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Studying Morphological, Physiological, and Molecular Regulation of Stomatal Conductance and Its Relationship to Water Use Efficiency in Alfalfa

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STUDYING MORPHOLOGICAL, PHYSIOLOGICAL, AND MOLECULAR REGULATION OF STOMATAL CONDUCTANCE AND ITS RELATIONSHIP TO WATER USE EFFICIENCY IN ALFALFA

BY

SURBHI GUPTA

A thesis submitted in partial fulfillment of the requirements for the

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THESIS ACCEPTANCE PAGE

Surbhi Gupta

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Yajun Wu
Advisor Date

Radhey Kaushik
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

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CONTENTS

LIST OF FIGURES……………………...…………………………………………….....vi

LIST OF TABLES……………...……………………………………………………viii

ABSTRACT……………………………………………………………………….....ix

1 CHAPTER 1 REVIEW OF LITERATURE 1

1.1 ALFALFA, A VERY IMPORTANT CROP!! 1
1.2 IMPORTANCE OF ALFALFA 2
1.2.1 Forage 2
1.2.2 Other use 3
1.3 CHALLENGES IN ALFALFA PRODUCTION 3
1.3.1 Biotic challenges 3
1.3.2 Abiotic challenges 4
1.3.2.1 Drought 4
1.4 WATER USE EFFICIENCY (WUE) 5
1.5 RELATION BETWEEN STOMATAL BEHAVIOR AND WUE... 5
1.5.1 Stomata density and transpiration rate 6
1.5.2 Stomatal conductance 7
1.5.3 ABA signalling 8
1.5.4 PYL family and drought tolerance 9
1.6 HYPOTHESIS AND OBJECTIVES: 10

2 CHAPTER 2 RELATIONSHIP BETWEEN STOMATAL DENSITY AND STOMATAL CONDUCTANCE IN ALFALFA 12

2.1 INTRODUCTION 12
2.1.1 Stomatal density and distribution in alfalfa 12
2.1.2 Regulation of stomatal density and behavior in drought conditions 13
2.1.3 Drought tolerance in alfalfa 14
2.2 MATERIALS & METHODS 14
2.2.1 Initial screening for variation in stomatal density 15
2.2.1.1 Plant Materials 15
2.2.1.2 Leaf impressions for stomatal density measurement 15
2.2.2 Impact of drought on stomatal density and conductance 16
2.2.2.1 Vegetative propagation of plants 16
2.2.2.2 Drought treatment 17
2.2.2.3 Stomatal density measurement 17
2.2.2.4 Stomatal conductance 18
2.2.3 Data analysis 18
2.3 RESULTS 18
2.3.1 Variation in stomatal density in alfalfa 18
2.3.2 Changes in stomatal density and conductance under drought 21

2.4 DISCUSSION 24

3 CHAPTER 3 IDENTIFICATION AND TRANSCRIPT ANALYSIS OF ABA BINDING PYL LIKE GENES IN *MEDICAGO SATIVA* FOR DROUGHT STRESS RESPONSE 27

3.1 INTRODUCTION 27
3.1.1 Abscisic acid an essential plant hormone 27
3.1.2 ABA in stomatal closure 27
3.1.3 ABA signaling pathway 27
3.1.4 Pyrabactin Resistance [PYR]/ [PYR1-Like (PYL] protein 29
3.2 RATIONALE AND HYPOTHESIS 30
3.3 MATERIALS & METHODS 31
3.3.1 Identification of homologs of AtPYLs in Medicago truncatula and Medicago sativa 31
3.3.2 Multiple sequence alignment and phylogenetic tree analysis 31
3.3.3 Conserved motifs 32
3.3.4 Primer designing 32
3.3.5 Plant materials and treatments 33
3.3.6 MsPYL gene expression in leaf, stem, and root of well-watered alfalfa plants 34
3.3.7 Drought treatment 34
3.3.8 Shoot dehydration treatment 35
3.3.9 RNA extraction and purification 35
3.3.10 cDNA synthesis and RT-PCR 36
3.3.11 Quantitative analysis using real time qPCR 36
3.3.12 Data analysis 37
3.4 RESULTS 37
3.4.1 Identification of PYLs in Medicago truncatula and Medicago sativa 37
3.4.2 Multiple sequence alignment and phylogenetic relationship of MtPYLs and MsPYLs with AtPYLs 41
3.4.3 Conserved motifs 48
3.4.4 MsPYL gene expression in different tissues of well-watered alfalfa 53
3.4.5 MsPYL gene expression due to drought stress 54
3.4.6 MsPYL gene expression under dehydration treatment 56
3.5 DISCUSSION 58
3.5.1 PYL-like genes in M. truncatula and M. sativa and phylogenetic analysis 58
3.5.2 PYL gene expression in different tissues of well-watered alfalfa 59
3.5.3 MsPYL gene expression under water stress conditions 60

4 CONCLUSION 61

5 REFERENCES: 62

LIST OF FIGURES

Figure 1 ABA signalling pathway showing the interaction of ABA and PYLs in the cascade in the presence of stress. 9

Figure 2 Leaf epidermal imprinting replica from adaxial surface used for stomatal counting. 19

Figure 3 Total stomatal density among 33 different genotypes in alfalfa. 20

Figure 4 Stomatal distribution in 9 different genotypes of alfalfa. 21

Figure 5 Effect of drought stress on stomatal density and conductance. 23

Figure 6 Correlation coefficient between Stomatal density and conductance of the 9 alfalfa genotypes in drought stressed conditions 24

Figure 7 Schematic representation of the regulation of osmolyte movements in guard cells in absence and presence of ABA 29

Figure 8 Linkage map showing distribution of MtPYL genes on 8 chromosomes. 40

Figure 9 Phylogenetic tree of PYL gene family in Arabidopsis thaliana. 42

Figure 10 Multiple sequence alignment of the PYL gene family in A. thaliana & Medicago sativa. 46

Figure 11 Phylogenetic tree of PYL genes in A. thaliana, M. truncatula & M. sativa. 47

Figure 12 Conserved motifs of MsPYL proteins in comparison to AtPYL protein family. 53

Figure 13 Relative transcript expression of the 15 genes in leaf, stem & root of well-watered RS genotype of alfalfa. 54

Figure 14 Relative Transcript level changes of the 15 MsPYL genes in well-watered and 14 days drought treated RS and AF shoot tissues. 55

Figure 15 Relative Transcript level changes of the 15 MsPYL genes in well-watered and 6 hour dehydrated RS and AF shoot tissues. 57

LIST OF TABLES

Table 1 Information about primers used for the selected PYL genes of interest in Medicago sativa. 33

Table 2 PYL gene family members names used in this study in Arabidopsis thaliana, Medicago truncatula and Medicago sativa with their Gene IDs according to NCBI database, Ensembleplants.org and alfalfatoolbox.org respectively 39

ABSTRACT

STUDYING MORPHOLOGICAL, PHYSIOLOGICAL, AND MOLECULAR REGULATION OF STOMATAL CONDUCTANCE AND ITS RELATIONSHIP TO WATER USE EFFICIENCY IN ALFALFA

SURBHI GUPTA

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Alfalfa (*Medicago sativa*), being a leguminous, highly fibrous, and proteinaceous plant has always been one of the top choices for the forage production but has high cost of irrigation in many dry and warm areas such as California and Arizona. Thus, a reduction of irrigation by using the higher water use efficiency (WUE) varieties can help the growers in reducing the cost and is critical for sustainable agriculture production. WUE is closely related to water loss through transpiring stomata. A study in our lab reported that Riverside (RS) an alfalfa genotype that has naturized in the national grassland in South Dakota, showed a higher WUE, compared to various commercial varieties and collections. Further studies in the lab revealed that RS showed a greater stomatal sensitivity to ABA in closure. In this study we examined if the stomatal density in different genotypes could play a role in the WUE of the plant by having impact on stomatal conductance. Alfalfa exhibits high density of stomata on adaxial surfaces. We found that variations in stomatal densities among genotypes exist, but stomatal density did not show correlation with the stomatal conductance, a contributing factor to WUE, emphasizing on the importance of stomatal sensitivity to ABA for higher WUE. One of the families of ABA receptors, Pyrobactin resistant like (PYL) in *Arabidopsis* is found to play essential role in drought conditions.

We hypothesized that homologs of PYL in alfalfa could be involved in regulating stomatal conductance and hence play an essential role in WUE of the plants. The current study involved identifying the PYL-like gene family in alfalfa and analyzing the change in gene expression levels during water stress conditions. The 15 identified MsPYL proteins showed conserved domains and ABA receptor properties with START-like sequences. We demonstrated that *MsPYL9* gene shows upregulation in RS genotype while showing no change in AF, genotype with lower WUE. This suggests that possibly, MsPYL9 could be related with higher WUE of RS. For this study we used two germplasms of alfalfa, but an understanding of candidate genes correlated with better WUE will bring new insights and potentially help improving alfalfa production in dry areas.

1 Chapter 1 Review of literature

1.1 Alfalfa, a very important crop

Alfalfa (*Medicago sativa* L.) is one of the most important forage crop and has been grown worldwide (Michaud et al. 1988). It is a perennial, outcrossing, and autotetraploid (2n=4x=32) leguminous crop of family Fabaceae with high nutritional value and cultivated for hay, pasture and silage (Radović et al. 2009; Acharya et al. 2020). In the United States, alfalfa is the fourth largest produced crop after corn, soy and wheat (Zhang et al. 2017b). According to the 2020 NASS report, total alfalfa hay and haylage produced was 53,067,000 tons with a value of approximately $8.8 billion.

Alfalfa has been recorded to be native from different parts of Asia. Documents from Southwestern Iran and Syria have records of charred remains of seeds from about 10,000 B.C. In USA, alfalfa earlier known as Lucerne, was introduced by English, French and German colonists as early as 1735 but the crop did not get commercially accepted until 19th century when Mexican alfalfa was introduced in southwest U.S. where the pH, drainage and soil composition was well suited for this crop. The alfalfa seeds introduced from Chile to California were the most successful using irrigation (Russelle 2001).

1.2 Importance of alfalfa

Alfalfa is a crop grown since ancient times worldwide and is extensively adapted to weather conditions ranging from winter to tropical as well as arid lands. Alfalfa has a symbiotic relationship with a nitrogen fixing soil bacterium *Sinorhizobium meliloti*. This relationship not only meet the needs of plant for nitrogen but also increases the soil fertility in terms of (N). Alfalfa can provide as high as 300 lb./acre/year of biologically fixed nitrogen in the soil which reduces the cost of nitrogen fertilizers for other crops (Kumar et al. 2018). Alfalfa has a unique deep root system and the taproots can grow up to 6m or more making it more drought resilient (Michaud et al. 1988). Alfalfa has been used in on-site phytoremediation because of the sponge nature of the extensive tap root system and the microorganisms associated with it which helps decomposing organic compounds like polyaromatic hydrocarbons and petroleum compounds some of which are carcinogenic as well. It is known to protect ground water quality as it absorbs excessive nitrates and other contaminants. According to USDA report, alfalfa was grown on site of Canadian Pacific train derailment in North Dakota to clean up a spill of 45,000 gallons. Transgenic alfalfa plants produced by ARS and University of Minnesota researchers have shown ability to breakdown atrazine, a widely used herbicide (Russelle 2001).

1.2.1 Forage

Alfalfa has a high nutritional value for animal feed as it has 15-22% of crude protein and ten kinds of vitamins including A, D, E, K, U, C, B1, B2, B6, B12 along with many

minerals (Soto-Zarazúa et al. 2016). Alfalfa is essentially grown to feed dairy cows, beef cattle and horses, but is also used for other farm animals like sheep, chickens, and turkeys.

1.2.2 Other use

Alfalfa shows many qualities which can be explored in different aspects. The sprouts of alfalfa have high antioxidants and phytoestrogen which may prevent impairments like osteoporosis, cancer, heart disease and menopausal symptoms. Transgenic alfalfa is used to produce monoclonal antibodies for human IgG (Khoudi et al. 1999) . Alfalfa with poly-b-hydroxybutyrate (PHB) gene has been used for producing biodegradable plastic polymer (Saruul et al. 2002).It was proposed that alfalfa be used for a biofuel crop since its high yield of cellulosic biomass with low input of fertilizers in biomass production (Monteros and Bouton 2009). Researchers have been working on finding many other uses of the plant in terms of nutrient and medicinal values.

1.3 Challenges in alfalfa production

Alfalfa is one of the highest biomass producing crop, but it faces many challenges from abiotic and biotic stresses as other crops.

1.3.1 Biotic challenges

Alfalfa is known to be home for around 1000 different insect species. Around 100-150 of these are pathogenic to the plant at different stages of their life. According to a USDA report, annually alfalfa production loses hundreds of million dollars due to insect pests.

Alfalfa weevil, blue and spotted aphids, alfalfa snout beetle are some of the most serious pests. Other than insects alfalfa is also susceptible to alfalfa mosaic virus, downy mildew, Fusarium wilt and many other diseases (Flanders and Radcliffe 2000).

1.3.2 Abiotic challenges

The major abiotic challenges alfalfa agriculture faces are salinity, drought and freezing. Being a tetraploid, alfalfa shows huge variation from susceptible to tolerant to stresses. These stresses not only greatly reduce biomass production but also the quality of the crop. Over the past century alfalfa has been bred by researchers to improve the yield and nutrition value as well as its ability to cope with the abiotic stresses (Kingston-Smith et al. 2013). In the current study, our emphasis is the drought stress and the strategies of alfalfa to cope up with water deficit conditions.

1.3.2.1 Drought

Water is the most essential resource for any living being. Water deficit leads to disruption of many cellular functions in plants, such as cell expansion, photosynthesis, development and hence affecting the overall growth of the plants (Chaves et al. 2003). Drought is the most common and detrimental abiotic challenge reducing the agricultural productivity around the world (Ghaderi and Siosemardeh 2011). Currently, maintenance of crops with high yields is depending on irrigation system as the irrigated crops produce 60% more yield than the rainfed crops around the globe (Rosegrant et al. 2009) but with rising population and scarcity of fresh water, the availability of water for irrigation has become increasingly

limited. Future climate forecasts increased global warming leading to longer and more frequent droughts which will further reduce the access to fresh water for irrigation (Joshua Elliott 2014). Thus developing and planting alfalfa with higher water use efficiency can be a solution to the forage industry (Gang et al. 2004).

1.4 Water use efficiency (WUE)

Water use efficiency can be defined in two ways, the biomass produced per unit water consumption or rate of CO_2 assimilation to transpiration (Farquhar and Sharkey 1982; Dawson et al. 2002). Biomass is considered the fresh/dry weight of the plant at the time of harvest. Being a forage, biomass in case of alfalfa is the whole shoot system including leaves and stems. During water deficit conditions, plants tend to close the stomatal pore to avoid excess loss of water which impacts the CO_2 intake hence, the biomass production and overall WUE (Zhang et al. 2017b). One of solution for this problem could be improving the moisture absorption from soil which is called efficient use of water. In theory, both enhancing photosynthesis and reducing transpirational water loss will result in greater WUE. As the molecular and biochemical processes of photosynthesis is quite complex, it is preferred by scientist to reduce the transpirational water loss from plants while maintaining the photosynthesis when improving WUE in plants (Blum 2009).

1.5 Relation between stomatal behavior and WUE

Stomata are the pores in the epidermal layer of the leaf formed by guard cells which are specialized cells to regulate the stomatal movement. The stomates are the site of gaseous

exchange between plant and environment. Opening of stomates facilitates the uptake of CO_2 by the plants and water loss due to transpiration (Kim et al. 2010). Thus, stomatal behavior directly impacts WUE.

1.5.1 Stomata density and transpiration rate

Stomatal density is generally measured in two ways. Number of stomata per unit area and number of stomata per unit epidermal cells. In past years there has been a lot of work done to understand the stomata formation and distribution (Torii 2012). The density on leaf surface are reported to vary due to ecosystems and environmental conditions along with genetic factors (Bertolino et al. 2019). The average stomatal density in corn and wheat is reported to be between 40 and 90/mm^2 whereas in rice and arabidopsis it is around 200/mm^2 suggesting the variation in different plant species (Zheng et al. 2013; Sakoda et al. 2020; Zhang et al. 2013; Kong et al. 2015). Recent study in our lab demonstrated that average stomatal density in alfalfa leaves is 225/mm^2 (Ghimire et al. 2021). A study in Israel on 32 indigenous plant species reported that xerophytic plants had significantly higher stomatal density as compared to irrigated plant species controlling the amount of water loss (Gindel 1969). Stomatal density is genetically controlled, and many genes involved have been identified. The plant protein epidermal patterning factors (EPFs) have been studied by number of researchers for the physiological implications in regulating stomatal density (Wang et al. 2016). By manipulating the EPF levels various lines of *A. thaliana* having stomatal densities from 20% to 325% of normal levels have been developed. The lines with lower stomatal density had lower transpiration rates and showed larger growth in the conditions of water deficit, resulting in a higher WUE (Hunt and Gray

2009; Doheny-Adams et al. 2012; Tanaka et al. 2013). It has been shown that transpiration decreased with reduction in abaxial stomatal density in *A. thaliana* overexpressing *PdEPF1* (Wang et al. 2016).

Regulation of stomatal density is a topic of interest when it comes to drought stress. The short and long term water deficit can cause some plastic modulations in the number of stomates allowing plant to adjust to the environment and regulate the gaseous exchanges (Bertolino et al. 2019). Differences among plant species responding to water deficit can be seen. For example, in a study, *Arabidopsis* plants did not show any changes in stomatal density when exposed to water stress condition (Xu and Zhou 2008; Doheny-Adams et al. 2012). In alfalfa, however, (Ghimire et al. 2021) reported an increase in stomatal density after drought treatment. Studies on *Arabidopsis* and barley have shown that overexpression of EPF2 decreases the stomatal density without any deleterious effects on yield. The transformed plants showed higher WUE as compared to control plants (Hughes et al. 2017; Franks et al. 2015).

1.5.2 Stomatal conductance

Stomatal conductance is another important factor determining transpirational water loss thus water use efficiency. In the conditions of reduced vapor pressure and drought stress the stomatal pores are closed by the guard cells to reduce excess water loss and in angiosperms this response is regulated by abscisic acid (Lange et al. 1971; McAdam et al. 2016). Along with ABA, stomatal movement is a quick response to factors like blue light, Ca^{2+}, CO_2, NO, H_2O_2 and ROS. These molecules essentially assist in the ABA signalling

pathway in stress response leading to the stomatal closure (Shimazaki et al. 2007; Kim et al. 2010; Chater et al. 2014).

1.5.3 ABA signalling

Abscisic acid (ABA) is the phytohormone involved in numerous vital aspects of plant growth and development starting from embryo maturation to cell division, seed dormancy and stress responses including cold, drought and salinity (Duarte et al. 2019; Miyakawa et al. 2013). ABA accumulates in leaves when the plant experiences stresses, especially drought and induces stomatal closure by modulating the solute efflux in guard cells along with regulating gene expression of many downstream proteins resulting in dehydration tolerance in the tissues (Miyazono et al. 2009; Corrêa de Souza et al. 2012).

In plants, ABA is perceived by the ABA receptors which initiates the signalling cascades for different responses. The genetic analysis of ABA receptors in *Arabidopsis* lead to the discovery of three major components of the cascade: the ABA receptor PYR/PYL/RCAR (PYL) protein family, the negative regulator type 2C protein phosphatase (PP2C) and the positive regulator class III SNF-1-related protein kinase 2 (SnRK2) (Figure 1) (Duarte et al. 2019). Once the ABA binds to PYL receptors, the complex inhibits the phosphatase activity of PP2C leading to autophosphorylation of SnRK2s (Sang-Youl Park and Nicholas J. Provart 2009). Activated SnRK2s induce stomatal closure by targeting NADPH oxidases, and ion channels (Miyakawa et al. 2013; Joshi-Saha et al. 2011). In *A. thaliana*, it is reported that the SnRK2 protein kinase activates the anion channel (SLAC1) for the efflux of anions and thus activating potassium efflux channel. SnRK2 also inhibits the

cation inward channel (KAT1) through phosphorylation. Efflux of ions eventually leads to an efflux of water and a decrease in turgor pressure in guard cells, resulting in stomatal closure (Miyakawa et al. 2013; Joshi et al. 2011).

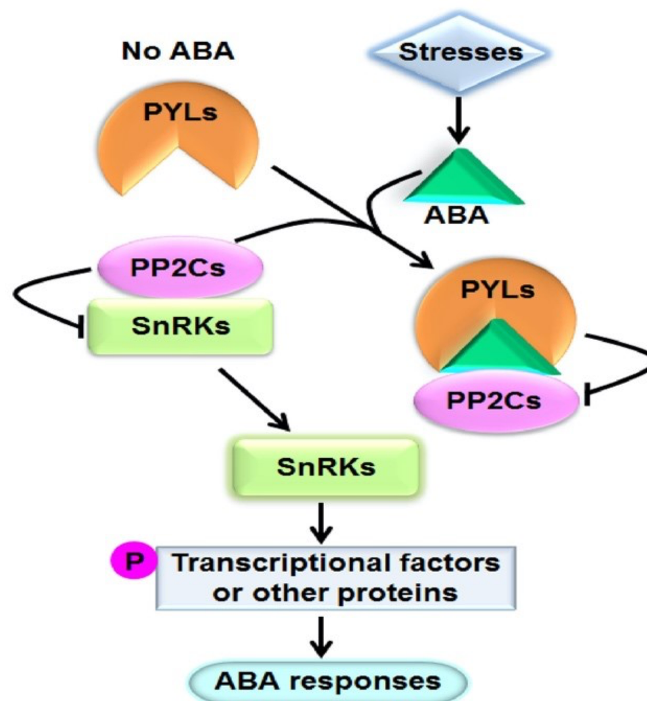


Figure 1 ABA signalling pathway showing the interaction of ABA and PYLs in the cascade in the presence of stress.

Binding of ABA to PYL receptors initiate the cascade by inhibiting PP2C proteins (Zhang et al. 2017a)

1.5.4 PYL family and drought tolerance

The PYR1(Pyrobactin Resistance 1) and PYL (Pyrobactin Resistance 1-like) proteins were first identified in *A. thaliana* for playing important role in ABA signalling. The 13 PYLs belong to START (Star-related lipid-transfer) protein superfamily of which AtPYR1,

AtPYL1, AtPYL2, AtPYL4, AtPYL5 and AtPYL8 play role in ABA dependent stomatal closure (Park et al. 2009; Gonzalez-Guzman et al. 2012). Different orthologs of AtPYLs have been reported to play crucial roles in drought and/or osmotic stress in many plants (Garcia-Maquilon et al. 2021; Bai et al. 2019; Nishimura et al. 2010; Zhang et al. 2017a; Di et al. 2018). Overexpression of AtPYL4 in *A. thaliana* has been reported to increase the WUE by improving the ABA dependent stomatal closure in drought conditions (Pizzio et al. 2013). Similar results were reported when overexpressing OsPYL5 increased the drought and stress tolerance in *Oryza sativa* with enhanced stomatal closure (Kim et al. 2014). The work done on PYLs strongly suggests the vital role of this protein family in tolerance and resistance to abiotic stresses.

1.6 Hypothesis and Objectives:

As an effort to understand WUE and its regulation in alfalfa, our lab conducted a study to examine the genotypic variations in WUE under drought conditions. In that study, we found that River side (RS), an alfalfa genotype that has naturized in the national grassland in South Dakota, showed a higher WUE, compared to various commercial varieties and collections (Anower 2015). Further studies in the lab revealed that while RS showed a less accumulation of ABA, it showed a greater stomatal sensitivity to ABA in stomatal closure compared to a genotype of lower WUE, Alfagraze (AF). RS also showed a surprising increase in stomatal density under drought (Ghimire et al. 2021). This raises two important questions: 1) how stomatal density is regulated and contributes to WUE in alfalfa; 2) how stomata in RS achieves greater sensitivity to ABA in closure. We hypothesize that 1) a

genotypical variation in stomatal density exists in alfalfa, and stomatal density, and thus WUE, is regulated by growth conditions; 2) a greater stomatal sensitivity to ABA in RS under drought is due to an enhanced ABA signaling, such as more ABA receptors or more sensitive receptors. Thus, the main objectives of this study are: 1) examine stomatal density among alfalfa genotypes and determine the relationship between stomatal density and conductance; 2) identify ABA receptor genes in alfalfa and examine their transcript levels under drought conditions, as a greater transcript level may lead to more ABA receptors. Our ultimate goal is to improve WUE in alfalfa by manipulating stomatal density and stomatal sensitivity to ABA.

2 Chapter 2 Relationship between stomatal density and stomatal conductance in alfalfa

2.1 Introduction

2.1.1 Stomatal density and distribution in alfalfa

Alfalfa production requires a high amount of water in comparison to other commercial crops. It is, however, considered a high WUE crop due to its higher biomass production, longer growing season, and dense canopy (Asseng and Hsiao 2000; Putnam 2012; Hanson et al. 2008). To sustain its high biomass production, irrigation is needed in many areas such as Arizona and California where alfalfa production is among the greatest while water supply is extremely limited. Thus, improving WUE in alfalfa is an urgent step for its sustainable production.

Plants are reported to lose 95 to 99% of the total absorbed water via transpiration through stomatal pores (McElrone et al. 2013). According to a report from University of Idaho during the June-September duration in alfalfa crop, the amount of water lost through stomatal evapotranspiration can reach up to 45 tons per acre per day, increasing the water usage hence affecting the WUE of the crop overall (Shewmaker et al. 2011). One of the strategies to improve WUE in alfalfa would be to optimize stomatal density i.e., changing the stomatal density without impacting photosynthesis since stomatal opening is needed for CO_2 fixation. Alfalfa shows several characteristics in stomatal density and distribution. First, it is an amphistomatous species, having stomates on both adaxial and abaxial surface. Second, unlike many other crops, alfalfa have more number of stomates on adaxial surface of leaf that is believed to be related with high CO_2 assimilation and biomass production

(Cole and Dobrenz 1970; Anderson and Briske 1990). Third, alfalfa leaf shows high stomatal density, 220/mm^2 in RS, and 210/mm^2 in AF (Ghimire et al. 2021) compared to other crops, for example, stomatal density in wheat and corn in the commercial varieties are reported be between 40 to 90/mm^2 (Kong et al. 2015; Zheng et al. 2013).

2.1.2 Regulation of stomatal density and behavior in drought conditions

Stomatal density and distribution vary from one species to another, clearly indicating a genetical control of these features. Stomatal density can also be adjusted under long-term drought conditions. Different studies on Banksia (Proteaceae) and wheat have shown that in arid conditions plants develop higher stomatal density with reduced size to help plants regulate the rate of transpiration as smaller stomata could open and close up to 6 times faster (Raven 2014; Drake et al. 2013; Yongping et al. 2006). Similar results were reported in longer drought conditions on Arabidopsis plants with mutated EPF2 genes by altering their stomatal density and size to reduce the conductance and transpirational water loss (Doheny-Adams et al. 2012). Plants with smaller stomates have shown higher water use efficiency in couple of studies (Aasamaa et al. 2001; Hetherington and Woodward 2003). Theoretically, reducing stomatal density together with closing stomata as a rapid response to drought, will reduce the transpirational water loss but will also reduce photosynthesis. It has been shown that the decline in transpiration rate is much higher than in photosynthesis (Edwards et al. 2012), suggesting WUE can be improved in plants without strongly lowering the yield. As the plants with higher WUE manage to loose less water, they are expected to be better solutions for dry and more arid conditions (Franco et al. 2004; Ares et al. 2000).

2.1.3 Drought tolerance in alfalfa

In past years numerous studies have been done on improving the abiotic stress tolerance of alfalfa using genomics, proteomics, and metabolomics. Many key genes responding to abiotic stresses have been identified in alfalfa (Song et al. 2019). Previous studies in our lab have identified a germplasm of alfalfa, Riverside (RS) naturally adapted to the Grand River National Grassland region in South Dakota. RS when exposed to water deficit conditions has demonstrated higher WUE compared to other commercial germplasms (Anower 2015). Further studies revealed that RS showed an increase in stomatal density under drought (Ghimire et al. 2021). This raised a question if stomatal density contributes to WUE in alfalfa. We hypothesize that a genotypical variation in stomatal density exists in alfalfa, and stomatal conductance, and thus WUE, is regulated by growth conditions. Thus, the main objectives of this study are 1) examine stomatal density among alfalfa genotypes; 2) find the impact of water deficit conditions on the stomatal density and stomatal conductance and to analyze if stomatal density as an independent variable has correlation with stomatal conductance in different germplasms of alfalfa.

2.2 Materials & methods

2.2.1 Initial screening for variation in stomatal density

2.2.1.1 Plant Materials

Thirty-three genotypes of alfalfa were used in this initial test. Some of these were commercial varieties, and some are from the stock center at Germplasm Resources Information Network (GRIN) at USDA-ARS. The genotypes are CS 15-2 14-5, Alfagraze (AF), SD201, BC11-1, Melone, PI-262-243, LC 46, U2948, CUF, Carib, Class, Wrangler, Renovator, Mesa, CS 153-14-3, LC 48, Mesasirsa, Salt, PI-26-2 18-45, Amergras, BC-79, Cimmarron, Sarnac, LC 047, CS 15-2 12-64, RS6, Forage, PI-539-49, LC004, Foster ranch (FR), PI 634 125, Apica, and PI 634 124. The plants were grown in one-gallon pots filled with potting mix (Sunshine mix #3, Sun Gro Horticulture Canada Ltd., MA, USA). All plants were grown in the greenhouse with 16 hours photoperiod and kept well-watered. Plants were fertilized with Miracle-Gro (Scotts Miracle-Gro Products, Inc., Marysville, Ohio, USA) slow-release fertilizer.

2.2.1.2 Leaf impressions for stomatal density measurement

Leaf impressions technique was used to obtain the epidermal imprint of adaxial and abaxial surfaces of the leaves (Randall 1984). A thin layer of commercially available clear nail polish (Seche Vite, American International Industries, Los Angeles, CA) was applied on the respective leaf surface with a brush. The nail polish was allowed to dry for 10 minutes. The dry film was taken off the leaf with the help of a clear tape. These tapes with imprints were mounted on the microscopic slides and observed under a light microscope (ATC 2000, Leica, IL, USA) with a total 400X magnification. The picture of total field of view was taken using a camera (COOLPIX 4500, Nikon, Melville, NY, USA). The number of

epidermal cells and stomata were counted for the area using ImageJ software. The stomatal density is calculated by:

$$Stomatal\ density = Number\ of\ stomates / Numer\ of\ epidermal\ cells$$

2.2.2 Impact of drought on stomatal density and conductance

From the initial screening nine genotypes were selected for studying the effect of drought treatment on stomatal density and stomatal conductance.

2.2.2.1 Vegetative propagation of plants

The selected nine genotypes, BC11-1, LC 46, Melone, CUF, Class, RS6, FR, PI 634-125, and Apica were vegetatively propagated. The cuttings were taken from healthy shoot by making a slanting cut under the third node and were quickly dipped into the rooting hormone IBA (Hormex rooting power no. 16, Brooker Chemical Chatsworth, Westlake Village, CA). The cuttings were planted into the potting mix pre-saturated with water (Sunshine Mix #3) and covered with a clear lid. The tray with cuttings were placed in the greenhouse. The lid was removed after 1 week as the cuttings started growing roots. The cuttings were allowed to grow for 3 weeks until the cuttings showed enough root growth. The young plants were then transplanted into cone containers of dimensions 3.8 x 21-cm filled with 38 grams of potting mix (Sunshine mix #3). The plantlets were grown in the greenhouse for 3 more weeks with 16-hour photoperiod and 70-75F temperature. The plantlets were watered every day and provided Miracle-Gro (Scotts Miracle-Gro Products, Inc.) nutrient solution (5 gm Miracle Gro/gallon of water) weekly (Anower et al. 2017a). After 3 weeks, 6 plants of each genotype with similar size were selected and used for the experiment.

2.2.2.2 Drought treatment

All the selected plants were watered to saturation on the day of starting the treatment. An aluminum foil was wrapped around the stem to cover the top surface of each container to prevent evaporation from the soil surface, only exposing shoots to the light and air. The containers with plants were weighed for the initial weight. Two water regimes were maintained for 30 days. Three plants of each genotype were kept **well-watered** by providing equal amount of water lost through transpiration whereas other three were given **drought stress**, providing only 50% of the water lost due to transpiration. The cone containers with plants were weighted every three days to estimate water loss. A syringe was used to slowly add required amount of water to each plant through aluminum foil, ensuring there is no dripping from the containers. Drought stress developed and became increasingly severe with time (Ghimire et al. 2021).

2.2.2.3 Stomatal density measurement

To determine stomatal density, the leaf impressions method as described above was used. The newly fully developed leaves of new shoots produced after drought stress treatment were used for leaf impression to examine whether drought stress impact the stomatal development. The images of leaf impressions were taken using Olympus BX53 Upright Compound Microscope and the images were analyzed using ImageJ for stomata and epidermal cells number.

2.2.2.4 Stomatal conductance

The stomatal conductance was measured using a portable leaf porometer (SC-1, Decagon devices, Inc., Pullman, WA) The porometer was calibrated prior to use according to the manual instructions. The youngest mature leaves of young shoots developed after drought stress treatment were selected for measuring stomatal conductance at the end of experiment. The stomatal conductance was recorded between 10 am to 2 pm on both adaxial and abaxial surface of the leaves. Three biological replicates were used for stomatal conductance measurement.

2.2.3 Data analysis

Statistical analysis was performed using Microsoft Excel 365 and R programming language. ANOVA was done using completely randomized design. Tukey's honestly significant difference test was performed to determine the significantly different mean values. Correlation test with a scatterplot was conducted to analyze the correlation between stomatal density and conductance.

2.3 Results

2.3.1 Variation in stomatal density in alfalfa

In order to understand the relationship between stomal density and WUE in alfalfa, it is necessary to identify alfalfa genotypes that differ in stomatal density. Thus, stomatal density was first surveyed among 33 genotypes. Figure 2 showed typical images of leaf epidermal imprinting replica used for stomatal counting. Both abaxial and adaxial surfaces

were examined for the stomatal density, and the average stomatal density of abaxial and adaxial surface were calculated and presented in Figure 3. The average stomatal density ranges from 0.217 to 0.343 stomates per epidermal cell with the lowest density for CS15-2, 14-5 and the greatest density for PI 634-124. To show the distribution of stomata on both leaf surfaces, 9 genotypes representing different average density were shown in Fig. 4. Two trends are noticeable. First, the adaxial surface has more stomates compared to the abaxial surface; second, the stomatal density on the adaxial surface appears to determine the order of the average stomatal density.

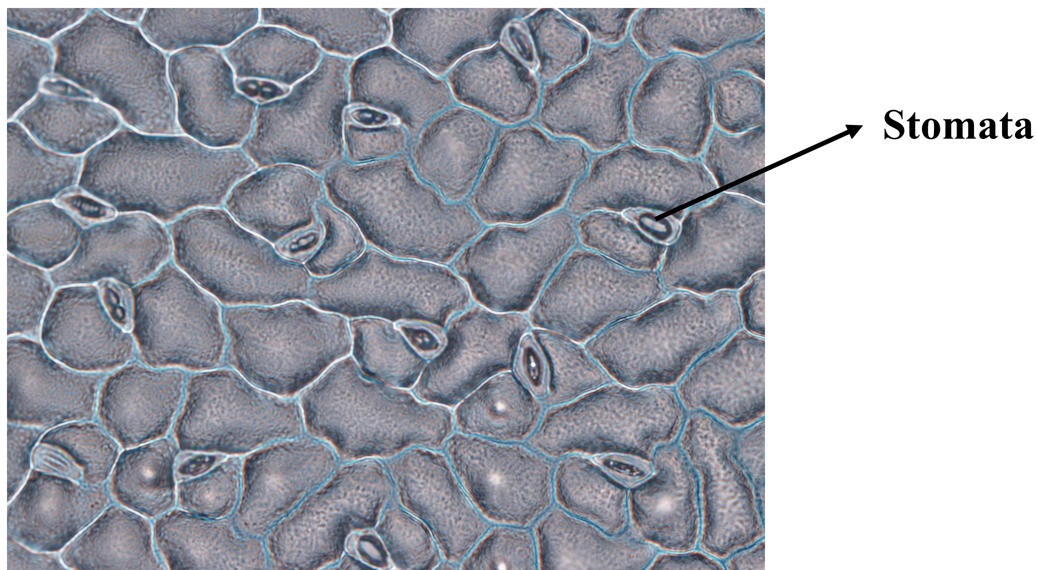


Figure 2 Leaf epidermal imprinting replica from adaxial surface used for stomatal counting.

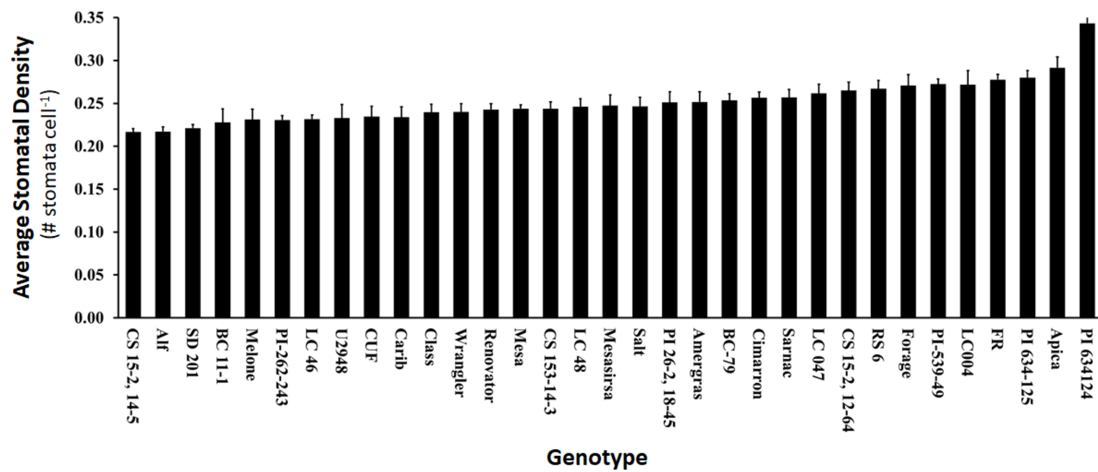


Figure 3 Average stomatal density among 33 different genotypes in alfalfa. The data is shown as mean ± S.E (n=3)

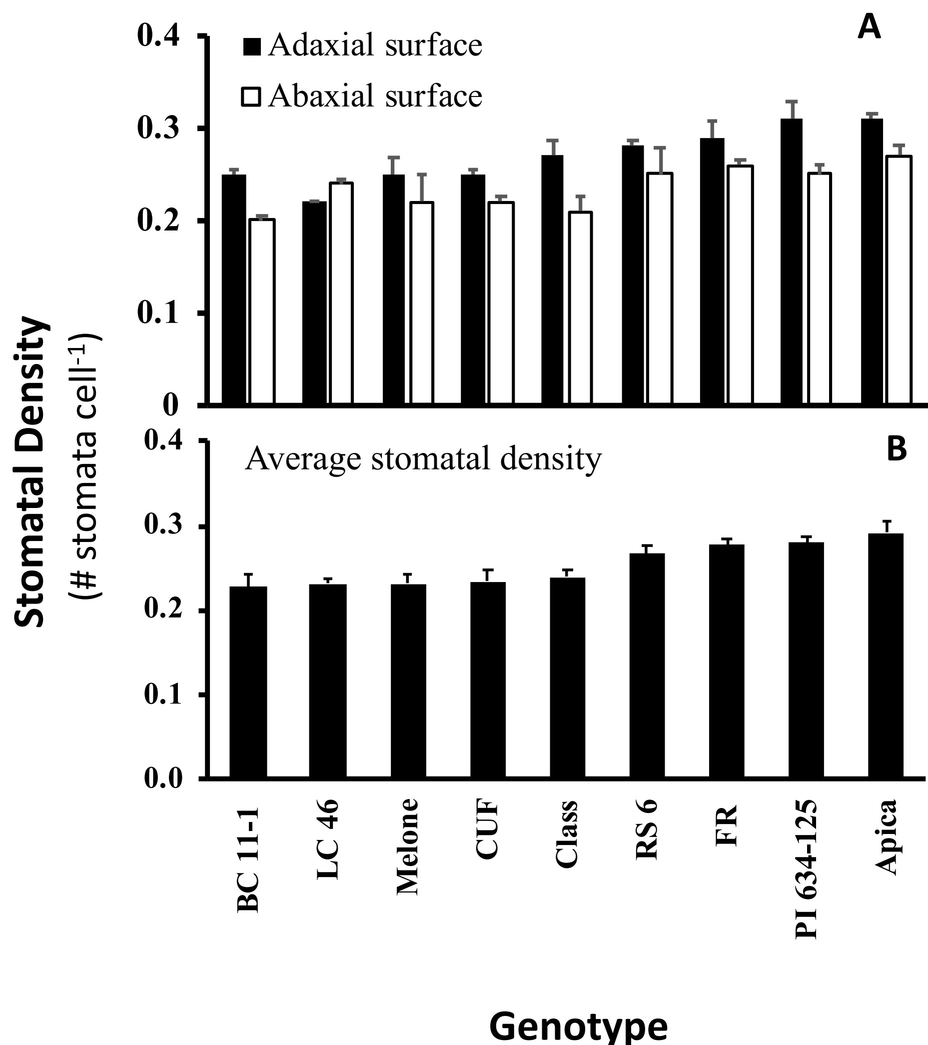


Figure 4 Stomatal distribution in 9 different genotypes of alfalfa.

The data is shown as mean ± S.E (n=3). A) Stomatal density on both surfaces of leaves. B) Average stomatal density for both the surfaces in the 9 genotypes.

2.3.2 Changes in stomatal density and conductance under drought

After a 30-days drought treatment we analyzed stomatal conductance and density in the control plants with 100% water replenishment and drought stressed plants with 50% water replenishment. The stomatal conductance was expected to decrease in the stressed plants. Most genotypes showed a significant decrease in stomatal conductance, while Apica,

PI634-125, and FR showed insignificant change under drought (Figure 5 A). Class, BC 11-1, LCO46, CUF, Malone and RS show more than 50% decline in stomatal conductance. Interestingly, none of the genotype showed a significant change in stomatal density due to drought (Figure 5B)

To study the impact of stomatal density on stomatal conductance a correlation test was performed using R programming. According to the scatter plot obtained and the correlation coefficient which is -0.14, it appears that stomatal density has very weak negative or no correlation with stomatal conductance in these genotypes (Figure 6).

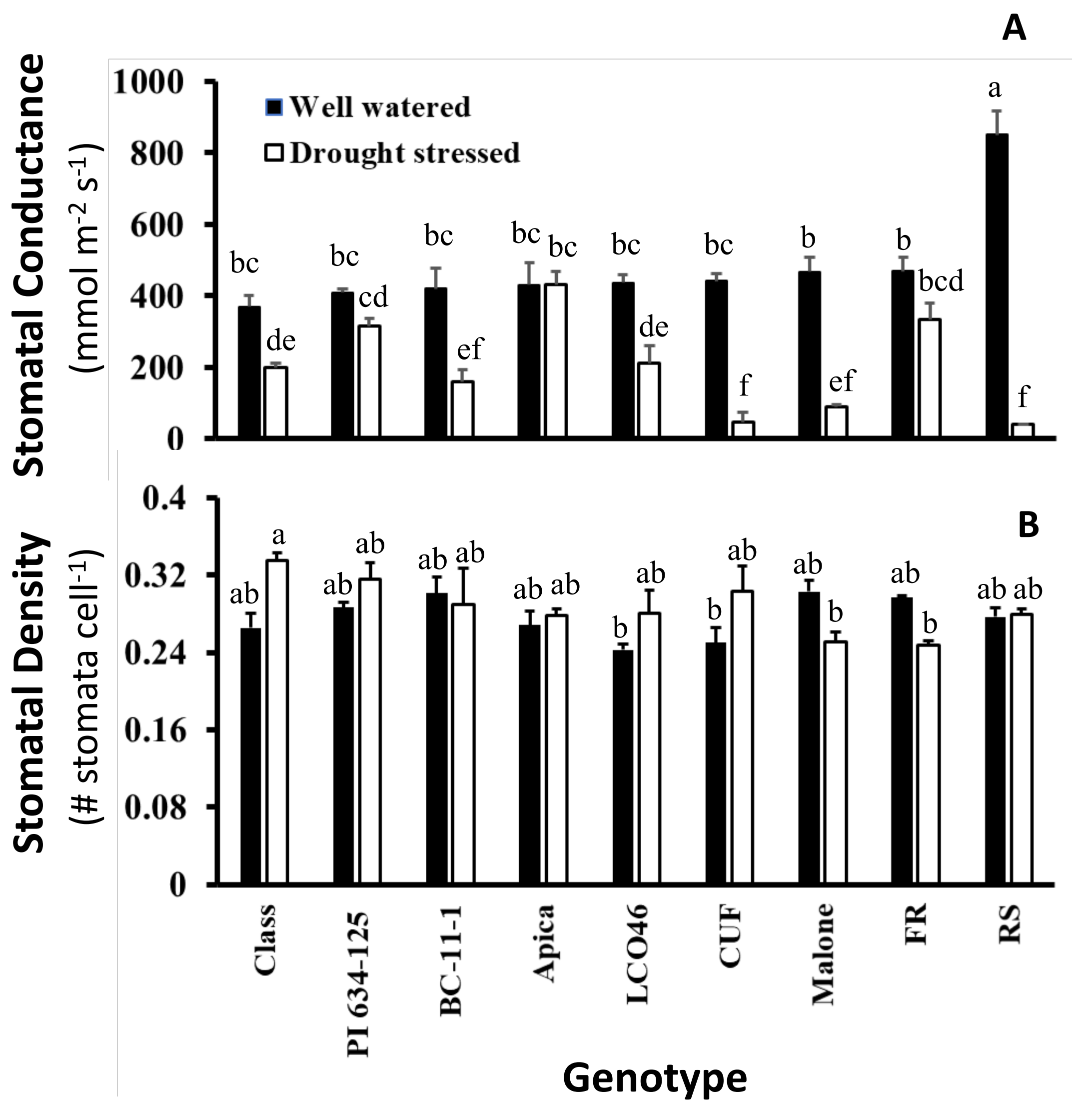
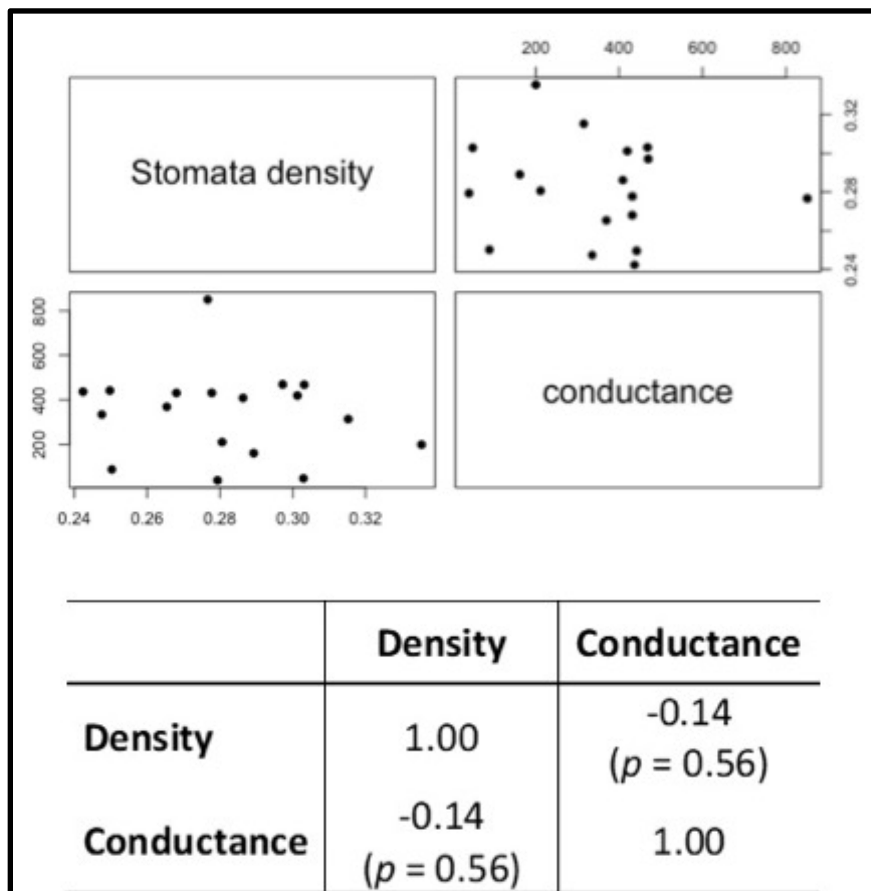


Figure 5 Effect of drought stress on stomatal density and conductance.

The data is shown as mean ± S.E (n=3). Different letters indicate significant difference (p<0.05). A) Stomatal conductance B) Stomatal density in well-watered and drought stressed conditions.



	Density	Conductance
Density	1.00	-0.14 (p = 0.56)
Conductance	-0.14 (p = 0.56)	1.00

Figure 6 Correlation coefficient between Stomatal density and conductance of the 9 alfalfa genotypes in drought stressed conditions

2.4 Discussion

While stomatal density and distribution directly impact the transpiration rate and thus potentially impact WUE in many plants, the genetical variation in stomatal density and its relation to WUE in alfalfa has not been examined. Our results from screening 33 accessions/genotypes of alfalfa in well-watered condition demonstrated that variations in the stomatal density exist among alfalfa genotypes. In terms of total stomatal density RS, FR, PI-634-125, Apica showed almost similar values around 0.275 stomates/epidermal cell

whereas CS15-2, SD 201, BC11-1, Melone, LC 46, CUF and Class showed less than 0.25 stomates/cell.

Further study was conducted on 9 different germplasms to evaluate the impact of drought stress on stomatal density and conductance in different germplasms and to evaluate the relationship of the stomatal density with stomatal conductance. None of the nine genotypes showed a significant change in stomatal density in new leaves developed after drought treatment. Surprisingly, RS had shown a significantly increase in stomal density under drought in our previous study (Ghimire et al. 2021). While the reason is not clear, it might have something to do with the growth conditions in the greenhouse which could impact how drought stress was developed. In this experiment, we noticed a rapid wilting developed in few days after stress treatment due to high light and temp in the summer. As a support, studies have shown that, rice and chinensis leaves show an increase in stomatal density while exposed to moderate drought stress but decrease the same in response to severe drought (Xu and Zhou 2008).

While stomatal density did not show a significant change in alfalfa under drought, most of the nine genotypes showed a significant decrease in stomatal conductance, including RS. The results suggest that controlling stomatal opening and closure can be the key to regulating transpirational water loss. Our results showed little to no correlation between stomatal density and conductance, further supporting the notion that controlling stomatal closure and opening is a more important process in regulating water loss and thus WUE.

To summarize, alfalfa genotypes demonstrated variation in stomatal density and behavior under well-watered and drought conditions. The stomatal conductance appears to be independent of the stomatal density which suggests the possibility of other factors like controlling stomatal pore size (closure) to be the key to transpirational water loss. This supports our previous study where a greater WUE in RS is related to greater sensitivity of stomatal closure to ABA. The next chapter is thus designed to address how this higher sensitivity of stomata to ABA is achieved.

3 Chapter 3 Identification and transcript analysis of ABA binding PYL like genes in *Medicago sativa* for drought stress response

3.1 Introduction

3.1.1 Abscisic acid an essential plant hormone

Abscisic acid (ABA) is an important phytohormone found in all the terrestrial plants and almost all the fungi. ABA plays diverse roles in plants from embryo development to cellular division and growth, seed dormancy and senescence to abiotic and biotic stresses responses (Cutler et al. 2010). In angiosperms, ABA is synthesized in the roots and transported to shoots through xylem in response to drought to regulate the transpirational loss (Hartung et al. 2002).

3.1.2 ABA in stomatal closure

ABA plays an essential role in closure of stomata by mediating solute efflux in the guard cells. Extensive studies in guard cells have revealed the core ABA signaling assembly used by plants to reduce water loss. It includes, the ABA, Pyrabactin Resistance [PYR]/[PYR1-Like (PYL] protein which is member of START protein family, Group-A protein phosphatases 2C (PP2C), and SNF1 related protein kinase 2 (SnRK2 or SRK2) (Weiner et al. 2010). In this assembly, PYR/PYL are the ABA receptor proteins, PP2C are the negative regulator and SnRK2 are the positive regulator in ABA dependent stomatal closing cascade.

3.1.3 ABA signaling pathway

ABA signaling pathway involves number of phosphorylation, ion channels and intermediate changes. In the subthreshold levels of ABA, PP2Cs inactivate the SnRK2

protein kinases and the S-type anion channel SLC1 by dephosphorylation. PP2C also downregulate the Ca^{2+} permeable cation channels (ICa). In the presence of ABA, PYR/PYL receptors bind with ABA and the complex binds and inhibits the PP2Cs. Inactivation of PP2Cs activates the SnRK2s by autophosphorylation. ICa channels released from downregulation results in increased Ca^{2+} concentration in cytoplasm which further activates CPKs. The activated SnRK2s and CPKs phosphorylate the SLAC1 channels and activate the anion efflux resulting in depolarization of the plasma membrane. Due to which K^{+} efflux initiates through voltage dependent GORK channel. The loss of osmolytes causes a decrease in osmotic potential in the guard cell or an increase in water potential. As a result, water leaves guard cells. A collapse of turgor pressure in guard cells closes the stomata as shown in Figure 7 (Munemasa et al. 2015).

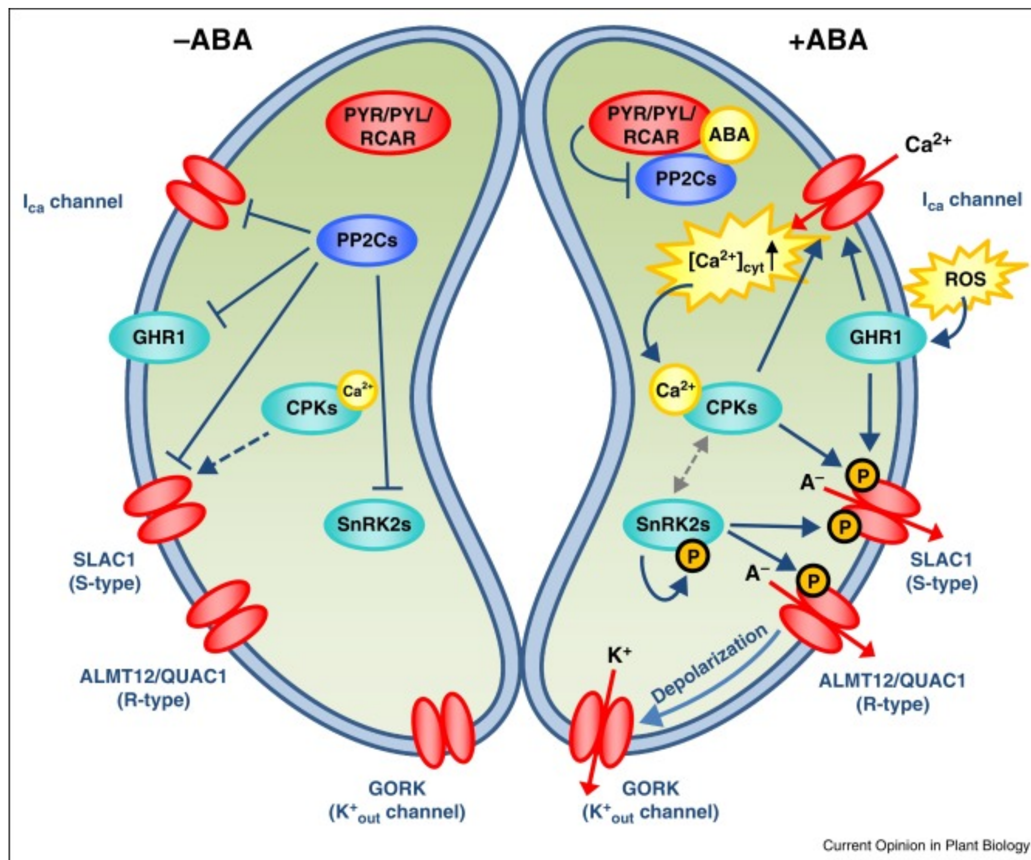


Figure 7 Schematic representation of the regulation of osmolyte movements in guard cells in absence and presence of ABA

(Munemasa et al., 2015)

3.1.4 Pyrabactin Resistance [PYR]/ [PYR1-Like (PYL] protein

PYL proteins being the receptors of ABA in guard cells play essential role in the signaling pathway, hence the stomatal closure. The different roles of PYR/PYL gene family were first studied in *A. thaliana* and until now fourteen PYLs have been discovered PYR1 and PYL1-13 (Park et al. 2009). Gonzalez-Guzman *et al.* in a study on *A. thaliana* demonstrated that recognition of ABA by PYR/PYL is essential for basic signal cascades involved in plant growth, seed production and stomatal regulation. The study on sextuple *pyr/pyl* mutants demonstrated that PYR1, PYL1, PYL2, PYL4, PYL5 and PYL8 members

of PYR/PYL family are required for stomatal closure to different degrees (Gonzalez-Guzman et al. 2012). Because of its significance in ABA-induced stomatal closure, expression of PYR and PYL genes have been studied frequently. In a study on *B. napus*, *PYL1* and *PYL8* like genes showed up-regulation in the drought stressed conditions (Di et al. 2018). In another study on *A. thaliana*, however, *PYR1*, *PYL2*, *PYL4*, and *PYL8* were downregulated when the plant was exposed to dry air (Dittrich et al. 2019). More complex regulation was seen in the study on tobacco plant when the seedlings had higher expression values of PYLs after short term dehydration and downregulation after long term dehydration (Bai et al. 2019).

3.2 Rationale and Hypothesis

A previous study in our lab demonstrated that RS germplasm, showed higher WUE as compared to several other alfalfa genotypes under drought conditions (Anower et al. 2017b). Further analysis showed the stomate in RS had higher sensitivity towards externally applied ABA (Ghimire et al. 2021). ABA accumulation or the number of stomate in RS appeared to be less important in relation to WUE. Since ABA receptors play a critical role in ABA signaling, we thus hypothesize that a higher sensitivity to ABA in RS in stomatal closure under drought is due to a higher level of PYR or PYL. As a first step, we examined the transcript level of *PYR* and *PYL* in the leaves of well-watered and water stressed RS.

3.3 Materials & methods

3.3.1 Identification of homologs of AtPYLs in *Medicago truncatula* and *Medicago sativa*

To find PYL genes in *Medicago sativa*, we first identified PYL like genes in a closely related plant species, *Medicago truncatula* using PYL protein sequences in *Arabidopsis* from NCBI database. These AtPYL protein sequences were used as queries in basic local alignment search tool (BLASTp) against *Medicago truncatula* genome in Ensembleplants.org with a scoring matrix set at BLOSUM 62 and E-value threshold at 1e-1. The 23 non-redundant proteins (MtPYL) obtained were further used as queries in the BLASTp and BLASTn tool on the Noble research institute's alfalfa breeder toolbox (alfalfatoolbox.org) against the alfalfa genome sequences. E-value cutoff for this search was 1e-5. PAM30 matrix was used for scoring the results. Redundant genes appeared due to the tetraploid complexity of the genome were removed manually.

3.3.2 Multiple sequence alignment and phylogenetic tree analysis

To see the evolutionary relationship of AtPYL gene family with the selected genes in *M. truncatula* and *M. sativa*, multiple sequence alignment was done using ClustalW in MegaX version 10.1.7 (Tamura et al. 2021). The phylogenetic tree of the protein sequences from the three plants was built using neighbor-joining method with bootstrap value of 1000.

3.3.3 Conserved motifs

To identify the conserved motifs, the protein sequence from *M. sativa* and *A. thaliana* were analyzed on Multiple EM for Motif Elicitation version 5.3.3 (MEME) (Bailey et al. 2009). Search was set for 20 motifs.

3.3.4 Primer designing

Primers for the selected 15 genes of *M. sativa* were designed using online tool, Integrated DNA technologies (IDT). The CDS sequences for each gene were used for primer designing and the intron locations were mapped manually based on the data from alfalfa breeder's toolbox (jbrowser) to design primers in flanking regions. To determine the efficiency and specificity of each primer, the PCR protocol was derived from previous study (Kanchupati et al. 2017). In brief, the primers were tested with genomic DNA of alfalfa (1 ng) in a 20 µl PCR reaction containing 2 µl of 10x PCR buffer, 2 µl of 2 mM deoxynucleotides, 1 µl each of 10 µM primers, 0.5 µl of Taq polymerase (5U μl^{-1}, BioLabs) and autoclaved MQ water to make 20 µl volume. The reactions were further run-on gradient thermocycler (Eppendorf Mastercycler) with initial denaturation at 94^0C for 3 mins, followed by 35 cycles of denaturation at 94^0C for 30 sec, annealing at the gradient temperature (R= 3 ^{0}C s^{-1}, G= ± 3 ^{0}C) for 30 sec, extension at 72^0C for 30 sec/1min (based on product size). A final extension at 72^0C for 10 mins, followed by 1 min at 22 ^{0}C in the end was programmed to allow the products to efficiently get double stranded. The PCR products were separated on 1.5% agarose gel for 45 mins at 120V with 10 µl of DNA

ladder (quick load 1kb DNA ladder, Biolabs) and visualized using LI-COR Odyssey Infrared imaging system premium at 600 nm. The primers which showed specificity were continued for further gene expression studies. All the primers used in the gene expression study are listed in Table 1.

Gene	Orientation	Primers	Length	Tm (⁰C)	Amplicon (bp)
MsPYL1	Forward	CTTCCACCTCCGATCAAGATTC	22	62	123
	Reverse	AGGTGTGGTGTGAGTTGATG	20	62	
MsPYL2	Forward	GTCAATGTCATCTCCGGTCTC	21	62	123
	Reverse	ATGGTAATTCTTCAAACGGTGTTC	24	62	
MsPYL3	Forward	CTCGAAGTCTTAGACGATGAACG	23	62	107
	Reverse	ATCGGACTAGGGTGAAGAGTAG	22	62	
MsPYL4	Forward	GATGGAAACGTTGGTAGCATTAG	23	62	134
	Reverse	CGATGTTCACCACCAACAAC	20	62	
MsPYL5	Forward	CGACAATCCACAAGGCTACA	20	62	113
	Reverse	CAGGTAGACCGGAAACAAGTC	21	62	
MsPYL6	Forward	CGACAATCCACAAGGCTACA	20	62	113
	Reverse	CAGGTAGACCGGAAACAAGTC	21	62	
MsPYL7	Forward	CACGGTGATAGTTGAGTCCTATG	23	62	131
	Reverse	GTGTAATGTTCTCTGCGGTTTG	22	62	
MsPYL8	Forward	CACGGTGATAGTTGAGTCCTATG	23	62	131
	Reverse	GTGTAATGTTCTCTGCGGTTTG	22	62	
MsPYL9	Forward	CACATCAAAGCACCAGTTCATC	22	62	133
	Reverse	CATTCACTTCTCTTACACTTCCAATAC	27	62	
MsPYL10	Forward	CAACAGCCTGGACAGAATCA	20	62	127
	Reverse	ACCTGCTCACGAATGGTTTAT	21	62	
MsPYL11	Forward	GATGTCTCTCCACCCTGAAATTAT	24	62	110
	Reverse	CGAAGTAGCAGGTTTCGTCTT	21	62	
MsPYL12	Forward	GTGGACCTGGAACCATCAAA	20	62	134
	Reverse	ATCCAACCCTGTTCCTCCTA	20	62	
MsPYL13	Forward	AGGAACAGGGTTGGATGAAAG	21	62	143
	Reverse	CATCACGGACTGCATCAGATAG	22	62	
MsPYL14	Forward	TCATCCCAAAGGTGATTCCAG	21	62	98
	Reverse	GCCACCTTCAGACATGGATAA	21	62	
MsPYL15	Forward	GCAGTTCTATCTGAAGCAGTA	21	59	102
	Reverse	TGCTTAATAATTAGGGTTTGCC	22	59	

Table 1 Primers used in PCR for PYL genes in Medicago sativa.

3.3.5 Plant materials and treatments

For the drought and dehydration treatment 60 plants each of RS and AF were grown in the cone containers with 38 g potting mix after vegetative propagation. All the plants were

grown in the greenhouse with 16 hours photoperiod. The plants were kept well-watered and were provided the Miracle Gro nutrient solution as described in the previous chapter.

3.3.6 MsPYL gene expression in leaf, stem, and root of well-watered alfalfa plants

For studying the gene expression in different tissues in RS, youngest mature leaves were collected from the first 2-3 nodes. The stem tissue was collected between 4-6 nodes. For the root samples, the washed roots were quickly dried with paper towel and the living, young root tip areas were collected. All the tissues were collected separately and immediately frozen in liquid Nitrogen to ultimately store in -80^{0}C.

3.3.7 Drought treatment

For drought stress treatment, 2 weeks old plants with uniform growth of each type were selected. Half of the plants of RS and AF were kept well-watered, i.e., replenishing 100% of the water lost each day. The other half were subjected to water stress, i.e., replenishing 50% of the water lost each day. We continued the treatment for 14 days and measured the stomatal conductance to monitor the stress condition along with morphological changes like wilting and ability of plants to recover from stress. The final harvest for both genotypes was done when the stomatal conductance reached the minimum level. Leaves from newly matured shoots were harvested from each of three biological replicates. All the harvested tissues were frozen in liquid nitrogen and stored at -80^{0}C.

3.3.8 Shoot dehydration treatment

For studying the change in gene expression with a short-term water deficit condition, we conducted a dehydration experiment on young shoots of the RS and AF plants. Eighteen young shoots (1-3 nodes) were cut from different well-watered plants for each of the RS and AF genotypes and divided into 3 groups of well-watered and 3 groups for dehydration with 3 shoots in each group (3 shoots * 3 replicates for each treatment). The well-watered shoots were dipped in water whereas the shoots used for dehydration were placed under white LED lights in a ventilated room with 26% humidity at 22^0C temperature. The weight of the shoots was monitored constantly. When 30% loss of weight was reached the samples were immediately frozen in liquid nitrogen and stored at -80^0C.

3.3.9 RNA extraction and purification

The extraction of total RNA from the tissues was performed using RNeasy plant mini kit, Qiagen. The isolated RNA samples were quantified for purity and concentration using a nanodrop, and RNA samples with 260/280 ratio between 1.8 to 2.2 were used for further analysis. To further test the integrity of RNA, the samples were separated on 1% agarose gel stained with ethidium bromide and the samples showing two clear bands were selected. To remove the trace genomic DNA in the RNA samples, 750 ng RNA was treated with TURBO DNase treatment and removal reagents in a 17.2 µl reaction (TURBO DNA-free kit, Invitrogen, fisher scientific, Carlsbad, California) following the instructions in manual. To check for genomic contamination, a PCR with the untreated and DNase treated RNA samples without reverse-transcription, as templates, was performed with genomic DNA

and cDNA as positive controls. Presence of bands in the untreated samples and positive controls while no amplification in DNase treated samples confirmed that RNA samples, after DNase treatment, are free of any genomic DNA contamination which was then used in gene expression analysis.

3.3.10 cDNA synthesis and RT-PCR

The DNase-treated RNA samples (~200 ng) were used for first strand cDNA synthesis using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen) in a 20 µl reaction. cDNA produced through this procedure was diluted 10 times before they were used for qPCR analysis.

3.3.11 Quantitative analysis using real time qPCR

We used Thermo Scientific DyNAmo Flash SYBR Green Hot Start qRT-PCR kit (ThermoFisher) for qPCR analysis. For each of the three biological replicate, 2 technical replicates were assayed in 10 µl reaction each, according to the manual. ABI 7900HT high-throughput Real Time Thermocycler (Applied Biosystems, MA, USA) was used according to standardized cycling steps with minor changes, as follows: 95 ^{0}C for 15 mins to activate the reaction, followed by 40 cycles of denaturation at 94 ^{0}C for 15 sec, annealing at 61 ^{0}C for 30 sec, extension at 72 ^{0}C for 30 sec, a final extension of 10 mins at 72 ^{0}C before melt curve step. The data was collected at each extension cycle step for the Ct value and at the melt curve step to determine the specificity of the reaction. *MsActin* gene was used to normalize the Ct value of all samples. The change in the transcripts of PYL like genes in

different treatments was calculated using the ΔΔCt method by calculating the fold change using $2^{\wedge-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001).

3.3.12 Data analysis

Microsoft Excel 365 was used for statistical analysis. T- test was performed to determine the significantly different mean values.

3.4 Results

3.4.1 Identification of PYLs in *Medicago truncatula* and *Medicago sativa*

We found 23 PYL protein family members in *Medicago truncatula* using 13 PYR/PYL protein sequences of *Arabidopsis thaliana* as query with scoring matrix BLOSUM 62 and E-value threshold of 1e-1 (Table 2). These proteins showed Polyketide cyclase/ dehydrase and START-like superfamily domains as identified on InterProScan tool. Using the physical location of the 23 MtPYLs, the genes were mapped on 8 chromosomes as shown in Figure 8. Although genes were found to be distributed on all 8 chromosomes, chromosome number 6, 7 and 8 had one gene on each whereas chromosome number 1 and 3 have two genes on each of them. Chromosome number 2, 4 and 5 showed tandem genes as well as distantly located genes. Chromosome 2 had 8 of the 23 genes, MtPYL10, 12-16, 18 and 20 in the tandem. Chromosome number 4 contained 5 of 23 genes, MtPYL11, 19, 21 in tandem and MtPYL6 and 22 distantly. Chromosome 5 had 2 genes in tandem, MtPYL4 and 23.

Similarly, 15 PYL proteins in *Medicago sativa* were obtained using 23 *M. truncatula* proteins as query by performing BLASTP against the alfalfa database with E-value cutoff 1e-5, BLASTp size 3 and PAM30 matrix for scoring the results (Table 2). These proteins are annotated to be involved in abscisic acid-activated signaling pathway and protein phosphatase inhibitor activity in the database. The 15 genes in alfalfa are found to be distributed on 7 of the 8 chromosomes but due to lack of genomic sequence of alfalfa, we were unable to locate the genes on the specific location on each chromosome.

Gene Name	Gene ID	Plant
AtPYL1	At5g46790	*Arabidopsis thaliana*
AtPYL2	At2g26040	*Arabidopsis thaliana*
AtPYL3	At1g73000	*Arabidopsis thaliana*
AtPYL4	At2g38310	*Arabidopsis thaliana*
AtPYL5	At5g05440	*Arabidopsis thaliana*
AtPYL6	At2g40330	*Arabidopsis thaliana*
AtPYL7	At4g01026	*Arabidopsis thaliana*
AtPYL8	At5g53160	*Arabidopsis thaliana*
AtPYL9	At1g01360	*Arabidopsis thaliana*
AtPYL10	At4g27920	*Arabidopsis thaliana*
AtPYL11	At5g45860	*Arabidopsis thaliana*
AtPYL12	At5g45870	*Arabidopsis thaliana*
AtPYL13	At4g18620	*Arabidopsis thaliana*
MtPYL1	MTR_5g030500	*Medicago truncatula*
MtPYL2	MTR_3g071740	*Medicago truncatula*
MtPYL3	MTR_7g070050	*Medicago truncatula*
MtPYL4	MTR_5g083270	*Medicago truncatula*
MtPYL5	MTR_1g016480	*Medicago truncatula*
MtPYL6	MTR_4g014460	*Medicago truncatula*
MtPYL7	MTR_8g027805	*Medicago truncatula*
MtPYL8	MTR_3g090980	*Medicago truncatula*
MtPYL9	MTR_1g028380	*Medicago truncatula*
MtPYL10	MTR_2g435310	*Medicago truncatula*
MtPYL11	MTR_4g120760	*Medicago truncatula*
MtPYL12	MTR_2g035150	*Medicago truncatula*
MtPYL13	MTR_2g035105	*Medicago truncatula*
MtPYL14	MTR_2g035100	*Medicago truncatula*
MtPYL15	MTR_2g035170	*Medicago truncatula*
MtPYL16	MTR_2g035130	*Medicago truncatula*
MtPYL17	MTR_6g033450	*Medicago truncatula*
MtPYL18	MTR_2g035190	*Medicago truncatula*
MtPYL19	MTR_4g120970	*Medicago truncatula*
MtPYL20	MTR_2g035320	*Medicago truncatula*
MtPYL21	MTR_4g120950	*Medicago truncatula*
MtPYL22	MTR_4g094532	*Medicago truncatula*
MtPYL23	MTR_5g081780	*Medicago truncatula*
MsPYL1	MSAD_307595	*Medicago sativa*
MsPYL2	MSAD_236253	*Medicago sativa*
MsPYL3	MSAD_291139	*Medicago sativa*
MsPYL4	MSAD_257700	*Medicago sativa*
MsPYL5	MSAD_276284	*Medicago sativa*
MsPYL6	MSAD_221395	*Medicago sativa*
MsPYL7	MSAD_264830	*Medicago sativa*
MsPYL8	MSAD_237211	*Medicago sativa*
MsPYL9	MSAD_224673	*Medicago sativa*
MsPYL10	MSAD_244845	*Medicago sativa*
MsPYL11	MSAD_280010	*Medicago sativa*
MsPYL12	MSAD_255399	*Medicago sativa*
MsPYL13	MSAD_255395	*Medicago sativa*
MsPYL14	MSAD_261603	*Medicago sativa*
MsPYL15	MSAD_255398	*Medicago sativa*

Table 2 PYL gene family members names used in this study in Arabidopsis thaliana, Medicago truncatula and Medicago sativa with their Gene IDs according to NCBI database, Ensembleplants.org and alfalfatoolbox.org respectively

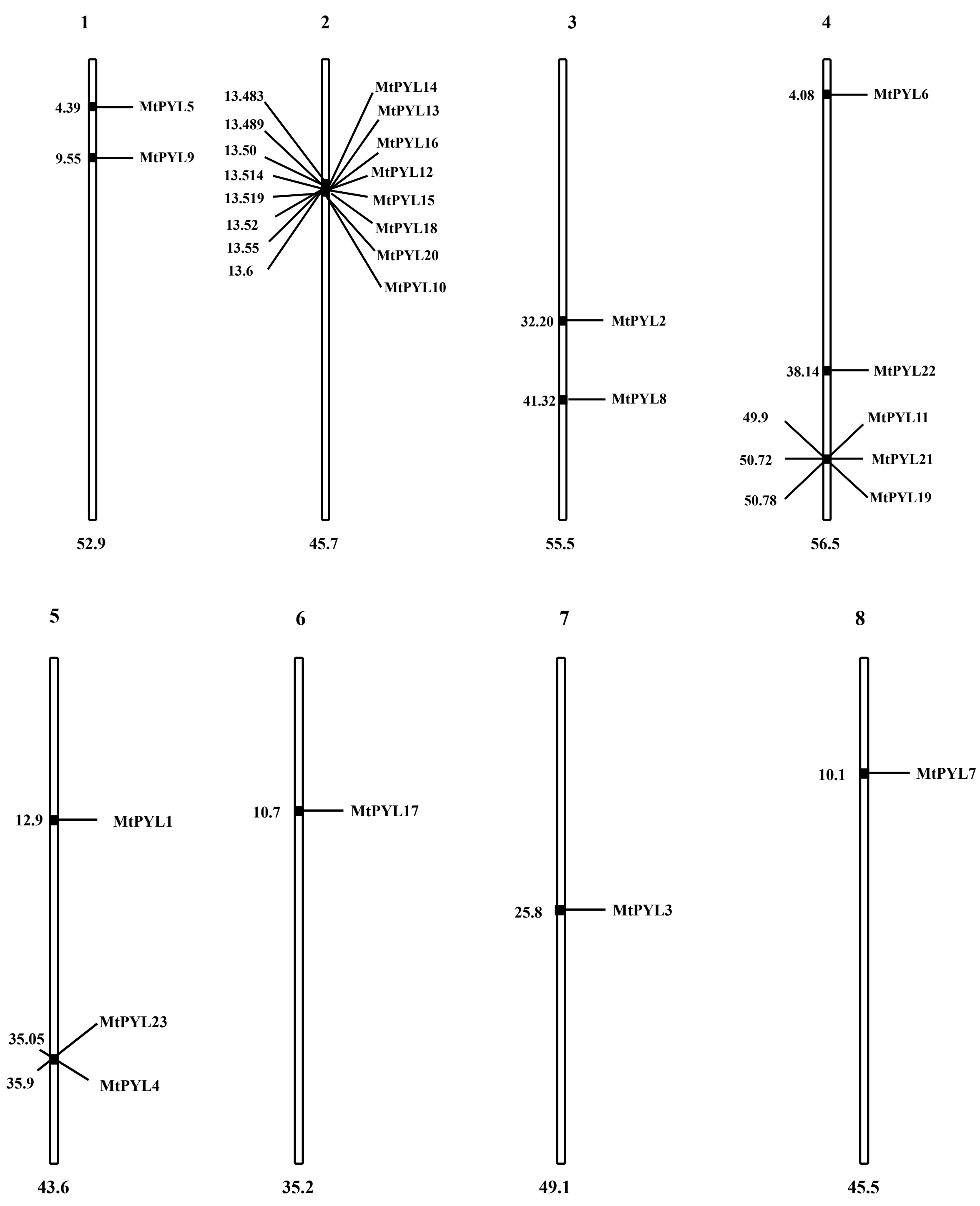


Figure 8 Distribution of MtPYL genes on 8 chromosomes.

The lengths of chromosomes are shown in Mbp. Medicago truncatula genes illustrated in this figure are: MtPYL1 (MTR_5g030500), MtPYL2 (MTR_3g071740), MtPYL3 (MTR_7g070050), MtPYL4 (MTR_5g083270), MtPYL5 (MTR_1g016480), MtPYL6 (MTR_4g014460), MtPYL7 (MTR_8g027805), MtPYL8 (MTR_3g090980), MtPYL9 (MTR_1g028380), MtPYL10 (MTR_2g435310), MtPYL11 (MTR_4g120760), MtPYL12 (MTR_2g035150), MtPYL13 (MTR_2g035105), MtPYL14 (MTR_2g035100), MtPYL15 (MTR_2g035170), MtPYL16 (MTR_2g035130), MtPYL17 (MTR_6g033450), MtPYL18 (MTR_2g035190), MtPYL19 (MTR_4g120970), MtPYL20 (MTR_2g035320), MtPYL21(MTR_4g120950), MtPYL22 (MTR_4g094532), MtPYL23 (MTR_5g081780)

3.4.2 Multiple sequence alignment and phylogenetic relationship of MtPYLs and MsPYLs with AtPYLs

To analyze the evolutionary relationship of the PYL genes of *M. truncatula* and *M. sativa* with PYL gene family of *Arabidopsis thaliana*, we performed the multiple sequence alignment and phylogenetic tree analysis on the protein sequences of the selected genes. The PYL proteins in *A. thaliana* are divided into three groups based on the divergence. Figure 9 shows that AtPYL1, 2 and 3 are in one group whereas AtPYL 4, 5, 6, 13, 11, 12, 13 form one group. Similarly, AtPYL7, 8, 9, 10 make a separate group. The sequence alignment of MsPYL proteins with AtPYL proteins is shown in Figure 10.

The rooted phylogenetic tree of AtPYLs, MtPYLs and MsPYLs with a bootstrap value of 1000 shows the closely related PYL proteins in *A. thaliana, M. truncatula* and *M. sativa* (Figure 11). Based on the distribution, the MtPYL and MsPYL proteins are divided into four subgroups. Subgroup I (indicated in red) contain the closely related orthologs of AtPYL4, 5 and 6, similarly subgroup II (indicated in violet) contains the closely related

orthologs of AtPYL7, 8, 9 and 10 and subgroup III (indicated in green) shows the close relatives of AtPYL1, 2 and 3. Subgroup IV contain no AtPYL protein but only MtPYL and MsPYL proteins. A rooted phylogenetic analysis was also conducted using tomato EPF1 sequence as outgroup, showing similar grouping of these PYL protein sequences among three species (data not shown). The proteins in similar group might perform similar functions.

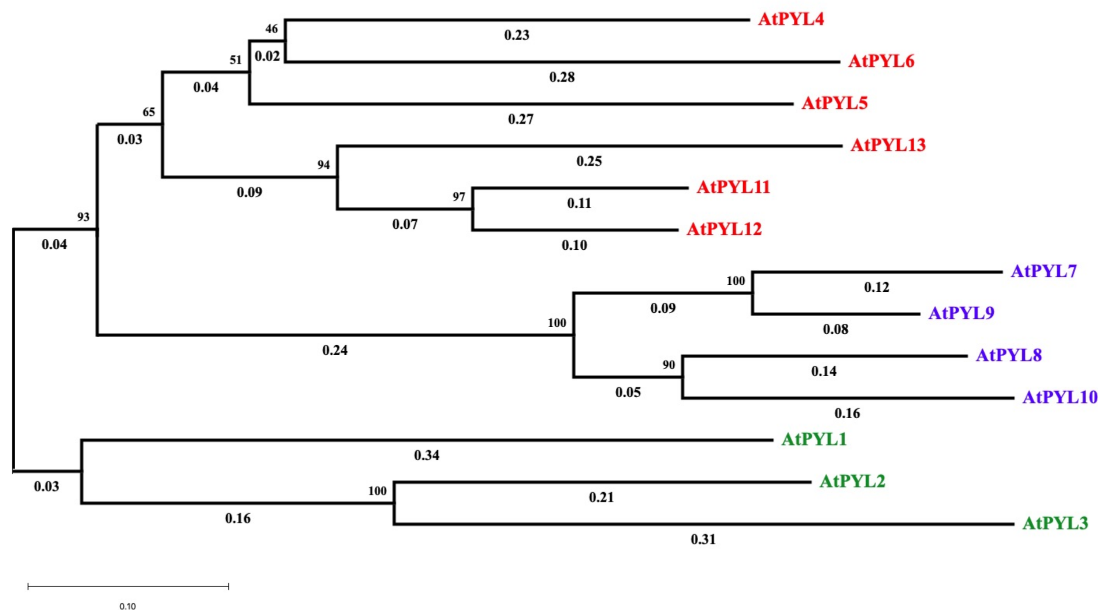
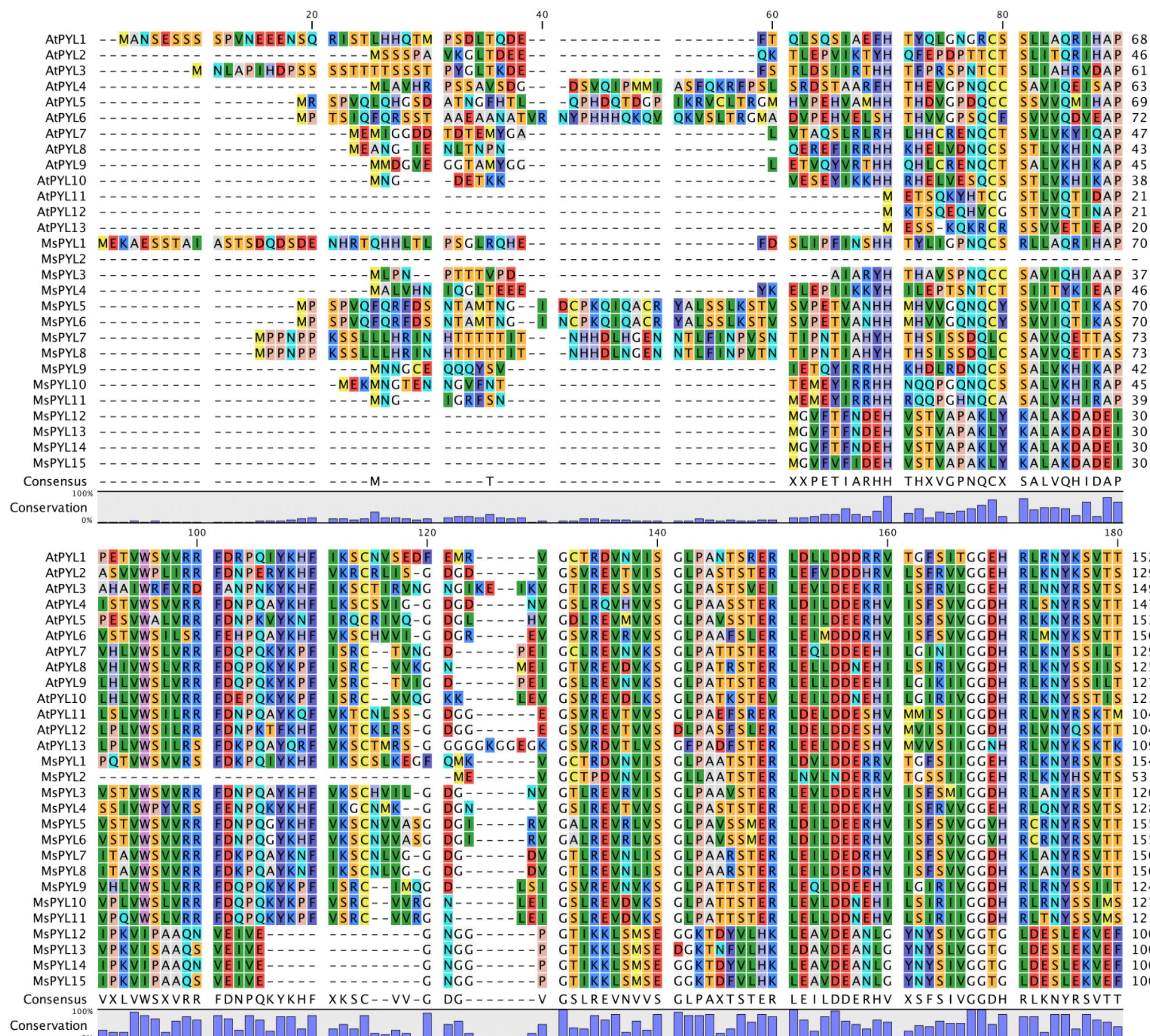


Figure 9 Phylogenetic tree of PYL gene family in Arabidopsis thaliana.

The Arabidopsis thaliana protein sequence include: AtPYL1 (At5g46790), AtPYL2 (At2g26040), AtPYL3 (At1g73000), AtPYL4 (At2g38310), AtPYL5 (At5g05440), AtPYL6 (At2g40330), AtPYL7 (At4g01026), AtPYL8 (At5g53160), AtPYL9 (At1g01360), AtPYL10 (At4g27920), AtPYL11 (At5g45860), AtPYL12 (At5g45870), AtPYL13 (At4g18620)


20
40
60
80
AtPYL1
AtPYL2
AtPYL3
AtPYL4
AtPYL5
AtPYL6
AtPYL7
AtPYL8
AtPYL9
AtPYL10
AtPYL11
AtPYL12
AtPYL13
MsPYL1
MsPYL2
MsPYL3
MsPYL4
MsPYL5
MsPYL6
MsPYL7
MsPYL8
MsPYL9
MsPYL10
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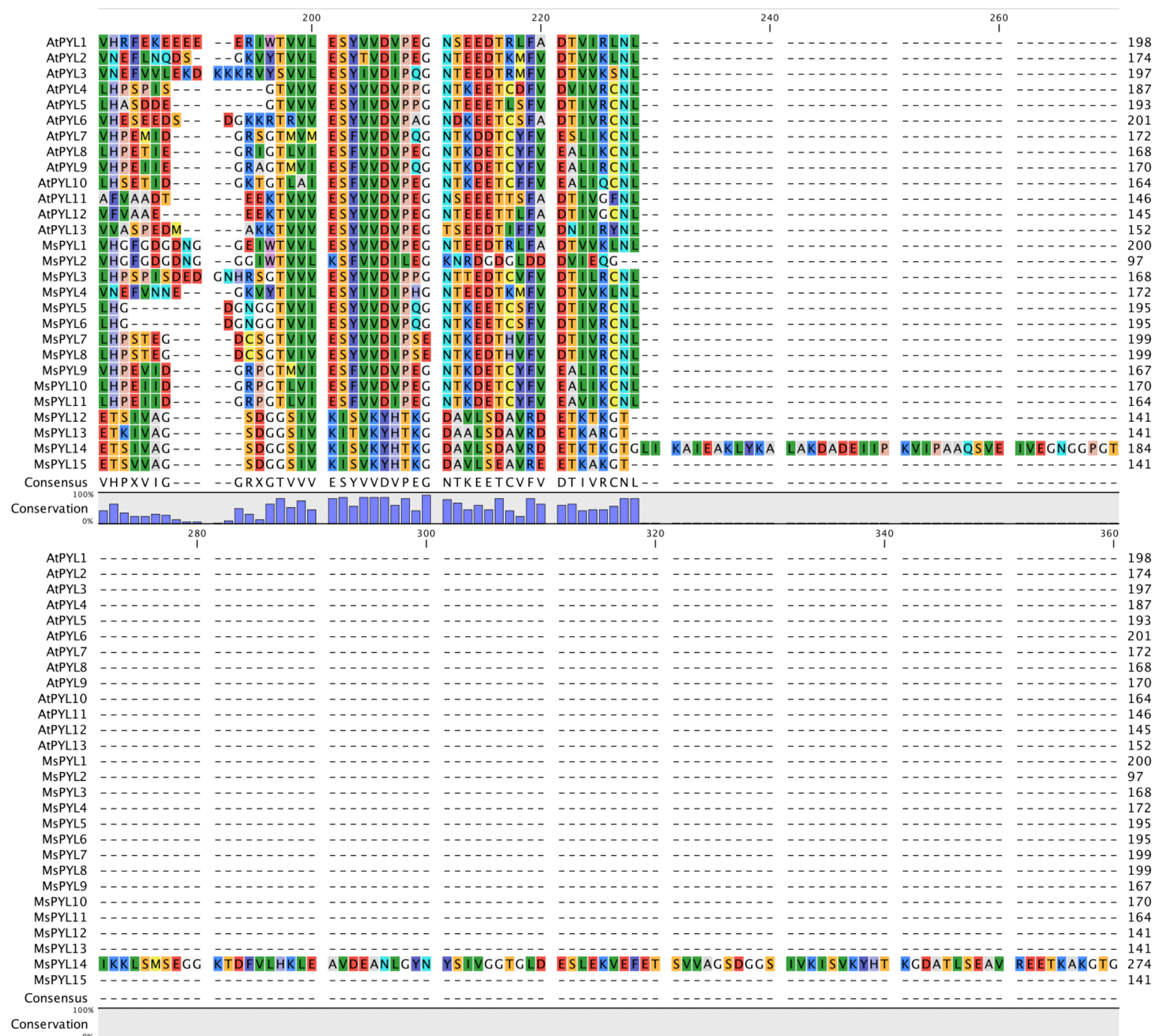
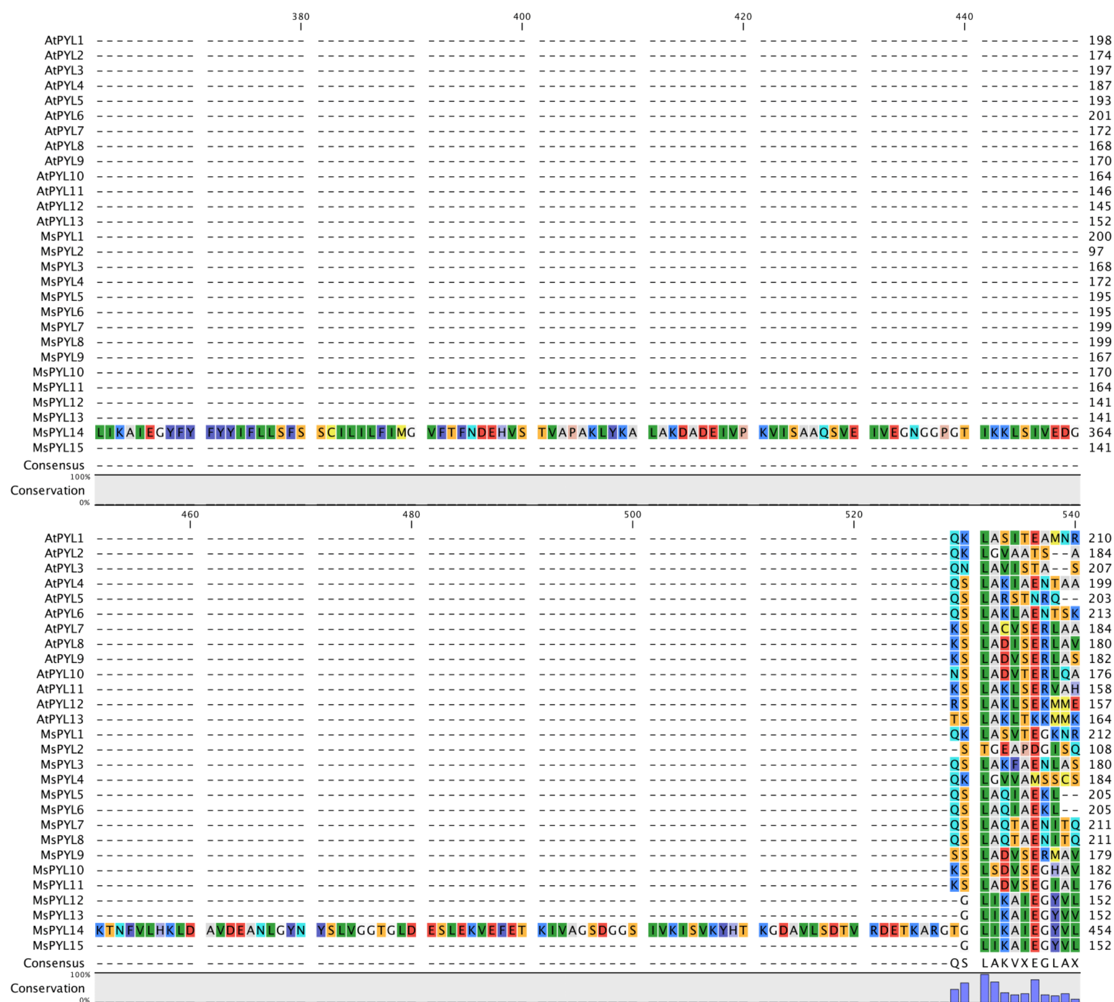
0%
100
120
140
160
180





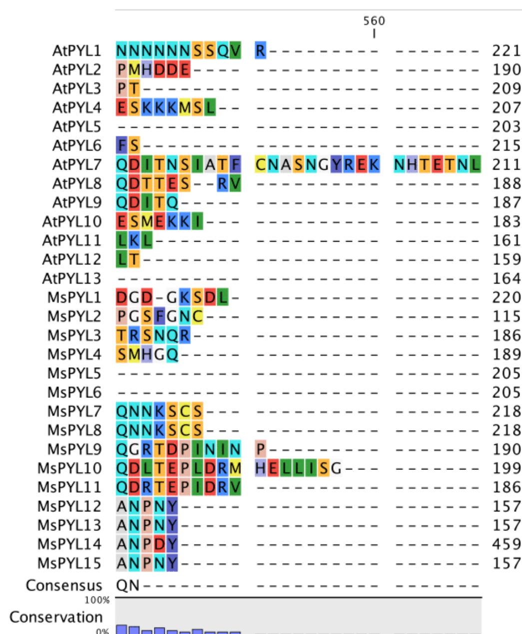


Figure 10 Multiple sequence alignment of the PYL gene family in A. thaliana & Medicago sativa.

The Arabidopsis thaliana protein sequence include: AtPYL1 (At5g46790), AtPYL2 (At2g26040), AtPYL3 (At1g73000), AtPYL4 (At2g38310), AtPYL5 (At5g05440), AtPYL6 (At2g40330), AtPYL7 (At4g01026), AtPYL8 (At5g53160), AtPYL9 (At1g01360), AtPYL10 (At4g27920), AtPYL11 (At5g45860), AtPYL12 (At5g45870), AtPYL13 (At4g18620)Medicago sativa sequences include: MsPYL1 (MSAD_307595), MsPYL2 (MSAD_236253), MsPYL3 (MSAD_291139), MsPYL4 (MSAD_257700), MsPYL5 (MSAD_276284), MsPYL6 (MSAD_221395), MsPYL7 (MSAD_264830), MsPYL8 (MSAD_237211), MsPYL9 (MSAD_224673), MsPYL10 (MSAD_244845), MsPYL11 (MSAD_280010), MsPYL12 (MSAD_255399), MsPYL13 (MSAD_255395), MsPYL14 (MSAD_261603), MsPYL15 (MSAD_255398)

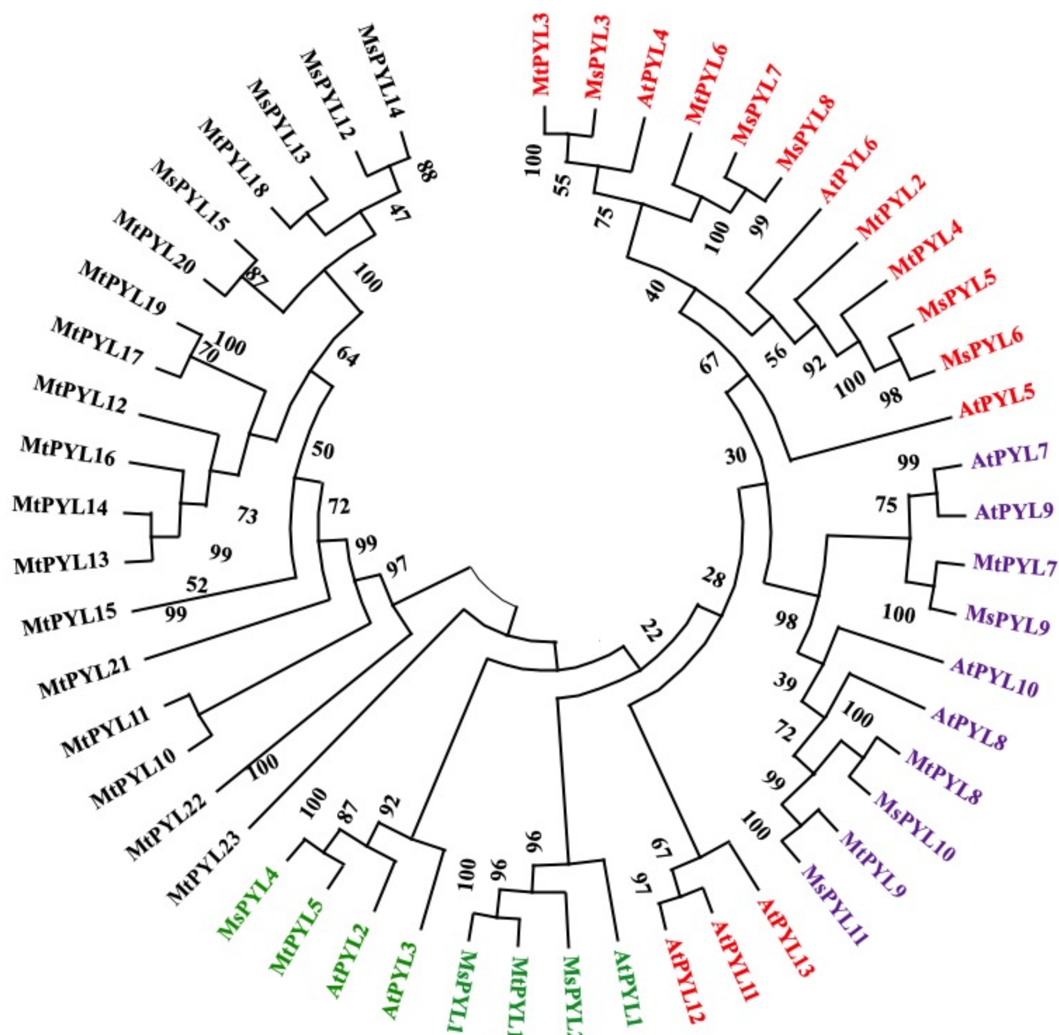


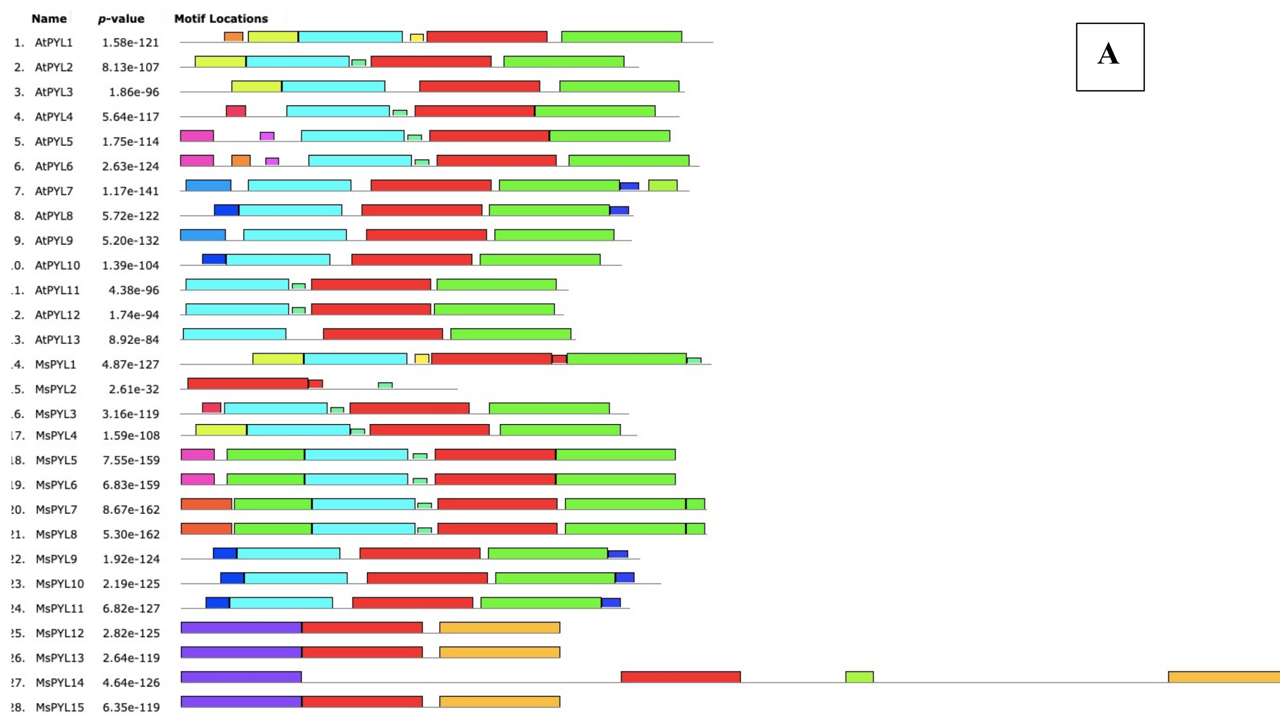
Figure 11 Phylogenetic tree of PYL Protein sequences in A. thaliana, M. truncatula & M. sativa.

The colors are representing the three subgroups of AtPYLs. The Arabidopsis thaliana protein sequence include: AtPYL1 (At5g46790), AtPYL2 (At2g26040), AtPYL3 (At1g73000), AtPYL4 (At2g38310), AtPYL5 (At5g05440), AtPYL6 (At2g40330), AtPYL7 (At4g01026), AtPYL8 (At5g53160), AtPYL9 (At1g01360), AtPYL10 (At4g27920), AtPYL11 (At5g45860), AtPYL12 (At5g45870), AtPYL13 (At4g18620)Medicago sativa sequences include: MsPYL1 (MSAD_307595), MsPYL2 (MSAD_236253), MsPYL3 (MSAD_291139), MsPYL4 (MSAD_257700), MsPYL5 (MSAD_276284), MsPYL6 (MSAD_221395), MsPYL7 (MSAD_264830), MsPYL8 (MSAD_237211), MsPYL9 (MSAD_224673), MsPYL10 (MSAD_244845), MsPYL11 (MSAD_280010), MsPYL12 (MSAD_255399), MsPYL13 (MSAD_255395), MsPYL14

(MSAD_261603), MsPYL15 (MSAD_255398) Medicago truncatula proteins illustrated in this figure are: MtPYL1 (MTR_5g030500), MtPYL2 (MTR_3g071740), MtPYL3 (MTR_7g070050), MtPYL4 (MTR_5g083270), MtPYL5 (MTR_1g016480), MtPYL6 (MTR_4g014460), MtPYL7 (MTR_8g027805), MtPYL8 (MTR_3g090980), MtPYL9 (MTR_1g028380), MtPYL10 (MTR_2g435310), MtPYL11 (MTR_4g120760), MtPYL12 (MTR_2g035150), MtPYL13 (MTR_2g035105), MtPYL14 (MTR_2g035100), MtPYL15 (MTR_2g035170), MtPYL16 (MTR_2g035130), MtPYL17 (MTR_6g033450), MtPYL18 (MTR_2g035190), MtPYL19 (MTR_4g120970), MtPYL20 (MTR_2g035320), MtPYL21(MTR_4g120950), MtPYL22 (MTR_4g094532), MtPYL23 (MTR_5g081780)

3.4.3 Conserved motifs

In this study, a total of 28 protein sequences including 13 AtPYL and 15 MsPYL proteins, were tested for conserved motifs using MEME software. 10 motifs were found with the E-value cutoff at 1.2e-004 (Figure 12). Three motifs showed higher conservation in most of the protein sequences as compared to other motifs. (Figure 12A) The same three motifs show START-like conserved domain. Motif 1 containing 50 amino acid residues “GSLREVNVVSGLPATTSTERLEILDDERHVJSFSIVGGDHRLKNYRSVTT” was found to be highly conserved in all proteins (Figure 12B). Motif 2 with 43 residues “HEVGPNQCSSAVVQHIKAPVSLVWSLVRRFDNPQKYKHFIKSC” and 3 with 50 amino acid residues “ETIDGRSGTVVVESYVVDVPEGNTKEETCYFVDTIVRCNLQSLAKVAERL” were conserved in 23 out 28 proteins (Figure 12C, D). The five other motifs found were short in length and were conserved in fewer proteins between *A. thaliana* and *M. sativa* (Figure 12E-G). Overall, the results suggest that PYL family has highly conserved domains hence might perform similar functions in alfalfa.


A
Name
p-value
Motif Locations
1. AtPYL1 1.58e-121
2. AtPYL2 8.13e-107
3. AtPYL3 1.86e-96
4. AtPYL4 5.64e-117
5. AtPYL5 1.75e-114
6. AtPYL6 2.63e-124
7. AtPYL7 1.17e-141
8. AtPYL8 5.72e-122
9. AtPYL9 5.20e-132
.0. AtPYL10 1.39e-104
.1. AtPYL11 4.38e-96
.2. AtPYL12 1.74e-94
.3. AtPYL13 8.92e-84
.4. MsPYL1 4.87e-127
.5. MsPYL2 2.61e-32
.6. MsPYL3 3.16e-119
17. MsPYL4 1.59e-108
18. MsPYL5 7.55e-159
19. MsPYL6 6.83e-159
20. MsPYL7 8.67e-162
21. MsPYL8 5.30e-162
22. MsPYL9 1.92e-124
23. MsPYL10 2.19e-125
24. MsPYL11 6.82e-127
25. MsPYL12 2.82e-125
26. MsPYL13 2.64e-119
27. MsPYL14 4.64e-126
28. MsPYL15 6.35e-119

E-value 2.5e-706

B

Name	Start	*p*-value	Sites		
16. MsPYL3	71	1.35e-49	CHVILGDGNV	GTLREVRVISGLPAAVSTERLEVLDDERHVISFSMIGGDHRLANYRSVTT	LHPSPISDED
21. MsPYL8	107	6.68e-49	CNLVGGDGDV	GTLREVNLISGLPAARSTERLEILDEDRHVISFSVVGGDHKLANYRSVTT	LHPSTEGDCS
20. MsPYL7	107	6.68e-49	CNLVGGDGDV	GTLREVNLISGLPAARSTERLEILDEDRHVISFSVVGGDHKLANYRSVTT	LHPSTEGDCS
6. AtPYL6	107	6.68e-49	HVVIGDGREV	GSVREVRVVSGLPAAFSLERLEIMDDDRHVISFSVVGGDHRLMNYKSVTT	VHESEEDSDG
22. MsPYL9	75	1.11e-48	RCIMQGDLSI	GSVREVNVKSGLPATTSTERLEQLDDEEHILGIRIVGGDHRLRNYSSIIT	VHPEVIDGRP
5. AtPYL5	104	1.11e-48	RIVQGDGLHV	GDLREVMVVSGLPAVSSTERLEILDEERHVISFSVVGGDHRLKNYRSVTT	LHASDDEGTV
17. MsPYL4	79	1.32e-48	GCNMKGDGNV	GSIREVTVVSGLPASTSTERLEILDDEKHVISFRVVGGEHRLQNYRSVTS	VNEFVNNEGK
14. MsPYL1	105	4.16e-48	SLKEGFQMKV	GCTRDVNVISGLPAATSTERLDVLDDERRVTGFSIIGGEHRLKNYRSVTS	VHGFGDGDNG
7. AtPYL7	80	4.16e-48	RCTVNGDPEI	GCLREVNVKSGLPATTSTERLEQLDDEEHILGINIIGGDHRLKNYSSILT	VHPEMIDGRS
4. AtPYL4	98	4.16e-48	SVIGGDGDNV	GSLRQVHVVSGLPAASSTERLDILDDERHVISFSVVGGDHRLSNYRSVTT	LHPSPISGTV
9. AtPYL9	78	4.89e-48	RCTVIGDPEI	GSLREVNVKSGLPATTSTERLELLDDEEHILGIKIIGGDHRLKNYSSILT	VHPEIIEGRA
23. MsPYL10	78	6.73e-48	RCVVRGNLEI	GSLREVDVKSGLPATTSTERLEVLDDNEHILSIRIIGGDHRLRNYSSIMS	LHPEIIDGRP
24. MsPYL11	72	1.08e-47	RCVVRGNLEI	GSLREVDVKSGLPATTSTERLELLDDNEHVLSIRIIGGDHRLTNYSSVMS	LHPEIIDGRP
8. AtPYL8	76	2.80e-46	RCVVKGNMEI	GTVREVDVKSGLPATRSTERLELLDDNEHILSIRIVGGDHRLKNYSSIIS	LHPETIEGRI
19. MsPYL6	106	1.90e-45	VVASGDGIRV	GALREVRLVSGLPAVSSMERLDILDEERHVISFSVVGGVHRCRNYRSVTT	LHGDGNGGTV
18. MsPYL5	106	1.90e-45	VVASGDGIRV	GALREVRLVSGLPAVSSMERLDILDEERHVISFSVVGGVHRCRNYRSVTT	LHGDGNGGTV
2. AtPYL2	80	1.33e-44	CRLISGDGDV	GSVREVTVISGLPASTSTERLEFVDDDHRVLSFRVVGGEHRLKNYKSVTS	VNEFLNQDSG
11. AtPYL11	55	3.63e-44	CNLSSGDGGE	GSVREVTVVSGLPAEFSRERLDELDDESHVMMISIIGGDHRLVNYRSKTM	AFVAADTEEK
1. AtPYL1	103	3.63e-44	NVSEDFEMRV	GCTRDVNVISGLPANTSRERLDLLDDDRRVTGFSITGGEHRLRNYKSVTT	VHRFEKEEEE
12. AtPYL12	55	7.17e-43	CKLRSGDGGE	GSVREVTVVSDLPASFSLERLDELDDESHVMVISIIGGDHRLVNYQSKTT	VFVAAEEEKT
10. AtPYL10	72	2.81e-42	CVVQGKKLEV	GSVREVDLKSGLPATKSTEVLEILDDNEHILGIRIVGGDHRLKNYSSTIS	LHSETIDGKT
3. AtPYL3	100	1.23e-40	NGNGIKEIKV	GTIREVSVVSGLPASTSVEILEVLDEEKRILSFRVLGGEHRLNNYRSVTS	VNEFVVLEKD
13. AtPYL13	60	5.18e-40	GGGGKGGEGK	GSVRDVTLVSGFPADFSTERLEELDDESHVMVVSIIGGNHRLVNYKSKTK	VVASPEDMAK
15. MsPYL2	4	1.62e-38	MEV	GCTPDVNVISGLLAATSTERLNVLNDERRVTGSSIIGGEHRLKNYHSVTS	VHGFGDGDNG
28. MsPYL15	51	1.57e-29	VEIVEGNGGP	GTIKKLSMSEGGKTDFVLHKLEAVDEANLGYNYSIVGGTGLDESLEKVEF	ETSVVAGSDG
27. MsPYL14	183	1.57e-29	VEIVEGNGGP	GTIKKLSMSEGGKTDFVLHKLEAVDEANLGYNYSIVGGTGLDESLEKVEF	ETSVVAGSDG
25. MsPYL12	51	5.21e-29	VEIVEGNGGP	GTIKKLSMSEGGKTDYVLHKLEAVDEANLGYNYSIVGGTGLDESLEKVEF	ETSIVAGSDG
26. MsPYL13	51	1.31e-27	VEIVEGNGGP	GTIKKLSMSEDGKTNFVLHKLDAVDEANLGYNYSLVGGTGLDESLEKVEF	ETKIVAGSDG

E-value 3.1e-533

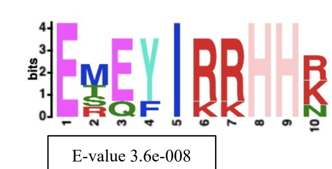
C

Name	Start	*p*-value	Sites
16. MsPYL3	19	2.64e-47	VPDAIARYHT HAVSPNQCCSAVIQHIAAPVSTVWSVVRRFDNPQAYKHFVKSC HVILGDGNVG
22. MsPYL9	24	1.33e-46	ETQYIRRHHK HDLRDNQCSSALVKHIKAPVHLVWSLVRRFDQPQKYKPFISRC IMQGDLSIGS
9. AtPYL9	27	1.96e-46	TVQYVRTHHQ HLCRENQCTSALVKHIKAPLHLVWSLVRRFDQPQKYKPFVSRC TVIGDPEIGS
23. MsPYL10	27	2.88e-46	EMEYIRRHHN QQPGQNQCSSALVKHIRAPVPLVWSLVRRFDQPQKYKPFVSRC VVRGNLEIGS
4. AtPYL4	45	2.17e-45	RDSTAARFHT HEVGPNQCCSAVIQEISAPISTVWSVVRRFDNPQAYKHFLKSC SVIGGDGDNV
7. AtPYL7	29	1.22e-44	TAQSLRLRHL HHCRENQCTSVLVKYIQAPVHLVWSLVRRFDQPQKYKPFISRC TVNGDPEIGC
8. AtPYL8	25	2.80e-44	EREFIRRHHK HELVDNQCSSTLVKHINAPVHIVWSLVRRFDQPQKYKPFISRC VVKGNMEIGT
24. MsPYL11	21	1.39e-43	EMEYIRRHHR QQPGHNQCASALVKHIRAPVPQVWSLVRRFDQPQKYKPFVSRC VVRGNLEIGS
19. MsPYL6	52	2.57e-43	VPETVANHHM HVVGQNQCYSVVIQTIKASVSTVWSVVRRFDNPQGYKHFVKSC NVVASGDGIR
18. MsPYL5	52	2.57e-43	VPETVANHHM HVVGQNQCYSVVIQTIKASVSTVWSVVRRFDNPQGYKHFVKSC NVVASGDGIR
10. AtPYL10	20	7.56e-42	ESEYIKKHHR HELVESQCSSTLVKHIKAPLHLVWSIVRRFDEPQKYKPFISRC VVQGKKLEVG
14. MsPYL1	52	4.26e-40	LIPFINSHHT YLIGPNQCSRLLAQRIHAPPQTVWSVVRSFDKPQIYKHFIKSC SLKEGFQMKV
1. AtPYL1	50	2.82e-39	LSQSIAEFHT YQLGNGRCSSLLAQRIHAPPETVWSVVRRFDRPQIYKHFIKSC NVSEDFEMRV
11. AtPYL11	3	5.06e-38	ME TSQKYHTCGSTLVQTIDAPLSLVWSILRRFDNPQAYKQFVKTC NLSSGDGGEG
5. AtPYL5	51	2.55e-37	VPEHVAMHHT HDVGPDQCCSSVVQMIHAPPESVWALVRRFDNPKVYKNFIRQC RIVQGDGLHV
21. MsPYL8	55	3.58e-37	IPNTIAHYHT HSISSDQLCSAVVQETTASITAVWSVVRRFDKPQAYKNFIKSC NLVGGDGDVG
20. MsPYL7	55	3.58e-37	IPNTIAHYHT HSISSDQLCSAVVQETTASITAVWSVVRRFDKPQAYKNFIKSC NLVGGDGDVG
6. AtPYL6	54	1.91e-36	VPEHVELSHT HVVGPSQCFSVVVQDVEAPVSTVWSILSRFEHPQAYKHFVKSC HVVIGDGREV
2. AtPYL2	28	2.81e-35	LEPVIKTYHQ FEPDPTTCTSLITQRIHAPASVVWPLIRRFDNPERYKHFVKRC RLISGDGDVG
12. AtPYL12	3	1.21e-34	MK TSQEQHVCGSTVVQTINAPLPLVWSILRRFDNPKTFKHFVKTC KLRSGDGGEG
17. MsPYL4	28	1.29e-32	LEPIIKKYHI LEPTSNTCTSIITYKIEAPSSIVWPYVRSFENPQKYKHFIKGC NMKGDGNVGS
13. AtPYL13	2	3.36e-32	M ESSKQKRCRSSVVETIEAPLPLVWSILRSFDKPQAYQRFVKSC TMRSGGGGGK
3. AtPYL3	43	2.19e-31	LDSIIRTHHT FPRSPNTCTSLIAHRVDAPAHAIWRFVRDFANPNKYKHFIKSC TIRVNGNGIK

E-value 6.0e-544

D

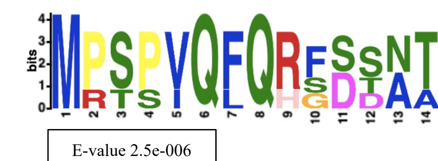
Name	Start	*p*-value	Sites
19. MsPYL6	156	1.50e-51	RCRNYRSVTT LHGDGNGGTVVIESYVVDVPQGNTKEETCSFVDTIVRCNLQSLAQIAEKL
18. MsPYL5	156	1.50e-51	RCRNYRSVTT LHGDGNGGTVVIESYVVDVPQGNTKEETCSFVDTIVRCNLQSLAQIAEKL
9. AtPYL9	131	3.55e-49	NYSSILTVHP EIIEGRAGTMVIESFVVDVPQGNTKDETCYFVEALIRCNLKSLADVSERL ASQDITQ
8. AtPYL8	129	9.52e-49	NYSSIISLHP ETIEGRIGTLVIESFVVDVPEGNTKDETCYFVEALIKCNLKSLADISERL AVQDTTESRV
22. MsPYL9	128	1.12e-48	NYSSIITVHP EVIDGRPGTMVIESFVVDVPEGNTKDETCYFVEALIRCNLSSLADVSERM AVQGRTDPIN
7. AtPYL7	133	8.56e-48	NYSSILTVHP EMIDGRSGTMVMESFVVDVPQGNTKDDTCYFVESLIKCNLKSLACVSERL AAQDITNSIA
24. MsPYL11	125	1.56e-47	NYSSVMSLHP EIIDGRPGTLVIESFVVDIPEGNTKDETCYFVEAVIKCNLKSLADVSEGI ALQDRTEPID
23. MsPYL10	131	2.09e-45	NYSSIMSLHP EIIDGRPGTLVIESFVVDVPEGNTKDETCYFVEALIKCNLKSLSDVSEGH AVQDLTEPLD
16. MsPYL3	129	7.60e-45	TTLHPSPISD EDGNHRSGTVVVESYVVDVPPGNTTEDTCVFVDTILRCNLQSLAKFAENL ASTRSNQR
10. AtPYL10	125	3.01e-44	NYSSTISLHS ETIDGKTGTLAIESFVVDVPEGNTKEETCFFVEALIQCNLNSLADVTERL QAESMEKKI
4. AtPYL4	148	1.15e-43	RLSNYRSVTT LHPSPISGTVVVESYVVDVPPGNTKEETCDFVDVIVRCNLQSLAKIAENT AAESKKKMSL
6. AtPYL6	162	1.64e-43	KSVTTVHESE EDSDGKKRTRVVESYVVDVPAGNDKEETCSFADTIVRCNLQSLAKLAENT SKFS
21. MsPYL8	160	4.06e-42	NYRSVTTLHP STEGDCSGTVIVESYVVDIPSENTKEDTHVFVDTIVRCNLQSLAQTAENI TQQNNKSCS
20. MsPYL7	160	4.06e-42	NYRSVTTLHP STEGDCSGTVIVESYVVDIPSENTKEDTHVFVDTIVRCNLQSLAQTAENI TQQNNKSCS
1. AtPYL1	159	8.76e-42	SVTTVHRFEK EEEEERIWTVVLESYVVDVPEGNSEEDTRLFADTVIRLNLQKLASITEAM NRNNNNNNSS
14. MsPYL1	161	8.25e-41	SVTSVHGFGD GDNGGEIWTVVLESYVVDVPEGNTEEDTRLFADTVVKLNLQKLASVTEGK NRDGDGKSDL
12. AtPYL12	106	1.02e-40	LVNYQSKTTV FVAAEEEKTVVVESYVVDVPEGNTEEETTLFADTIVGCNLRSLAKLSEKM MELT
5. AtPYL5	154	2.10e-40	RLKNYRSVTT LHASDDEGTVVVESYIVDVPPGNTEEETLSFVDTIVRCNLQSLARSTNRQ
11. AtPYL11	107	1.21e-38	VNYRSKTMAF VAADTEEKTVVVESYVVDVPEGNSEEETTSFADTIVGFNLKSLAKLSERV AHLKL
17. MsPYL4	133	6.46e-37	YRSVTSVNEF VNNEGKVYTIVLESYIVDIPHGNTEEDTKMFVDTVVKLNLQKLGVVAMSS CSSMHGQ
2. AtPYL2	135	7.07e-37	KSVTSVNEFL NQDSGKVYTVVLESYTVDIPEGNTEEDTKMFVDTVVKLNLQKLGVAATSA PMHDDE
13. AtPYL13	113	1.72e-36	NYKSKTKVVA SPEDMAKKTVVVESYVVDVPEGTSEEDTIFFVDNIIRYNLTSLAKLTKKM MK
3. AtPYL3	158	2.05e-36	TSVNEFVVLE KDKKKRVYSVVLESYIVDIPQGNTEEDTRMFVDTVVKSNLQNLAVISTAS PT



E

E-value 3.6e-008

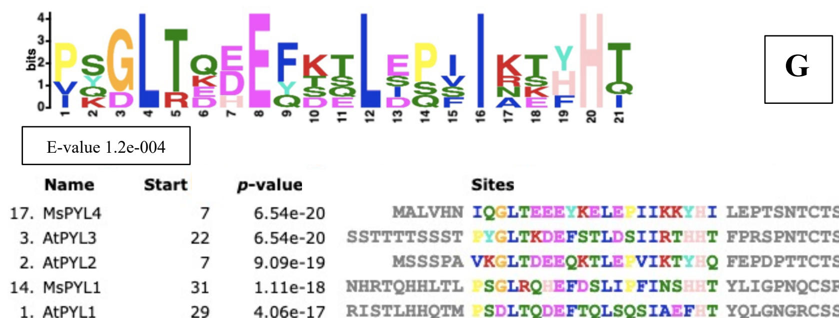
Name	Start	*p*-value	Sites
24. MsPYL11	11	4.87e-14	MNGIGRFSNM EMEYIRRHHR QQPGHNQCAS
23. MsPYL10	17	7.01e-14	TENNGVFNTT EMEYIRRHHN QQPGQNQCSS
8. AtPYL8	15	1.95e-12	GIENLTNPNQ EREFIRRHHK HELVDNQCSS
22. MsPYL9	14	4.68e-12	GCEQQQYSVI ETQYIRRHHK HDLRDNQCSS
10. AtPYL10	10	2.52e-10	MNGDETKKV ESEYIKKHHR HELVESQCSS



F

E-value 2.5e-006

Name	Start	*p*-value	Sites
19. MsPYL6	1	1.57e-19	MPSPVQFQRFDSNT AMTNGINCPK
18. MsPYL5	1	1.57e-19	MPSPVQFQRFDSNT AMTNGIDCPK
6. AtPYL6	1	1.06e-13	MPTSIQFQRSSTAA EAANATVRNY
5. AtPYL5	1	2.82e-12	MRSPVQLQHGSDAT NGFHTLQPHD



Name	Start	p-value	Sites		
17. MsPYL4	7	6.54e-20	MALVHN	IQGLTEEEYKELEPIIKKYHI	LEPTSNTCTS
3. AtPYL3	22	6.54e-20	SSTTTTSSST	PYGLTKDEFSTLDSIIRTHHT	FPRSPNTCTS
2. AtPYL2	7	9.09e-19	MSSSPA	VKGLTDEEQKTLEPVIKTYHQ	FEPDPTTCTS
14. MsPYL1	31	1.11e-18	NHRTQHHLTL	PSGLRQHEFDSLIPFINSHHT	YLIGPNQCSR
1. AtPYL1	29	4.06e-17	RISTLHHQTM	PSDLTQDEFTQLSQSIAEFHT	YQLGNGRCSS

Figure 12 Conserved motifs of MsPYL *proteins in comparison to* AtPYL *protein family.*

A) Boxes with same color represent conserved motifs. B-D) Highly conserved motifs found in most of the selected proteins. E-G) Motifs conserved in specific protein sequences

3.4.4 MsPYL gene expression in different tissues of well-watered alfalfa

In order to identify the genes mainly expressed in leaves and understand the potential functions of these genes, the expression of the 15 MsPYL genes in healthy leaf, stem, and root tissues of RS genotype was analyzed. The expression of all the genes in stem and root tissue were normalized using expression levels in leaf as the base for the comparison (Figure 13). MsPYL1-4, 7-11showed relatively higher expression in leaves as compared to stems and roots. MsPYL5, 6, 13 and 15 showed multiple folds in roots as compared to leaves. Gene MsPYL5, 6, 13-15 were highly expressed in stem as compared to leaves. Overall, the results suggest that all the 15 genes are expressed in leaves but to a variable degree. This gives an idea that genes expressed in leaves could be involved in the ABA sensitivity hence closure of stomata.

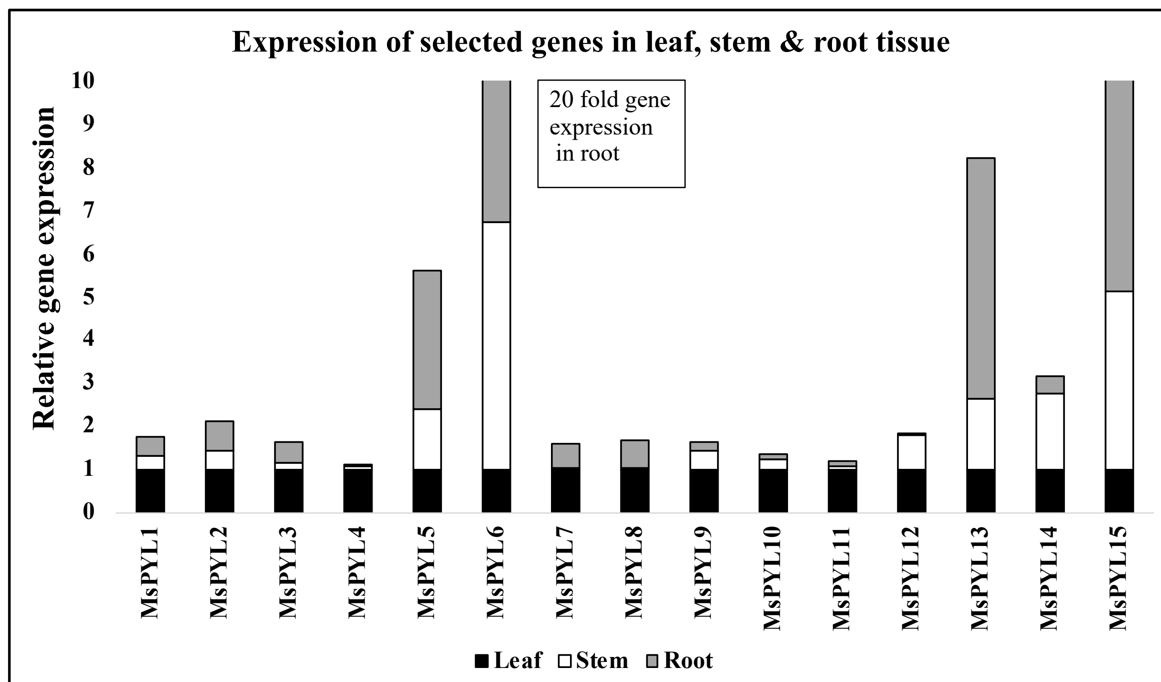


Figure 13 Relative transcript level of the 15 genes in leaf, stem & root of well-watered RS genotype of alfalfa.

The expression values in each tissue represent the mean fold change when compared to the leaf

3.4.5 MsPYL gene expression due to drought stress

In order to examine the changes in the level of gene expression due to drought stress, two genotypes of alfalfa, RS and AF were given drought treatment for 14 days and the newly matured leaves were collected and examined for real time transcript level changes. The $2^{\wedge-\Delta\Delta Ct}$ values for all the samples were normalized with the expression in well-watered leaves for each sample. Figure 14 shows the comparison of the relative transcript levels in well-watered, and drought stressed RS and AF leaves. MsPYL1, 2, and 4 did not show any change in both the genotypes whereas MsPYL5 showed downregulation in both the genotypes and MsPYL10 showed upregulation in both RS and AF upon drought treatment. MsPYL 3, 6-8 showed downregulation in RS and MsPYL9 showed more than 1.5-fold

increase in transcript level in RS whereas in AF the expression for these genes stayed the same. MsPYL11-15 on the other hand showed multiple fold increase in drought treated AF shoot tissues where in RS they did not show any change. Overall, it suggests that different genes might perform different functions hence are expressed to different levels at the time of stress.

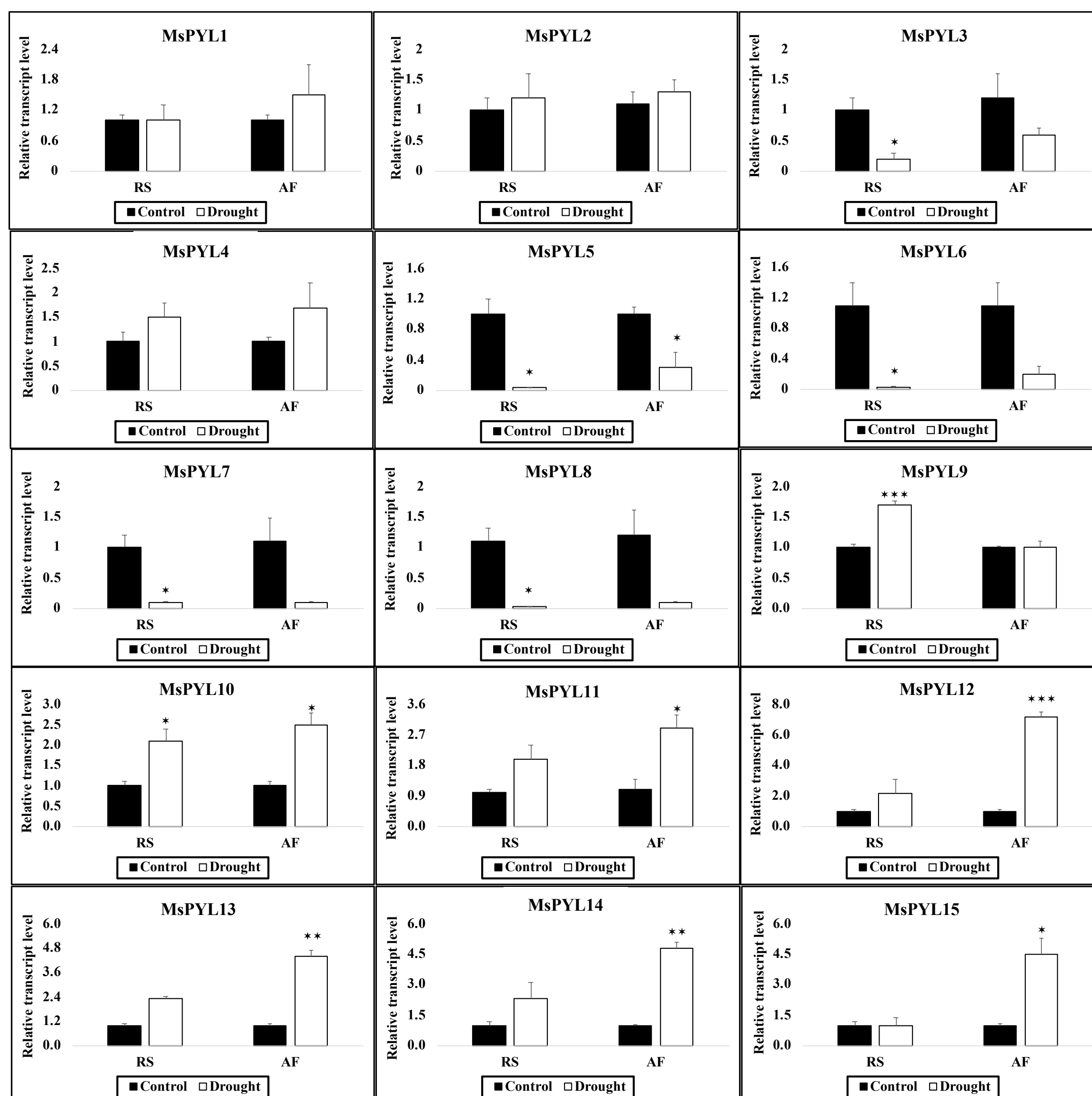


Figure 14 Relative Transcript level of the 15 MsPYL genes in well-watered and 14 days drought treated RS and AF leaf tissues.

*The expression values represent the mean fold change±S.E (n=3) when compared to the expression in well-watered tissue for the each gene. *,**, *** signify P value ≤ 0.05, 0.01, 0.001 respectively representing statistically significant difference*

3.4.6 MsPYL gene expression under dehydration treatment

To examine the changes in the level of gene expression due to short term water stress, young shoots (1-3 nodes) of two genotypes of alfalfa, RS and AF were exposed to dehydration for 6 hours and were collected and examined for real time transcript level changes. The $2^{\wedge-\Delta\Delta Ct}$ values for all the samples were normalized using expression in well-watered shoots of similar sizes. Figure 15 shows the comparison of the relative transcript levels in well-watered and dehydrated RS and AF shoots. Eight of the fifteen genes, MsPYL1-8 showed downregulation in the dehydrated conditions in both the genotypes. MsPYL9 and 13 showed upregulation in RS whereas in AF it did not show any change. MsPYL10-12, 14 and 15 did not show any change in both the genotypes.

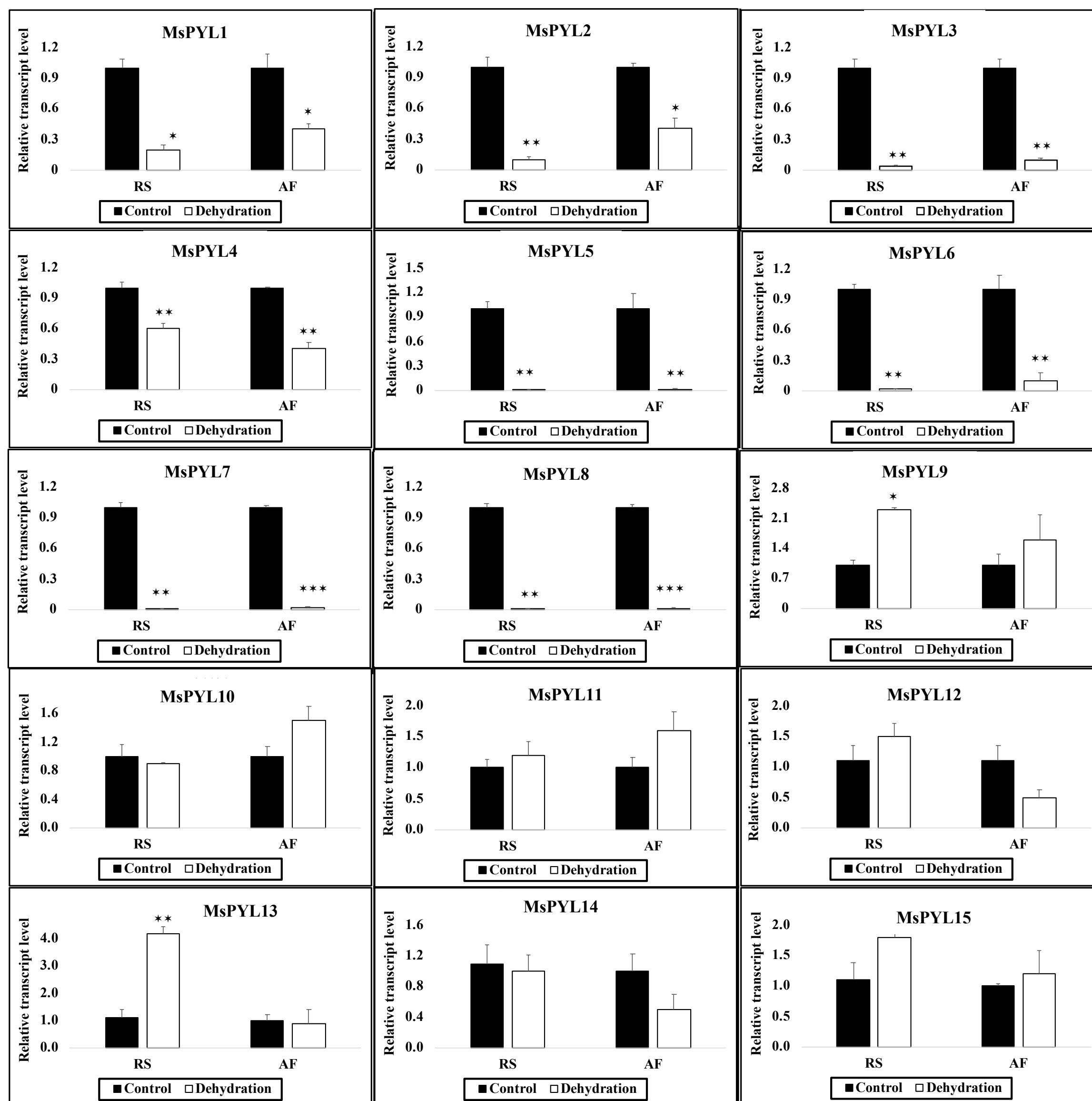


Figure 15 Relative Transcript level of the 15 MsPYL genes in well-watered and 6 hour dehydrated RS and AF shoot tissues.

*The expression values represent the mean fold change±S.E (n=3) when compared to the expression in well-watered tissue for each gene. *,**, *** signify P value ≤ 0.05, 0.01, 0.001respectively, representing statistically significant difference*

3.5 Discussion

A previous study in our lab demonstrated that RS germplasm, shows higher WUE and a potential reason was found to be the higher sensitivity of RS stomate towards ABA as compared to AF when drought treated (Ghimire et al. 2021; Anower 2015). To understand molecular mechanism underlying the higher sensitivity to ABA in RS, we examined expression of ABA receptor genes in alfalfa.

3.5.1 PYL-like genes in *M. truncatula* and *M. sativa* and phylogenetic analysis

We identified 23 and 15 PYL-like proteins in *M. truncatula* and *M. sativa*, respectively. The reason for fewer PYL-like proteins found in alfalfa is probably due to incomplete genome sequence in the alfalfa genome database. These proteins contain START-like superfamily domains which are known to play role as ABA receptor. For these 15 genes, we designed primers and were able to amplify gene sequences from alfalfa tissues. The 23 genes for the identified proteins in *M. truncatula* are located on 8 chromosomes and 8 out of these 23 genes show tandem cluster on chromosome 2 which suggests the chances of gene duplication. The 15 genes in *M. sativa* are annotated to be present on 7 of the 8 chromosomes and like *M. truncatula*, here also we see the chances of gene duplication but due to unavailability of exact location, it cannot be concluded. As most genotypes of *M. sativa* are tetraploid, we can expect presence of more than 15 genes in different genotypes. Looking at the physical properties of proteins, most of the proteins show high similarity among the three plants. Three motifs of length 50, 43 and 50 with START like domains

were found to be conserved in all the selected proteins of alfalfa, suggesting that these proteins can be the potential ABA receptors. The lengths of MsPYL proteins also have high resemblance with AtPYLs as most of them are around 150-200 aa, except MsPYL14 which is 459 amino acids long. In *Arabidopsis* the PYL family could be divided into three subgroups based on their phylogenetic relation and when we added the *M. truncatula* and *M. sativa* protein sequences in the phylogenetic study it appeared that some of the MtPYLs and MsPYLs were more closely related to AtPYLs. Subgroups 1, 2 and 3 contain AtPYLs, MtPYLs and MsPYLs showing high conservation and similarity in these proteins. Subgroup 4 however contains genes from *M. truncatula* and *M. sativa*, suggesting that these genes evolved after the divergence. This suggest that some of the PYL-like genes might act as ABA receptors and be involved in stomatal closure whereas others might be expressed in different tissues and perform different functions. As previously studied in *Arabidopsi*s, in the family of 13 PYLs, AtPYR1, AtPYL1, AtPYL2, AtPYL4, AtPYL5 and AtPYL8 have been found to play role in ABA dependent stomatal closure (Park et al. 2009; Gonzalez-Guzman et al. 2012). It can be implied from this study that the genes and proteins with high similarity and conserved domains might have higher probability of having same functions.

3.5.2 PYL gene expression in different tissues of well-watered alfalfa

PYL-like genes have been studied and reported to express in different tissues in different plants like seeds in soybean, latex of rubber tree (Di et al. 2018). In this study, we analyzed the expression of 15 *MsPYL* genes in leaves, stem and root tissues of healthy RS plants and found that all the studied MsPYLs are expressed in leaves but to a different degree. *MsPYL1-4* and *7-11* were highly expressed in leaves as compared to the tissues, suggesting

that these proteins have high probability of being ABA receptors and might involve in stomatal closure. Whereas *MsPYL5, 6* and *13-15* had a higher level of expression in stem and roots and very slightly expressed in leaves. This indicates that these PYLs might be involved in other roles in roots and stem tissues.

3.5.3 MsPYL gene expression under water stress conditions

Drought and dehydration stress have been reported to impact the expression of PYL in plants. In a study on *B. napus*, *PYL1* and *PYL8* like genes showed up-regulation in the drought stressed conditions (Di et al. 2018). The gene expression also changed in some of *PYL* genes in *M. sativa* under water stress conditions. In short term dehydration stress, 8 of 15 genes showed downregulation in both the genotypes whereas in 14 days water stress condition, fewer genes were downregulated in both the plants. Similar results were reported when arabidopsis plants were exposed to dry air, number of genes were downregulated (Dittrich et al. 2019). Although, a study on tobacco showed that short term duration led to higher expression levels and long-term dehydration, resulted in downregulation (Bai et al. 2019). This suggests that different genes can express differently depending on the plants. In our study, *MsPYL11-15* showed upregulation in drought stressed AF plants while showing no significant changes in RS, but these genes showed no change in the dehydrated AF tissues. *MsPYL9* is the one gene which showed higher expression levels in RS and did not show any change in AF plants when treated with both short term dehydration as well as in long term drought stress. This suggests that possibly, *MsPYL9* is the key candidate gene that contributes to higher sensitivity to ABA and thus higher WUE in RS.

4 Conclusion

While alfalfa is an important forage crop known for its drought resilience and higher water use efficiency than other forage crops (Michaud et al. 1988), its high yield in many areas requires irrigation. Thus, improving WUE in alfalfa can save water and improve the economic return for producers. WUE in alfalfa has been closely related to the stomatal conductance and sensitivity to ABA in RS, a genotype showing high WUE (Ghimire et al. 2021). In this study, we reveal that, while there is a variation in stomatal density among alfalfa genotypes, stomatal density does not correlate with stomatal conductance and thus WUE, further suggesting stomatal sensitivity to ABA play a more important role in controlling stomatal conductance. After examining 15 PYL like genes in alfalfa, we identified *MsPYL9* is the only gene specifically upregulated in RS compared to AF. Our results may have identified a key player in controlling WUE in alfalfa, since higher expression of *MsPYL9* may lead to more receptor proteins and higher sensitivity to ABA. Further study is needed to identify other contributing factors and molecular mechanisms underlying high WUE in alfalfa.

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