotic stress, heat stress, antioxidants, alfalfa

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List of Abbreviations

A	absorbance
ABA	abscisic acid
AGO	argonaute
AGP	*ADP GLUCOSE PYROPHOSPHORYLASE*
ANOVA	Analysis of variance
ATPC1	*ATP SYNTHASE GAMMA CHAIN1*
APX	ascorbate peroxidase
AP2	*APETELA2*
BHT	butylated hydroxy toluene
bZIP	basic leucine zipper
CAD	Canadian dollars
CAT	catalase
CBC	cap-binding complex
CCD	carotenoid cleavage dioxygenase
CCD1	*CAROTENOID CLEAVAGE DIOXYGENASE 1*
cDNA	complementary DNA
CI	confidence interval
CSD	Cu/Zn SOD

DCL	dicer-like
DCL1	dicer-like 1
DDL	dawdle
DF	dilution factor
DFR	dihydroflavanol 4-reductase
DNA	deoxyribonucleic acid
ds	double stranded
DW	dry weight
EIF4A	*ELONGATION INITIATION FACTOR 4A*
ETC	electron transport chain
eTM	endogenous target mimic
ER	endoplasmic reticulum
EV	empty vector
FLS	flavonol synthase
FT	*FLOWERING LOCUS T*
FW	fresh weight
GPP	gross primary product
GPX	glutathione peroxidase
GR	glutathione reductase

HAG1	*HISTONE ACETYLTRANSFERASE OF THE GNAT FAMILY 1*
HAT	histone acetyltransferase
HEN1	HUA enhancer 1
HESO1	HEN1 suppressor 1
HPLC	High Performance Liquid Chromatography
HPLC-DAD	HPLC with diode array detection
hr	hour
HSF	heat shock transcription factor
HSP	heat shock protein
HST	hasty
HYL1	hyponasty leaves 1
LHCB	*CHLOROPHYLL A/B-BINDING PROTEIN*
lncRNA	long non-coding RNA
LWP	leaf water potential
MeOH	methanol
min	minute
MIR	miRNA gene
miRNA	microRNA
miRNA*	microRNA antisense passenger strand

miR156	microRNA156
miR156OE	microRNA156 overexpression
MRE	microRNA responsive elements
MTBE	methy tert-butyl ether
MW	molecular weight
MYB	*MYELOBLASTOSIS*
m^7G	7-methylguanosine
NCED3	*NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3*
ncRNA	non-coding RNA
NPP	net primary product
OEC23	*PSII OXYGEN EVOLVING COMPLEX23*
PA	peak area
PAP1	production of anthocyanin pigment 1
PCR	Polymerase Chain Reaction
pl	path length
PPL1	*PSBP-LIKE PROTEIN1*
Pri-miRNA	primary miRNA
Pre-miRNA	precursor miRNA
PSBD	*PSII D2 PROTEIN*
PSBF	*CYTOCHROMEB559*

PSBK	*PHOTOSYSTEM II REACTION CENTER PROTEIN K*
PSBQ	*OXYGEN EVOLVING ENHANCER3*
PSI	photosystem I
PSII	photosystem II
PTGS	post-transcriptional gene silencing
P5CR	*PYRROLINE-5-CARBOXYLATE REDUCTASE*
qRT-PCR	Quantitative Reverse Transcription PCR
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA intereference
ROS	reactive oxygen species
RT	reverse transcription
Rubisco	ribulose-1,5-bisphophosphate carboxylase/oxygenase
RWC	relative water content
s	second
SBP	Squamosa promoter binding protein
SD	standard deviation
SDN	*SMALL RNA DEGRADING NUCLEASE*
se	serrate
sHSP	small HSP

siRNA	small interfering RNA
SOC1	*SUPPRESSOR OF CONSTANS1*
SOD	superoxide dismutase
SPL	*SQUAMOSA PROMOTOR BINDING PROTEIN-LIKE*
SPL13i	SPL13 RNAi
SPL9i	SPL9 RNAi
SPS	*SUCROSE PHOSPHATE SYNTHASE*
ss	single stranded
TEAC	trolox equivalent antioxidant capacity
TFC	total flavonoid content
TGA1	*TEOSINTE GLUME ARCHITECTURE1*
TGA3	*TGA1A-RELATED GENE3*
TMA	total monomeric anthocyanin
TPC	total phenolic content
TW	turgid weight
USD	U.S. dollars
w/v	weight/volume
WT	wildtype

Chapter 1

1 Introduction

1.1 The importance of alfalfa

Medicago sativa (alfalfa) is a forage and fodder crop widely grown in Canada and around the world (Annicchiarico et al., 2015); plants in the genus *Medicago* are commonly referred to as medics. Alfalfa is one of the most important forage crops and the most widely cultivated forage legume, grown on an estimated 30 Mha globally (Annicchiarico et al., 2015), and the alfalfa market was valued at $17.5 billion USD in 2017 (NAAIC, 2017). While alfalfa is the most widely grown, various other medics are also used as forage (Small, 2011). For example, barrel medic (*Medicago truncatula*) is a well-studied model legume (Young et al., 2011), but is also grown for forage.

Alfalfa has relatively deep roots with a maximum rooting depth of 3.7 m (Canadell et al., 1996) with 95% of roots in the top 1.36 m (Fan et al., 2016), the deepest of various common temperate crops including wheat, corn, various beans, cereals, and oilseed crops (Fan et al., 2016). Deep-rooting crops have better access to ground water, reducing irrigation requirements, and may help reduce water stress susceptibility (Putnam et al., 2007). Additionally, as is the case with many legumes, alfalfa and other medics form symbiotic relationships with nitrogen-fixing Rhizobia in root nodules, fixing atmospheric nitrogen (N_2) and reducing fertilizer requirements for alfalfa and crops following it in the rotation (Blesh and Drinkwater, 2013; Small, 2011). Alfalfa's associations with these bacteria can fix up to 560 kg/ha/year of nitrogen, replenishing soil for years to come and may help reduce cost and improve energy efficiencies of farms (COPA-COGECA, 2007; Small, 2011). The use of leguminous crops to help replenish soil nitrogen is also a more sustainable method than fertilizer-based strategies alone and is more capable of balancing nitrogen inputs and outputs to reduce fertilizer runoff and nitrogen leaching (Blesh and Drinkwater, 2013).

Medics, including alfalfa, are also of interest for use as cover crops, an important area of research for sustainable agriculture (Gabriel et al., 2012; Small, 2011; Verhallen et al.,

2001). Cover crops limit soil erosion and water runoff, physically interfering with surface runoff and allowing increased water infiltration while adding to the soil organic matter without negatively impacting soil salinity (COPA-COGECA, 2007; Gabriel et al., 2012; Lu et al., 2000; Strock et al., 2004). Cover crops including alfalfa may be utilized as green manure, further increasing soil fertility when ploughed into the soil (Small, 2011), and, in the case of alfalfa and other legumes, after the crop has replenished the soil through its symbiotic relationship with nitrogen-fixing bacteria (Entz et al., 1995).

Alfalfa is a perennial crop and, weather permitting, can grow year round, increasing the potential yield compared to other crops. Additionally, due to alfalfa's relatively strong competition with weed growth and production of allelopathic compounds, its use as a smother crop can inhibit the growth of undesirable weeds and reduce herbicide usage (Small, 2011). Medics may in some cases reduce the yield of crop plants for which it is employed to shield from the effects of weeds (Sheaffer et al., 1991). It may not be advisable to grow alfalfa on the same land for extended periods of time without removing the old stand and reseeding on the same land due to alfalfa's production of autotoxic and allelopathic secondary metabolites (Bagg, 2001; Chon et al., 2002). However, alfalfa's use as a cover crop with corn did not reduce corn yield compared to unweeded corn plots and the total yield of plots with alfalfa used as a cover crop was not greatly reduced compared to fields with just corn that were carefully weeded (Ghosheh et al., 2005). Alfalfa as a cover crop provides a usable crop and also increases the water utilization of the land, probably due to its deep roots (Ghosheh et al., 2005). Alfalfa is recommended for use as a cover crop with corn and cereals by the Ontario government (Verhallen et al., 2001). Due to its relatively high yield and hardy nature, alfalfa is a viable biofuel crop, increasing its potential value as new sustainable energy production protocols and alternative fuels become more economically viable (Sanderson and Adler, 2008).

In addition to its importance worldwide, alfalfa is an agricultural mainstay in Canada, as forage crops had been the most widely grown crop type in Canada for many years before they were recently supplanted by oilseed crops (Statistics Canada, 2011). Alfalfa, colloquially referred to as the king of fodders and queen of the forages (Small, 2011), was grown on over 3.75 Mha on over 60,000 farms in Canada in 2016 (Statistics Canada,

2018), and can yield up to 49 tonnes/ha of dry alfalfa with irrigation or 20 tonnes/ha without irrigation (Small, 2011). Although alfalfa's yield per ha is lower than those of grasses such as switchgrass (*Panicum vigatum*) and miscanthus (*Miscanthus* spp.) (Aung, 2014), it is the best feed for large livestock, including ruminants and horses (Bauchan and Greene, 2000), due to its nutrient composition and overall well balanced (~20%) protein content (Small, 2011).

Alfalfa is a hardy crop, relatively resistant to drought and heat damage in comparison to many other crops, most likely born out of its heritage from the dry Mediterranean climate (Small, 2011). Heat waves and drought are correlated phenomena and are expected to rise in the near future (Kirtman et al., 2013). Similarly, alfalfa's resistance to drought, heat and cold are thought to be correlated, as physiological characteristics of cultivars with resistance to drought and heat conditions tend to be similar to known cold-tolerant cultivars. These physiological characteristics include smaller leaves, reduced internode length, increased pubescence, and increased biomass allocation to roots (Small, 2011). Cold and winter tolerance (overwintering) is a trait alfalfa has been selectively bred for in order to allow for longer growing seasons and higher yield (Small, 2011), with variable success in part due to variation in winter climates across the areas in which alfalfa is grown (Volenec et al., 2002). Alfalfa breeding has also focused on disease resistance due to its susceptibility to microbial organisms. Once again, breeding has been successful in producing cultivars that have some tolerance, but only locally as susceptibility was observed when cultivars were transferred to other locations (Volenec et al., 2002).

Traditional selective breeding is generally time consuming and labor intensive, and especially so in alfalfa where it is made even more difficult by the plant's obligate outcrossing nature (Lesins and Lesins, 1979) and its tetraploid genome (McCoy and Bingham, 1991), both of which contribute to increasing genetic diversity and difficulty of selective breeding. While traditional selective breeding is difficult in alfalfa, its relative ease of transformation and propagation mean it may be more suitable for genetic improvement using biotechnological approaches rather than classical breeding (Small, 2011). Additionally, the identification of selectable markers associated with abiotic stress tolerance may aid in breeding more resilient and desirable alfalfa cultivars.

1.2 Plant heat stress

Heat stress negatively impacts crop growth and development, ultimately reducing production (Hall, 2001). The negative effects of extreme heat on crop production result in serious economic and humanitarian consequences both globally (Mendelsohn et al., 2000; Nelson et al., 2014; Piao et al., 2010; Rosenzweig et al., 2014; Schlenker and Lobell, 2010) and locally (AAFC, 2015). Global mean temperatures are rising, and extreme temperature events are expected to increase in incidence and severity, particularly in most of Latin America, Southern and Central parts of Europe, and Canada, which may experience severe effects as a result of climate change (Lotze-Campen and Schellnhuber, 2009). Increased temperature will result in increased potential and actual evapotranspiration, further limiting water accessibility (Rind et al., 1990).

Even the most conservative estimates predict an increase in Canada's mean annual temperatures coast to coast (NRC, 2008). Changes in mean temperatures depend on many factors and are not uniform across Canada; however, even conservative estimates predict increases between 3 and 8°C across Canada by the 2080s compared to mean temperatures from the last half of the 20th century (Bush et al., 2014). Climate change is generally expected to have more dramatic effects at higher latitudes (Trenbeth et al., 2007). Canada, therefore, can expect dramatic increases in temperature, especially in northern and Arctic Canada. Projections suggest northern Canada will have the largest increases in winter temperatures while southern Canadian summers will have the greatest increase (Bush et al., 2014).

These expected increases will have some positive and negative consequences on Canadian agriculture, including longer growing seasons and increased arable land, but also increased crop loss due to extreme weather events and increased biotic stresses and invasive species (Campbell et al., 2014). While it is possible the current predictions for climate change in the near future will increase the potential opportunities for agricultural production by decreasing the length of the frost season (AAFC, 2015), with these possible beneficial changes comes increased incidence of heat waves (Deryng et al., 2014; Donat et al., 2013; Horton et al., 2015; Kirtman et al., 2013; Teixeira et al., 2013),

negatively affecting crop yield (Lesk et al., 2016; Lobell and Gourdji, 2012; Lobell et al., 2013; Lobell et al., 2012; Moriondo et al., 2010; Moriondo et al., 2011) and endangering livestock and livestock productivity (Nardone et al., 2010). Additionally, with the expected increase in mean temperatures comes increased variability in climate. Increasing climate variability will make it difficult to accurately choose crops better suited to expected weather patterns (Campbell et al., 2014). Developing hardier crops to withstand the expected increase in mean temperatures and variability in conditions will better serve Canadian farmers. The losses due to extreme heat are expected to be compounded by the increased occurrence of the total number of extreme weather events and their coincidence in the near future (Lesk et al., 2016).

Heat waves and drought in Europe in 2003 provides a recent example of the global effects of extreme temperatures on agricultural yield, during which European temperature records were broken (WMO, 2004). This extreme weather event affected nearly the entire continent, with different countries affected by drought, extreme heat, or a combination of the two. This allows for a unique opportunity to isolate and investigate the effects on crop production in countries unaffected by drought and look at the particular effects of heat waves. Italy and parts of France had increases in mean temperatures around 2ºC but were unaffected by drought conditions and each suffered significant decreases in crop net primary product (NPP) compared to the previous five years (Ciais et al., 2005). Reduction in gross primary product (GPP) was also negatively correlated with increased temperatures (Ciais et al., 2005). Locally, Canada was also affected by a heat wave and drought in 2002 resulting in a reduction of approximately $2 billion CAD in agricultural production (AAFC, 2013). The impact of climate change (including heat waves) on an already precarious food security situation and increasing population (Porter et al., 2014; Schmidhuber and Tubiello, 2007) could lead to a humanitarian disaster. Reduced crop production due to extreme weather events would further exacerbate what is already an existing crisis (FAO; WFP; EU, 2018). The impact of heat stress on crop production would also extend to fodder stores, limiting livestock and dairy production, resulting in scarcity of meat and dairy food products (AAFC, 2015; Nardone et al., 2010).

Heat stress increases evapotranspiration and leads to a decrease in water availability (Rind et al., 1990), and also adversely affects a suite of developmental and physiological processes in plants (Hasanuzzaman et al., 2013). Elevated temperatures affect cellular function through protein misfolding and denaturation (Bernstam, 1978; Wu and Wallner, 1985) and increased membrane fluidity, requiring changes to the saturation of phospholipids in cellular and organellar membranes (Zheng et al., 2011). These adverse effects result not only in losses in crop production (Chen et al., 1982), but also reduced nutritional quality of the crop (Undersander et al., 1993). To reduce solar warming under high heat, plants, including alfalfa (Reed and Travis, 1987), undertake paraheliotropic leaf movements to reduce the surface area of the leaf that is exposed to the sun (Gamon and Pearcy, 1989). Additionally, plants increase transpiration under heat stress to control leaf temperature by evaporative cooling, which can significantly reduce leaf temperatures up to 9°C (Urban et al., 2017). Complicating this, drought and heat stress often coincide, resulting in reduced stomatal conductance to conserve water, restricting the plant's ability to use transpiration and evaporative cooling as stress avoidance mechanisms (Zandalinas et al., 2018).

Extreme temperatures also lead to oxidative damage through an increase in the production of reactive oxygen species (ROS), which can result in cellular damage at high concentrations. ROS production leads to damage to nucleic acids (Jena, 2012; Kong and Lin, 2010), proteins (Costa et al., 2007), and cell membranes (Stark, 2005). Cell membranes and membrane-bound organelles may be damaged directly through lipid peroxidation or by oxidative damage to membrane-bound proteins, both resulting in a loss of cellular function (Stark, 2005). Products of lipid peroxidation are also highly reactive and may further damage nucleic acids or proteins (Møller and Wallin, 1998). Oxidative damage at the cellular level manifests itself physiologically, disrupting plant growth and development (Cheikh and Jones, 1994). ROS damage adversely affects chloroplast function and morphology (Xing et al., 2013) and leads to a decrease in chlorophyll content and photosynthetic capacity, resulting in chlorosis (Briantais et al., 1996; Larkindale and Knight, 2002; Liu and Huang, 2000). ROS are utilized by plant cells as signalling molecules to respond to stress; ROS induce changes to deal with stress

conditions at lower levels while high ROS concentrations induce developmental delays, apoptosis or may damage cells enough to cause necrosis (Petrov et al., 2015).

In response to heat stress, plants regulate genes associated with photosynthesis, carbon fixation, nutrient metabolism, osmolyte biosynthesis, antioxidant production and increase the expression of genes encoding heat shock proteins (HSPs) (Hu et al., 2014; Sailaja et al., 2014). HSPs are broadly categorized based on size and, while originally discovered in relation to heat stress (Ritossa, 1962), are responsive to various stresses (Park and Seo, 2015). The five HSP classes based on molecular weight are HSP100, HSP 90, HSP70, HSP40 and small HSPs (sHSPs). HSPs generally function as chaperones in preventing misfolding and denaturation, staving off cellular apoptosis due to stress-related damage (Al-Whaibi, 2011). HSPs may function individually or by forming complexes with other HSPs of the same or different class, or with other types of chaperones (Al-Whaibi, 2011; Park and Seo, 2015). sHSPs form oligomers and bind to misfolded proteins, preventing further misfolding and protein aggregation (Sun and MacRae, 2005), and assist in the removal of denatured proteins by making the protein complexes insoluble (Jiao et al., 2005). Of all HSPS, sHSPs are the most abundant in plants (Wang et al., 2004).

Heat shock transcription factors (HSFs) are generally responsible for inducing *HSP* expression and the expression of other heat stress-associated genes in plants (Nover et al., 2001). *HSF* gene expression is broadly associated with heat stress tolerance; increased *HSF* expression increases thermotolerance and vice versa (Mishra et al., 2002; Schramm et al., 2008). Osmolytes accumulate in response to heat stress to help regulate water relations and maintain cellular turgor (Wahid et al., 2007) and are associated with HSF-mediated heat stress response (Sailaja et al., 2014). The upregulation of proline biosynthesis genes, such as *PYRROLINE-5-CARBOXYLATE REDUCTASE* (*P5CR*), are associated with thermotolerance (De Ronde et al., 2004). In addition to regulating cellular turgor, osmolytes including proline help maintain mitochondrial function (Hamilton and Heckathorn, 2001) and scavenge ROS (Trovato et al., 2008).

Oxidative damage is induced under heat stress conditions (Allakhverdiev et al., 2008; Mathur et al., 2011), and plants employ both enzymatic and non-enzymatic antioxidants

to scavenge ROS and reduce oxidative damage. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) (Ahmad et al., 2010). Non-enzymatic antioxidants include tocopherols, ascorbic acid, glutathione, carotenoids and phenolics (Ahmad et al., 2010). Carotenoids are pigments important to chloroplast function and accumulate in the chloroplast. In addition to their antioxidant properties, they assist in light harvesting for use in photosynthesis (Young, 1991) and are hormone precursors (Al-Babili and Bouwmeester, 2015; Nambara and Marion-Poll, 2005). The most important function of carotenoids is as antioxidants that scavenge the ROS produced naturally through photosynthesis, such as singlet oxygen and hydroxyl radicals (Young, 1991). Carotenoids are found bound to chloroplast membranes and may protect membranes from lipid peroxidation by scavenging ROS produced *in situ* (Frank and Cogdell, 1993). Among the diverse non-enzymatic phenolic antioxidants, anthocyanins are a particularly strong antioxidant pigment (Agati et al., 2012; Ferreres et al., 2011; Nakabayashi et al., 2014) and are products of the phenylpropanoid pathway (Shi and Xie, 2014). Anthocyanins are synthesized at the endoplasmic reticulum (ER) and are stored in the vacuoles (Zhao and Dixon, 2010). Anthocyanins protect chloroplasts from excess light by absorbing some of the energy, alleviating the negative effects of extreme excitation on photosystem II (PSII) (Agati et al., 2013; Close and Beadle, 2003). By throttling the energy inputs to PSII, anthocyanins and other flavonoids can help reduce the excess production of ROS (Landi et al., 2015). Of all phenolic antioxidants, anthocyanins show the highest ROS scavenging capacity (Bi et al., 2014). Although anthocyanins are stored remote from regular ROS production, in vacuoles rather than in plastids, hydrogen peroxide (H_2O_2) easily diffuses across membranes, especially under highly stressful conditions (Yamasaki et al., 1997). To this end, it is believed anthocyanins can help regulate ROS signalling in plants in addition to ROS scavenging (Mittler et al., 2004).

Photosynthesis is reduced at the thylakoid and stroma when plants are exposed to high temperatures (Wise et al., 2004), interrupting the function of PSII and the electron transport chain (ETC) (Mathur et al., 2011; Wise et al., 2004). Heat increased the expression of *CHLOROPHYLL A/B-BINDING PROTEIN* (*LHCB*) and many

photosynthesis-associated genes in *Populus simonii*, including *CYTOCHROMEB559* (*PSBF*), *PHOTOSYSTEM II REACTION CENTER PROTEIN K* (*PSBK*), *ATP SYNTHASE GAMMA CHAIN1* (*ATPC1*) and *PSII D2 PROTEIN* (*PSBD*) while decreasing the expression of *PSII OXYGEN EVOLVING COMPLEX23* (*OEC23*), *PSBP-LIKE PROTEIN1* (*PPL1*) and *OXYGEN EVOLVING ENHANCER3* (*PSBQ*) (Song et al., 2014). Additionally, heat stress induced transcription of genes associated with the thylakoid, photosystem I (PSI), PSII and chlorophyll binding in *Brachypodium distachyon* (Chen and Li, 2016). Furthermore, heat destabilizes Rubisco, impairing carbon fixation (Feller et al., 1998). As a response to this, genes encoding enzymes associated with carbon fixation and storage such as *SUCROSE PHOSPHATE SYNTHASE* (*SPS*) (Chaitanya et al., 2001), *ADP GLUCOSE PYROPHOSPHORYLASE* (*AGP*) (Linebarger et al., 2005) and various others are negatively regulated by heat (Sailaja et al., 2014). Moreover, a number of non-coding RNAs (ncRNAs) have also been shown to be differentially regulated by heat stress, including many miRNA family members (Sailaja et al., 2014).

1.3 Plant miRNAs

First discovered in *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993), microRNAs (miRNAs) are small ncRNA molecules that regulate gene expression post-transcriptionally, also known as post-transcriptional gene silencing (PTGS) (Vaucheret et al., 2001), and may also regulate the function of other ncRNAs such as small interfering RNAs (siRNAs) (Manavella et al., 2012) and long non-coding RNAs (lncRNAs) (Hirsch et al., 2006; Zhang et al., 2018). Plant small ncRNAs can generally be grouped into two categories: siRNAs, which are further divided into minor categories, and miRNAs. While siRNAs are derived from dsRNA molecules, miRNAs are derived from hairpin ssRNAs and are usually independent transcription units encoded by miRNA genes (*MIR*) (Allen et al., 2004), but may be transcribed in tandem (Boualem et al., 2008) or intronically (Rajagopalan et al., 2006). miRNA targets are recognized at the target's miRNA response element (MRE), which is highly complementary to the miRNA sequence in plants, and almost always result in cleavage due to the high degree of complementarity, but may also regulate genes without cleavage by physically stalling translation (Millar and

Waterhouse, 2005). miRNAs play a key role in the regulation of transcription factors, as most conserved plant miRNAs target transcription factors (Samad et al., 2017; Sunkar et al., 2005).

miRNAs are transcribed as *MIR* genes by RNA polymerase II (Lee et al., 2004) and may be 5′ capped with 7-methylguanosine (m^7G) (Xie et al., 2005) and poly-A tailed (Lu et al., 2009). These primary miRNAs (pri-miRNAs) contain stem-loop secondary structures to be processed into precursor miRNAs (pre-miRNAs). After transcription and while still in the nucleus, dawdle (DDL) stabilizes pri-miRNAs before being processed (Yu et al., 2008), and helps recruit dicer-like (DCL) ribonuclease (Ren and Yu, 2012). pri-miRNAs are processed into pre-miRNAs by a complex comprised of a DCL ribonuclease (Park et al., 2002; Schauer et al., 2002; Vazquez et al., 2008), a hyponastic leaves 1 (HYL1) dsRNA binding protein (Vazquez et al., 2004), a serrate (SE) zinc-finger (Yang et al., 2006) and the nuclear cap-binding complex (CBC) (Laubinger et al., 2008). While not required, HYL1 and SE aid DCL in pri-miRNA cleavage (Dong et al., 2008). Most miRNAs are 21 nt in length but may range from 19-26 nt and are processed primarily by dicer-like 1 (DCL1) (Guleria et al., 2011; Park et al., 2002; Schauer et al., 2002); however, other DCL paralogs may contribute to miRNA processing, which is thought to be dependent on miRNA product length (Vazquez et al., 2008). Unlike animal miRNAs, the cap and tail as well as the loop are cleaved by DCLs inside the nucleus (Bologna et al., 2013). Pri-miRNA processing takes place at dicing bodies, which are located in the nucleus and are comprised of other DCL interacting proteins (Fang and Spector, 2007; Liu et al., 2012).

After cleavage from pri-miRNA to pre-miRNA, which still contain the loop structure, and pre-miRNA to miRNA/miRNA* duplex, miRNA have 3′ overhangs that are methylated by HUA enhancer 1 (HEN1) to prevent degradation (Yu et al., 2005). Methylated miRNA/miRNA* duplexes are then exported to the cytoplasm by hasty (HST) (Park et al., 2005). Non-methylated pre-miRNAs are targeted for degradation by HEN1 suppressor 1 (HESO1) uridylation and degraded by exoribonuleases encoded by *SMALL RNA DEGRADING NUCLEASE* (*SDN*) genes (Zhao et al., 2012). After export, the miRNA/miRNA* duplex comprised of a methylated miRNA strand and an miRNA*

antisense strand, dissociates and the miRNA strand binds to argonaute (AGO) proteins to form the RNA-induced silencing complex (RISC) (Maunoury and Vaucheret, 2011; Vaucheret et al., 2004) with the assistance of HSP90 (Iki et al., 2012; Iki et al., 2010). The miRNA is then used to target mRNAs and other single stranded RNA molecules for cleavage by RISC (Brodersen et al., 2008; Manavella et al., 2012; Zhang et al., 2018).

In addition to having their expression controlled by miRNAs, lncRNAs may also control the function of miRNAs by having a high degree of complementarity and sequestering the miRNA-RISC complex, preventing it from downregulating other targets (Wu et al., 2013). miRNAs require perfect complementarity from the 9^{th} to 11^{th} nucleotides and some lncRNAs have evolved to have mismatches in these specific sites (Dai and Zhao, 2011; Wu et al., 2013). In this case, lncRNAs bind to the RISC complex but are not cleaved; lncRNAs with this function are known as endogenous target mimics (eTMs) (Dai and Zhao, 2011; Wu et al., 2013; Ye et al., 2014). lncRNAs may also encode pri-miRNAs themselves (Hirsch et al., 2006; Zhang et al., 2018).

Since their discovery, miRNAs have been closely associated with organismal development (Lee et al., 1993; Wienholds and Plasterk, 2005; Wightman et al., 1993). Many plant miRNAs are similarly associated with developmental processes (Li and Zhang, 2016). More recently, many miRNAs associated with stress responses have been investigated. Research has revealed a range of miRNAs that are differentially expressed in response to abiotic and biotic stresses (Sunkar et al., 2012). Some miRNAs appear to be more generally stress-responsive and may be associated with general consequences of stresses, whereas others are more specific and appear only under specific stress-related conditions (Sunkar et al., 2012). For example, decreased *miR398* expression was associated with heat stress response in *Arabidopsis* (Guan et al., 2013), cotton (*Gossypium hirsutum*) (Wang et al., 2016), and wheat (*Triticum aesitivum*) (Kumar et al., 2015). miR398 targets the transcript of Cu/Zn SOD (CSD) antioxidant enzymes *CSD1* and *CSD2*; the downregulation of *miR398* functions indirectly to decrease the cellular damage to plants caused by heat stress conditions via the antioxidant activity of CSDs (Guan et al., 2013). miRNAs regulated under heat stress affect plant development through hormone biosynthesis-associated transcription factors (Kruszka et al., 2014; Meng et al.,

2010; Wang et al., 2012) and other development-associated transcription factors (Barku et al., 2014; Giacomelli et al., 2012; Kruszka et al., 2014; Kumar et al., 2015). Additionally, some miRNAs, including miR529, miR396, miR160 and miR164, directly or indirectly regulate *HSP*s (Guan et al., 2013; Kumar et al., 2015). *miR168* regulates the expression of *AGO1*, which encodes a RISC complex component, and was downregulated in response to heat stress in *Populus trichocarpa* (Lu et al., 2005), suggesting a more global change in the post-transcriptional regulation by siRNAs and miRNAs in response to heat. Among others, *miR156* was upregulated in response to heat in wheat, while *miR172* was downregulated (Xin et al., 2010) and two *miR156* precursors were upregulated in *Brassica rapa* (Yu, X. et al., 2012). In *Arabidopsis*, *miR156* was upregulated in response to heat and was necessary for heat stress memory (Stief et al., 2014). Furthermore, the downregulation of conserved miR156 targets was required for HS memory and resulted in the differential regulation of *sHSP*s among other genes (Stief et al., 2014).

1.4 miR156 and *SPL* genes

miR156 is a highly conserved miRNA, found in all embryophytes (Luo et al., 2013) and its targets, the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, are conserved in all Viridiplantae (Preston and Hileman, 2013). Generally, miR156 downregulates *SPL* genes post-transcriptionally via transcript cleavage or translational repression (He et al., 2018) (Figure 1), but has also shown direct regulation of genes outside the highly conserved *SPL* gene family (Aung et al., 2015c), including a basic leucine zipper (bZIP) transcription factor in corn (Chuck et al., 2007). *miR156* expression is important for juvenile plant development and slows phase transition (Schwarz et al., 2008) while its target *SPL* genes are associated with phase transition and adult development such as flowering and fruiting (Wu and Poethig, 2006).

SPL transcription factors contain the conserved Squamosa promoter binding protein (SBP)-domain, made up of two zinc-finger like motifs (Yamasaki et al., 2004) and regulate genes with the corresponding SBP-binding consensus sequence NNGTACR characterized by the GTAC core (Cardon et al., 1999). Beyond the SBP-domain, SPL

transcription factors can vary greatly in size and function (Cardon et al., 1999). The *miR156/SPL* network contributes to diverse aspects of plant development (Aung et al., 2015c; Nonogaki, 2010). *miR156* and *SPLs* regulate leaf development and shape (Aung et al., 2015a; Aung et al., 2015b; Bhogale et al., 2014; Martin et al., 2010a, b; Shikata et al., 2009; Zhang et al., 2011); shoot maturation and branching (Aung et al., 2015b; Fu et al., 2012; Gao et al., 2018; Schwarz et al., 2008; Shikata et al., 2009; Wang, 2014; Wei et al., 2012); phase transition, flowering and fruiting (Aung et al., 2015a; Aung et al., 2015b; Bhogale et al., 2014; Cui et al., 2014; Fu et al., 2012; Gao et al., 2018; Wang, 2014; Wang et al., 2011; Wu and Poethig, 2006; Xie et al., 2006; Yu, S. et al., 2012; Zhang et al., 2011); trichome development (Aung et al., 2015b; Shikata et al., 2009); and root development and architecture (Aung et al., 2015b; Wang, 2014; Xie et al., 2012; Yan et al., 2013; Yu et al., 2015).

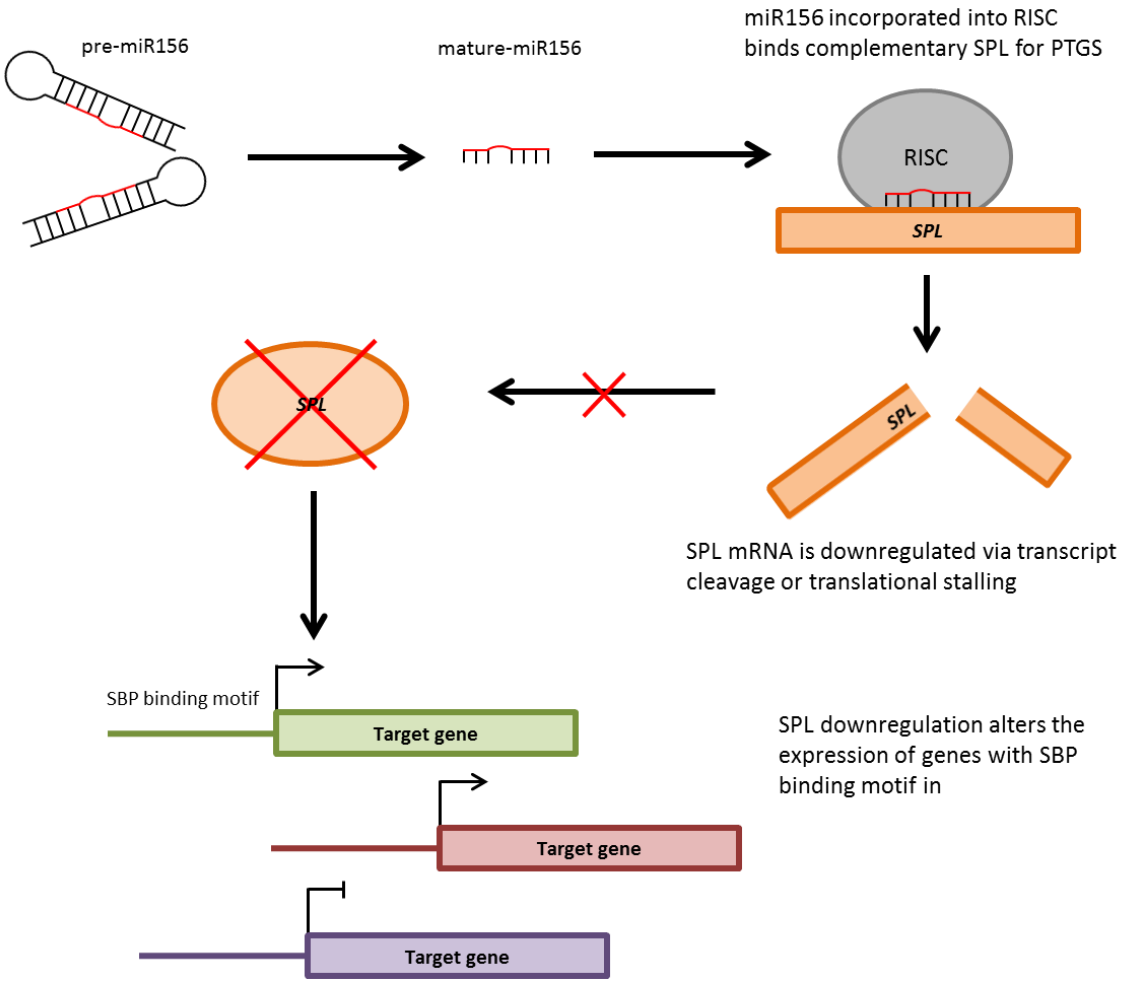

Figure 1. Mode of action of the miR156/SPL gene regulatory network

miR156 and *miR172* are inversely regulated during plant development (Wu et al., 2009), with *SPL9* and *SPL10* upregulating *miR172* as their expression increases during plant development (Wu et al., 2009). While miR156 supresses phase transition, miR172 promotes it (Jung et al., 2007; Lauter et al., 2005; Martin et al., 2010a). Wu et al. (2009) also suggest phase transition influences on *miR156*/*miR172* are stabilized by negative feedback loops mediated by *SPL* genes upregulating *miR172* while downregulating *miR156*.

miR156 is differentially expressed in plants under various biotic and abiotic stresses and is associated with various stress responses (Aung et al., 2015c). *miR156* is responsive to drought (Arshad et al., 2017a; Cui et al., 2014; Liu et al., 2017), salinity (Arshad et al., 2017b; Cui et al., 2014), heat (Kumar et al., 2015; Mangrauthia et al., 2017; Stief et al., 2014; Xin et al., 2010; Yu, X. et al., 2012), metal toxicity (Lima et al., 2011; Zhang et al., 2017), oxidative stress (Jia et al., 2017) and bacterial infection (Padmanabhan et al., 2013).

miR156 expression increased under heat stress in *Arabidopsis* (Stief et al., 2014). Anthocyanin content was downregulated by SPL9 in *Arabidopsis* and increased with increased *miR156* expression (Gou et al., 2011). The increase in anthocyanin content in *Arabidopsis* also contributed to abiotic stress tolerance (Cui et al., 2014), a finding that was also reported for *Oryza sativa* (rice) (Cui et al., 2014). Recent research shows enhanced *miR156* expression (*miR156OE*) and reduced *SPL13* expression (*SPL13i*) led to increased *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) (unpublished data, Hannoufa lab), a gene encoding an enzyme in the phenylpropanoid pathway that produces anthocyanin precursors (Davies et al., 2003). Additionally, Gao et al. (2018) found *SPL13* overexpression altered the expression of R2R3 *MYELOBLASTOSIS* (*MYB)* genes *MYB53* and *MYB112*. Many R2R3 MYB transcription factors are associated with the phenylpropanoid pathway (Borevitz et al., 2000; Gou et al., 2011; Kranz et al., 1998). *miR156* overexpression in *Brassica napus* also increased seed carotenoid content (Wei et al., 2010). The increase in carotenoid content in *miR156* overexpression plants may be due to decreased expression of *CAROTENOID CLEAVAGE DIOXYGENASEs* (*CCDs*)

and decreased carotenoid catabolism as a result, as *CCD8* was downregulated in *SPL13*-silenced alfalfa plants (Gao et al., 2018).

In alfalfa, seven *SPL* genes have been identified as targets of silencing by miR156 via transcript cleavage (Gao et al., 2016). *miR156OE* alfalfa showed increased forage yield and improved quality characteristics (Aung et al., 2015b) as well as increased tolerance to drought and salinity (Arshad et al., 2017a; Arshad et al., 2017b). As drought and water stress tolerance mechanisms and phenotypes are correlated with temperature stress tolerance in medics (Small, 2011), it was hypothesized that *miR156OE* alfalfa may also have improved heat tolerance.

1.5 Proposed Research

miR156 was induced under high temperature in model plants (Stief et al., 2014) and crops (Kumar et al., 2015; Mangrauthia et al., 2017; Xin et al., 2010; Yu, X. et al., 2012). As *miR156* and its *SPL* targets are highly conserved and *miR156OE* alfalfa have shown increased drought tolerance (Arshad et al., 2017a), a stress tolerance that is correlated with temperature stress in *Medicago* spp. (Small, 2011), I proposed that *miR156* expression is enhanced under heat stress in alfalfa while at least some *SPL* genes would show decreased expression. To investigate this hypothesis, wild type (WT) alfalfa plants were exposed to heat stress (40ºC) and the expression of *miR156* and target *SPL* genes was determined by qRT-PCR and compared to those of plants grown under greenhouse conditions. Additionally, I predicted that *miR156OE* alfalfa and alfalfa with silenced expression of one target *SPL* would have increased heat stress tolerance. The alfalfa genotypes were exposed to heat stress and were phenotypically monitored and compared to empty vector (EV) controls to determine differences in heat stress tolerance. Plants were monitored and samples taken after varying length of heat stress to determine physiological, biochemical and molecular differences between *miR156OE* alfalfa and empty vector (EV) controls. Biochemical analyses included the concentrations in leaf tissue of various classes of antioxidants associated with plant stress response. Physiological variables included relative water content, midday leaf water potential, and proline concentrations. The transcript levels of various stress-related genes and genes

previously associated with miR156 were also measured in order to determine differences in gene expression under heat stress in *miR156OE*.

1.6 Hypotheses and Objectives

I hypothesized that because *miR156* regulates *SPL* gene expression in alfalfa and *miR156* is upregulated in response to heat in *Arabidopsis* and other crops, *miR156* would be upregulated and its targets would be downregulated in response to heat stress in alfalfa. Additionally, as increased *miR156* expression and the corresponding downregulation of target *SPL* genes improved heat stress tolerance in *Arabidopsis* and drought tolerance in alfalfa, *miR156OE* would improve heat stress tolerance in alfalfa through the downregulation of some of its target *SPL* genes.

The objectives of this study are:

- Evaluate the transcription of *miR156* and its target *SPL* genes under heat stress conditions in alfalfa
- Determine the effects of *miR156OE* and target *SPL* RNAi knockdown on heat stress tolerance in alfalfa
- Identify physiological, biochemical or molecular characteristics that might contribute to heat tolerance in *miR156OE* and *SPL* RNAi alfalfa
- Conduct phenotypical characterization of *SPL9* RNAi (*SPL9i*) plants

Chapter 2

2 Materials and Methods

2.1 Plant materials

2.1.1 Alfalfa genotypes

Alfalfa clone N4.4.2 (Badhan et al., 2014) was used to produce transgenic genotypes and served as wildtype alfalfa for the investigation of *miR156* and *SPL* genes under heat stress. *miR156OE* (Aung et al., 2015b) and *SPL13i* plants (Arshad et al., 2017a) that were previously developed in the Hannoufa lab, as well as newly developed *SPL9i* plants, were utilized to investigate the role of *miR156* and its target *SPL* genes in heat stress response in alfalfa. *miR156OE* genotypes A8a (66-fold increase in *miR156* transcript abundance), A8 (200-fold increase in *miR156* transcript abundance) and A11 (490-fold increase in *miR156* transcript abundance) were used to investigate the role of *miR156* (Aung et al., 2015b). *SPL13i* genotypes 02 (22-fold decrease in *SPL13* transcript abundance), 05 (5-fold decrease in *SPL13* transcript abundance) and 06 (3.5-fold decrease in *SPL13* transcript abundance) were used to investigate the role of *SPL13* (Arshad et al., 2017a). *SPL9i* genotypes 5A, 5C, 6C and 11 were used to investigate the role of *SPL9*. *miR156OE* and *SPL13RNAi* alfalfa were obtained from previous research in the Hannoufa lab (Arshad et al., 2017a; Aung et al., 2015b) while *SPL9i* plants were developed in the Hannoufa lab according to previously described methods (Arshad et al., 2017a; Aung et al., 2015b; Tian et al., 2002). All transgenic alfalfa plants were generated via *Agrobacterium tumefaciens*-mediated gene transfer (Arshad et al., 2017a; Aung et al., 2015b; Tian et al., 2002). The previously unpublished *SPL9i* transgene construct can be found in Appendix A.

2.1.2 Plant transformation

miR156OE and *SPL13i* plants were previously generated in the Hannoufa lab (Arshad et al., 2017a; Aung et al., 2015b). *SPL9i* plants were generated in a similar manner by Banyar Aung (Hannoufa's lab) and Mimmie Lu (Tian's lab). A 300 bp fragment of the *SPL9* gene was amplified from alfalfa cDNA samples. Primers used for amplification can

be found in Appendix B. The amplified fragments were then cloned into pHELLSGATE12 destination vector (Helliwell and Waterhouse, 2003) using Gateway cloning. The *SPL9i* construct was then transferred to *A. tumefaciens* (GV3101) by electroporation and confirmed by colony PCR.

N4.4.2 alfalfa plants were then transformed by Mimmie Lu (Tian's lab) according to Tian et al. (2002) with slight modifications (Aung, 2014). Trifolia and petiole were excised and pre-cultured in SH2K (Schenk and Hildebrandt, 1972) medium before infection with *A. tumefaciens* in liquid media. Transformed explants were then grown on timentin-supplemented SH2K for callus induction for a further two days before being transferred to kanamycin-supplemented callus induction media. Six weeks after infection, calli were transferred to embryo induction BOi2Y media (Bingham et al., 1975; Tian et al., 2002) with timentin and kanamycin for 6-8 weeks. Embryos were then transferred to germination and development media and allowed to develop. Developed plants were then planted in PRO-MIX BX medium (Premier Horticulture Inc., Quakertown, PA) and covered in plastic domed enclosures for a week before finally being transferred to larger pots and grown under greenhouse conditions.

2.2 Plant growth conditions

Alfalfa genotypes were reared and maintained in light intensity-controlled greenhouse conditions (Table 1) at approximately 21°C with a relative humidity of approximately 80% under a 16/8 hr light/dark photoperiod. To maintain alfalfa genotypes, alfalfa plants were vegetatively propagated using stem cuttings. CleanStart® Oasis© 3.8 cm root cubes (Smithers-Oasis North America, Kent, OH) were used for alfalfa propagation by stem cutting inside domed enclosures. Stem cuttings were made from lengths of stock plant stems with two nodes at developmentally similar stages and were watered approximately once a week depending on water loss from the enclosure. After rooting (3-4 weeks), stem cuttings were potted in PRO-MIX BX medium (Premier Horticulture Inc.) in 14 × 14 cm pots for all experiments except when alfalfa were grown to the flowering stage, where alfalfa plants were potted in 28 cm tall pots with a diameter of 23 cm.

Table 1. Light intensity comparison of greenhouse and growth chamber conditions

Conditions	Daytime light intensity (µmol/m^2/s) (mean ± S.D.)	Maximum (µmol/m^2/s)	Minimum (µmol/m^2/s)	N	t-test
Greenhouse	245.9±37.13	291.2	162.4	10	t = 1.573 *p* = 0.133
Growth Chamber	212.9±50.73	301.4	140.6	10	

2.3 Heat treatment

Plants reared in the greenhouse were transferred to Percival I66VL (Percival, Perry, IA) growth chambers for heat stress treatment at 40°C while maintaining the same photoperiod as the greenhouse with similar light intensity (Table 1) (Li et al., 2013). Before treatment, soil water content was established at 40% using a Fieldscout soil sensor reader (Spectrum Technologies Inc., Aurora, IL) in control and heat-treated pots. While the growth chamber was not humidity-controlled, growth chamber humidity was monitored using a hygrometer and maintained between 60-80%. Treatments with more plants (e.g. survival analysis) had higher humidity than those with fewer plants (e.g. expression analysis). Experimental control plants remained under greenhouse conditions and were subjected to the same watering regimen as heat-treated plants. Pots were rotated randomly to different spots within the growth chamber at 12 hr intervals during treatments to reduce the effects of microclimates. Control plants were similarly rotated on greenhouse benches.

2.4 Sample collection

Penultimate apical trifolia (~90 mg FW) excised from the primary plant stem were taken as leaf samples for RNA extraction and flash frozen in liquid nitrogen in 2.8 mm ceramic bead tubes (Mo Bio Laboratories, Carlsbad, CA) and used for tissue lysis and stored at -80°C. Samples for RNA extractions were taken after 0.5-24 hr of heat stress. Leaf samples for metabolite extractions were flash frozen in liquid nitrogen along with shoot

tissue after the course of the survival analysis experiment. Leaves were separated from shoots while frozen then ground using a mortar and pestle. Aliquots of 50-100 mg of ground leaf tissue were weighed in Eppendorf tubes and stored at -80°C for use in various extractions.

2.5 RNA extractions

2.5.1 Filter-based total RNA isolation

Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer using lysis buffer RLC for tissue lysis due to the relatively high concentrations of polyphenols in alfalfa that solidify when using lysis buffer RLT.

2.5.2 Organic extraction/filter hybrid total RNA isolation for small RNAs

Due to the insufficient retention of small RNAs by simple filter-based RNA isolation methods, a mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) was used to isolate total RNA including small RNAs according to the total RNA isolation protocol (Thermo Fisher Scientific). Plant tissue lysis was conducted with the addition of 0.1 volumes of Plant RNA isolation aid (Thermo Fisher Scientific).

2.5.3 DNase treatment and cDNA synthesis

After isolation, RNA samples were treated with Turbo DNA-*free*™ DNase I (Thermo Fisher Scientific) according to the rigorous DNase treatment protocol, where half of the DNase was added initially followed by the second half after 30 min. The DNase reaction was terminated after one hr. After DNase treatment, RNA integrity was examined on a 1% agarose gel. For the analysis of mRNA transcription, cDNA from 1 µg of RNA template was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) according to the provided protocol. For the analysis of microRNA (miRNA) abundance, cDNA synthesis was carried out with 2 µg of RNA template using the Superscript™ IV (Thermo Fisher Scientific) cDNA synthesis kit utilizing random

hexamer primers in addition to 2 µL of stem-loop 1 µM reverse transcription (RT) primers in a 20 µL reaction (Varkonyi-Gasic et al., 2007).

2.6 qRT-PCR

Relative transcript levels were determined by qRT-PCR in 96 well plates on CFX96 Real Time System/C1000 Touch thermal cyclers (Bio-Rad Laboratories) using Ssofast^{TM} Evagreen® Supermix (Bio-Rad Laboratories). Primer efficiencies were determined using serial dilutions of pooled cDNA from all samples to generate a standard curve to confirm acceptable primer efficiencies (90-110%). Sample cDNA was diluted with milli-Q water to concentrations within acceptable primer efficiencies based on the standard curve. PCR reactions consisted of 5 µL of Ssofast^{TM} EvaGreen® Supermix, 0.5 µM of forward primer, 0.5 µM of reverse primer, 1 µL of diluted cDNA and brought to a total reaction volume of 10 µL using milli-Q water. Annealing temperatures for qRT-PCR were determined using gradient annealing temperatures ranging from 52°C to 64°C. PCR reactions consisted of a 30 s enzyme activation step at 95°C followed by 45 cycles of a 5 s 95°C denaturation step followed by a 15 s 58°C annealing and extension step. Melt curves from 65°C-95°C were used to ensure reaction specificity. Appropriate reference genes for transcription analysis across heat stress and control conditions were investigated by determining the M-values and standard deviations of potential reference genes (Guerriero et al., 2014). Based on reference gene stability, *ELONGATION INITIATION FACTOR 4A* (*EIF4A*) and *CYCLOPHILIN* were used as reference genes for mRNA transcript abundance analysis under heat stress. *U6* small nuclear RNA (*U6 snRNA*) and *CYCLOPHILIN* were used as reference genes for miRNA transcription analysis to include a small RNA reference gene in standardization of small RNA transcription (Shu et al., 2016). All primers used for gene transcription experiments are listed in Appendix B. Normalized transcript abundance of genes was calculated in triplicate using the ΔΔCT method and Bio-Rad CFX Manager (Bio-Rad Laboratories).

2.7 Tissue scoring and survival analysis

Individual plants subjected to heat stress were visually scored on a scale of 1-4 for chlorosis, wilting and scorching at 12-hr intervals over approximately one week (144-192

hr). Plants were scored for heat stress symptoms in a manner adapted from commonly used methods for tracking the progression of plant disease intensity (Bock et al., 2008) and abiotic stress tolerance (Xu et al., 2008). The symptoms corresponding to each score were 1=<25% chlorotic, wilted or scorched tissue; 2=25-50% chlorotic, wilted or scorched tissue; 3=50-75% chlorotic, wilted or scorched tissue; 4=>75% chlorotic, wilted or scorched tissue (Figure 2). After each scoring session, the plants were photographed and after the stress treatment was finished the in vivo plant scores were reviewed and confirmed using the photographs. After heat stress treatment, tissue was collected for biochemical and phenotypic analysis. Survival analysis was visualized using Kaplan-Meier curves constructed using Prism 6 software (GraphPad Software, La Jolla, CA) on plants with chlorotic tissue scores of 3 or greater (Schandry, 2017).

2.8 Trolox equivalent antioxidant capacity (TEAC) assay

Non-enzymatic antioxidants were extracted from 100 mg of ground leaf tissue twice using 1 mL of 1:1 MeOH:H_2O, first by vortexing for 10 min at 500 rpm followed by re-extraction by shaking at 200 rpm overnight at 4°C under dark condition (Li et al., 2010). Pooled supernatants were filtered through 0.45 µm syringe filters and evaporated by vacuum. TEAC was determined using the Sigma-Aldrich antioxidant assay kit (Sigma-Aldrich, St. Louis, MO) according the manufacturer's protocol after reconstituting extracts in 1× Assay buffer and were aliquoted onto transparent 96 well plates. Samples were measured spectrophotometrically using a Multiskan Go plate reader (Thermo Fisher Scientific). TEAC was determined on a per g basis compared to a vitamin E analog Trolox standard curve.

2.9 Carotenoid and chlorophyll quantification using HPLC-DAD

2.9.1 Extraction procedure

Leaf chlorophylls and carotenoids were extracted according to Kormendi et al. (2016) using the unsaponified extraction method to retain leaf chlorophylls. Chlorophyll and carotenoids were extracted thrice from 50 mg of ground leaf tissue using 500 µL of 2:1:1 hexane:acetone:ethanol by vortexing at 1000 rpm at room temperature for 2 min. Supernatants were collected after centrifugation at 2655 *g* for 2 min at 4°C then pooled

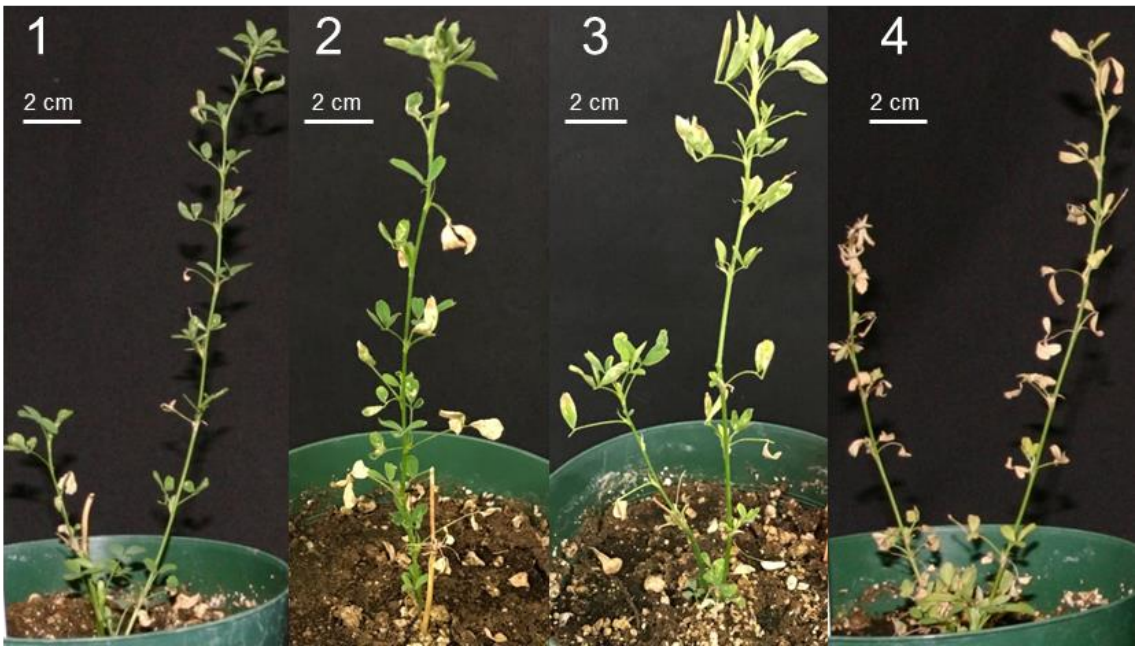

Figure 2. Representative plants for survival analysis tissue scoring

Representative plants from *miR156OE* survival analysis showing chlorotic, wilted or scorched tissue scores within scoring ranges **1**, <25%; **2**, 25-50%; **3**, 50-75%; **4**, >75%.

and evaporated under a stream of nitrogen. Extracts were then reconstituted in 1 mL 5:4:1 acetonitrile:dichloromethane:MeOH with 0.5% BHT (Kormendi et al., 2016). Extracts were stored at -20°C. The reconstitution buffer was used as a blank for HPLC.

2.9.2 HPLC conditions

Reconstituted chlorophyll/carotenoid extracts were filtered through 0.2 µm syringe filters into 2 mL HPLC vials. A total of 5 µL extract was injected into an Agilent 1200 Series HPLC and separated on a Poroshell 120, EC-C18, 4.6 mm × 75 mm, 2.7 µm particle size column with a Poroshell 120 Fast guard column (Agilent Technologies, Mississauga, Canada). HPLC gradient conditions were run according to (Kormendi et al., 2016) with mobile phases consisting of methanol (solvent A), methy *tert*-butyl ether (solvent B, MTBE) and water (Solvent C) (Table 2).

2.9.3 Determination of chlorophyll content

Chlorophylls a and b were monitored at 645 and 663 nm using a diode array detector (DAD). Chlorophyll concentrations (mg/g) were calculated based on ratios of peak areas of each chlorophyll at 645 and 663 nm (Ni et al., 2009) as per calculations below:

$$\text{Chlorophyll a mg/g} = (12.7 \times PA_{663nm} - 2.69 \times PA_{645nm}) \times V\ (ml)/1000 \times FW\ (mg)$$

$$\text{Chlorophyll b mg/g} = (22.9 \times PA_{645nm} - 4.86 \times PA_{663nm}) \times V\ (ml)/1000 \times FW\ (mg)$$

$$\text{Total Chlorophyll mg/g} = (8.02 \times PA_{663nm} + 20.20 \times PA_{645nm}) \times V\ (ml)/1000 \times FW\ (mg)$$

PA = peak area; V = reconstitution buffer volume; FW= leaf tissue fresh weight

2.9.4 Determination of carotenoid content

Lutein and β-carotene were monitored at 450 nm using DAD. Samples were run alongside lutein and β-carotene standards, which were used to create standard curves for the quantification of carotenoids on a per gram basis.

Table 2. HPLC solvent gradient for leaf chlorophyll and carotenoid content analysis

Time (min)	% A (MeOH)	% B (MTBE)	% C (H_2O)
0	80	0	20
7	100	0	0
7.5	100	0	0
8.5	5	95	0
10.5	5	95	0
11	100	0	0
11.5	100	0	0
12	80	0	20
14	80	0	20

2.10 Determination of total phenolic content (TPC), total flavonoid content (TFC) and total monomeric anthocyanin (TMA) contents

2.10.1 Extraction procedure

Crude extracts were prepared by treating 100 mg of ground leaf tissue with 1 mL of acidified methanol (0.2% HCl) (Cheok et al., 2013). Samples were sonicated for 15 min at 40 KHz followed by vortexing for 1 hr at 1000 rpm at room temperature and centrifuging for 2 min at 2655 *g* at 4°C. Supernatants were then filtered through 0.2 µm syringe filters to produce the crude extracts for TPC, TFC and TMA and were aliquoted in appropriate amounts as indicated below. Extracts were stored at 4°C in the dark for up to 12 hr.

2.10.2 Determining total phenolic content (TPC)

The Folin-Ciocalteu method was used to determine TPC content in leaf extracts (Folin and Ciocalteu, 1927; Singleton et al., 1999). Aliquots of 100 µL of crude extract were mix-vortexed with 500 µL of Folin-Ciocalteu reagent for 3 min at 1000 rpm followed by mixing with 400 µL of 7.5% w/v sodium carbonate. The solution was then incubated for 30 min under dark condition before transferring to 1 mL cuvettes. TPC was measured spectrophotometrically at 765 nm using SmartSpecTM Plus spectrophotometer (Bio-Rab

Laboratories). Gallic acid was used to construct standard curves for TPC quantification. A solution substituting 100 µL of acidified methanol for crude extract was used as a blank.

2.10.3 Determining total flavonoid content (TFC)

The aluminum complexation reaction method was used to determine the TFC of leaf extracts (Chang et al., 2002). A total of 100 µL of crude extracts was mixed with 300 µL of methanol, 20 µL of 10% (w/v) aluminum chloride, 20 µL of 1 M potassium acetate and 540 µL of water. Blank samples were prepared by substituting aluminum chloride with water. The solution was then incubated in the dark at room temperature and transferred to 1 mL cuvettes. TFC was measured at 415 nm spectrophotometrically and compared to a quercetin standard curve (Chang et al., 2002).

2.10.4 Determining total monomeric anthocyanin (TMA) content

TMA was determined using the pH differential method (Cheok et al., 2013). Aliquots of 250 µL of crude extracts were subjected to pH-dependent TMA extraction using pH 1 0.025 M potassium chloride and pH 4 0.4 M sodium acetate at a 1:3 extract:solvent ratio. Solutions were mixed and transferred to 1 mL cuvettes. Absorbance was measured at 520 nm and 700 nm under both pH conditions and TMA was determined according to the equation below. Mill-Q water was used as a blank. TMA was then converted to a per g basis.

$$\text{TMA (mg/L)} = (A \times MW \times DF \times 1000) / (\varepsilon \times pl)$$

A (absorbance) = $(A510\text{ nm} - A700\text{ nm})_{pH1}$-$(A510\text{ nm} - A700\text{ nm})_{pH4.5}$; MW (molecular weight of cyanidin-3-glucoside) =449.2 g/mol; DF (dilution factor) = 4; ε (cyanidin-3-glucoside molar absorptivity coefficient L/mol/cm) = 26,900; pl (path length cm) = 1

2.11 Determining proline content

Proline was determined using the colorimetric assay of Bates et al. (1973) as per the protocol in Abraham et al. (2010). Proline was extracted from 100 mg of ground leaf tissue using 500 µL of 3% sulfosalicylic acid. Aliquots of 100 µL of extract was mixed

with 100 µL of 3% sulfosalicylic acid, 200 µL of glacial acetic acid, and 200 µL of acidic ninhydrin followed by incubation at 96°C for 60 min. Acidic ninhydrin was prepared by mixing 1.25 g of ninhydrin, 30 mL of acetic glacial acetic acid, and 20 mL of 6 M phosphoric acid. After reaction termination on ice, samples were extracted with 1 mL of toluene. The chromophore containing phase was transferred to a transparent 96 well plate and measured spectrophotometrically at 520 nm. Proline content was determined using a proline standard curve.

2.12 Measurement of plant water status

2.12.1 Relative water content (RWC)

Leaf tissue was harvested from each plant after 96 hr of heat stress for relative water content (RWC) calculations. Relative water content was determined by harvesting approximately 0.5 g of fresh leaves and weighing to determine the exact fresh weight (FW). After determining fresh weight, the turgid weight (TW) was determined by soaking leaves in deionized water for 24 hr under dark condition in 20 mL vials and pat-drying before weighing. Finally, dry weight (DW) of the leaves was determined by drying the leaves at 60°C for at least 48 hr. RWC was calculated using the following equation:

$$RWC = ((FW - DW) / (TW - DW)) \times 100\%$$

FW = fresh weight, DW = dry weight, TW = turgid weight

2.12.2 Midday leaf water potential (LWP)

Midday leaf water potential (LWP) was determined after 96 hr of heat stress using a SAPS II Portable Plant Water Status Console (Soilmoisture Equipment Corp., Santa Barbara, CA) pressure chamber to apply pressure to the leaf until liquid emerges from the end of the petiole (Rapaport et al., 2015; Turner, 1988). Leaves with appropriately long petioles were excised using a razor blade to avoid crushing the petiole and the restriction of water flow. One A11 plant was excluded from LWP analysis due to its short petiole length being incompatible with the pressure chamber.

2.12.3 Stomatal conductance

Leaf stomatal conductance was measured after 96 hr of heat stress using the Decagon Devices, Inc. SC-1 Leaf Porometer (METER Environment, Pullman, WA). The stomatal conductance of antepenultimate leaves of all plants was measured to reduce variability between plants.

2.13 Phenotypic characterization of alfalfa

Newly developed transgenic alfalfa plants were characterized 30 and 60 days after potting and maintained under greenhouse conditions. Variables measured included antepenultimate leaf length, antepenultimate leaf width, stem length, stem diameter, number of nodes, average internode length, shoot fresh weight, shoot dry weight, primary branches, secondary branches, root fresh weight and root dry weight. Flowering time was monitored at 24 hr intervals after cutting plants back after an initial two months of growth post potting.

Primary branches were considered to be all branches originating from the bottom of the stem at the soil level while secondary branches were those originating aboveground from primary branches. Stem length was measured from the longest primary stem and nodes were counted on the longest primary stem. Average internode length was calculated from the stem length and number of nodes on the longest primary stem. All lengths/widths were measured using digital calipers to the nearest 0.01 mm except stem lengths, which were measured to the nearest mm using a ruler. For measuring dry weight, plant tissues were dried at 60°C for at least 48 hr.

2.14 Statistical analysis

Two-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was used to determine significant differences in transcription levels of genes in heat stress-treated and control condition WT alfalfa. Molecular and physiological differences between transgenic and EV alfalfa were determined using one- or two-way ANOVAs. Sample sizes ranged from 3-20 biological replicates as indicated in figure legends. Where significant effects were found, Dunnett's test was used because significant differences

between transgenic and EV control alfalfa under the same condition could be determined. Differences in heat stress tolerance based on chlorotic/wilted/scorched tissue were determined by Bonferroni corrected log-rank tests using Prism 6 (Graphpad Software, La Jolla, CA) (Bland and Altman, 2004).

Chapter 3

3 Results

3.1 Effect of heat stress on expression of *miR156* and *SPL* genes

The effects of heat stress on the expression of *miR156* and *SPL* genes was investigated by subjecting WT alfalfa to 40°C heat stress or greenhouse control conditions, extracting total RNA from these plants and then determining the transcript levels of mature *miR156* and its seven target *SPL* genes by qRT-PCR.

3.1.1 *miR156* transcript abundance under heat stress

To quantify mature *miR156* transcript abundance under heat stress conditions, I extracted RNA from the tissue of WT plants exposed to heat stress with sufficient small RNA retention to allow for stem-loop reverse transcription of mature *miR156* and compared transcript levels to the levels of control plants under greenhouse conditions. *miR156* had 105-fold higher transcript abundance under heat stress conditions at the earliest time point (30 min) (Figure 3A). While the abundance of mature *miR156* transcript was still numerically higher than in the control at 2 hr, the increase was not statistically significant.

3.1.2 *SPL* transcript abundance under heat stress

To investigate how miR156-targeted *SPL* genes are expressed under heat stress, I extracted RNA from the tissue of WT plants exposed to heat stress for qRT-PCR analysis and data were compared to control plants under greenhouse conditions. Both *SPL13* and *SPL9* were downregulated by 65-70% after 3 hr of heat stress (Figures 3B and 3C). *SPL13* retained the low transcription level after 12 hr of stress compared to the 3 hr control, but was not different than the 12 hr control plants. Other *SPL* genes showed no significant response to heat stress after 3 hr or 12 hr (Figures 3D, 3E, 3F, 3G). While *SPL6* showed no differences between individual groups or in response to heat stress, ANOVA revealed that there was a significant difference between time points ($F_{1,11}$=6.846, p=0.0225) (Figure 3F). *SPL2* was undetectable in all samples.

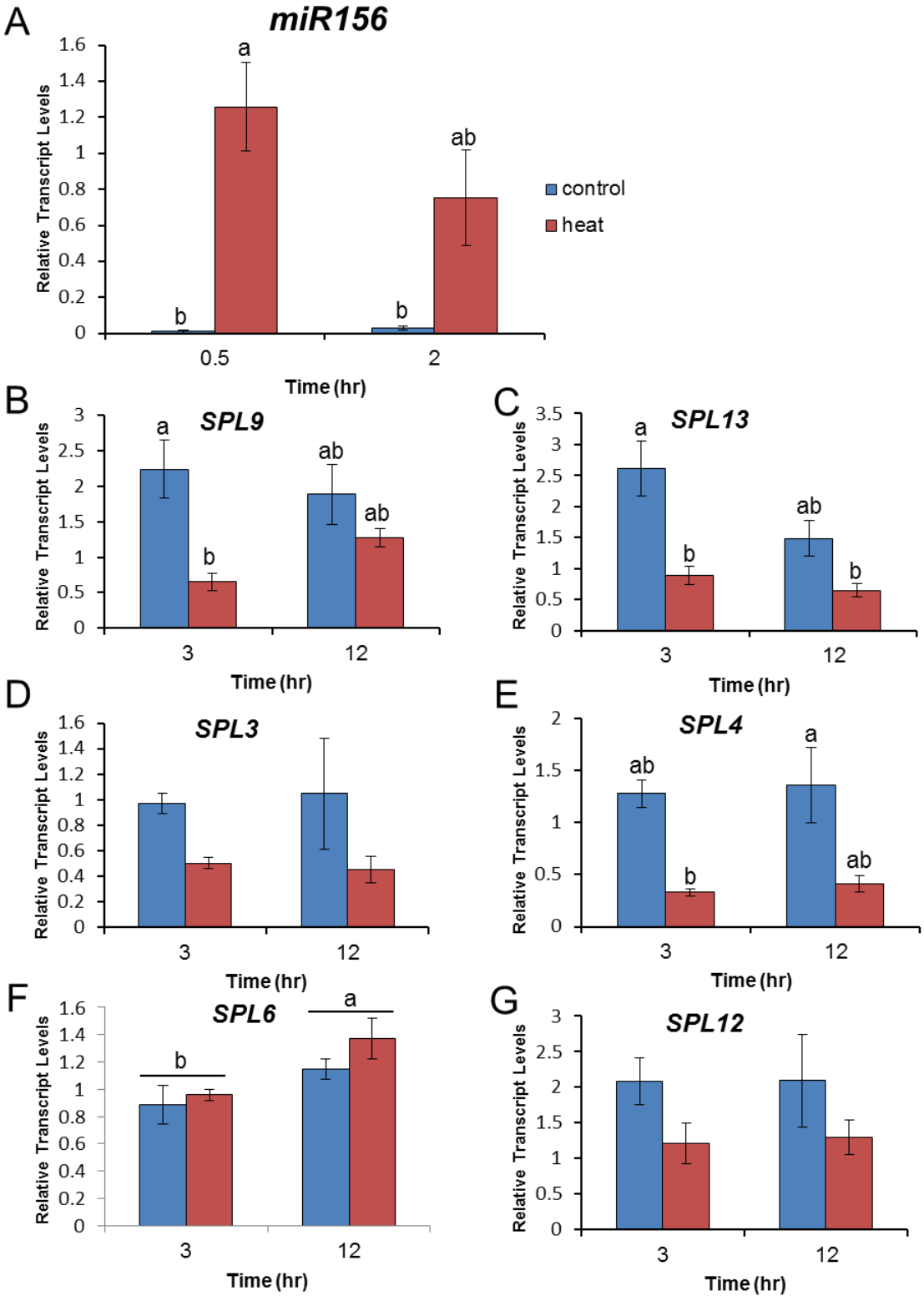
A
miR156
Relative Transcript Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
ab
b
0.5
2
Time (hr)
control
heat
B
SPL9
Relative Transcript Levels
3
2.5
2
1.5
1
0.5
0
a
b
ab
ab
3
12
Time (hr)
C
SPL13
Relative Transcript Levels
3.5
3
2.5
2
1.5
1
0.5
0
a
b
ab
b
3
12
Time (hr)
D
SPL3
Relative Transcript Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3
12
Time (hr)
E
SPL4
Relative Transcript Levels
2
1.5
1
0.5
0
ab
b
a
ab
3
12
Time (hr)
F
SPL6
Relative Transcript Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
a
3
12
Time (hr)
G
SPL12
Relative Transcript Levels
3
2.5
2
1.5
1
0.5
0
3
12
Time (hr)

Figure 3. Effect of heat stress on the transcript abundance of *miR156* and its target *SPL* genes in WT alfalfa

Normalized transcript levels of **A**, mature *miR156*; **B**, *SPL9*; **C**, *SPL13*; **D**, *SPL3*; **E**, *SPL4*; **F**, *SPL6*; **G**, *SPL12* genes in WT alfalfa under control and 40°C heat stress conditions. Transcript levels determined by qRT-PCR were normalized using **A** reference genes *CYCLOPHILIN* and snRNA *U6* or **B-G** reference genes *CYCLOPHILIN* and *EIF4A*. Normalized transcript levels are reported as mean ± standard error of 4 biological replicates. Bars with the same letter are not significantly different at $p<0.05$ as determined by ANOVA followed by Tukey's HSD.

3.2 Survival analysis of *miR156OE* and *SPL* RNAi alfalfa

To investigate how miR156 and SPLs influence heat stress tolerance, I subjected *miR156OE, SPL13i* and *SPL9i* plants to 40°C heat stress treatment over the course of 6-8 days and monitored individual plants at 12 hr intervals for chlorosis, wilting and scorching. A threshold of 50% chlorotic, wilted or scorched tissue was used for survival curve construction and statistical analysis. Kaplan-Meier curves provide the estimates of survival over the course of the entire survival experiment rather than just at a final time point as commonly seen in the analysis of plant tolerance to stress (Schandry, 2017), and, similarly, the log-rank test compares the survival of populations over the entire course of the experiment (Bland and Altman, 2004).

3.2.1 Heat stress survival of *miR156OE* alfalfa

The ability of plants to survive stress exposure is a key aspect of stress tolerance and necessary for crop yield. Heat stress induces leaf and stem scorching, damaging the crop quality, and reducing future yield. To investigate whether increased expression of *miR156* under heat stress affects alfalfa survival, *miR156OE* genotype A8a, A8 and A11 plants and EV control plants were exposed to prolonged (144 hr) heat stress and monitored and scored at specific intervals for chlorosis, wilting and scorching, which are major symptoms of heat stress (Li et al., 2013; Vollenweider and Gunthardt-Goerg, 2005). Comparing scores showed *miR156OE* plants were significantly less affected by heat stress over time compared to EV controls (Figure 4A). I found that the onset of chlorosis and wilting began earlier in EV plants, as early as 48 hr into the experiment, and plants steadily fell out of the healthy population (Figure 4A). *miR156OE* genotypes then began scoring over 50% chlorosis at a slower rate, beginning with the highest *miR156* expresser, A11, followed by the other genotypes in descending order of *miR156* expression levels (Aung et al., 2015b).

3.2.2 Heat stress survival of *SPL13i* and *SPL9i* alfalfa

As expression analysis revealed that *SPL13* is silenced under heat, I hypothesized that maintaining low *SPL* expression is needed to alleviate heat stress in alfalfa. To that end, *SPL13i* genotypes 02, 05 and 06 and EV control plants were subjected to prolonged heat

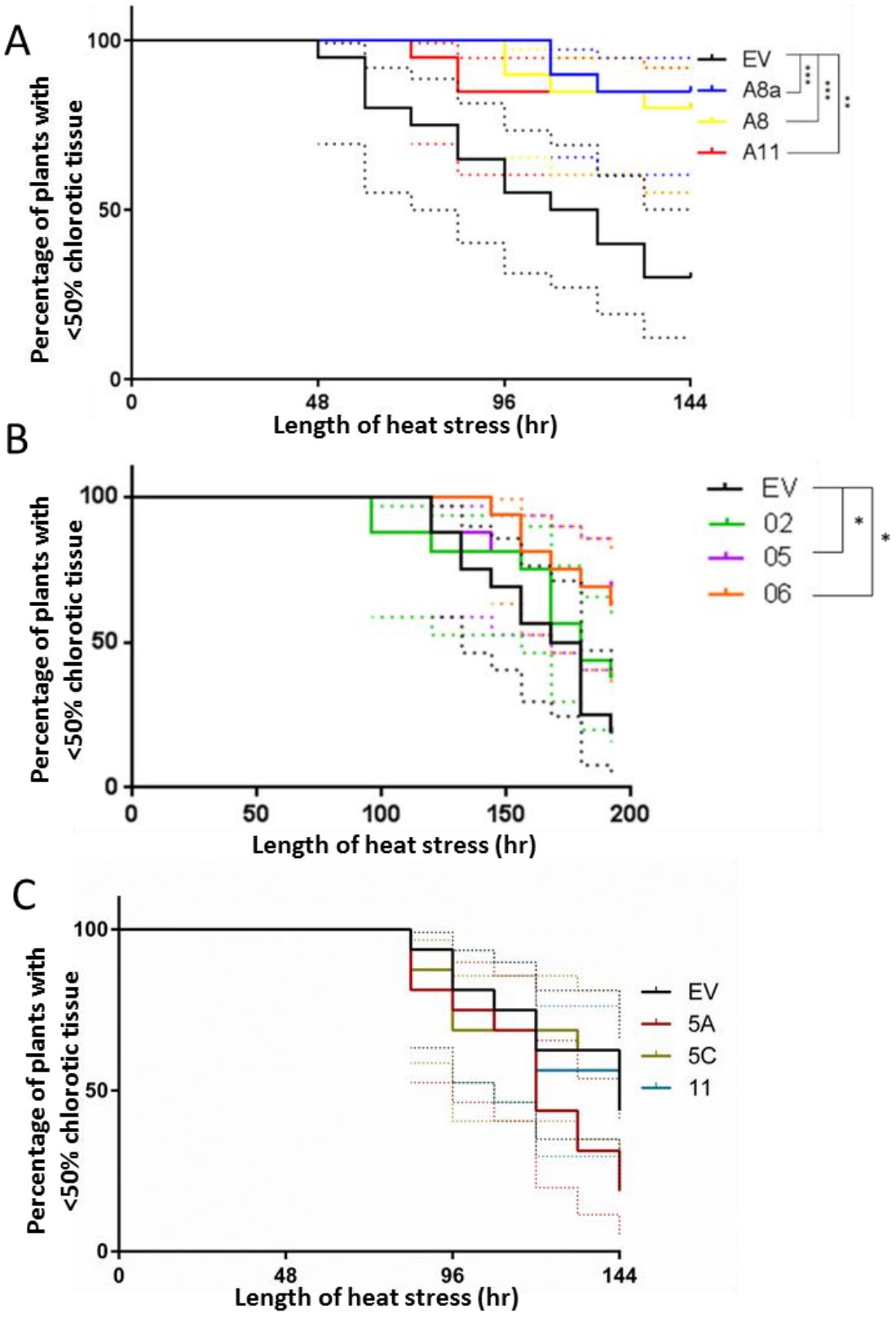
A
100
50
0
0
48
96
144
Percentage of plants with <50% chlorotic tissue
Length of heat stress (hr)
EV
A8a
A8
A11
***
***
**
B
100
50
0
0
50
100
150
200
Percentage of plants with <50% chlorotic tissue
Length of heat stress (hr)
EV
02
05
06
*
*
C
100
50
0
0
48
96
144
Percentage of plants with <50% chlorotic tissue
Length of heat stress (hr)
EV
5A
5C
11

Figure 4. Alfalfa survival under heat stress conditions in *miR156OE*, *SPL13i* and *SPL9i* transgenic alfalfa

Kaplan-Meier curves representing survival of alfalfa estimated by scores of greater than 50% chlorosis/wilting/scorching of **A**, *miR156OE* genotypes A8a, A8 and A11; **B**, *SPL13i* genotypes 02, 05 and 06; **C**, *SPL9i* genotypes 5A, 5C and 11 compared to EV control under 40°C heat stress over the course of 6-8 days. Percentage of scored living plants ± 95% CI (dashed lines) of **A** 20 or **B**, **C** 16 biological replicates. Significant differences were determined by Bonferroni corrected log-rank tests where * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

stress (192 hr) and monitored and scored for chlorosis, wilting and scorching. Based on stress scores, *SPL13i* genotypes 05 and 06 were less affected by heat stress over time compared to EV control (Figure 4B). Unlike the results for *miR156OE* plants, *SPL13i* genotype 02 plants were the first to fall out of the healthy population and, despite ending the experiment with a numerically higher population of healthy plants, were not significantly different from EV control (Figure 4B). The genotype 05 plant population initially had the same chlorotic onset as EV controls, but the healthy population fell more slowly than the EV population towards the end of the experiment (Figure 4B). Finally, the 06 population chlorotic onset was delayed and stress symptoms developed more slowly than EV control (Figure 4B).

Similarly, as *SPL9* is silenced under heat, I also examined *SPL9i* plants for survival. For that, *SPL9i* genotype 5A, 5C and 11 alfalfa plants and EV controls were subjected to heat stress for 144 hr and analyzed as described for *miR156OE* and *SPL13i* plants. Stress scores did not differ between *SPL9i* and EV control with no particular trend (Figure 4C). The onset of chlorosis and wilting began at the same time, with genotypes 5C and 11 ending the experiment with about the same healthy population percentage as EV whereas 5A had the smallest healthy population (Figure 4C).

3.2.3 Effect of heat stress on the chlorophyll contents of *miR156OE* and *SPL13i* plants

Reductions in chlorophyll fluorescence (Moffatt et al., 1990) and increases in chlorophyll degradation (Karim et al., 1999) under high temperatures are quintessential symptoms of heat stress. Chlorophyll quantification is therefore commonly used to measure plant heat stress response and tolerance (Baker and Rosenqvist, 2004). To confirm the results of chlorotic/wilted/scorched scores, I measured the chlorophyll concentrations of a subset of *miR156OE* and *SPL13i* plants; values were then normalized to the chlorophyll contents of control plants to determine if there were obvious differences in chlorophyll content between the experimental and control plants. *miR156OE* genotypes A8a and A8 had 50-140% greater relative chlorophyll content indexes compared to EV controls (Figure 5A). *miR156OE* plants had indexes around 1, suggesting they had chlorophyll content similar to control plants after 144 hr of heat stress whereas heat-stressed EV plants had

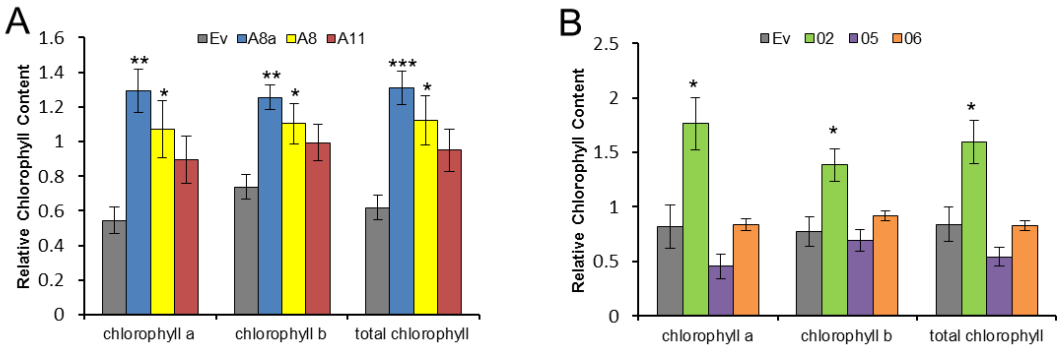

Figure 5. Chlorophyll content of heat stress treated *miR156OE* and *SPL13i* alfalfa

Chlorophyll content indexes of a subsample of **A**, *miR156OE* plants; **B,** *SPL13i* plants under 40°C heat stress over the course of the **A** 144 or **B** 192 hr survival experiment normalized to control plant chlorophyll contents. Data are reported as mean ± standard error of 8 biological replicates (A) or 4 biological replicates (B). Asterisks indicate significant differences between *miR156OE* or *SPL13i* genotypes and EV controls as determined by ANOVA followed Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

chlorophyll concentrations approximately half of their corresponding control plants (Figure 5A). In contrast to the results of the survival analysis, only *SPL13i* genotype 02 plants, the only *SPL13i* genotype that did not perform well in the survival analysis, showed a significant increase in relative chlorophyll content after 192 hr of heat stress (Figure 5B). Genotype 02 also had indexes much greater than 1, suggesting higher chlorophyll concentrations in heat stress-treated 02 than control 02 plants (Figure 5B). Otherwise, genotypes EV, 05 and 06 all had slight reduction in chlorophyll compared to their control plants and were not significantly different from one another (Figure 5B).

3.3 Heat stress-induced changes in water status of alfalfa

Plant response to abiotic stress involves changes at the physiological and morphological levels that provide the plant with coping strategies (Fahad et al., 2017; Sullivan and Eastin, 1974; Tozzi et al., 2013). Plants must retain water under heat stress but can also alleviate heat stress by increasing transpiration rates (Urban et al., 2017). Appropriately balancing water retention with evaporative cooling may help plants survive heat stress conditions under varying levels of water stress (Zandalinas et al., 2018). To determine if modulating the expression of *miR156* and *SPL* genes induces physiological changes that alleviate heat stress and reduce the effects of increased evapotranspiration under extreme heat, *miR156OE, SPL13i* and *SPL9i* plants were subjected to 40°C heat stress for a moderate length of time (96 hr) and compared to greenhouse control plants.

3.3.1 *miR156OE* water status under heat stress

The ability of a plant to retain water during heat and drought plays a significant role in its ability to withstand stress (Sullivan and Eastin, 1974; Tozzi et al., 2013). I calculated the RWC of heat stress-treated and control plants to determine if *miR156OE* plants retained more water under heat stress. A11 plants alone had 4% higher RWC under control conditions; however, no differences were seen among *miR156OE* genotypes and EV controls under heat stress (Figure 6A). I then investigated the midday LWP to gain an insight into the gas exchange of the *miR156OE* alfalfa and infer the level of stress. Under heat stress, *miR156OE* genotypes A8a and A8 had 0.2 MPa higher LWP compared to EV

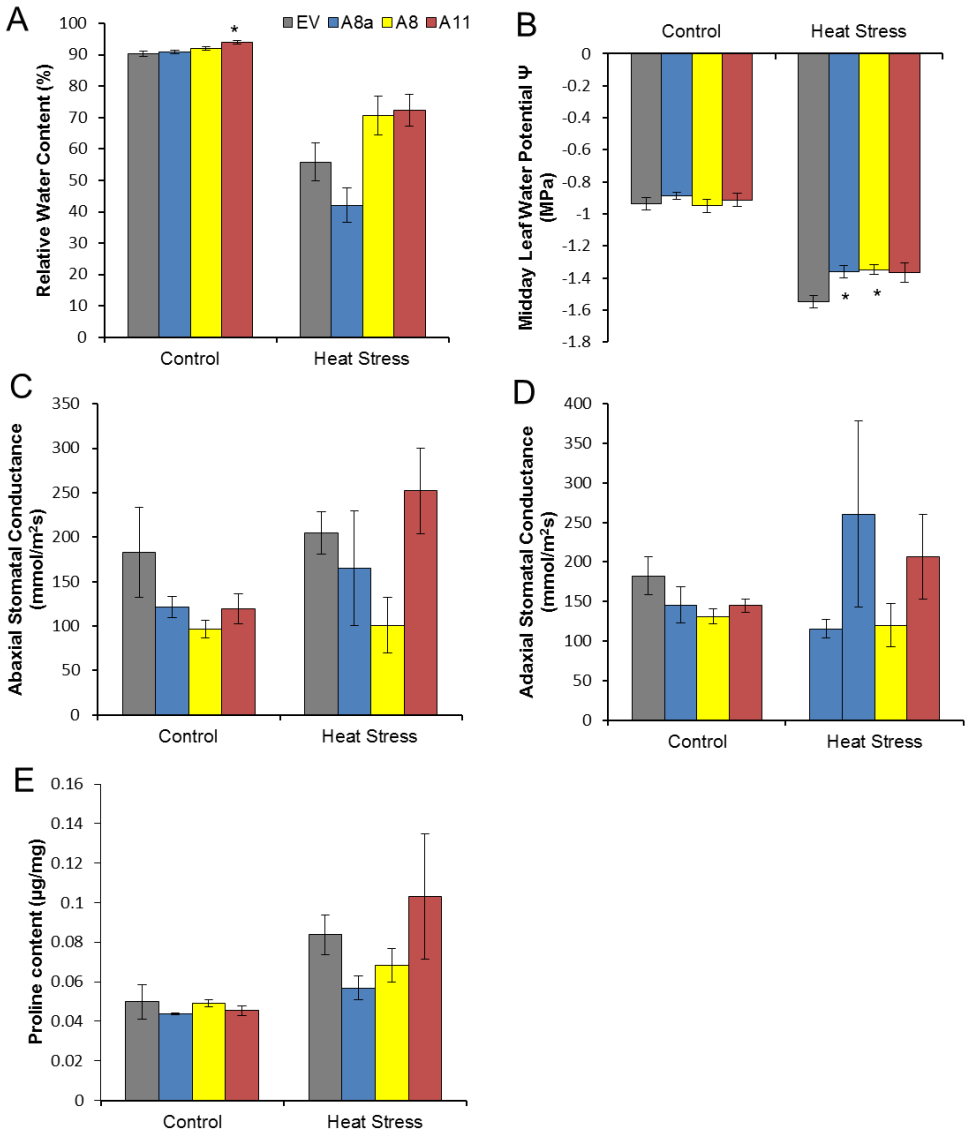

Figure 6. Water status and proline content in *miR156OE* alfalfa under heat stress

A, Relative water content; **B**, midday leaf water potential; **C**, abaxial stomatal conductance; **D**, adaxial stomatal conductance; **E**, proline concentration of *miR156OE* genotypes A8a, A8 and A11 after 40°C heat stress or control conditions for **A**-**D** 96 hr or **E** 144 hr. Data are reported as mean ± standard error of 3-4 biological replicates. Asterisks indicate significant differences between *miR156OE* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed Dunnett's test where $p<0.05$.

controls, suggesting increased gas exchange and reduced stress than EV controls (Figure 6B). One A11 replicate was excluded from this analysis due to short petiole lengths. Stomatal conductance was also determined to measure gas exchange in heat stress and control plants but no significant differences were detected and variances were both large and unequal, even after log transformation (Figure 6C, 6D). To determine if compatible solutes were contributing to differences in LWP, I measured the proline concentration of plants under heat stress and control conditions from samples taken at the conclusion of the survival analysis experiment. No significant differences in proline concentrations were found between the genotypes (Figure 6E).

3.3.2 *SPL13i* water status under heat stress

To determine if *SPL13* downregulation conferred physiological advantages under heat stress similar to those observed in *miR156OE* alfalfa, RWC, LWP and stomatal conductance were determined for *SPL13i* plants. I calculated the RWC for *SPL13i* plants under heat stress and control conditions and compared them to EV controls to determine if *SPL13* downregulation in alfalfa resulted in increased water retention. Similar to the results in *miR156OE* plants, the lowest expressing *SPL13i* genotype, 06 had 4% higher RWC under control conditions (Figure 7A). Also, similar to the *miR156OE* results, no differences were observed under heat stress although all *SPL13i* genotypes had higher means numerically (Figure 7A). Additionally, *SPL13i* plants had significantly decreased LWP under control conditions (Figure 7B). Similar to the results of *miR156OE* plants, *SPL13i* genotypes 02 and 06 had significantly increased LWP under heat stress (Figure 7B). Log-transformed stomatal conductance showed that all *SPL13i* genotypes had increased abaxial stomatal conductance (Figure 7C) and genotypes 05 and 06 had increased adaxial stomatal conductance (Figure 7D). Proline concentrations were determined under heat stress and control conditions using samples collected for the survival analysis to determine if compatible solutes were contributing to differences in LWP and stomatal conductance. No differences in proline concentrations were found and samples were not homoscedastic (Figure 7E). Proline content was abnormally high in EV, 02 and 05 genotypes (Abraham et al., 2010; Bates et al., 1973).

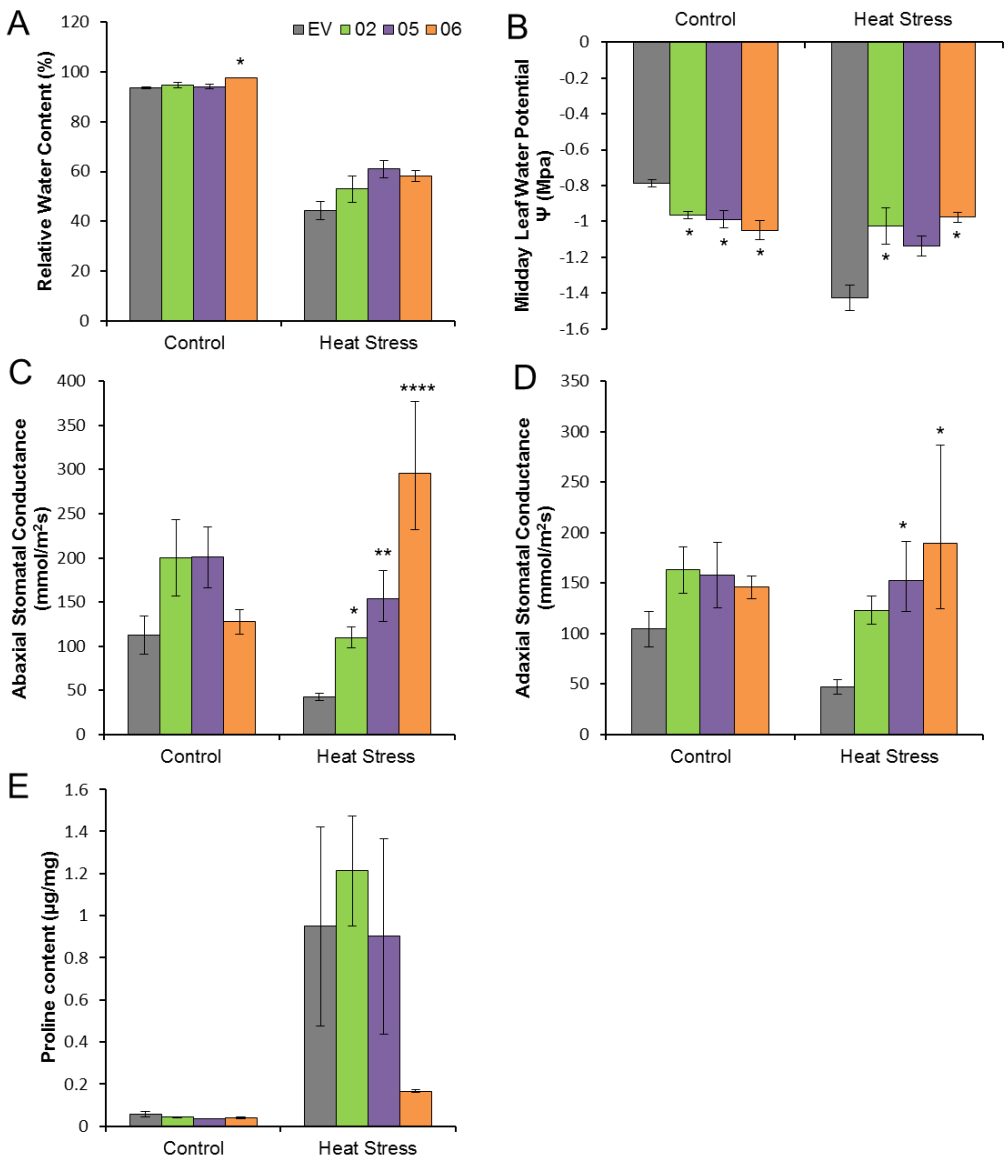

Figure 7. Water status and proline content in *SPL13i* alfalfa under heat stress

A, Relative water content; **B**, midday leaf water potential; **C**, abaxial stomatal conductance; **D**, adaxial stomatal conductance; **E**, proline concentrations of *SPL13i* genotypes 02, 05 and 06 after 40°C heat stress or control conditions for **A-D** 96 hr or **E** 192 hr. Data are reported as mean ± standard error of 4 biological replicates. Asterisks indicate significant differences between *SPL13i* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$ and **** indicates $p<0.0001$.

3.3.3 *SPL9i* water status under heat stress

Before investigating any contributions by *SPL9* downregulation to physiological advantages in heat stress tolerance, I first investigated if and to what extent *SPL9* was downregulated in the newly generated *SPL9i* alfalfa plants. qRT-PCR revealed that the four genotypes I tested had between 1.5- and 2.2-fold decreases in *SPL9* transcript abundance (Figure 8). Next, I determined the RWC, LWP and stomatal conductance for *SPL9i* genotypes 5A, 5C, 6C and 11. Contrary to results in *miR156OE* and *SPL13i* experiments, genotype 6C plants had lower RWC under control conditions (Figure 9A); however, no differences were seen in RWC under heat stress conditions similar to *miR156OE* and *SPL13i* plants. No differences were found in LWP (Figure 9B) or abaxial stomatal conductance (Figure 9C) between the different genotypes and EV controls. Due to the lack of differences in water status and survival, I abandoned further investigation of the role of *SPL9* in heat stress response.

3.4 Effects of heat stress on antioxidant capacity and content in alfalfa

Abiotic stress can cause production of ROS, and thus plants respond to stress in part by producing antioxidants, such as carotenoids and phenolic compounds, to scavenge ROS (Apel and Hirt, 2004; Foyer and Noctor, 2005). As a previous studies showed changes in antioxidant content and capacity in *miR156OE* alfalfa plants when exposed to drought (Arshad et al., 2017a), I decided to measure them under heat stress.

3.4.1 Effect of heat stress on antioxidant capacity and content of *miR156OE*

To determine if increased *miR156* expression under heat stress improves alfalfa's ability to scavenge the increased ROS due to stress conditions, I compared the antioxidant potential of non-enzymatic antioxidants in *miR156OE* leaf extracts to EV controls after prolonged heat stress. While there were no differences detected under control conditions, *miR156OE* genotypes A8 and A11, respectively, had 44% and 100% higher antioxidant

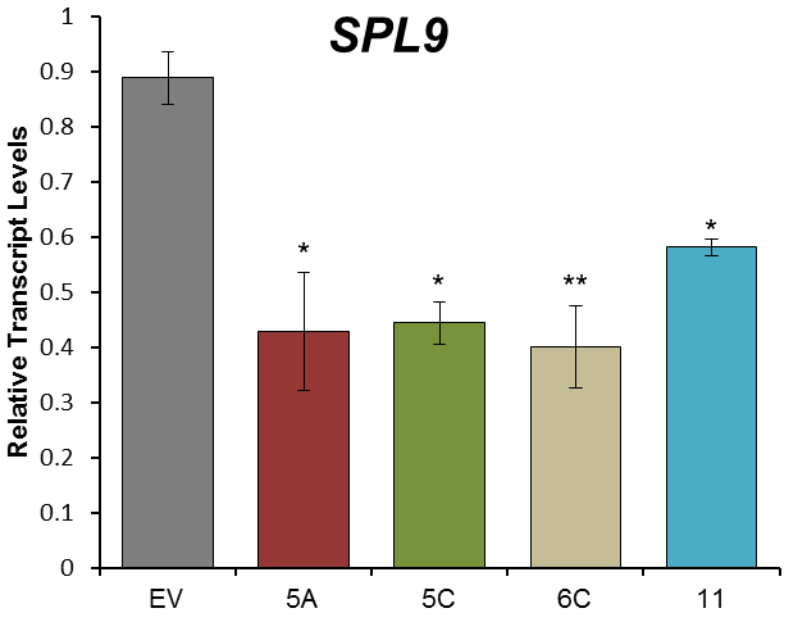

Figure 8. Transcript abundance of *SPL9* in *SPL9i* alfalfa

Normalized transcript levels of the *SPL9* gene in EV control plants and *SPL9i* genotypes 5A, 5C, 6C and 11 under greenhouse conditions after one month of growth. Normalized transcript levels were determined by qRT-PCR using reference genes *CYCLOPHILIN* and *ACTIN*. Normalized transcript levels are reported as mean ± standard error of 3 biological replicates. Asterisks indicate significant differences between *SPL9i* genotypes and EV controls as determined by ANOVA followed by Dunnett's test where * indicates $p<0.05$ and ** indicates $p<0.01$.

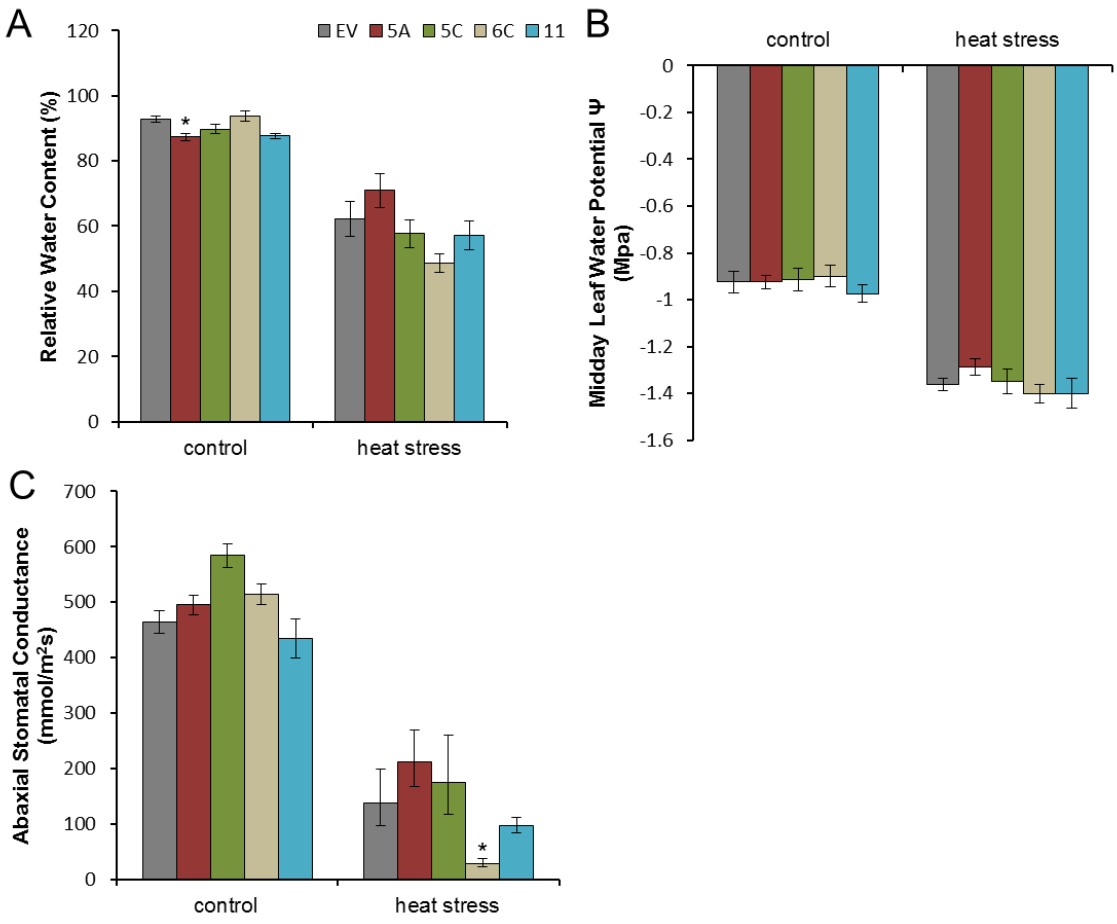

Figure 9. Water status in *SPL9i* alfalfa under heat stress

A, Relative water content; **B**, midday leaf water potential; **C**, abaxial stomatal conductance of *SPL9i* genotypes 02, 05 and 06 after 40°C heat stress or control conditions for 96 hr. Data are reported as mean ± standard error of 4 biological replicates. Asterisks indicate significant differences between *SPL9i* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed Dunnett's test where $p<0.05$.

capacity than EV controls under heat stress (Figure 10A). Next, carotenoid and phenolic compound contents were investigated to determine what antioxidants might be contributing to differences in antioxidant potential. No differences were seen in the concentrations of the carotenoids lutein (Figure 10B) or β-carotene (Figure 10C) under control or heat stress conditions; however, the mean carotenoid content of all three *miR156OE* genotypes had numerically higher values than EV controls under heat stress. While no differences were detected in TPC under control conditions, A8a plants had 17% higher TPC than EV controls under heat stress (Figure 10D). No change in TFC was observed under control conditions, but contrary to expectations based on associations with the phenylpropanoid pathway (Cui et al., 2014), A8a and A8 plants had 35-47% decreased TFC under heat stress (Figure 10E). Finally, all *miR156OE* genotypes had 109-150% increased TMA content compared to EV control under heat stress (Figure 10F).

3.4.2 Effect of heat stress on antioxidant capacity and content of *SPL13i*

To determine if the downregulation of *SPL13* under heat stress improves alfalfa's ability to scavenge the stress-induced ROS, I compared the antioxidant potential of non-enzymatic antioxidants in *SPL13i* and EV leaf extracts after prolonged heat stress and under control conditions. *SPL13i* genotypes were not significantly different than EV plants under control conditions but genotypes 02 and 05 had 157% and 187% higher antioxidant capacity than EV control, respectively, under heat stress (Figure 11A). Unlike *miR156OE* plants, *SPL13i* genotype 02 had an 80-100% increase in leaf carotenoid contents while no significant differences detected under control conditions (Figure 11B, 11C). There were no differences in TPC between *SPL13i* and EV genotypes under control or heat stress conditions (Figure 11D). Unlike results obtained with *miR156OE*, *SPL13i* 05 plants had 53% higher TFC under heat stress while the other genotypes did not differ (Figure 11E). The TMA values for *SPL13i* mirror those of *miR156OE*, with *SPL13i* 06 plants having 97% higher TMA under heat stress conditions (Figure 11F).

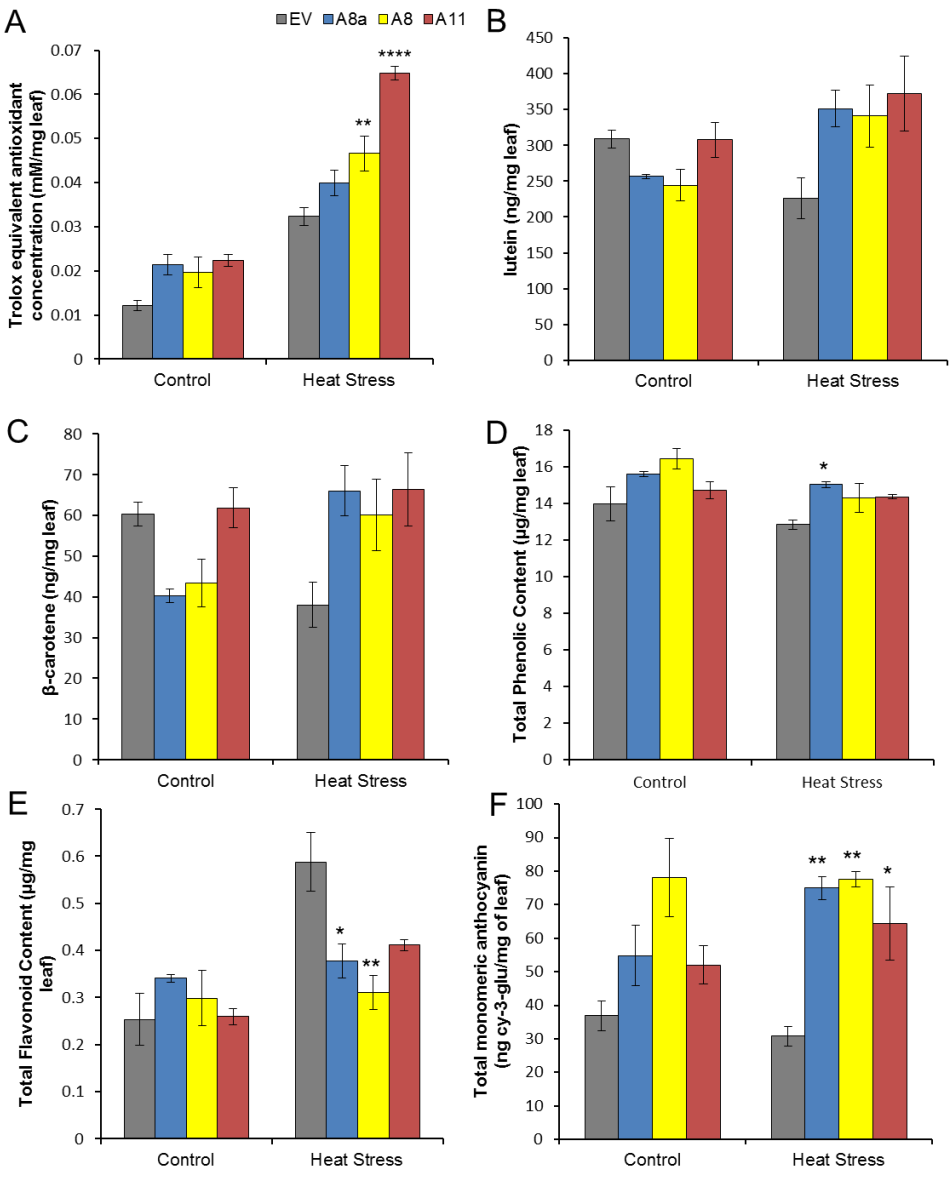

Figure 10. Antioxidant analyses of *miR156OE* alfalfa under heat stress

A, antioxidant capacity; **B**, β-carotene; **C**, lutein; **D**, total phenolic content; **E**, total flavonoid content; **F**, total monomeric anthocyanin of *miR156OE* genotypes A8a, A8 and A11 compared to EV controls after 144 hr of 40°C heat stress or control conditions. Data are reported as mean ± standard error of **D**-**F** 4 or **A**-**C** 8 biological replicates. Asterisks indicate significant differences between *miR156OE* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$ and **** indicates $p<0.0001$.

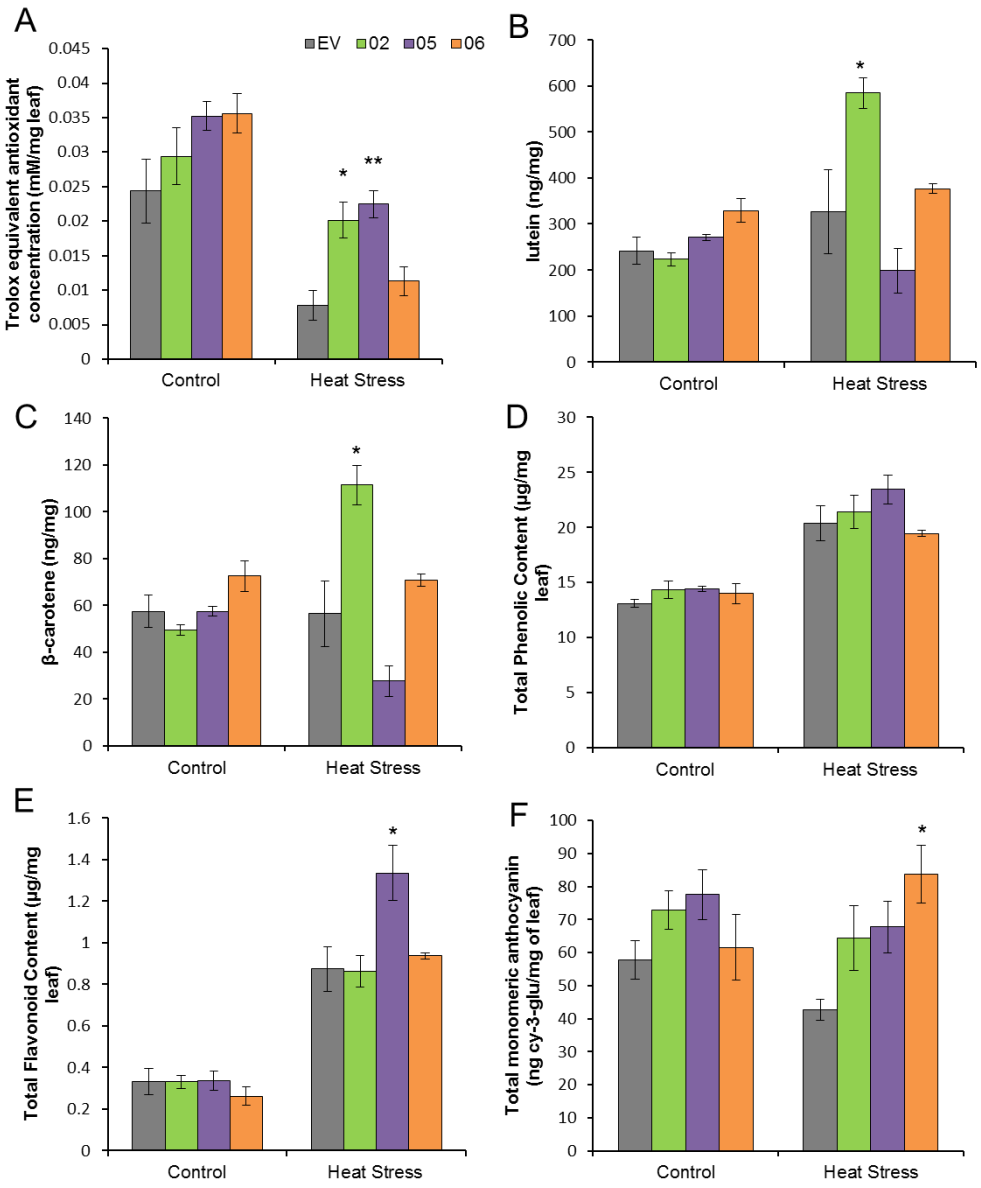

Figure 11. Antioxidant analyses of *SPL13i* transgenic alfalfa under heat stress

A, antioxidant capacity; **B**, β-carotene; **C**, lutein; **D**, total phenolic content; **E**, total flavonoid content; **F**, total monomeric anthocyanin of *SPL13i* genotypes 02, 05 and 06 compared to EV controls after 192 hr of 40°C heat stress or control conditions. Data are reported as mean ± standard error of 4 biological replicates. Asterisks indicate significant differences between *SPL13i* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed Dunnett's test where * indicates $p<0.05$ and ** indicates $p<0.01$.

3.5 Effect of heat stress on gene expression in *miR156OE* and *SPL13i* alfalfa

I investigated the relative transcription levels of select abiotic stress-regulated genes and genes that were previously associated with miR156 (Arshad et al., 2017a; Arshad et al., 2017b; Gao et al., 2018) in alfalfa. RNA was extracted from *miR156OE* and *SPL13i* plants after 24 hr of heat stress exposure or regular growth under control conditions to determine if molecular changes induced by *miR156* overexpression and *SPL13* silencing contribute to the differences in heat stress tolerance observed. The 18 genes were chosen based on previous associations with alfalfa *miR156*/*SPL13* abiotic stress tolerance (Arshad et al., 2017a; Arshad et al., 2017b; Gao et al., 2018) and/or their association with heat stress tolerance (Sailaja et al., 2014; Stief et al., 2014). A full list of genes investigated can be found in Appendix A.

3.5.1 Transcriptional changes in *miR156OE* under heat stress

Of the 18 genes investigated, four showed differential transcript abundance between *miR156OE* genotypes and EV control. Two carotenoid catabolism-associated genes were differentially regulated under heat stress. *CAROTENOID CLEAVAGE DIOXYGENASE 1* (*CCD1*) was upregulated 10-fold and 14-fold in genotypes A8a and A11, respectively, under heat stress (Figure 12A). *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) was reduced by about 80% in A8a and A8 plants compared to EV plants under control conditions but increased by 180-235 % in A8a and A8 plants after heat exposure (Figure 12B). *MYB53* was unaffected under control conditions but increased 6-fold under heat in A11 (Figure 12C). Similarly, while no significant differences were observed under control conditions, *bZIP* family gene *TGA1A-RELATED GENE3* (*TGA3*) showed an 18-fold increase in transcription in A11 plants compared to EV control under heat stress conditions (Figure 12D).

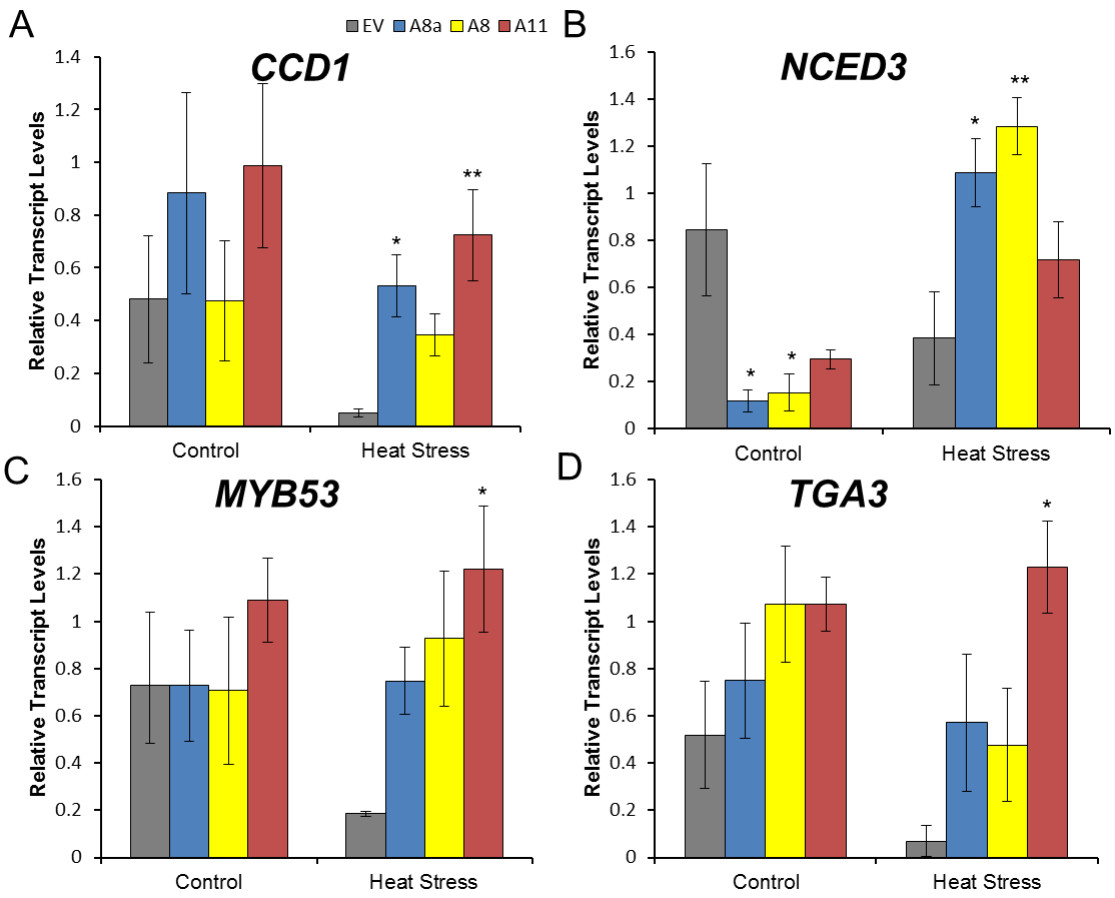

Figure 12. Transcriptional changes in *miR156OE* alfalfa under heat stress

Normalized transcript levels of **A**, *CCD1*; **B**, *NCED3*; **C**, *MYB53*; **D**, *TGA3* genes in *miR156OE* and EV control alfalfa under control and 40°C heat stress conditions. Transcript levels determined by qRT-PCR were normalized using reference genes *CYCLOPHILIN* and *ACTIN*. Normalized transcript levels are reported as mean ± standard error of 3 biological replicates. Asterisks indicate significant differences between *miR156OE* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed by Dunnett's test where * indicates $p<0.05$ and ** indicates $p<0.01$.

3.5.2 *SPL13i* transcriptional changes under heat stress

None of the 18 genes selected for analysis showed significantly different transcription levels under heat stress conditions, although two genes were differentially regulated in *SPL13i* plants under control conditions (Figure 13). The *CCD1* carotenoid metabolism gene was downregulated by 80% and 63% in genotypes 05 and 06, respectively (Figure 13A). *WD40-2* was downregulated by 78% under control conditions in 02 plants (Figure 13B*).*

3.6 Effects of *SPL9* silencing on alfalfa phenotypes

In addition to investigating the effects of *SPL9* downregulation on heat stress, I also characterized *SPL9i* knockdown plants at the phenotypic level after one and two months of growth. I first monitored *SPL9i* alfalfa plants one month after potting to determine the effects of silencing *SPL9* on earlier stages of development. All four *SPL9i* plants that were selected for characterization had significant downregulation of *SPL9* (Figure 8). Surprisingly, all four genotypes also had 37-51% longer primary stems (Figure 14A) while 6C had 20% smaller stem diameter (Figure 14B). All *SPL9i* genotypes had about 20% shorter antepenultimate leaf lengths (Figure 14C) while no significant differences were observed in antepenultimate leaf width (Figure 14D). Genotypes 5C and 11 had 20-25% longer internodes (Figure 14E) but no differences were detected in the number of nodes on the primary stem (Figure 14F). Genotype 6C had twice as many shoots as EV control (Figure 14G). All genotypes had 40-70% higher vegetative (forage) yield (Figure 14H, 14I). Genotypes 5A and 5C had enhanced root biomass, with 20-55% greater FW and 50-90% greater DW (Figure 14J, 14K), but no significant differences were found in root length (Figure 14L).

SPL9i shoot morphology was also monitored after two months of growth. All genotypes had 30-48% longer stems after two months of growth (Figure 15A), recapitulating results obtained from the 1 month study (Figure 14A), and no differences were seen in stem diameter (Figure 15B). Leaf lengths were up to 25% shorter in all genotypes (Figure 15C)

and genotype 6C had narrower leaf width (Figure 15D), whereas no differences were previously observed at 1 month.

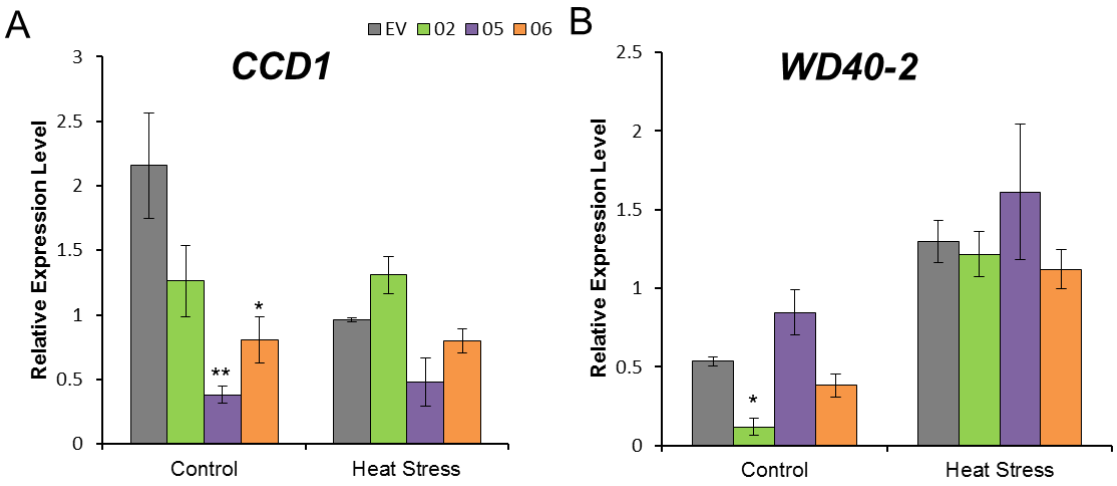

Figure 13. Transcriptional changes in *SPL13i* alfalfa under heat stress

Normalized transcript levels of **A**, *CCD1*; **B**, *WD40-2* genes in *SPL13i* and EV control alfalfa under control and 40°C heat stress conditions. Transcript levels determined by qRT-PCR were normalized using reference genes *CYCLOPHILIN* and *ACTIN*. Normalized transcript levels are reported as mean ± standard error of 4 biological replicates. Asterisks indicate significant differences between *SPL13i* genotypes and EV controls within greenhouse control and heat treated plants as determined by ANOVA followed by Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

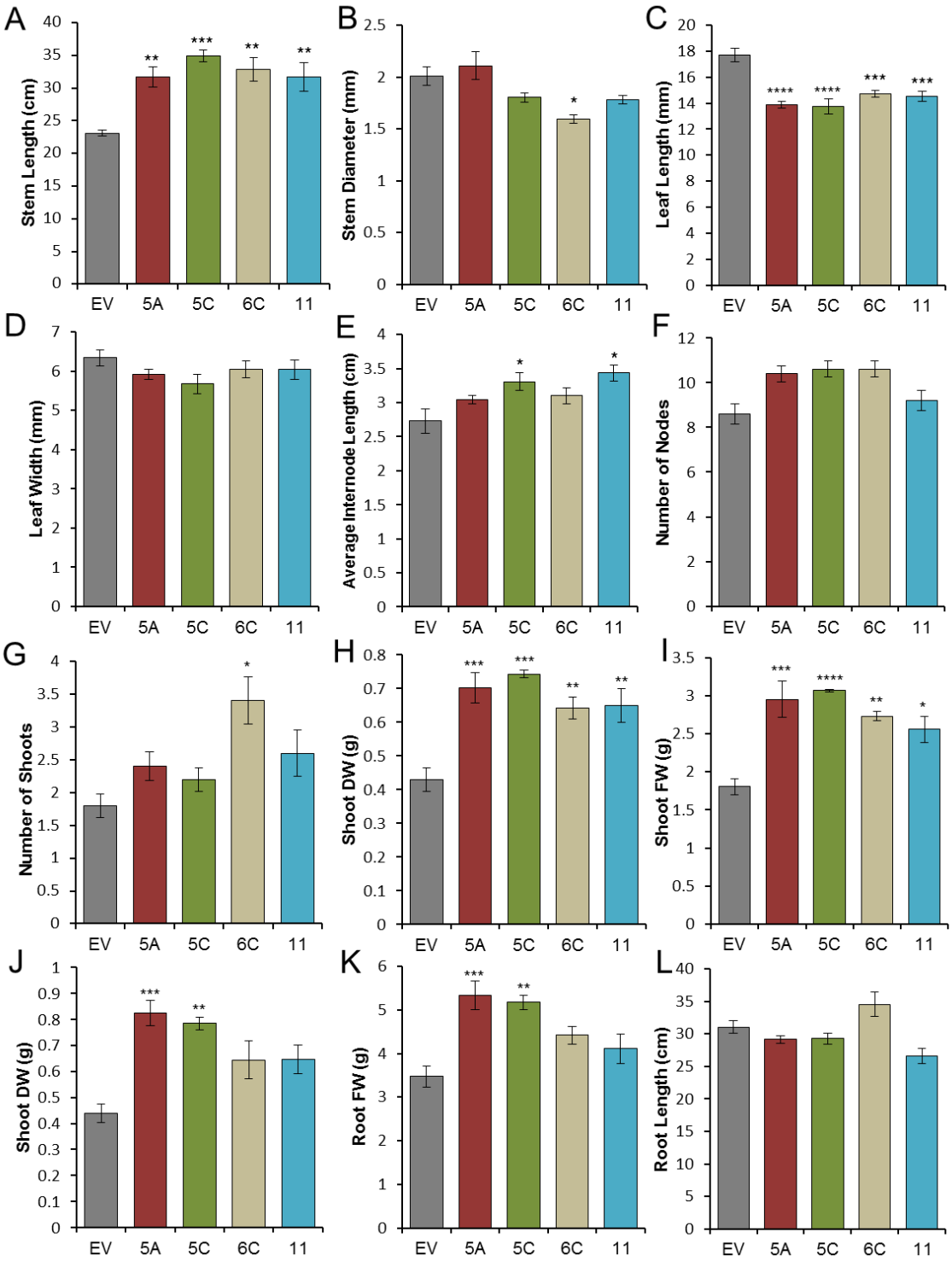
A
Stem Length (cm)
B
Stem Diameter (mm)
C
Leaf Length (mm)
D
Leaf Width (mm)
E
Average Internode Length (cm)
F
Number of Nodes
G
Number of Shoots
H
Shoot DW (g)
I
Shoot FW (g)
J
Shoot DW (g)
K
Root FW (g)
L
Root Length (cm)
EV
5A
5C
6C
11

Figure 14. Phenotypic characterization of one-month-old *SPL9i* alfalfa plants

A, stem length; **B**, stem diameter; **C**, leaf length; **D**, leaf width; **E**, average internode length; **F**, number of nodes; **G**, number of shoots; **H**, shoot dry weight; **I**, shoot fresh weight; **J**, root dry weight; **K**, root fresh weight; **L**, root length of *SPL9i* and EV control plants after 1 month of growth under greenhouse conditions. Data are reported as mean ± standard error of 5 biological replicates. Asterisks indicate significant differences between *SPL9i* genotypes and EV controls as determined by ANOVA followed by Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

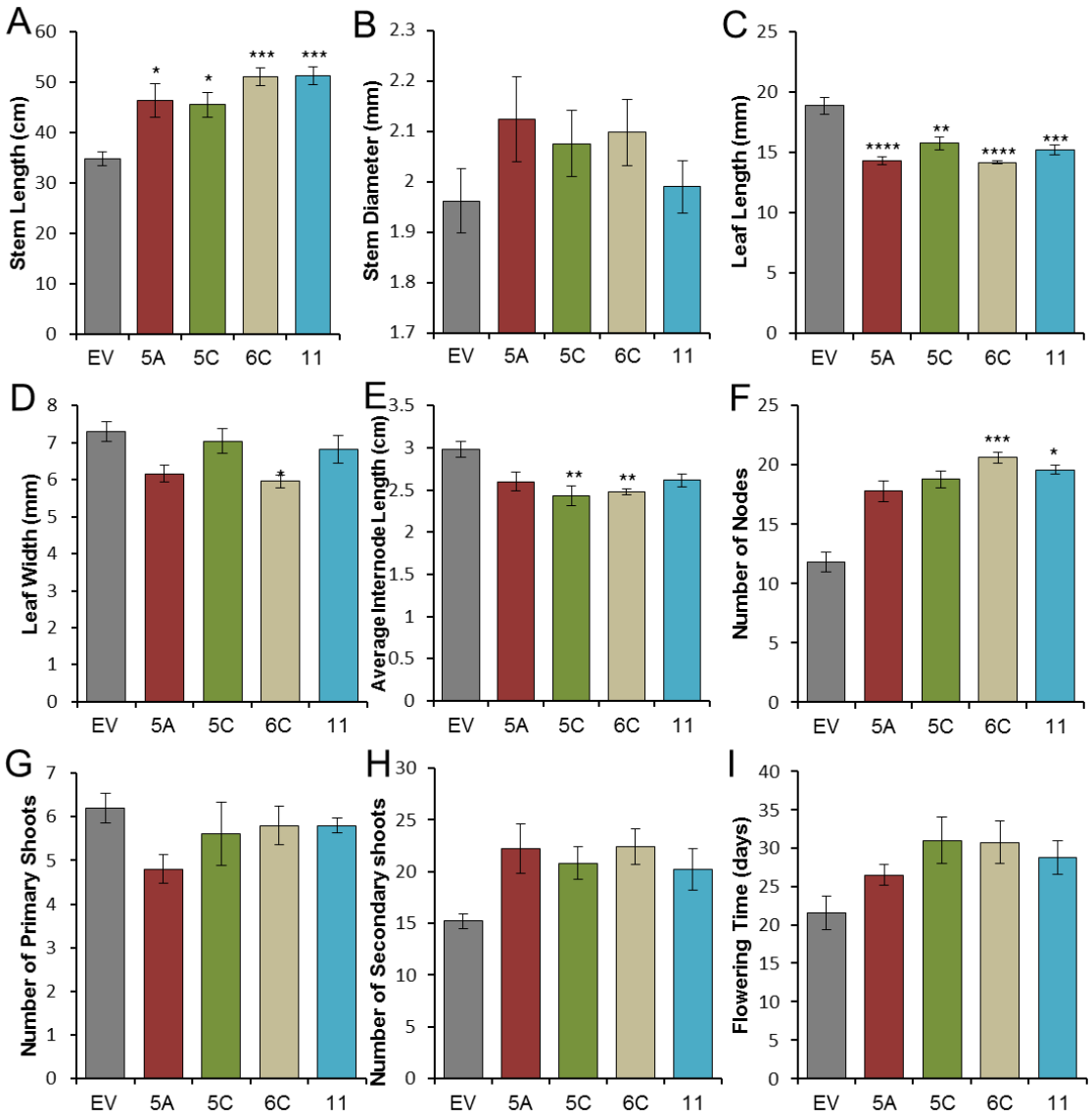

Figure 15. Phenotypic characterization of two-month-old *SPL9i* alfalfa plants

A, stem length; **B**, stem diameter; **C** leaf length; **D** leaf width; **E** average internode length; **F** number of nodes; **G** number of primary shoots; **H**, number of secondary shoots; **I**, flowering time of *SPL9i* and EV control plants after **A-G** 2 months of growth under greenhouse conditions. Data are reported as mean ± standard error of **H** 4 or **A-G** 5 biological replicates. Asterisks indicate significant differences between *SPL9i* genotypes and EV controls as determined by ANOVA followed by Dunnett's test where * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and **** indicates $p<0.0001$.

Genotypes 5C and 11 previously had 20-25% longer internodes (Figure 14E), but genotypes 5C and 6C had 18% shorter internode lengths after 2 months while EV plants had the highest average internode length (Figure 15E). Genotypes 6C and 11 had an increased 7-8 more nodes after two months (Figure 15F). No significant differences were observed in the number of primary or secondary shoots (Figures 15G and 15H) or in flowering time (Figure 15I).

While I did not investigate *SPL9i* plant antioxidant content or TMA, I did notice that *SPL9i* plants grown under control conditions appeared to accumulate more pigments in stems and occasionally on the abaxial surfaces of leaves (Figure 16). The pink and purple pigmentation suggests that *SPL9* downregulation increased the accumulation of anthocyanins and/or proanthocyanidins. Stem pigmentation was consistent and appeared earlier than leaf pigmentation, as seen in nearly all plants of the four genotypes after a month while EV plants remained green (Figure 16A). More dramatic differences in pigmentation were observed in the senescing leaves of some *SPL9i* genotype plants, most notably in genotype 5C (Figure 16B). The leaf pigmentation did not appear consistently after two months of growth but was never observed in EV control plants.

Figure 16. Increased pigmentation in leaves and stems of *SPL9i* alfalfa plants

Increased pigment accumulation in *SPL9i* genotypes. **A**, one-month-old stems; **B**, two-month-old leaves.

Chapter 4

4 Discussion

4.1 Research overview

miR156 expression plays an important role in heat stress tolerance and memory in *Arabidopsis* (Stief et al., 2014), and *miR156OE* increased drought tolerance (Arshad et al., 2017a) as well as yield under salinity stress (Arshad et al., 2017b) in alfalfa. Based on this previous research, I hypothesized that the overexpression of *miR156* in alfalfa would confer increased heat stress tolerance. Due to the conserved nature of the *SPL* genes downregulated by miR156 (Aung et al., 2015c; Gao et al., 2016; Wang and Wang, 2015), I hypothesized that if increased heat stress tolerance in *miR156OE* alfalfa was conserved, it would be through this conserved gene regulation pathway. Recent data within the Hannoufa lab have suggested that the *miR156*/*SPL* gene network may be contributing to the regulation of antioxidants such as anthocyanins (unpublished data, Hannoufa lab) and carotenoids (Arshad et al., 2017a; Wei et al., 2012; Wei et al., 2010).

To investigate the role of miR156 in heat stress response in alfalfa, I first investigated the regulation of *miR156* and its target *SPL* genes under high heat conditions. This analysis revealed that *miR156* was upregulated under heat, leading to the downregulation of some *SPL* genes, which were pursued to further investigate heat stress tolerance. Following that, *miR156OE* and alfalfa plants with RNAi-silenced *SPL* genes were tested for heat stress survival to determine whether the observed expression patterns were contributing to heat stress tolerance. Based on the increased survival of *miR156OE* and *SPL13i* plants, mechanisms that may be contributing to heat stress tolerance were investigated by assessing various parameters of water status, accumulation of antioxidants and gene expression profiles. This revealed that *miR156OE* and *SPL13i* plants were less stressed compared to EV controls and increased gas exchange may be reducing the effects of heat stress by increasing transpiration rates without negatively impacting water retention. Furthermore, differences in non-enzymatic antioxidants and genes associated with antioxidant biosynthesis might be contributing to the increased stress tolerance observed

in *miR156OE* and *SPL13i* alfalfa by reducing the effects of oxidative stress under heat stress conditions. *SPL9i* plants were phenotypically characterized before determining that *SPL9* had no effect on heat stress survival or tolerance.

4.2 *miR156OE* and *SPL13i* contribute to heat stress tolerance in alfalfa

Expression analysis by qRT-PCR revealed that mature *miR156* matching the product from the *miR156d* precursor was indeed upregulated in response to heat stress. This upregulation occurred soon (0.5 hr) after the exposure to heat stress, but then expression reverted to a level similar to that in unstressed control plants. These results suggest miR156 may act relatively early in the heat stress signalling, but may not retain the role of heat stress memory in alfalfa as was observed in *Arabidopsis* (Stief et al., 2014). This expression pattern mirrors that which was found in *Arabidopsis* where an initial large increase after 4 hr of exposure was followed by a tapered but still increased expression of some *miR156* precursors up to 52 hr later (Stief et al., 2014). These results are mirrored in the downregulation of *SPL9* and *SPL13* with a decrease in expression at earlier time points (3 hr) but with no differences after 12 hr. The expressions of *miR156*, *SPL13* and *SPL9* cannot be directly correlated because *miR156* expression was reduced to no significant difference in expression before the first time point of *SPL* gene investigation, but generally followed the same pattern with an earlier change in expression followed by no difference at later time points. These results suggest that miR156 may not be solely responsible for regulating *SPL* genes under extreme heat conditions, especially considering the lack of downregulation in a number of other target *SPL* genes despite an increase in *miR156* expression. This is consistent with other reports that point to *miR156* and *SPL* genes being subject to epigenetic regulation (Kim et al., 2015; Lafos et al., 2011; Xu et al., 2016a; Xu et al., 2016b). For example, Kim et al. (2015) found that the expression of eight *Arabidopsis* SPL genes were downregulated in *HISTONE ACETYLTRANSFERASE OF THE GNAT FAMILY 1* (*HAG1*) mutants in a *miR156* independent manner. HAG1 is a component of histone acetyltransferase (HAT) complexes (Pandey et al., 2002) responsible for acetylating histone lysine residues and is generally associated with transcriptional activation (Berger, 2007). These results directed

my attention towards the manipulation of the downregulated *SPL* genes using RNAi to investigate their role in heat stress tolerance.

The difference between time points in *SPL6* expression suggests there may be diurnal regulation of *SPL6*. Unpublished data from the Hannoufa lab have not yet conclusively connected *SPL6* to any photoperiod-dependent phenotypes or flowering. *SPL6* was not influenced by the *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CONSTANS1* (*SOC1*) genes in *Arabidopsis* and did not show photoperiod sensitive regulation whereas *SPL3*, *SPL4* and *SPL5* did (Jung et al., 2012). I did not find that the alfalfa orthologs of the *FT*/*SOC1* regulated *SPL*s from *Arabidopsis* were differentially regulated over time in this experiment; however, closer inspection of their regulation over a large set of time points would be necessary to definitively determine any diurnal regulation in alfalfa. It is possible *SPL6* may play a role in other photoperiod-dependent traits in alfalfa. *miR156* expression has been associated with light intensity in *Arabidopsis* (Xie et al., 2017) and it is possible that this may be contributing to the differential regulation of *SPL6* over time. Further profiling of *SPL6* regulation over time in WT alfalfa may help determine if it is indeed regulated in response to the photoperiod.

Following expression analysis, I investigated the effects of *miR156* overexpression using *miR156OE* alfalfa that were exposed to 40°C over the course of 6 days compared to EV controls based on phenotypically scoring the plants for damage. These results conclusively showed that the overexpression of *miR156* increased heat stress tolerance in alfalfa. Taken with the significantly increased chlorophyll content in A8a and A8 relative to the EV control, the increased survival of *miR156OE* genotypes suggest that the moderate *miR156* expression levels in the two genotypes (Aung et al., 2015b) confer more heat stress tolerance than is found in controls and extremely high *miR156* expressers (e.g. A11). Similar results were also observed in alfalfa plants under drought stress (Arshad et al., 2017a), where moderate expressers A8 and A16 (Aung et al., 2015b) had increased survival compared to WT plants whereas higher expressers A11 and A17 (Aung et al., 2015b) did not. It is possible that large increases in the expression of *miR156* too quickly increase biomass, limiting the other positive effects of other phenotypic advantages such as small leaf size and lower internode length. A8a and A8

genotypes do not have significantly increased yield until later stages of development, whereas A11 plants showed increased yield under control conditions after 2 months (Aung et al., 2015b). There may also be levels of *miR156* overexpression/*SPL* downregulation that severely disrupt the transcriptome and inhibit stress response or development in general.

Previous research showed that *SPL13i* plants had increased drought tolerance (Arshad et al., 2017a). This finding combined with the reduction in *SPL13* expression under heat stress led me to investigate the survival of *SPL13i* plants under heat. The results of the heat stress survival suggest that *SPL13* downregulation may be contributing to the miR156-regulated heat stress tolerance. Interestingly, the subsample of heat stress-treated plants taken for chlorophyll determination showed that chlorophyll was higher only in genotype 02, the only genotype that did not have significantly better heat stress tolerance. This may be due to randomly subsampling from populations in which approximately half of the plants suffered heavily from chlorosis and scorching while the other half did not. While this result does not overwhelmingly support the results of the chlorotic/wilted/scorched scoring analysis, it also does not discount it entirely. It is possible the increased chlorophyll concentrations were a result of normalizing the chlorophyll content to FW rather than DW. Similar to my results observed with *miR156* expression, it is possible that differences in the *SPL13* expression levels of individual *SPL13i* genotypes are contributing to heat stress tolerance in a similar manner as they respond under drought stress (Arshad et al., 2017a) and that the expression of *SPL13* in genotype 02 plants is reduced beyond the expression levels that are beneficial for abiotic stress tolerance. Genotype 02 plants did not survive as well as other *SPL13i* plants and it is likely after 192 hr of heat stress they are drier than the other *SPL13i* genotypes. As a result one would expect a lower FW to DW ratio, increasing the concentration of chlorophyll per gram of FW than in the plants that are less wilted. If these 02 plants have lower water content but have sufficiently protected chloroplasts from oxidative damage, it is possible they might retain higher chlorophyll content per gram fresh weight compared to unstressed controls than the more chlorotic EV plants or the healthier 05 and 06 genotypes.

While SPL9 is associated with drought and salinity stress response in *Arabidopsis* and rice (Cui et al., 2014), my investigation of *SPL9i* plants did not reveal differences in heat stress tolerance. *SPL9i* plants also did not display many of the phenotypes observed in *miR156OE* plants other than increased yield and reduced leaf size. Average internode length was not reduced until two months of growth. While there was increased root mass in some cases, the increase in shoot mass was more consistent. I found no differences in LWP or stomatal conductance, so it is possible that the positive effects of evaporative cooling that *miR156OE* and *SPL13i* may have experienced with increased gas exchange would not have affected *SPL9i* plants. It is possible that any biochemical advantages *SPL9i* plants have in heat stress tolerance, such as the apparent increase in accumulation of anthocyanins, were negated by their observed developmental and morphological disadvantages. Alfalfa and other medics are believed to be more resilient to water stress than other plants due to the evolution of smaller leaves, reduced internode length, and reduced stem height (which reduces water loss), and increased biomass allocation to roots, increasing access to available water in the soil (Small, 2011). *SPL9i* plants were taller and appeared to allocate increased biomass to shoots more consistently than roots after one month, which may have contributed to increased water loss and susceptibility to heat stress. Despite having smaller leaves, there are also more leaves transpiring, with increased transpiration required to move water up the taller shoots.

Taken together, these results suggest that *miR156* is involved in the heat stress tolerance of alfalfa through the regulation of *SPL13*, while *SPL9* does not appear to contribute significantly to the heat stress tolerance observed in *miR156OE* alfalfa. While *SPL9* expression has been associated with decreased *DFR* expression and stress tolerance in *Arabidopsis* and rice (Cui et al., 2014; Gou et al., 2011), recent unpublished results from Dr. Hannoufa's lab suggest that SPL13 acts as a transcriptional repressor of *DFR* and the phenylpropanoid pathway-associated MYB transcription factors in a similar manner. This suggests that *SPL13* may play a significant role in increasing the accumulation of flavonoid antioxidants observed in *miR156OE* alfalfa. Taken with the apparent anthocyanin accumulation in *SPL9i* plants, it is possible that *SPL13* and *SPL9* negatively regulate anthocyanin biosynthesis redundantly.

It is possible that some of the inconsistent and inconclusive responses to heat stress in this study resulted from high variation within genotypes and low statistical power due to small sample sizes (3-8). These negative results should be interpreted with caution. To elucidate the physiological, biochemical and molecular effects of modulating the miR156/SPL network in alfalfa's heat stress response, the experiments should be repeated with larger sample sizes.

4.3 Effects of modulating the *miR156*/*SPL* gene network on alfalfa water status

To determine the extent to which miR156 and target *SPL* genes alleviate heat stress symptoms associated with water loss, various measures of water status were investigated. While no differences were detected in response to heat-stress in any of the transgenic plants, the highest *miR156* expresser, A11, and the lowest *SPL13* expresser, 06 (Arshad et al., 2017a), had increased RWC under control conditions. The initial increase in RWC may contribute to heat stress survival; however, it is unlikely that a difference of around 3% is biologically significant given the eventual loss of greater than 50% RWC. I found there was high variation in RWC within genotypes under heat stress conditions, with the symptoms of heat stress progressing unevenly in the small sample size. LWP is used to infer the degree to which plants are stressed under water-limiting conditions (Morgan, 1984), including the increased potential evapotranspiration associated with high temperatures (Rind et al., 1990). Increased transpiration also results in evaporative cooling that leads to marked reductions in leaf temperatures compared to temperatures of leaves with limited transpiration (Urban et al., 2017). LWP of heat-stressed plants revealed that *miR156OE* plants appeared less stressed compared to EV controls; however, these results were not supported by higher stomatal conductance that would be expected to increase leaf water potential. Results with *SPL13i* plants supported data obtained from *miR156OE,* where I found increased LWP and increased stomatal conductance under heat stress. Additionally, *SPL13i* plants had reduced LWP under control conditions, possibly suggesting that *SPL13i* plants would have an initial advantage in controlling water loss under heat stress conditions. In addition to being less stressed, increased gas exchange may be reducing leaf temperatures by evaporative cooling, which would reduce the

impacts of heat stress and reduce ROS production. Measuring differences in leaf temperatures would help definitively determine if evaporative cooling plays a substantial role in the heat stress tolerance of *miR156OE* and *SPL13i* alfalfa.

The lack of difference, or reduced performance in the case of genotype 6C, between *SPL9i* and control plant water status measurements further support that the differences in heat stress tolerance in *miR156OE* plants were not mediated by the differential expression of *SPL9* seen in the expression analysis, supporting the results of the survival analysis. Without increases in LWP or stomatal conductance, it is unlikely that the *SPL9i* plants would have benefitted from evaporative cooling provided by increased transpiration. Increasing transpiration in *SPL9i* plants would also have been costly considering that they did not have higher RWC than EV controls either. No beneficial differences were observed for *SPL9i* plants in RWC or LWP, while *miR156OE* and *SPL13i* plants maintained similar RWC compared to EV controls while they maintained increased gas exchange.

It is possible that, since ROS plays a key role in hydroactive guard cell closure (Kohler et al., 2003; Zhang et al., 2001) and therefore stomatal conductance (Kaiser, 2009), the alleviation of oxidative stress in *miR156OE* plants and *SPL13* may be contributing to the increased LWP and increased stomatal conductance observed in these plants under heat stress conditions. It is also possible that increased antioxidant capacity is reducing the amount of ROS production, leading to reduced signalling and increased stomatal aperture. Regardless of which is a cause and which is an effect, if any, increased LWP would suggest reduced oxidative stress in alfalfa and increased evaporative cooling.

4.4 *miR156* and *SPL13* influence the accumulation of antioxidants in alfalfa under heat stress

Due to the increased survival of *miR156OE* and *SPL13i* alfalfa despite the lack of difference in their RWC, I investigated the concentrations of antioxidants to determine if differences in survival may be influenced by the ability of these plants to deal with the oxidative stress associated with heat. The *miR156*/*SPL* network had previously been associated with stress response via antioxidant production (Arshad et al., 2017a; Arshad

et al., 2017b), including increased biosynthesis of anthocyanins (Gou et al., 2011) and carotenoids (Wei et al., 2012; Wei et al., 2010). The role of anthocyanins in *miR156*-mediated abiotic stress tolerance has further been supported by unpublished results from the Hannoufa lab that directly connect *SPL*s to phenylpropanoid pathway-associated genes such as *DFR*.

The observed increase in the antioxidant capacity of *miR156OE* and *SPL13i* plants suggests they are better equipped to deal with the oxidative damage induced by heat stress. Generally, antioxidant potential and TPC are correlated (Piluzza and Bullitta, 2011); however, in this investigation I found no differences in TPC other than in one *miR156OE* genotype under heat stress compared to the EV control. It is possible that the shifting of groups or individual compounds within the broad class of phenolic compounds is resulting in increased antioxidant potential. Specific classes of antioxidants were subsequently investigated.

More closely investigating the reliability of TFC measurement by aluminum complexation revealed a bias toward flavonols and flavones rather than flavanones or flavanonols (dihydroflavonols) (Chang et al., 2002; Pękal and Pyrzynska, 2014). Unpublished results from the Hannoufa lab suggest that *SPL13* binds the promoter region of *DFR* and directly represses its expression. DFR plays a vital role in anthocyanin production by processing dihydroflavonols into leucoanthocyanidins (Davies et al., 2003), an anthocyanin precursor. DFR competes for its substrate with flavonol synthase (FLS), which directly produces flavonols (Davies et al., 2003). It is possible that the observed reduction of TFC in *miR156OE* plants is due to the increased processing of dihydroflavonols by DFR, reducing the production of flavonols by FLS that are better detected by aluminum complexation. The increases observed in anthocyanin content despite the reduction of TFC support the idea that these results may be due to the difference in quantification of flavonoid rather than real differences in TFC. While qRT-PCR results did not significantly confirm this relationship, *DFR* expression was numerically highest in *miR156OE* genotypes.

Additionally, anthocyanin biosynthesis is regulated in part by MYB transcription factors, including production of anthocyanin pigment 1 (PAP1) in *Arabidopsis* (Gou et al., 2011). In this case, SPL9 regulates *DFR* and other anthocyanin-associated genes by disrupting the formation of the MYB-bHLH-WD40 complex. The MYB-bHLH-WD40 complex activates genes such as *DFR* and *FLAVONOID 3'-HYDROXYLASE* (*F3'H*) and SPL9 can interfere by binding to the promoter regions of these genes in close proximity to the MYB transcription factor, competing with the bHLH and WD40 components of the complex (Gou et al., 2011). Further studying the effects of miR156 on anthocyanin biosynthesis and stress tolerance, Cui et al. (2014) found that *miR156* overexpression reduced *SPL9* expression, increased anthocyanin content and resulted in the increased abiotic stress tolerance of *Arabidopsis*. Furthermore, this effect was conserved in rice as the increased expression of *miR156* in rice resulted in increased tolerance to salinity and drought (Cui et al., 2014).

Phenotypically, *SPL13i* plants and *SPL9i* plants had increased pigment accumulation. The red/purple pigments accumulated in *SPL13i* plant shoots are most likely anthocyanins or proanthocyanidins. *SPL9i* plants had similar accumulation of pigment in their stems and also had purple pigmentation on the adaxial surfaces of senescing leaves. While the *SPL9* plants had pigmentation suggesting accumulation of phenylpropanoid pathway products such as anthocyanins, they did not fare better under heat stress conditions than EV controls, suggesting there is more to heat stress survival than just antioxidant capacity. It is possible that the increased antioxidant potential is contributing while evaporative cooling is reducing the overall effects of heat stress by lowering tissue temperatures and together result in the increased heat stress tolerance observed in *miR156OE* and *SPL13i* plants. Further investigating the TEAC and contents of various antioxidants in *SPL9i* plants would help determine the role of *SPL9* in antioxidant accumulation.

While I observed no differences in β-carotene or lutein concentrations, and the presence of other xanthophylls was inconsistent in retention time and therefore unable to be quantified, it is possible that the insignificant increases that were seen in β-carotene and lutein contents in heat-treated *miR156OE* plants were contributing to the total antioxidant

capacity that was significantly increased. Optimizing HPLC conditions to better analyze xanthophylls such as zeaxathin and neoxanthin, both of which are substrates for *NCED3* and precursors of xanthoxin, may better elucidate the role of the *miR156*/*SPL* network in carotenoid regulation and its relationship to abiotic stress in alfalfa.

Based on these results, the increased biosynthesis of the powerfully antioxidant anthocyanins (Agati et al., 2012; Ferreres et al., 2011; Nakabayashi et al., 2014) within the total phenolic compound pool resulting in increased antioxidant potential supports the explanation for the ability of *miR156OE* and to a lesser extent *SPL13i* to withstand heat stress. It is possible that carotenoids as well as some other uninvestigated antioxidants also contribute to the observed differences in antioxidant capacity. None of the genes encoding antioxidant enzymes investigated in this study had differences in expression, and metabolite extractions yielded differences in antioxidant potential, suggesting that the differences in the ability of *miR156OE* and *SPL13i* plants would be due to non-enzymatic antioxidants.

4.5 Effects of *miR156*/*SPL* gene network on the regulation of stress- associated genes in alfalfa

The more consistent and stress responsive nature of genes differentially regulated in *miR156OE* support the roles of these genes in stress tolerance of *miR156OE* plants. The heat stress-induced transcriptional changes observed in *miR156* and *SPL13i* plants suggest that differences in the metabolism of non-enzymatic antioxidants may contribute to differences in heat stress tolerance observed in *miR156OE* and *SPL13i* genotypes.

CCD1 and *NCED3* are carotenoid dioxygenases responsible for the cleavage of carotenoids to produce apocarotenoids (Harrison and Bugg, 2014). Such apocarotenoids include the abscisic acid (ABA) precursor xanthoxin (Iuchi et al., 2001; Qin and Zeevaart, 1999; Schwartz et al., 1997), a product of the more specific *NCED3*. ABA production via *NCED3* plays a vital role in water stress response (Iuchi et al., 2001; Qin and Zeevaart, 1999). Increased *NCED3* expression under heat should yield increased ABA and therefore induce many physiological changes associated with reducing water loss including hydroactive guard cell closure (Sah et al., 2016). While this result contrasts

with the water status results obtained after four days of heat stress, it is possible that the increased expression of *NCED3* after 24 hr of heat stress may result in reduced long-term water loss and allow for relatively increased levels of transpiration and leaf temperature. Reduced long-term water loss could allow for less stressed plants after 96 hr and result in the significantly increased LWP/stomatal conductance of *miR156OE* and *SPL13i* plants and therefore increased evaporative cooling.

Other apocarotenoids produced by the cleavage of the more promiscuous CCDs, such as CCD1, often yield molecules vital to cell-to-cell signalling as well as volatile semiochemicals (Hou et al., 2016). Carotenoid cleavage products such as β-ionone, β-cyclocitral and other emerging apocarotenoid signalling molecules (ACSs) (Hou et al., 2016; Rubio-Moraga et al., 2014) are products of enzymatic cleavage as well as the oxidative cleavage of carotenoids (Sommerburg et al., 2003). β-cyclocitral and other ACSs act as signalling molecules in response to oxidative stress, inducing transcriptional changes (Hou et al., 2016; Ramel et al., 2012). It has also been suggested that products of *CCD*s are used for chloroplast status signalling and for inducing the regulation of genes associated with chloroplast function (Rottet et al., 2016). Ordoudi et al. (2009) also showed that extracts high in apocarotenoids had similar *in vivo* antioxidant and ROS scavenging potential to that of phenolic compounds, suggesting that the processing of the antioxidant carotenoids through increased cleavage may not reduce the antioxidant capacity of cells. Previously, *CCD8* was found to be downregulated in *SPL13i* alfalfa and upregulated in alfalfa overexpressing *SPL13*, further supporting a role for the *miR156*/*SPL* network in carotenoid biosynthesis in alfalfa (Gao et al., 2018).

The upregulation of *MYB53* in *miR156OE* plants supports previous findings that *SPL13OE* reduces *MYB53,* despite not being supported in *SPL13i* plants in this study or previous studies (Gao et al., 2018). It is possible that *MYB53* is regulated redundantly by miR156-targeted *SPL* transcription factors, similar to other miR156-induced phenotypes such as flowering time in *Arabidopsis* (Xu et al., 2016b). Considering that SPL transcription factors share a common consensus DNA binding element, it might be expected that there is some redundancy to the regulation of downstream genes (Mao et al., 2016). The R2R3 class of MYB transcription factors regulate various developmental

and metabolic processes (Kranz et al., 1998), including phenylpropanoid metabolism and anthocyanin biosynthesis. A BLAST search of *MYB53* in *M. truncatula* sequences revealed that they are closely related to *Arabidopsis* R2R3 *MYB* genes in subgroup 10 (Kranz et al., 1998). In *Arabidopsis*, *myb9* and *myb107* mutants, members of subgroup 10, accumulated relatively high levels of proanthocyanidins in seed coats (Lashbrooke et al., 2016) while peach (*Prunus persica*) *PpMYB9* was identified as a potential anthocyanin activator (Zhou et al., 2016). These results suggest that R2R3 *MYB* subgroup 10 genes, including *MYB53*, are associated with the phenylpropanoid pathway and the biosynthesis of anthocyanins but may not be definitively categorized as activators or repressors of anthocyanin synthesis. Taken with the increased monomeric anthocyanin contents in *miR156OE* genotypes under heat stress, the upregulation of *MYB53* under heat stress in *miR156OE* plants supports *MYB53* as an anthocyanin activator in alfalfa. It is possible that MYB53 plays a similar role in alfalfa to that of PAP1 and other anthocyanin-associated MYB transcription factors in the accumulation of anthocyanin (Cui et al., 2014; Gou et al., 2011). In addition to regulating phenylpropanoid pathway-associated genes by interacting with MYB transcription factors (Gou et al., 2011), the upregulation of *MYB53*, and previous studies in the lab connecting *SPL13* and *MYB53* (Gao et al., 2018), suggest that miR156 also contributes to anthocyanin biosynthesis by indirectly regulating *MYB53*.

bZIP genes are a diverse group of transcription factors found across Eukaryota (Amoutzias et al., 2007). *bZIP* genes in plants serve diverse roles clustered into 10 groups in *Arabidopsis* with a few unique *bZIP*s as well (Jakoby et al., 2002). Different *bZIP* genes are associated with growth and development; abiotic and biotic stresses; cell signalling transduction; and photomorphogenesis (Jakoby et al., 2002). *bZIP*s in plants include the *TGA3* gene investigated here; *TGA3* is part of the *bZIP* group D genes that are associated with development and biotic stress response (Jakoby et al., 2002). This *bZIP* gene had increased expression in *miR156OE* alfalfa under salinity stress (Arshad et al., 2017b). *TGA3* is also closely related to *TEOSINTE GLUME ARCHITECTURE1* (*TGA1)*, which is targeted directly by miR156 in corn (Chuck et al., 2007). The differential expression of *TGA3* under heat stress would likely contribute to a suite of transcriptional

changes in response to heat stress (Jakoby et al., 2002). Considering the difference in plant development associated with *miR156OE* (Aung et al., 2015c), it follows that transcription factors associated with developmental regulation would be differentially expressed.

The regulation of various *WD40* genes has been associated with stress responses (Mishra et al., 2014; Mishra et al., 2012), the accumulation of anthocyanins through the MYB-bHLH-WD40 complex (Gou et al., 2011) and regulation by miR156 in alfalfa (Arshad et al., 2018). Recently, Arshad et al. (2018) showed that the regulation of *WD40-2* by miR156 under drought stress contributed to drought tolerance in alfalfa. Therein a potential miR156 recognition site was identified and cleavage was detected albeit upstream of the recognition site, a phenomenon previously seen in canonical miR156 targets (Gao et al., 2016; Jiao et al., 2010). While *WD40-2* expression was unaffected in *miR156OE* in this experiment, its downregulation in *SPL13i* genotype 02 suggests miR156 may regulate *WD40-2* both directly and indirectly. Stress-responsive *WD40* genes may also be indirectly regulated by miR156 by the flowering associated *APETELA2* (*AP2*) genes (Mishra et al., 2012), which are in turn regulated by miR172 (Aukerman and Sakai, 2003; Schmid et al., 2003). As previously discussed, expression of *miR156* and *miR172* are linked and inversely proportionate. It is possible the regulation of this *WD40* gene is influenced indirectly by miR156 through SPL13 but many *WD40* genes are influenced by several environmental stimuli and are responsive to ABA (Mishra et al., 2014). This may also connect the expression of *WD40-2* to the expression of *NCED3*; however, no differences in *NCED3* expression were seen in *SPL13i* plants. Despite *WD40-2* contributing to *miR156OE*-associated drought stress tolerance in alfalfa (Arshad et al., 2018), it was only regulated in the single heat-susceptible *SPL13i* genotype under control conditions suggesting it is not contributing to *miR156OE*-associated heat stress tolerance. Due to the large number of *WD40* genes in plants (e.g. more than 250 in *Arabidopsis*) (van Nocker and Ludwig, 2003), a more thorough molecular investigation under heat stress in alfalfa would be required to determine if and to what extent their expression plays in heat stress tolerance definitively.

The regulatory patterns of investigated genes in response to heat stress that were observed in *miR156OE* plants were not detected in *SPL13i* plants. This suggests that miR156 is not regulating these heat stress responsive genes through *SPL13* and that the contributions to heat stress tolerance by *SPL13* downregulation may not be heat stress-specific but rather a result of molecular and developmental changes providing inherent stress tolerance advantages. However, the regulation of *WD40-2* suggests that miR156 may be indirectly regulating *WD40-2* through SPL13 in addition to regulating it directly (Arshad et al., 2018). Since targets outside of the *SPL* gene family, such as *WD40-2* (Arshad et al., 2018), appear to affect abiotic stress tolerance in alfalfa, the regulation of genes outside the *SPL* gene family may be similarly contributing to heat stress tolerance.

While *sHSP*s and *APX2* were downregulated in response to the overexpression of heat stress memory-associated *SPL* genes in *Arabidopsis* (Stief et al., 2014), I observed no differences in any of the *sHSP*s investigated in this study. The *SPL*s that Stief et al. (2014) found necessary for heat stress memory in *Arabidopsis* are more closely related to the alfalfa *SPL12* (Gao et al., 2016), which was not differentially regulated under heat stress in alfalfa. While the list of genes investigated here was by no means exhaustive, it was targeted to genes that had previously been associated with miR156-mediated stress tolerance in alfalfa (Arshad et al., 2017a; Arshad et al., 2017b; Gao et al., 2018) or heat stress tolerance in *Arabidopsis* (Stief et al., 2014). My results suggest that the effect of *miR156* modulation in alfalfa heat stress tolerance is not necessarily through HSF-mediated heat stress response or via the regulation of antioxidant enzymes, but by the regulation of physiological responses and the accumulation of non-enzymatic antioxidants. Transcriptome analysis through RNA sequencing and extensive gene ontology analysis would be necessary to draw this conclusion definitively, but none of the results here contradict it.

4.6 *SPL9* influences on alfalfa growth and morphology

SPL9 expression appears to be associated with shoot and leaf development, as well as with flowering time in alfalfa. Previous research showed SPL9 to affect shoot development (Schwarz et al., 2008), plastochron (Wang et al., 2008) and leaf morphology

(Wu et al., 2009) while influencing phase changes (Xu et al., 2016b) and flowering (Wu et al., 2009; Xu et al., 2016b) via its downstream activation of *miR172* (Wu et al., 2009). Here, stem length was increased after one and two months of growth in all genotypes. This change in stem length was unexpected considering *miR156OE* plants reduce stem length while increasing node number, resulting in reduced internode length (Aung et al., 2015b). These results suggest SPL9 has an effect on the plastochron in alfalfa as well, as after two months of growth, two genotypes had an increased number of nodes, and controls consistently had the lowest number of nodes. Average internode changes were not consistent, with initial increases followed by decreases after two months. It is possible that SPL9 is affecting the internode length by regulating the plastochron and shoot development, as increases in stem length were more consistent. Differences observed in stem diameter that were detected after one month were likely influenced by the stem diameter of the initial cutting.

Results from this study suggest that, similar to previous results in *Arabidopsis* (He et al., 2018), SPL9 affects alfalfa trifolia leaf length, therefore reducing the leaf area, confirming its role in leaf morphology in the model legume *Lotus japoicus* (Wang et al., 2015). Previous results in *L. japonicus* also showed that *miR156* overexpression resulted in smaller leaves (Wang et al., 2015). The effects of *SPL9* downregulation appear to affect the leaf length more than width, with shorter leaf length in all genotypes investigated after both one and two months of growth, whereas only 6C plants had narrower leaf width and only after two months of growth.

Despite the numerical increase in flowering time across all genotypes, I found no significant differences in flowering time in *SPL9i* alfalfa. Results from Xu et al. (2016b) suggest that effects of *SPL* genes may act redundantly to control flowering time in *Arabidopsis*, with significant differences in flowering time requiring double or triple mutant lines. It is possible that similar redundancy in *SPL* gene function prevents the delays in flowering time that *miR156OE* alfalfa showed (Aung et al., 2015b).

While the concentration of antioxidants was not investigated in *SPL9i* plants, phenotypically, leaves of *SPL9i* also turned purple, similar to unpublished results from

the Hannoufa lab in which *SPL13i* had red/purple pigmented stems. Purple pigmentation is a hallmark of anthocyanin and proanthocyanidin accumulation. The stress response associations of *SPL9* have largely been attributed to increased anthocyanin production via increased *DFR* expression in *Arabidopsis* (Gou et al., 2011).

4.7 Conclusion

While miR156 was known to be involved in alfalfa drought and salinity tolerance (Arshad et al., 2017a; Arshad et al., 2017b), the results of this study suggest that *miR156* expression is naturally induced to mitigate the effects of heat stress in alfalfa (Figure 17). *miR156OE* alfalfa have phenotypes associated with heat stress tolerance and increased potential evapotranspiration such as reduced plant height and increased root length that may contribute to heat stress tolerance. The *miR156*-associated heat stress tolerance appears to be at least in part a result of the downregulation of *SPL13* by miR156 while there is no evidence to support the contribution of *SPL9* to heat stress tolerance despite its regulatory patterns and apparent accumulation of anthocyanins.

Previous research has suggested that the miR156/SPL network improves stress tolerance by relieving oxidative stress induced by abiotic stress (Arshad et al., 2017a; Arshad et al., 2017b; Cui et al., 2014) which is supported by my results that *miR156OE* and *SPL13i* alfalfa accumulate higher antioxidant capacity and anthocyanin content under heat stress, similar to results in *Arabidopsis* under control conditions (Gou et al., 2011). In addition to biochemical advantages that may assist in heat stress tolerance, the upregulation of *miR156* and subsequent downregulation of *SPL13* appear to reduce the stress response in alfalfa, allowing plants to continue to transpire at higher rates, potentially reducing the severity of heat stress by evaporative cooling, while not affecting water content. It’s

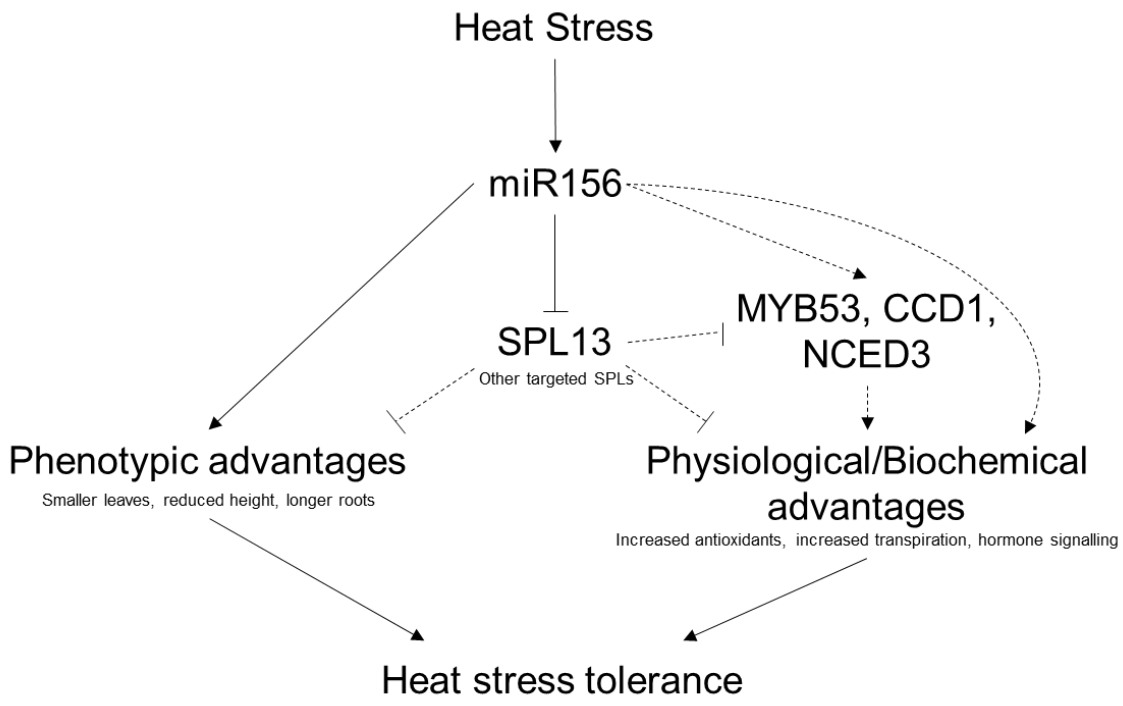

Figure 17. A Proposed model of *miR156*/*SPL*-regulated heat stress response in alfalfa

Solid lines indicate an evidence-supported pathway. Dashed lines indicate a suggested pathway. Arrows indicate upregulation or enhancement and bars indicate downregulation or repression.

possible that the induced biochemical and physiological changes that contribute to increased heat stress tolerance are mediated in part by genes that regulate antioxidant biosynthesis or genes associated with hormone signalling. Together, these results suggest that while the *miR156*/*SPL* gene network does not affect the typical HSF-mediated heat stress response, its effect on physiology and metabolism help mitigate the effects of heat stress at the physiological level. Furthermore, the miR156/SPL network may be useful for alfalfa with increased tolerance to heat stress in addition to other previously investigated abiotic stresses.

Chapter 5

5 Future Directions

Looking forward, further research on *SPL* target genes, and specifically *SPL13*-regulated genes associated with stress response would provide a better understanding of the role of the *miR156*/*SP*L network in stress response. While a good start, the genes tested in this study were by no means an exhaustive list. Next-generation sequencing data comparing the transcriptome profiles of *miR156OE* or *SPL13i* genotypes would better identify the genes and pathways associated with *miR156*/*SPL* network stress response.

The analysis of various metabolites in the phenylpropanoid pathway in *miR156OE* and *SPL13i* plants by more specific techniques, such as LC-MS, might help elucidate the role of the *miR156*/*SPL* gene network in anthocyanin biosynthesis. Holistic and precise metabolomic investigation may identify antioxidants other than anthocyanins contributing to differences in antioxidant potential and also uncover the role of *miR156*/*SPL* in carotenoid metabolism. It may also be useful to analyze the antioxidant content of stems, as *SPL13i* and *SPL9i* both show pigmentation of maturing stems. The accumulation of anthocyanins, especially proanthocyanidins, in alfalfa forage would represent a major advance in quality improvement.

Modulating the expression of multiple *SPL*s at once may help identify any functional redundancies within the *SPL* gene family in alfalfa. Using the expression of *miR156*/*SPL* genes for as molecular markers for the selective breeding or the genetic improvement of alfalfa may generate plants that have better yield and improved stress tolerance. Investigating *miR156OE* and *SPL13i* plants for resistance to other common abiotic and biotic stresses would determine if there are any serious pitfalls to increasing *miR156* expression or silencing *SPL13*, and thus such analysis should be undertaken before applying this system to commercial field production of alfalfa.

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Appendices

Appendix A

SPL9 RNAi construct

Destination vector: pHellgate12

Promoter: 35SCaMV

Host strain: Agrobacterium (GV3101)

Antibiotic Resistance: Kanamycin and Rifampicin

Insert Description: SPL9 gene specific primers (SPL9RNAi_F-CACC CTC TCT CTT CTG TCA AAT CAA ACA TGG G and SPL9RNAi_R-TTA CAG TGA CCA TTG AGA AGA TTC ATA GG) were designed based on M. sativa NGS sequences. These primers were used to amplify SPL9 (300 bp) sequence from alfalfa (*M. sativa*), and the fragment was finally cloned into pHELLGATE12 destination vector for to generate SPL9RNAi construct.

SPL9RNAi (Clone 9)

attR1 and attR2 REGIONS REVERSE REPEATS OF SPL9 (300 bp) PART OF PDK INTRON *PART OF CAT INTRON*

Note: Whole sequences of PDK and Cat introns were not sequenced (/…/ refers to gaps)

GAAGGATCTTACTAAGAGTCATTTCATTTGGAGAGGACACGCTCGAGACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCGCCCCCTTCA
CCATGGAGACAAGAAGGTCAGAGGGAAAAATAAGTTTGAAGTACAAAGAGGATCATGAGGAGGAAGAAGAGGAAGAGGATACGGATTTTG
AAGAGGAAGAGGATGGTAGAAGGAAGAGGGTAGTGACAGATCTCTATAGCAAGAGAAGTTCCAAAAAAGCTGGAAGCTCAAATATTCCAC
CTTGTTGTCAAGTGGAGAATTGTGATGCTGATCTTAGTGAAGCTAAGCAATATCACCGGAGACATAAGGTCTGTGAGTATCATGCCAAAG
CAAGGGTGGGCGCGCCGACCCAGCTTTCTTGTACAAAGTGGTCTCGAGGAATTCGGTACCCCAGCTTGGTAAGGAAATAATTATTTTCTT
TTTTCCTTTTAGTATAAAATAGTTAAGTGATGTTAATTAGTATGATTATAATAATATAGTTGTTATAATTGTGAAAAAATAATTTATAAA
TATATTGTTTACATAAACAACATAGTAATGTAAAAAAATATGACAAGTGATGTGTAAGACGAAGAAGATAAAAGTTGAGAGTAAGTATAT
TATTTTTAATG/……../*CCATAACCAATCAATTAAAAAATAGATCAGTTTAAAGAAAGATCAAAGCTCAAAAAAATAAAAAGAGAAAAGG*
*GTCCTAACCAAGAAAATGAAGGAGAAAAACTAGAAATTTACCTGCACAAGCTTGGATCCTCTAGACCACTTT*GTACAAGAAAGCTGGGTC
GGCGCGCCCACCCTTGCTTTGGCATGATACTCACAGACCTTATGTCTCCGGTGATATTGCTTAGCTTCACTAAGATCAGCATCACAATTC
TCCACTTGACAACAAGGTGGAATATTTGAGCTTCCAGCTTTTTTGGAACTTCTCTTGCTATAGAGATCTGTCACTACCCTCTTCCTTCTA
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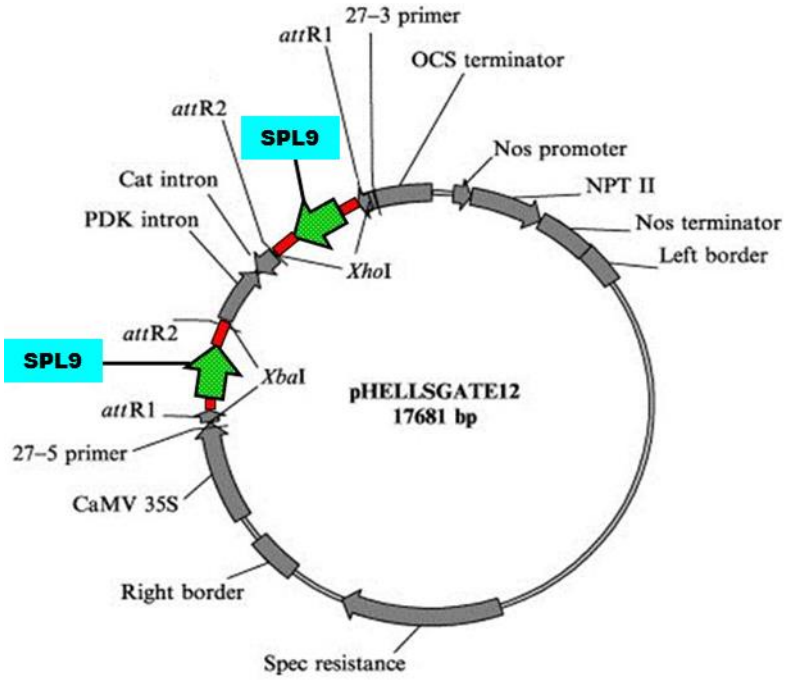
CGCCTATGATCGCATGATATTTGCTTTCAATTCTGTTGTGCACGTTGTAAAAAACCTGAGCATGTGTAGCTCAGATCCTTACCGCCGGTT
TCGGTTCATTCTAATGAATATATCACCCGTTACTATCGTATTTTTATGAATAATATTCTCCGTTCAATTTACTGATTGTACCCTACTACT
TTATATGTACAATATTAAAATGAAAACAATATATTGTGCTGAATAGGTT

Map



Appendix B

Primers used in this study

Reference genes			
Name	Sequence	length	target
ms_CyclophF	CAAACTTTCCTGACGAGTCACC	74	cyclophilin
ms_CyclophR	ACGGTCAGCAATTGCCATTG		
ms_GAPDHqF	CCCTCTCCCTGTACAAAACTC	90	GAPDH
ms_GAPDHqR	ACACGTAACACCAACCTTCC		
ms_ADF1qF	TCAAGGCGAAAAGGACACAC	149	actin-depolymerizing protein
ms_ADF1qR	AAAACAGCATAGCGGCACTC		
ms_eIF4AqF	TGCTAAGTTGCCTGAAACCG	127	elongation initiation factor 4A
ms_eIF4AqR	TGCCCATGTTTTCACCTTGC		
Ms-Actin-Fma	AGCAAAAGATGGCAGATGCT	156	Actin
Ms-Actin-Rma	CCATACCAACCATGACACCA		
BA-Acc1-F	GATCAGTGAACTTCGCAAAGTAC	91	Acetyl CoA Carboxylase
BA-Acc1-R	CAACGACGTGAACACTACAAC		
ms-U6Fq1	TCGGGGACATCCGATAAAAT	75	snRNA U6
ms-U6Rq1	TTTGTGCGTGTCATCCTTGC		
miR156/SPL genes			
Name	Sequence	length	target
MsmiR156dRT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCTC	61	miR156d
MsmiR156dF	GCGGCGGTGACAGAAGAGAGT		
Universal R	GTGCAGGGTCCGAGGT		
qMsSPL2F	GTAGAAGAGGAGGAGGGTGATG	108	SPL2
qMsSPL2R	GCAACAGGGTGGAGAAACAC		
qMsSPL3F	TGGCTGGACATAACGAGAGG	171	SPL3
qMsSPL3R	GTTTGATGACAGAAGAGAGAGAGC		

qMsSPL4F	ATGCCAAAGCACCTACCGT	137	SPL4
qMsSPL4R	CCTCTCATTATGCCCAGCCA		
MsSPL6rtpcr-F	CTCGGCCGATACATCAAACTG	191	SPL6
MsSPL6rtpcr-R	CCTCTGTTCAACACCATTGACG		
qMsSPL9F	GGAAGAGGTGGGTCAGTTCA	140	SPL9
qMsSPL9R	ACAGTAACAGTTGGGCACTTAG		
MsSPL12-Fq1	CCTCAGCCTGAAGCAGTGAA	135	SPL12
MsSPL12-Rq1	CTTGCTGTTGGGCATGTCTG		
MsSPL13rtpcr-F	CACTTCATAGCCAAACCACACCTCTT	186	SPL13
MsSPL13rtpcr-R	AGGGCTGCATAAGAGACATTGAATGA		
Stress associated/miR156 associated genes			
Name	Sequence	length	target
Ms-bZIP-F	TGCTTCCCCAATGTTGAAGA	143	bZIP family protein TGA3
Ms-bZIP-R	TCTGTGCTTGACGTCGTAGT		
qMsLOBF	AGATGGTGATTCGTGACCCG	142	lateral organ boundaries (LOB) domain protein 36
qMsLOBR	CTGTTGCTGCTGTTGTTGATTC		
DFR-F	GTTTGTGTCACAGGGGCTTC	107	Dihydroflavonol 4-reductase
DFR-R	TTCAAGTTTTCTGGGTCGCG		
qMsCCD1F	CTCATCATTGGCTTGCTGGTAA	127	carotenoid cleavage dioxygenase1
qMsCCD1R	CGGATTTGGTCCCACCCTAA		
qMsCCD4a-6F	GGGTTGTTATGGTGGTGAGC	109	carotenoid cleavage dioxygenase 4a-6
qMsCCD4a-6R	ACCTTGACTCTCCCTTCTTCTC		
qMsCCD8F	GAGAAATGGGAAGGAGAACTGC	116	carotenoid cleavage dioxygenase 8
qMsCCD8R	ACCCGTCAAAGAGATGCCTG		
qMsHIRAF	GAGGCGAAAGAACTTGGTCAG	112	Histone transcription regulator HIRA protein
qMsHIRAR	TGTGCTGGACTCTCTGCTAAG		
qMsMYB53F	CAACGCTCCTCTTGTTCTTGG	144	MYB53
qMsMYB53F	CGCCTCTTTCTTATGTGCTGC		
MYB112-F	TGGGTGATGGAGGTGAAGAA	120	myb transcription factor 112
MYB112-R	CACCGTTGTTTGAGGTTTGG		
NCED-F-qPCR	CTCGAGGTTCGGGGTTTTAT	164	9-cis-epoxycarotenoid

NCED-R-qPCR	ATATCGAATCAGCCGGTGTC		dioxygenase 3
MsHSP17.7-q-F	CACCACATAATGGACCTCACAGAT	172	17.7 kDA Heat Shock Protein
MsHSP17.7-q-R	TGATGTCACCTGATTTCAACCCTG		
Hsp17F	TGCTTGTTATAAGCGGAGAG	166	17 kDA Heat Shock Protein
Hsp17R	ACAGTCAACACACCATCTTG		
Hsp23F	GGATGCGAAAGAGACAGAGG	123	23 kDA Heat Shock Protein
Hsp23R	TCTTTGGCACCTTCACCTTT		
qMsPOD-F	CTCTTTTCTTTGAAGCTTTTAAGGC	152	Peroxidase pxdC
qMSPOD-R	ATCCTCTGACGACTCTCTGATGACT		
qMsZEP-F	CGTCTATGTTGAGCTGGATCTT	97	zeaxanthin epoxidase ZEP
qMSZEP-R	CGTAGTTGTCCGTTTGCTTTG		
PP2C-F-qPCR	ATGCGATCTTCTGTGGTGTG	130	Protein Phosphatase 2C
PP2C-R-qPCR	GATTCATTTCGCCTCGACTC		
WD40-1-F-qPCR	GCTCTCGTGAGGACCTTGAC	189	WD40-1
WD40-1-R-qPCR	ATGAGGACCAATGCATCACA		
WD40-2-F-qPCR	GTTGCTCCGTGTGAGAAACA	193	WD40-2
WD40-2-R-qPCR	CATTTGTGATCGCATTGTCC		
Plant transformation gene specific primers			
Name	Sequence	length	target
SPL9RNAi_F	CACCCTCTCTCTTCTGTCAAATCAAACA TGGG	300	SPL9
SPL9RNAi_R	TTACAGTGACCATTGAGAAGATTCATAGG		

Curriculum Vitae

Name:	Craig Matthews
Post-secondary Education and Degrees:	University of Western Ontario London, Ontario, Canada 2011-2016 B.Sc. The University of Western Ontario London, Ontario, Canada 2016-2018 M.Sc.
Honours and Awards:	the Western Scholarship of Excellence 2011 George H. Duff Travel Bursary 2017
Related Work Experience	Teaching Assistant University of Western Ontario 2011-2018

Publication:

Matthews C, Arshad M, Hannoufa A. (2018) Alfalfa response to heat stress is modulated by microRNA156. *Physiologia Plantarum* [in press, DOI: 10.1111/ppl.12787]

Presentations:

Matthews C, Amyot L, Smith DR, Hannoufa A. (2017) Investigating the role of the *miR156-SPL* network in heat stress response in *Medicago sativa*. Plants From Sea to Sky. A joint meeting of the Canadian Society of Plant Biologists and the Canadian Society for Horticultural Science; Vancouver, British Columbia. [Poster].

Hannoufa A. Arshad N, Gao R, Feyissa B, Matthews C, Aung B, Amyot L, Croft M (2017). The miR156/SPL module is a hub for regulating alfalfa traits. 13th International Symposium on Biocatalysis and Agricultural Biotechnology, Taichung, Taiwan. [Speaker: Hannoufa A]

Hannoufa A. Arshad N, Gao R, Feyissa B, Matthews C, Aung B, Amyot L, Croft M (2018). Connecting the dots: How miR156 regulates multiple traits in alfalfa. 11th meeting of The Canadian Plant Biotechnology Conference (CAPB 2018), Saskatoon, Saskatchewan. [Speaker: Hannoufa A]