7-2020

Genetics of Physiological Traits Associated with Drought Tolerance in Soybean (Glycine max)

Sumandeep Kaur Bazzer
University of Arkansas, Fayetteville

Genetics of Physiological Traits Associated with Drought Tolerance in Soybean (*Glycine max*)

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Crop, Soil, and Environmental Sciences

by

Sumandeep Kaur Bazzer
Punjab Agricultural University
Bachelor of Science in Biotechnology, 2013
Punjab Agricultural University
Master of Science in Plant Breeding and Genetics, 2015

July 2020
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Larry C. Purcell, Ph.D.
Dissertation Director

_____
Jeffery D. Ray, Ph.D.
Committee Member

_____
Richard E. Mason, Ph.D.
Committee Member

_____
Mary C. Savin, Ph.D.
Committee Member

_____
Ken Korth, Ph.D.
Committee Member

Abstract

Soybean (*Glycine max* L.) is one of the major row crops in the United States, and its production is often limited by drought stress. Physiological traits from exotic germplasm that confer drought tolerance may be useful in improving commercial soybean production. For example, carbon isotope ratio ($\delta^{13}C$) is positively correlated with water use efficiency (WUE), and nitrogen isotope ratio ($\delta^{15}N$) is negatively correlated with N_2 fixation; canopy temperature (CT) is an indicator for genetic variation in transpiration and stomatal conductance. Therefore, the objectives of this research were to identify the genomic regions associated with: (1) $\delta^{13}C$ and $\delta^{15}N$ using a population of 196 F_6-derived recombinant inbred lines (RIL) from PI 416997 × PI 567201D that was phenotyped in four environments, (2) CT and $\delta^{13}C$ using a population of 168 F_5-derived RILs from KS4895 × Jackson that was phenotyped in multiple environments and irrigation treatments. In the PI 416997 × PI 567201D population, $\delta^{13}C$ and $\delta^{15}N$ had a wide phenotypic range in all environments, and PI 416997 had higher $\delta^{13}C$ and lower $\delta^{15}N$ values than PI 567201D. $\delta^{13}C$ had high heritability (90%) whereas the heritability of $\delta^{15}N$ was relatively lower (35%), indicating that $\delta^{15}N$ was more affected by the environment. QTL mapping identified eight loci on seven chromosomes associated with $\delta^{13}C$, and these loci explained between 2.5 to 30% of the phenotypic variation. There were 13 loci on 10 chromosomes associated with $\delta^{15}N$, explaining 1.7 to 14.4% of the phenotypic variation. There were strong interactions between QTLs and environments for $\delta^{15}N$. In the KS4895 × Jackson RIL population, Jackson had a cooler canopy than KS4895, and the heritability of CT had low heritability (31%) across environments. There were 11 loci present on eight chromosomes associated with CT that individually explained 4.6 to 12.3% of the phenotypic variation. The heritability of $\delta^{13}C$ in KS4895 × Jackson RIL population heritability was 83% when estimated over environments and

over irrigation treatments. A total of 24 QTLs associated with $\delta^{13}C$ were identified and clustered in nine genomic loci on seven chromosomes. The identified QTLs for $\delta^{13}C$, $\delta^{15}N$, and CT were co-localized with genomic regions associated with drought tolerance-related traits from previous studies. These genomic regions may be important resources in soybean breeding programs to improve tolerance to drought. Further research is needed to fine map the identified QTLs and validate markers linked with these regions

.

Acknowledgments

I would like to thank my advisor, Dr. Larry C. Purcell, for his continued and selfless encouragement and support throughout my graduate studies. I am also very thankful for the faith that he has given me in conducting this research project. He always gave me valuable and precious advice whenever I encountered any research or personal problems. I appreciate him sharing his research experience and opinions with me. He often imparted me knowledge by example and through his dedication and passion for research. I will always keep the lessons he has taught me in mind and use them in my research career.

I would also like to thank my advisory committee members: Dr. Jeffery D. Ray, Dr. Richard E. Mason, Dr. Mary C. Savin, and Dr. Ken Korth. I appreciate their understanding, precious suggestions, and patient help on my research project.

Additionally, I would like to thank the members in our research group for their support: Dr. Andy King for his help with my lab, field work, and experimental design of my research project; Marilynn Davies for her help, suggestions, and her patient guidance on my research or my personal problems; Dr. Avjinder Kaler and Dr. Sadal Hwang for their help with building my research background; Caio dos Santos, Flávia Werner, Letícia Fontana, and Akshita Mishra for their help with my research work and for their friendship. I enjoyed the time working together with them, and I will never forget them.

I would like to thank the Department of Crop, Soil and Environmental Sciences at the University of Arkansas for supporting my graduate studies and everyone working there. Finally, I would like to thank the United Soybean Board and the Arkansas Soybean Promotion Board for their financial support of my research project.

Dedication

I dedicate my dissertation to my family. My loving grandparents, Surjit Kaur and Santokh Singh, and parents, Nirmal Singh and Mohinder Kaur, who have done everything for me which I could never pay back. My sister and brother also gave tremendous support and love to me. My close friends', Avjinder Kaler and Mohini Prabha Singh, support have given me great courage to face any difficulty and hardship in my life. And lastly, but far from least, I would like to thank my whole Bazzer family, for their support and blessing in my life.

Table of Contents

CHAPTER I. Introduction and Literature Review 1

Introduction 2

Literature Review ….....6

Soybean Yield and Impact of Drought 8

Mechanisms to Ameliorate Drought Impacts …….……..……..……..9

Morphological Responses to Water Stress ….……...……..…..…10

Physiological Responses of Water Stress 11

Breeding for Drought Tolerance 12

Traits Associated with Drought Tolerance ……..….....13

Carbon Isotope Ratio 13

Canopy Temperature 15

Nitrogen Fixation 16

Mapping QTLs for Drought Tolerance Related Traits 18

Objectives 21

References 23

CHAPTER II. Identification of Quantitative Trait Loci for Carbon Isotope Ratio ($\delta^{13}C$) in a Recombinant Inbred Population of Soybean 38

Abstract 39

Introduction 40

Materials and Methods 44

Field Experiments …….....44

Phenotypic Evaluations 45

Statistical Analysis ..…….....46
GBS Library Construction, Genotyping and SNP Calling 48
Marker Quality Control 48
Construction of Linkage 49
QTL Analysis 49
Identification of Putative Candidate Genes and their Functions ……..50
Results 52
Genetic Map Construction 53
QTL Analysis 53
QTL × Environment Interaction for $\delta^{13}C$ 55
QTL × QTL Interaction 55
Discussion 57
Co-localization of QTL for $\delta^{13}C$ with QTLs for other Agronomic Traits ……..….60
Conclusions 63
References 74
CHAPTER III. Mapping Quantitative Trait Loci (QTL) for Plant Nitrogen Isotope Ratio ($\delta^{15}N$) in Soybean 82
Abstract 83
Introduction 84
Materials and Methods 88
Development of RIL Population ……..88
Field Trials ……..…….88
Data Collection 89

Statistical Analysis 90
Selection of Lines with Extreme Values for $\delta^{13}C$ and $\delta^{15}N$ 91
Genotyping-by-Sequencing and Construction of Linkage Map 91
QTL Analysis 92
Identification of Putative Candidate Genes 93
Results 94
Phenotype Evaluation 94
QTL Analysis 95
QTL × environment and QTL × QTL interactions analysis 96
Identification of Putative Candidate Genes 97
Discussion 98
Conclusions 103
References 112
CHAPTER IV. Mapping and Confirmation of Quantitative Trait Loci (QTLs) Associated with Carbon Isotope Ratio ($\delta^{13}C$) in Soybean 117
Abstract 118
Introduction 119
Materials and Methods 123
Field Experiments 123
Phenotypic Evaluations 124
Statistical Analysis 125
QTL Analysis 127
Results 129

QTL Analysis 131
QTL Analysis by Environment ……..……132
QTL Analysis by Irrigation Treatment ……..133
QTL Analysis over Environment and Irrigation Treatment (AEI) ……..……134
Discussion 136
References 155
CHAPTER V. Identification of Quantitative Trait Loci Associated with Canopy Temperature in Soybean ……..……165
Abstract 166
Introduction 167
Materials and Methods 170
Phenotypic Evaluation 171
Statistical Analysis 171
QTL Analysis 172
Candidate Gene Identification 174
Results 175
Phenotype Evaluation 175
QTL Analysis 176
Candidate Gene Identification 177
Discussion 178
Conclusions 183
References 189
CHAPTER VI. Conclusions 195

List of Tables

CHAPTER II. Identification of Quantitative Trait Loci for Carbon Isotope Ratio ($\delta^{13}C$) in a Recombinant Inbred Population of Soybean 38

Table 2_1. Phenotypic data of $\delta^{13}C$ (‰) for parents (PI 416997 and PI 567201D) and recombinant inbred lines (RILs) evaluated at Stoneville, MS in 2016 (ST16) and in 2017 (ST17), Columbia, MO in 2017 (CO17), and Fayetteville, AR in 2017 (FAY17) 64

Table 2_2. Pearson correlation coefficients between $\delta^{13}C$ of RILs derived from PI 416997 × PI 567201D at Stoneville, MS in 2016 (ST16) and in 2017 (ST17), Columbia, MO in 2017 (CO17), and Fayetteville, AR in 2017 (FAY17) 64

Table 2_3. Analysis of variance and heritability (h^2) of $\delta^{13}C$ across environment and for individual environment 65

Table 2_4. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ detected in four environments (ST16, ST17, CO17, and FAY17) in the RIL population derived from the cross of PI 416997 and PI 567201D using ICIM mapping 66

Table 2_5. QTLs identified across environments for $\delta^{13}C$ and QTL × environment interaction detected in four environments using MET functionality 68

Table 2_6. Epistatic QTLs identified for $\delta^{13}C$ in RIL population derived from cross of PI 416997 × PI 567201D by the ICIM-EPI method implemented in QTL IciMapping ..……..69

CHAPTER III. Mapping Quantitative Trait Loci (QTL) for Plant Nitrogen Isotope Ratio ($\delta^{15}N$) in Soybean 82

Table 3_1. Phenotypic variation for $\delta^{15}N$ (‰) in the parents (PI 416997 and PI 567201D) and RIL population grown in four environments: Stoneville in 2016 (ST16), Stoneville in 2017 (ST17), Fayetteville in 2017 (FAY17), and Columbia in 2017 (CO17) 104

Table 3_2. Analysis of variance (ANOVA) for $\delta^{15}N$ in the RIL population along with parents evaluated in four environments (ST16, ST17, FAY17, and CO17) 104

Table 3_3. RILs with high $\delta^{13}C$ and low $\delta^{15}N$ phenotypic values or RILs with low $\delta^{13}C$ and high $\delta^{15}N$ phenotypic values in individual environments (ST16: Stoneville in 2016, ST17: Stoneville in 2017, FAY17: Fayetteville in 2017, and CO17: Columbia in 2017) and across environments (AE). Values in parentheses are the phenotypic values of $\delta^{13}C$ and $\delta^{15}N$ for that RIL. Bold values indicate the RILs appeared within multiple individual environments as well as across environments 105

Table 3_4. Quantitative trait loci (QTLs) associated with $\delta^{15}N$ detected in individual environments (ST16, ST17, FAY17, and CO17) and across environment (AE) in the RIL population of PI 416997 and PI 567201D using BIP functional module of ICIM mapping 106

Table 3_5. QTLs showing QTL × environment interaction in four environments detected using MET functional module of ICIM mapping …….…….……...108

Table 3_6. Epistatic QTLs identified for $\delta^{15}N$ in the RIL population of PI 416997 × PI 567201D by the ICIM-EPI method of BIP functional module …….....109

CHAPTER IV. Mapping and Confirmation of Quantitative Trait Loci (QTLs) Associated with Carbon Isotope Ratio ($\delta^{13}C$) in Soybean …….....117

Table 4_1. Descriptive statistics of $\delta^{13}C$ (‰) of parents and recombinant inbred lines (RILs) evaluated at Keiser, AR in 2013 (KS13) and Stuttgart, AR in 2012 and 2013 (ST12 and ST13) under rainfed (RF) and irrigated (IRR) conditions, and at Pine Tree (PT17) and Rohwer (RH17) in 2017 under RF conditions 143

Table 4_2. Pearson correlation coefficients between $\delta^{13}C$ of RILs derived from KS4895 x Jackson in different environments including Stuttgart in 2012 (ST12) and 2013 (ST13), Keiser in 2013 (KS13), Pine Tree in 2017 (PT17), and Rohwer in 2017 (RH17) under rainfed (RF) and irrigated (IRR) conditions. Correlation coefficients were calculated using RIL means (n=166-168) 144

Table 4_3. Analysis of variance and heritability (h^2) of $\delta^{13}C$ over environment and irrigation treatment, by environments (KS13, ST12, and ST13), by irrigation treatment (rainfed, RF; irrigated, IRR) and for individual environments within irrigation treatment ….....145

Table 4_4. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ identified by composite interval mapping (CIM) using $\delta^{13}C$ BLUP values by environments (ST12, ST13, and KS13) averaged over irrigation treatment, by irrigation treatment (RF and IRR) averaged over environments, and averaged over both environments and irrigation treatments (AEI) ……...…….146

Table 4_5. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ identified by multiple interval mapping (MIM) using $\delta^{13}C$ data by environments (ST12, ST13, and KS13) averaged over irrigation treatment, by irrigation treatment (rainfed, RF; irrigated, IRR) averaged over environments, and averaged over both environments and irrigation treatments (AEI) in a KS4895 × Jackson population ……..148

Table 4_6. Reported genomic regions associated with other traits overlapping with the quantative trait loci positions for the $\delta^{13}C$ identified in the KS4895 × Jackson population …..150

CHAPTER V. Identification of Quantitative Trait Loci Associated with Canopy Temperature in Soybean ……..165

Table 5_1. Planting date and weather data including maximum temperature (MaxT), minimum temperature (MinT), No. of days without rainfall, estimated vapor pressure deficit (VPD), and estimated soil moisture deficit at the time of canopy temperature measurements for the field experiments conducted at Pine Tree (PT) and Rohwer (RH) in 2017, 2018, and 2019 ….……184

Table 5_2. A summary statistic of canopy temperature (CT) in the parents (KS4895 and Jackson) and RILs (n=168) population of KS4895 and Jackson evaluated at Pine Tree and Rohwer in 2017, 2018 and 2019 …….184

Table 5_3. The QTLs associated with canopy temperature identified by composite interval mapping (CIM) and multiple interval mapping (MIM) in a RIL population of KS4895 and Jackson which were evaluated at Pine Tree and Rohwer in 2017, 2018, and 2019 …..……185

List of Figures

CHAPTER II. Identification of Quantitative Trait Loci for Carbon Isotope Ratio ($\delta^{13}C$) in a Recombinant Inbred Population of Soybean 38

Figure 2_1. Total cumulative rainfall (mm) for the four environments (ST16, ST17, CO17, and FAY17). At ST16, ST17, and FAY17 irrigation was applied (stars indicate the time of irrigation) as needed whereas CO17 was rainfed. Sampling dates for $\delta^{13}C$ for each environment are indicated by a filled circle ….....71

Figure 2_2. Frequency distribution showed broad range of $\delta^{13}C$ within each environment. Vertical lines indicate the mean of the parental genotypes PI 416997 (Red) × PI 567201D (Blue). Mean of RILs and Standard deviation (SD) were shown on the top corner of each histogram. A, B, C, and D indicated the environment ST16, ST17, CO17, and FAY17, respectively ..……72

Figure 2_3. Physical position of SNPs on soybean chromosomes and position of loci associated with $\delta^{13}C$ identified by ICIM mapping. The physical positions of SNP markers indicated in base pairs are shown on the x-axis and the y-axis represents chromosome number. The solid blue diamond represents the centromere location. The numbers in the black circles represent the locus numbers on a specific chromosome. The QTL positions for individual loci are designated by a blue bar above the respective chromosome. The length of the blue bar represents the distance between flanking markers ….....73

CHAPTER III. Mapping quantitative trait loci (QTL) for Plant Nitrogen Isotope Ratio ($\delta^{15}N$) in Soybean 82

Figure 3_1. Distribution of $\delta^{15}N$ among recombinant inbred lines and parental genotypes at Stoneville, MS in 2016 (a), Stoneville, MS in 2017 (b), Fayetteville, AR in 2017 (c), and Columbia, MO in 2017 (d) ……..…….....110

Figure 3_2. Physical position of SNPs on soybean chromosomes and position of loci (horizontal red bars) associated with $\delta^{15}N$ identified by ICIM mapping for additive QTLs. The numbers in the black circles represent the loci number on a specific chromosome. Green, pink, and light-blue vertical bars indicate QTLs found at the same positions in previous studies, and yellow circles indicate the position of nodulation genes ….…….…….111

CHAPTER IV. Mapping and Confirmation of Quantitative Trait Loci (QTLs) Associated with Carbon Isotope Ratio ($\delta^{13}C$) in Soybean ……......117

Figure 4_1. Cumulative rainfall (mm) starting from planting to sampling period for the five environments (Stuttgart in 2012 (ST12) and in 2013 (ST13), Keiser in 2013 (KS13), Pine Tree in 2017 (PT17), and Rohwer in 2017 (RH17)). Stars indicate dates of irrigation for the irrigated treatment ……..152

Figure 4_2. Frequency distribution of $\delta^{13}C$ (‰) averaged over environments for rainfed (A) and irrigated conditions (B) in RILs derived from a cross between KS4895 and Jackson. Values of $\delta^{13}C$ for KS4895 were greater than for Jackson in both rainfed ($P \leq 0.01$) and irrigated ($P \leq 0.05$) treatments ……..……..153

Figure 4_3. Position of QTLs associated with $\delta^{13}C$ based on composite interval mapping (CIM) in the KS4895 × Jackson population ……..154

CHAPTER V. Identification of Quantitative Trait Loci Associated with Canopy Temperature in Soybean ……165

Figure 5_1. The box plots showed a broad range of canopy temperature in KS4895 × Jackon RIL population within each environment. Environment prefixes PT and RH denotes Pine Tree and Rohwer, respectively followed by 17 (2017), 18 (2018), and 19 (2019) for years. Box edges represent the upper and lower quartile with a median (bold line in the middle of box) and mean value (cross in the middle of box) ...……187

Figure 5_2. Physical position of SNPs on soybean chromosomes and position of loci (red downward triangle) associated with CT. The numbers in the black circles represent the loci number on a specific chromosome. Vertical colored bars (except blue) indicate the other QTLs found at the same positions in previous studies. CW, CT, CW denote canopy wilting, canopy temperature, and canopy wilting, respectively……....188

List of Publications

CHAPTER II. Bazzer, S.K., A.S. Kaler, J.D. Ray, J.R. Smith, F.B. Fritschi, and L.C. Purcell (2020) Identification of quantitative trait loci for carbon isotope ratio ($\delta^{13}C$) in a recombinant inbred population of soybean. Theor. Appl. Genet. 133:2141-2155

CHAPTER III. Bazzer, S.K., J.D. Ray, J.R. Smith, F.B. Fritschi, and L.C. Purcell (2020) Mapping quantitative trait loci (QTL) for plant nitrogen isotope ratio ($\delta^{15}N$) in soybean. Euphytica (in review)

CHAPTER IV. Bazzer, S.K., A.S. Kaler, C.A. King, J.D. Ray, S. Hwang, and L.C. Purcell (2020) Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean. Crop Sci. https://doi.org/10.1002/csc2.20240.

CHAPTER V. Bazzer, S.K., and L.C. Purcell (2020) Identification of quantitative trait loci associated with canopy temperature in soybean. Scientific Reports (in review)

CHAPTER I

Introduction and Literature Review

Introduction

Soybean [*Glycine max* (L.) Merr.] is one of the most important economic legume crops. It is widely cultivated around the world due to its high protein and oil concentration. Soybean faces many challenges posed by various environmental stresses, and drought is a major constraint to global soybean production and yield (Boyer, 1982; Hufstetler et al., 2007). Specht et al. (1999) reported that insufficient moisture and unpredictable rainfall can reduce soybean yield by 36% in the US. Drought stress affects soybean yield by reducing photosynthesis, leaf area, leaf number, and symbiotic N_2 fixation (Serraj et al., 1999a; Sinclair and Serraj, 1995). Also, N_2 fixation is more sensitive to water stress conditions than other physiological processes (Serraj and Sinclair, 1997; Sinclair, 1986). Therefore, there is a need for the development of soybean cultivars with high yield potential under water stress to maintain or increase soybean productivity (Polania et al., 2016).

The direct selection of soybean genotypes with high yield stability under water deficit conditions is difficult due to its polygenic nature, low heritability, and significant genotype × environment (G × E) interactions (Ceccarelli et al., 1991; Teulat et al., 2002). As an alternative, yield can be improved by identifying morpho-physiological traits that are genetically associated with yield under water deficit conditions (Babu et al., 2003; Slafer et al., 2005; Teulat et al., 2002). Among those traits, water use efficiency (WUE), cooler canopy, and insensitivity of N_2 fixation to drought have been considered important physiological traits and indicators of drought tolerance. Dissecting the genetic basis of these traits may help to improve yield under drought stress conditions in soybean and other crops.

Under water-limited conditions, crop yield can be expressed as a function of the water transpired, WUE, and harvest index (Passioura, 1996). The improvement of any of these traits

may ultimately leads to increased yield in water deficit conditions. The use of the WUE trait in drought tolerance breeding programs is often limited by difficulties associated with its measurement in large populations. Farquhar et al. (1982) and Farquhar and Richards (1984) proposed a promising screening for WUE based on carbon isotope composition present in plant samples. The ratio of ^{13}C to ^{12}C ($^{13}C/^{12}C$) isotope in plants is less than that of $^{13}C/^{12}C$ in the atmosphere because there is discrimination of the heavier isotope of carbon (^{13}C) over the lighter isotope of carbon (^{12}C) during carboxylation process of photosynthesis in plants. The plant carbon isotope composition is either expressed as the carbon isotope ratio ($\delta^{13}C$) or carbon isotope discrimination ($\Delta^{13}C$), and it provides an integrated measurement of WUE in C_3 plants (Farquhar and Richards, 1984). The $\delta^{13}C$ is directly proportional to WUE, whereas $\Delta^{13}C$ is inversely proportional to WUE (Farquhar et al., 1982). The $\delta^{13}C$ or $\Delta^{13}C$ have been used widely for the selection of genotypes with improved WUE in various crops (Cattivelli et al., 2008; Condon et al., 2004; Dhanapal et al., 2015a; Kaler et al., 2017b).

Canopy temperature (CT) is an important physiological trait and can be used as a surrogate measurement of transpiration, stomatal conductance, and leaf water potential (Jones et al., 2009; Rebetzke et al., 2013). Under sufficient soil moisture, increased air temperature and vapor pressure deficit lead to an increased transpiration, resulting in canopy cooling (Tanner, 1963). However, under water deficit conditions, reduced stomatal conductance and transpiration results in high CT (Gates, 1968; Tanner, 1963). Therefore, selecting genotypes that maintain lower CT compared with other genotypes under water deficit conditions could be useful to improve drought tolerance (Blum, 2004). Manual recording of CT of a large number of genotypes is difficult and tedious due to temporal variation in air temperature, solar radiation, wind, and soil moisture. Therefore, high throughput phenotyping through aerial thermal infrared

imaging could be one of the approaches to measure relative CT differences among genotypes under different environmental conditions (Knipling, 1970; Merlot et al., 2002). Various studies have shown that thermal infrared imaging is an effective tool to evaluate CT differences among genotypes (Kaler et al., 2018a; O'Sgaughnessy et al., 2011; Zia et al., 2011).

The symbiotic relationship between soybean and N_2-fixing soil bacteria is sensitive to drought conditions, resulting in decreased N accumulation and crop yield (Márquez-García et al., 2015; Purcell et al., 1997; Serraj et al., 1999a). Differences among genotypes for their sensitivity to N_2 fixation under drought conditions can evaluated by ^{15}N natural abundance. Nitrogen isotope ratios between ^{15}N and ^{14}N ($\delta^{15}N$) can serve as an index of N_2 fixation differences among soybean genotypes, as $\delta^{15}N$ is negatively associated with N_2 fixation (Andrews and Lea, 2013; Barrie et al., 1995; Letolle, 1980). This method compares the abundance of ^{15}N isotope to ^{14}N isotope in plant tissue, the atmosphere, and soil environment. The atmosphere has a lower concentration of the ^{15}N isotope compared to the soil, and biological N_2 fixation dilutes the ^{15}N in plant tissue as compared to plants that depend on mineral N as a N source (Doughton et al., 1995). Various studies have shown that $\delta^{15}N$ can be used to estimate the amount of N fixed by genotypes via N_2 fixation (Dhanapal et al. 2015b; Steketee et al. 2019).

The physiological traits associated with drought tolerance are complex quantitative traits, controlled by a large number of genes and environmental factors (Blum, 2011; Reynolds and Tuberosa, 2008). Understanding the genetic basis of drought tolerance may help breeders and geneticists to develop cultivars with high yield potential and improved drought tolerance (Chen et al., 2011; Zhang et al., 2001). Recent advances in genome sequencing and genotyping platforms along with a continuous decline in genotyping cost, facilitate the development of high density linkage maps in various crops (Byrne et al., 2013; Chen et al., 2014; Poland et al., 2012;

Rafalski, 2010) including soybean (Cregan et al., 1999; Hyten et al., 2010; Song et al., 2004, 2013). Single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) markers have been widely used and have high applicability in breeding and genetic studies. With the development of high-density linkage maps in soybean, linkage mapping and genome wide association mapping (GWAM) have been conducted to identify the genomic regions associated with drought tolerance related traits (Abdel-Haleem et al., 2012; Bazzer et al., 2020a, 2020b, 2020c; Charlson et al., 2009; Dhanapal et al., 2015a, 2015b; Hwang et al., 2013, 2014, 2015; Kaler et al., 2017a, 2017b, 2018a, 2018b).

Linkage mapping, or QTL mapping, provides opportunities to identify the number of QTLs controlling the phenotypic variation in a trait, the effect of each QTL, and the interaction of QTLs with the environment (Tanksley, 1993). Advanced populations such as recombinant inbred lines (RILs) are frequently used for QTL mapping in soybean (Abdel-Haleem et al., 2012; Bazzer et al., 2020a, 2020b, 2020c; Charlson et al., 2009; Hwang et al., 2013, 2014, 2015) to identify the QTLs and underlying genes related to drought tolerance traits. The molecular markers that are closely linked to identified QTLs/genes facilitate the selection of superior genotypes and have potential advantage for marker assisted selection (MAS) (Collard and Mackill, 2008; Ribaut and Hoisington, 1998).

Previous studies have investigated the genomic regions associated with drought-tolerance-related traits by using GWAM and linkage mapping in soybean. More studies are needed to map new QTLs across various environments and across different genetic backgrounds, as well as to confirm QTLs identified previously mapped in different soybean populations. The findings from these studies provide useful information to improve drought tolerance in soybean.

Literature Review

Soybean [*Glycine max* (L.) Merr.] is a major feed crop which belongs to the genus *Glycine*, family *Fabaceae* (*Leguminosae*), subfamily *Faboideae*, order *Fabales*, and tribe *Phaseoleae*. The genus *Glycine* was divided by Hermann (1962) into three subgenera: *Leptocyamus* (Benth.), *Glycine*, and *Soja* (Moench). The subgenus *Glycine* consists of 26 wild perennial species, whereas *Glycine soja* (wild progenitor) and *Glycine max* (cultivated soybean) belong to subgenus *Soja* (Chung and Singh, 2008; Kim et al., 2010a). The cultivated soybean (*Glycine max* (L.) Merr.) was domesticated from its annual wild relative (*G. soja* Seib. & Zucc.) around 5000 years ago, and the Yellow River region of China is the origin of cultivated soybean (Carter et al., 2004; Dong et al., 2001; Li et al., 2008). The major differences between the wild and cultivated soybean are seed color, seed size, seed oil and protein concentrations, grain yield, and tolerance to different biotic and abiotic stresses (Joshi et al., 2013). Soybean was first introduced into North America in 1765 (Hymowitz and Harlan, 1983), and it is now widely grown and consumed in the US for food primarily as feed and various industrial products.

Cultivated soybean is paleopolyploid in nature. There was an ancient whole genome duplication that occurred approximately 59 million years ago in the legume lineage, and a second genome duplication that occurred approximately 13 million years ago specific in the soybean lineage (Cannon et al., 2006). *Glycine max* and *Glycine soja* behave like diploids cytogenetically and both have 20 chromosomes (2n=40). The genome size of soybean is 1.1 Gb (Schemitz et al., 2010).

Bottleneck events include inbreeding, domestication, founding effect, and selective breeding over the past 75 years decreased the genetic diversity that resulted in narrow genetic diversity of soybean (Hyten et al., 2006). In North America, 35 ancestors contributed around

95% of the genes found in modern cultivars (Cui et al., 2001; Delannay et al., 1983; Gizlice et al., 1994, 1996; Salado-Navarro et al., 1993). The narrow genetic base of soybase cultivars and loss of tolerance genes during domestication increases their susceptibility to various biotic and abiotic stresses.

Soybean is grown and consumed worldwide due to its high protein (~40%) and edible oil (~18-19%) concentrations. Globally, soybean has annual production of 341.8 million metric tons. It is the world's leading economic oilseed crop as it contributes about 59% to world oilseed production (www.soystats.com). Depending upon global oil markets, soybean has also been at times an attractive crop for biodiesel production (Pimentel and Patzek, 2008).

The symbiotic association of soybean with *Bradyrhizobium japonicum* soil bacteria reduces atmospheric N_2 into ammonium (Strodtman and Emerich, 2009). This association decreases or eliminates the requirement of N fertilizer for soybean (Giller, 2001; Jensen and Hauggaard-Nielsen, 2003). Depending upon soil N concentration, N_2 fixation may provide from 50% to 85-90% of the N requirement (Mastrodomenico and Purcell, 2012).

Soybean is a short-day plant, which begins flowering in response of short photoperiods (Garner and Allard, 1920). Soybean growing season ranges from 70 to 140 days depending on the cultivar and regions where it is planted. Flowering in soybean is sensitive to photoperiod, which results into divisions of cultivars into different maturity groups according to latitudes. Soybean is classified into different maturity groups (MGs) that ranging from 000 to X (McWilliams et al., 1999). Soybean grown on lower latitudes initiate flowering at shorter daylength (e.g. 10 hours) and those belong to late maturity groups. Whereas, soybean grown at higher latitudes initiate flowering at longer daylengths (e.g. 13 hours) and those belong to earlier maturity groups. Arkansas typically grows cultivars belonging to MGs III, IV, and V.

Soybean Yield and Impact of Drought

Drought stress is a major constraint on the production and yield stability of soybean (Heatherly and Elmore, 1986; Manavalan et al., 2009) and leads to the reduction of 5 to 50% soybean yield (Sadeghipour and Abbasi, 2012). In the US, approximately 90% of the agricultural area is non-irrigated (Board and Kahlon, 2011). In Arkansas, approximately 80% of the soybean crop is irrigated; however, the depletion of ground water brings into question the long-term viability of irrigation. There was a linear increase in soybean yield by 22.6 kg ha^{-1} $year^{-1}$ in the US from 1924 to 1998 (Specht et al., 1999). In the US, a severe drought stress in 2012 resulted in a 1594 kg ha^{-1} yield reduction under non-irrigated conditions compared with irrigated production (USDA, 2013). In 2013, a significant difference in soybean yield was reported under irrigated (3,531 kg ha^{-1}) compared with non-irrigated (2596 kg ha^{-1}) conditions in Arkansas (USDA, 2014).Therefore, there is need for development of soybean cultivars that can better withstand water deficit conditions.

Gleick (2003) reported that 70 to 85% of water withdrawals worldwide are used for agricultural irrigation. This level of water consumption by agriculture is not sustainable into the future. As the result, drought seriously threatens crop production and food security in the 21st century (Tuberosa et al., 2002). Drought is generally accepted as a major abiotic stress on crop plants and is increasingly becoming a severe problem in many regions of the world. It is assumed that by the year 2025, around 1.8 billion people will face absolute water shortage and 65% of the world's population will live under water-deficit environments (Nezhadahmadi et al., 2013).

Drought alone accounts for global annual crop yield losses, ranging from 0 to 40%. Up to 45% of the world's agricultural lands are subjected to continuous or frequent drought (Ashraf and Foolad, 2007). Drought stress has been reported to severely reduce germination and seedling

stand in a large number of plant species (Kaya et al., 2006). The nature of drought is complex, and it is considered as a set of climatic pressures that are produced by several phenomena such as heat, water deficiency, evaporative demand, and high irradiance. Plant response to drought stress is complex, because it reflects combining the effects of stress, which influences almost all processes of plant growth, and important factors controlling crop yield (Bahari, 2014). Most of the soybean hectarage in the USA is grown in areas where erratic and low rainfall often reduces yield (Dogan et al., 2007; Hufstetler et al., 2007).

Mechanisms to Ameliorate Drought Impacts

Mechanisms of drought tolerance in plants can be grouped into three broad categories: drought escape, dehydration avoidance, and dehydration tolerance (Carrow, 1996; Turner et al., 2001). Drought escape is the mechanism in which plants complete their life cycle before the onset of drought stress. The Early Soybean Planting System (ESPS) is example of drought escape, and it is widely used in the southern US. In ESPS, the early maturing cultivars are planted in March or early April. These cultivars start flowering in late April to early May and set seed by mid-July to early August. Plentiful rainfall early in the season avoids drought that occurs most years in August (Heatherly and Elmore, 2004).

Drought avoidances strategies result in the maintenance of high-water status during water deficit conditions by reducing evapotranspiration and stomatal conductance, increasing water use efficiency and root growth, and limiting vegetative growth. In dehydration tolerance, plants maintain their cell turgor pressure by accumulation of osmolytes or osmoprotectants (Nguyen et al., 1997). Drought tolerant genotypes are able to continue the primary growth and development activities (Chandler and Bartels, 1999).

Drought stress has significant effects during both vegetative and reproductive stages. In Nebraska, Specht et al. (1999) reported that there can be up to 40% losses in soybean yield under drought during both the vegetative and the reproductive stages. During vegetative stages, drought stress leads to reduction in leaf number, leaf curling, reduced plant growth and ultimately reduction in soybean yield. During reproductive stages, there is increased flower abortion, shriveled seeds, and a 24 to 50% in yield reduction (Frederick et al., 2001; Boyer, 1983).

Morphological Responses to Water Stress

Different severity levels of drought result in differential responses by plants to maintain their growth and development. The morphological traits related to leaves (shape, expansion, size, pubescence, waxiness, senescence, and cuticle) and roots (length, area, density, and dry weight) offer potential mechanisms to lessen the impact of drought. Leaf area or leaf expansion is reduced under water stress to maintain the balance between water absorbed by roots and water transpired by leaves (to achieve less transpiration per unit leaf area). Reduced tillering, branching, and number of leaves per branch in response to drought lead to reduced leaf area (Kim et al., 2010b). In grasses, leaf rolling is a common response to drought stress and leads to a 50 to 70% reduction in water loss by transpiration (Sirault et al., 2004).

Root development increases in response to moderate stress due to an increased allocation of carbon to roots (Smith and De Smet, 2012). Under terminal drought stress, extensive primary root development and greater root density leads to improved/greater extraction of available soil water, resulting in increased seed yield (Ludlow and Muchow, 1990; Kashiwagi et al., 2005; Subbarao et al., 1995; Turner et al., 2001). In soybean, root-related traits such as root density, volume, and root distribution in the soil profile induced by drought stress have been proposed as indicators of drought tolerance (Garay and Wilhelm, 1982; Liu et al., 2005). In soybean, slow

canopy wilting was found to be correlated with seed yield under water deficit conditions (Bai and Purcell, 2018b).

Physiological Responses to Water Stress

Drought stress affects various physiological processes, mainly cell growth due to a reduction in cell turgor pressure (Taiz and Zeiger, 2006). Plant water balance is impacted by transpiration rate, stomatal conductance (*Gs*), leaf water potential, and uptake of soil moisture (Gerhards et al., 2018). Stomatal closure and stomatal conductance are sensitive plant responses to soil moisture status across plant species (Jones, 2004; Lawlor and Cornic, 2002). A reduction in transpiration and stomatal conductance under water deficit conditions result in increased canopy temperature (Turner et al., 2001).

Genotypes with drought tolerance can maintain a favorable water balance by limiting water loss while soil-moisture is still plentiful or improving soil moisture extraction. Water use efficiency (defined as ratio of the dry matter accumulated to the water consumed) is an important physiological trait that is highly affected by drought conditions (Monclus et al., 2006). The closing of stomata and reduced transpiration in response to water deficit conditions leads to improved WUE (Abbate et al., 2004). Under drought, closure of stomata and decreased stomatal conductance lead to a rapid decline in photosynthesis and limits the contribution of current assimilates to grain (Akram 2011; Sueedipour and Moradi, 2011).

A number of genes are activated in response to drought stress, and these genes are involved in the perception of drought and transmission of stress stimuli to other plant parts. Some genes over-expressed in early response to drought are involved in signal transduction, regulatory transcriptional and translational factors, and production of plant regulatory compounds (abscisic acid, salicylic acid, and brassinosteroids) (Kulkarni et al., 2017; Reddy et

al., 2004; Schachtman and Goodger, 2008; Shinozaki et al., 2003; Verma et al., 2016). Genes related to osmotic balance, water transport, and oxidative stress are activated through signal transduction late in the response to stress (Knight and Knight, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000). Abscisic acid (ABA) plays a significant role in response to water stress and is linked with rapid stomatal closure, reduced transpiration, increased water flux into the root system, and rapid accumulation of osmotic solutes (proline and betaine) (Davis and Mansfield, 1983; Prasad et al., 2008).

Breeding for Drought Tolerance

To overcome the devasting effects of drought and improve production efficiency in the face of a burgeoning world population, more stress tolerant crops must be developed (Khush, 1999). Improved cultivars with drought tolerance is the best solution to lessen the impact of drought on crop productivity. For developing varieties for drought conditions, conventional breeding methods are time consuming and labor intensive due to the quantitative nature of yield and drought tolerance (McWilliam, 1989; Ribaut et al., 1997). This problem is compounded in soybean because human selection has reduced the genomic diversity of cultivated soybean (Carter et al., 2004), limiting the availability of favorable alleles that could improve drought tolerance in adapted germplasm (Hyten et al., 2006; Lam et al., 2010). Understanding the genetic basis of drought tolerance in soybean is a prerequisite for developing genotypes with high yield potential under drought.

An alternative strategy of improving drought tolerance in crops is based upon identifying specific physiological traits that improve the crop adaptation to water deficit conditions (Subbarao et al., 1995). Dissecting the physiological basis of drought tolerance will help elucidate the mechanisms controlling drought tolerance and seed yield (Chen et al., 2011).

Therefore, a detailed understanding of the genetics and physiology of drought tolerance as well as the use of the proper germplasm and selection methods will facilitate the development of drought tolerant cultivars (Mohammadi et al., 2004). Moreover, identifying the genes controlling the physiological responses of plants to drought may lead to targeted genetic improvement strategies.

Traits Associated with Drought Tolerance

Measurement of physiological traits associated with drought tolerance should discriminate tolerant and susceptible genotypes. Traits associated with plant water status that have demonstrated genetic differences include canopy wilting (Abdel-Haleem et al., 2012; Charlson et al., 2009; Hwang et al., 2015; Kaler et al., 2017a; Sloane et al., 1990), canopy temperature (Bai and Purcell, 2018b; Blum, 1988; Ludlow and Muchow, 1990; Kaler et al., 2018a), carbon isotope ratio (Bai and Purcell, 2018a; Bazzer et al., 2020a, 2020b; Dhanapal et al., 2015a; Kaler et al., 2017b), canopy coverage (Kaler et al., 2018b), rate of excised leaf water loss (Basal et al., 2005), leaf relative water content (Babu et al., 2003), relative electrical conductivity (Lafitte and Courtois, 2002), and malondialdehyde content (Sairam et al., 1997). A better understanding of the physiological basis for yield improvements under water-deficit conditions will help to identify targets for soybean yield improvement in the future (Koester et al., 2014).

Carbon Isotope Ratio:

Water use efficiency (WUE), which is defined as the amount of dry matter produced per unit of water transpired, is one among several physiological traits that impart drought tolerance (Condon et al., 2004). Direct measurement of WUE depends either on extensive leaf gas-exchange data or long-term measures of plant water consumption and biomass production

(Manavalan et al., 2009). Thus, WUE is closely associated with total biomass yield (Chen et al., 2011; Passioura, 1996; Wright, 1996). Passioura (1996) proposed a simple model to view yield as a multiplicative function of water transpired (T, m^3), water use efficiency (WUE, kg Biomass m^{-3}), and harvest index (HI, kg grain kg^{-1} Biomass) under water limited environments:

$$Y = T \times WUE \times HI$$

In short, crop performance could be improved through increases in T, WUE, or HI (Wright, 1996). Richards et al. (2002) pointed out that these three components are likely independent of each other. Thus, an improvement in any one of these components could result in an increase in yield. Greater WUE can be achieved by coordination between photosynthesis and transpiration (Chen et al., 2011). Genetic variability for WUE has been found in many cultivars or lines of several crop species including soybean (Mian et al., 1996), peanut (Wright et al., 1994), cowpea (Ashok et al., 1999; Ismail and Hall, 1992), cotton (Quisenberry and McMichael, 1991; Saranga et al., 1999), sorghum (Donatelli et al., 1992), barley (Hubick and Farquhar, 1989), and wheat (Ehdaie and Waines, 1993; Van Den Boogaard et al., 1997).

The application of WUE for improving drought tolerance, however, has been limited by lack of suitable screening methods in large populations under field conditions (Chen et al., 2011, 2012). To avoid the difficulty of measuring WUE of field grown plants, Farquhar et al. (1982) and Farquhar and Richards (1984) proposed that the discrimination of the heavier isotope of carbon (^{13}C) over the lighter isotope of carbon (^{12}C) in C_3 plants, could serve as a proxy for WUE**.** This discrimination occurs in the carbon assimilation process, mainly at the initial carboxylation step catalyzed by Rubisco (Xu et al., 2009). The extent of this carbon isotope discrimination ($\Delta^{13}C$) is related to the ratio of the internal to external concentrations of CO_2 (Ci/Ca) which is further controlled by stomatal conductance and photosynthetic capacity

(Brugnoli and Farquhar, 2000; Farquhar et al., 1989). An approximate expression for the overall $\Delta^{13}C$ in leaves for C_3 plants during photosynthesis has been described by Farquhar et al. (1989) as:

$$\Delta^{13}C = a + (b-a)\ Ci/Ca$$

where 'a' is the discrimination that occurs during diffusion of CO_2 into the intercellular airspaces and 'b' is the discrimination associated with carboxylation by Rubisco.

Therefore, $\Delta^{13}C$ is positively correlated with the ratio of internal leaf CO_2 concentration to ambient CO_2 concentration (Ci/Ca) and has a negative relationship with WUE (Ehdaie et al., 1991; Farquhar and Richards, 1984; Johnson and Bassett, 1991). Thus, a high Ci/Ca leads to a higher $\Delta^{13}C$ and a lower WUE (Farquhar and Richards, 1984). This negative correlation with WUE has been used for indirect selection of improved WUE in C_3 plants under selected environments (Cattivelli et al., 2008). The linkage between $\Delta^{13}C$ and WUE was predicted on the concept that both are functionally dependent on Ci (Farquhar et al., 1989). Thus, $\Delta^{13}C$ provides an integrated measurement of WUE in C_3 plants (Farquhar and Richards, 1984). The alternative expression of $\Delta^{13}C$ is carbon isotope ratio ($\delta^{13}C$) which is directly proportional to WUE, whereas $\Delta^{13}C$ is inversely proportional to WUE.

Canopy Temperature

It is also difficult to measure transpiration (T) on a large number of genotypes in a field experiment, but canopy temperature (CT) variation due to water stress can be used as an indicator for transpiration differences among genotypes. Under sufficient soil moisture, transpiration results in canopy cooling (Tanner, 1963). Under water deficit conditions, there is a decrease in stomatal conductance and closure of stomata which results in increased CT due to lack of transpirational cooling (Gates, 1968; Tanner, 1963). Thus, CT can be used as a surrogate

measure of stomatal conductance, plant transpiration, and plant water status (Amani et al., 1996; Araus et al., 2003; Jones et al., 2009; Rebetzke et al., 2013). Therefore, selecting genotypes that maintain lower CT compared with other genotypes under water deficit conditions may be useful to improve drought tolerance (Blum, 2004).

In wheat, Olivares-Villegas et al. (2007) reported that lower CT is a highly heritable (h^2=0.65) drought-adaptive trait that significantly contributed to improved crop performance. They suggested that lower CT as a dehydration-avoidance mechanism can be used as a selection criterion to identify high yielding genotypes or as an important predictor of yield performance under drought (Fischer et al., 1998).

Field measurement of CT of a large number of genotypes is difficult because of temperature variation over time due to solar radiation, wind, soil moisture, and air temperature. Therefore, high throughput phenotyping through aerial thermal infrared imaging could be one of the approaches to measure relative CT differences among genotypes (Knipling, 1970; Merlot et al., 2002). In recent years, unmanned aerial systems (UAS) with thermal infrared imaging have become an advanced field phenotyping platform to monitor crop water status, improving irrigation, and managing irrigation (Jackson et al., 1981; Jones, 2004; Martínez et al., 2016; Santesteban et al., 2017; Zhang et al., 2018).

Nitrogen Fixation

Legumes have the ability to fix atmospheric N_2 by establishing a symbiotic relationship with N_2-fixing soil bacteria. This symbiotic interaction between soybean and rhizobia is the most significant natural pathway for the introduction of atmospheric N into the biosphere. But growth of legume plants and number of nodules is depressed under drought conditions, which results in decreased N accumulation and crop yield (Márquez-García et al., 2015; Serraj et al., 1999a).

Proposed mechanisms decreasing N_2 fixation during drought include carbon shortage, oxygen limitation, and feedback regulation by nitrogen accumulation (Purcell, 2009; Serraj et al., 1999b). The process of root hair infection by *Rhizobium* and the formation of infection threads have also been found to be seriously inhibited by water deficit (Graham, 1992; Purcell et al., 1997).

Differences among genotypes in the sensitivity of N_2 fixation to drought can be evaluated by ^{15}N natural abundance, which provides a snapshot of the fraction of N derived from the atmosphere (NDFA) (Shearer et al., 1986) and serves as an index of N_2 fixation (Andrews and Lea 2013; Barrie et al., 1995; Letolle, 1980). The ^{15}N natural abundance ($\delta^{15}N$) method compares the abundance of ^{15}N isotope to ^{14}N isotope in plant tissue, the atmosphere, and soil environment. The atmosphere has lower concentration of ^{15}N isotope compared to the soil. As a result, biological N_2 fixation dilutes the ^{15}N in plant tissue as compared to plants that depend on mineral nitrogen as a nitrogen source (Doughton et al., 1995). The difference in ^{15}N and ^{14}N concentration between soil and atmosphere is expressed in terms of parts per thousand (‰) and is referred to as the N isotope ratio ($\delta^{15}N$) (Peoples et al., 1989). The fraction of NDFA from $\delta^{15}N$ is determined as (Kohl and Shearer, 1981):

$$NDFA = (\delta^{15}N_{ref} - \delta^{15}N_{samp})/(\delta^{15}N_{ref} - \delta^{15}N_0)$$

where $\delta^{15}N_{ref}$ and $\delta^{15}N_{samp}$ are the ^{15}N composition of the reference plant (non-nodulating soybean) and the plant sample, respectively, and $\delta^{15}N_0$ is a constant (-1.30 for soybean, Bergersen et al., 1989) that represents the $\delta^{15}N$ value of soybean totally dependent upon N_2 fixation. Genetic variability for NDFA or $\delta^{15}N$ has been found in soybean genotypes/accessions and has been used to study the inheritance of N_2 fixation (Dhanapal et al., 2015b; Steketee et al.,

2019). These studies have shown that NDFA or $\delta^{15}N$ can be used to estimate the amount of N fixed by genotypes via N_2 fixation.

Mapping QTLs for Drought Tolerance Related Traits

Drought tolerance is a complex and quantitatively inherited trait that is governed by several complex factors including genotype, environment, and genotype × environment interactions (Carter et al., 1999). Quantitative trait loci (QTL) are regions in the genome that are associated with a quantitative trait. Identification of QTLs related to drought tolerance via molecular markers is one approach to improve selection efficiency (Pathan et al., 2007).

The availability of high density molecular marker maps allows the genetic analysis of physiological traits and provides additional information on the identification of the number of QTLs affecting the trait, genomic location of the QTLs, and which parent contributes the favorable allele for each QTL (Mian et al., 1996). Linkage mapping is a statistical procedure to identify QTLs associated with a trait in a segregating bi-parental population across multiple environments.

The biparental segregating populations such as recombinant inbred lines (RIL), backcross, double haploid line, and F2 populations have been widely used for QTL mapping and these populations have several advantages and disadvantages over other (Collard et al., 2005; McCouch and Doerge, 1995; Paterson, 1996). The RIL population is derived from a cross of two homozygous (inbreds) parents follow by continuous selfing over several generations resulting homozygous lines known as RILs. Time required for development of RILs (usually 6-8 generations) is the major disadvantage of this population. The RIL population is permanent and immortal as the composition of this population is not altered over time, which allows to replicate the experiments across multiple environments.

Analysis of QTLs in replicated, segregating RIL populations under different environments allows the variance to be partitioned into genotype and environment components, together with an estimate of the number of genetic loci controlling traits (Kearsey and Hyne, 1994). Recent advances in high throughput genotyping and phenotyping platforms have revolutionized the dissection of the genetic basis of traits associated with drought tolerance and will accelerate soybean breeding research for drought tolerance.

In soybean, a large number of QTLs have been identified for agronomic and physiological traits, seed composition, and responses to both biotic and abiotic stresses (Manavalan et al., 2009). To date, only a few QTLs associated with drought tolerance have been reported in soybean, and these identified QTLs explained less than 10 % of the phenotypic variation for those traits (Manavalan et al., 2009; Mian et al., 1998; Monteros et al., 2006). In soybean, QTLs associated with WUE have been identified using an F_4-derived population developed from a cross between "Young" (PI 508266) and PI 416937 (Mian et al., 1996). A total of four QTLs were associated with WUE and when combined, these QTLs explained 38% of the variability for WUE. Mian et al. (1998) identified two independent QTLs associated with WUE in a F_2- derived population from a cross of S100 × Tokyo.

In a field experiment, Specht et al. (2001) evaluated $\Delta^{13}C$ RILs developed from a cross between 'Minsoy' and 'Noir 1' and identified five QTLs associated with $\Delta^{13}C$ in soybean. Dhanapal et al. (2015a) and Kaler et al. (2017b) used GWAM to identify SNPs associated with $\delta^{13}C$ using a panel of 373 diverse soybean accessions. A total of 39 SNPs was significantly associated with $\delta^{13}C$ and likely tagged 21 different loci (Dhanapal et al., 2015a). Kaler et al. (2017b) identified 54 SNPs for $\delta^{13}C$ that likely tagged 46 loci. Kaler et al. (2018a) conducted the first GWAM study for CT using a diverse panel of 345 maturity group IV soybean accessions

and association analysis identified 34 loci associated with CT. Bai and Purcell (2018b) found that slow wilting genotypes under water deficit conditions had a cooler canopy than fast wilting genotypes and that a cooler canopy has a positive correlation with grain yield.

In soybean, GWAM and linkage mapping studies have dissected the genetic basis of several morpho-physiological traits such as canopy wilting (Abdel-Haleem et al., 2012; Charlson et al., 2009; Hwang et al., 2015, 2016; Kaler et al., 2017a), oxygen isotope ratio ($\delta^{15}O$, Kaler et al., 2017b), and canopy coverage (Kaler et al., 2018b). Charlson et al. (2009) evaluated soybean canopy wilting in a population of 92 F_3- and F_5- derived recombinant inbred lines (RILs) generated from cross of KS4895 and Jackson and identified four putative QTLs linked with canopy wilting on Gm08, Gm14, Gm17, and Gm13. Du et al. (2009) used a mapping population of 184 RILs developed from a cross of Kefeng1 and Nannong1138-2 and identified two QTLs (Gm08 and Gm20) for wilting coefficient. Abdel-Haleem et al. (2012) identified seven QTLs for wilting on Gm02, Gm04, Gm05, Gm12, Gm14, Gm17, and Gm19.

Hwang et al. (2015, 2016) used multiple bi-parental populations to identify QTLs linked with canopy wilting and found eight QTL clusters associated with canopy wilting. Previously, GWAM experiments in soybean identified loci associated with canopy coverage, canopy wilting, and oxygen isotope ratio. GWAM identified between 33 and 50 putative loci associated with canopy coverage (Kaler et al., 2018b), 23 putative loci associated with canopy wilting (Kaler et al., 2017a), and 31 loci with oxygen isotope ratio (Kaler et al., 2017b). Steketee et al. (2020) also conducted a GWAM for canopy wilting using a different diverse panel of soybean accessions and identified 44 loci were associated with canopy wilting under rainfed conditions.

Various QTL analyses have been performed to identify the genomic regions associated with N_2 fixation related traits under drought. Hwang et al. (2013) conducted QTL analysis for

shoot ureide and N concentration by using a mapping population derived from the cross of KS4895 and Jackson and identified two QTLs for ureide and shoot N concentration under drought conditions. Hwang et al. (2014) were the first to map QTLs for nodule number, nodule size, and nodule weight in field experiments. Dhanapal et al. (2015b) conducted GWAM on a panel of 374 maturity group 4 accessions and identified 17 and 19 SNPs significantly associated with NDFA and N concentration, respectively. Steketee et al. (2019) were the first to perform association mapping for $\delta^{15}N$ using a panel of 211 diverse soybean accessions and found 23 and 26 SNPs significantly associated with $\delta^{15}N$ and N concentration, respectively.

To improve drought tolerance in soybean, more studies are needed to identify and confirm QTLs associated with drought tolerance, to map new QTL(s)/gene(s), and to determine gene action under drought. High-density genetic maps and confirmed QTLs/genes, which are screened across the various environments and across genetic backgrounds, are the most important criteria for developing drought-resistant soybean through marker-assisted selection (Manavalan et al., 2009). Subsequently, the next steps will be to confirm these QTLs in different genetic backgrounds and in different environments to evaluate the efficacy of identified QTLs in selecting drought tolerant/resistant genotypes.

Objectives

The objective of this research was to identify the genomic regions (QTLs) associated with drought-related traits. In this research, two different biparental populations were phenotyped on field scale experiments for $\delta^{13}C$, $\delta^{15}N$, and CT at multiple locations under different environmental conditions. We have been able to identify genomic regions and link molecular markers with identified genomic loci responsible for these targeted traits. A deeper understanding of genetic control of physiological traits associated with drought tolerance will

help improve drought tolerance in soybean. The overall objective of this study was to identify the QTLs associated with drought tolerance-related traits and to confirm the identified QTLs from previous studies. This dissertation is divided into four chapters that detail the specific aims of this research:

- Identification of quantitative trait loci for carbon isotope ratio ($\delta^{13}C$) in a recombinant inbred population of soybean
- Mapping quantitative trait loci (QTL) for plant nitrogen isotope ratio ($\delta^{15}N$) in soybean
- Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean
- Identification of quantitative trait loci associated with canopy temperature in soybean

References

Abbate, P.E., J.L. Dardanelli, M.G. Cantarero, M. Maturano, R.J.M. Melchiori, and E.E. Suero. 2004. Climatic and water availability effects on water-use efficiency in wheat. Crop Sci. 44:474-483.

Abdel-Haleem, H., T.E. Carter Jr, L.C. Purcell, C.A. King, L.L. Ries, P. Chen, W. Schapaugh Jr, T.R. Sinclair, and H.R. Boerma. 2012. Mapping of quantitative trait loci for canopy wilting trait in soybean (*Glycine max* L. Merr). Theor. Appl. Genet. 125:837-846.

Akram, M. 2011. Growth and yield components of wheat under water stress of different growth stages. Bangl. J. Agric. Res. 36:455-68.

Amani, I., R.A. Fischer, and M.P. Reynolds. 1996. Canopy temperature depression association with yield of irrigated spring wheat cultivars in hot climate. J. Agron. Crop Sci. 176:119-129.

Andrews, M., and P.J. Lea. 2013. Our nitrogen "footprint": the need for increased crop nitrogen use efficiency. Ann. Appl. Biol. 163:165-169

Araus, J.L., G.A. Slafer, M.P. Reynolds, and C. Royo. 2002. Plant breeding and drought in C3 cereals: what should we breed for? Ann. Bot. 89:925-940.

Ashok, I.S.A.H., T.G. Prasad, M.U. Kumar, R.C.N. Rao, and G.C. Wright. 1999. Variation in transpiration efficiency and carbon isotope discrimination in cowpea. Aust. J. Plant Physiol. 26:503-510.

Ashraf, M., and M.R. Fooland. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 9:206-216.

Babu, R.C., B.D. Nguyen, V. Chamarerk, P. Shanmugasundaram, P. Chezhian, P. Jeyaprakash, S.K. Ganesh, A. Palchamy, S. Sadasivam, S. Sarkarung, L.J. Wade, and H.T. Nguyen. 2003. Genetic analysis of drought resistance in rice by molecular markers association between secondary traits and field performance. Crop Sci. 43:1457-1469.

Bahari, N. 2014. Evaluation of yield and some morphological traits of wheat under drought stress. Indian J. Fund. Appl. Sci. 4:42-46.

Bai, H., and L.C. Purcell. 2018a. Response of carbon isotope discrimination and oxygen isotope composition to mild drought in slow- and fast-wilting soybean genotypes. J. Crop Improv. 32:239-253.

Bai, H., and L.C. Purcell. 2018b. Aerial canopy temperature differences between fast- and slow-wilting soybean genotypes. J. Agron. Crop Sci. 204:243-251.

Barrie, A., S. Debney, C.T. Workman, and C. Pullan. 1995. Recent developments in high productivity stable isotope analysis. In: International symposium on nuclear and related

techniques in soil-plant studies for sustainable agricultural and environmental preservation. Vienna, Austria, 17-21 Oct 1994, pp 29-61.

Basal, H., C.W. Smith, P.S. Thaxton, and J.K. Hemphill. 2005. Seedling drought tolerance in upland cotton. Crop Sci. 45:766-771.

Bazzer, S.K., A.S. Kaler, C.A. King, J.D. Ray, S. Hwang, and L.C. Purcell. 2020b. Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean. Crop Sci. https://doi.org/10.1002/csc2.20240.

Bazzer, S.K., A.S. Kaler, J.D. Ray, J.R. Smith, F.B. Fritschi, and L.C. Purcell. 2020a. Identification of quantitative trait loci for carbon isotope ratio ($\delta^{13}C$) in a recombinant inbred population of soybean. Theor. Appl. Genet. 133:2141-2155.

Bazzer, S.K., J.D. Ray, J.R. Smith, F.B. Fritschi, and L.C. Purcell. 2020c. Mapping quantitative trait loci (QTL) for plant nitrogen isotope ratio ($\delta^{15}N$) in soybean. Euphytica (in review)

Bergersen, F.T., J. Brockwell, R.R. Gault, L. Morthorpe, M.B. Peoples, and G.L. Turner. 1989. Effects of available soil nitrogen and rates of inoculation on nitrogen fixation by irrigated soybeans and evaluation of delta 15N methods for measurement. Aust. J. Agric. Res. 40:763-780.

Blum, A. 1988. Drought resistance. In: Plant Breeding for Stress Environment. CRC Press, Boca Raton, Florida, USA. pp 43-76.

Blum, A. 2004. Sorghum physiology. In: Physiology and biotechnology integration for plant breeding. Ed: Nguyen, H.T., and A. Blum. Marcel Dekker, New York. pp 141-223.

Blum, A. 2011. Drought resistance - is it really a complex trait? Funct. Plant Biol. 38:753-757.

Board, J.E., and C.S. Kahlon. 2011. Soybean yield formation: what controls it and how it can be improved. In: Soybean Physiology and Biochemistry. Ed: El-Shemy, A. H. In Tech. pp 1-36.

Boyer, J.S. 1982. Plant productivity and environment. Science 218:443-448.

Boyer, J.S. 1983. Environmental stress and crop yields. In: Crop reaction to water and temperature stressors in humid, temperate climates. Ed: Raper, C.D., and P.J. Kramer. West view Press, Boulder, pp 3-7.

Brugnoli, E, and G.D. Farquhar. 2000. Photosynthetic fractionation of carbon isotopes. In: Photosynthesis: Physiology and Metabolism. Ed: Leegood, R.C., T.D. Sharkey, and S. von Caemmerer. Kluwer Academic Publishers, Dordrecht, pp 399-434.

Byrne, S., Czaban, A., Studer, B., Panitz, F., Bendixen, C., and Asp, T. (2013). Genome wide allele frequency fingerprints (GWAFFs) of populations via genotyping by sequencing. PLoS ONE 8:e57438.

Cannon, S.B., L. Sterck, S. Rombauts, S. Sato, F. Cheung, J. Gouzy, X. Wang, J. Mudge, J. Vasdewani, T. Schiex, M. Spannagl, E. Monaghan, C. Nicholson, S.J. Humphray, H. Schoof, K.F.X. Mayer, J. Rogers, F. Quétier, G.E. Oldroyd, F. Debellé, D.R. Cook, E.F. Retzel, B.A. Roe, C.D. Town, S. Tabata, Y. Van de Peer, and N.D. Young. 2006. Legume genome evolution viewed through the *Medicago truncatula* and *Lotus japonicus* genomes. Proc. Natl. Acad. Sci. 103:14959-14964.

Carrow, R. 1996. Drought avoidance characteristics of diverse tall fescue cultivars. Crop Sci. 36:371-377.

Carter Jr, T.E., R. Nelson, C.H. Sneller, and Z. Cui. 2004. Genetic diversity in soybean. In: Soybeans: Improvement, Production, and Uses Eds: Boerma, H.R., and J.E. Specht, third ed. ASA-CSSA-SSSA, Madison, WI. pp 303-416.

Carter Jr, T.E., P.I. De Souza, and L.C. Purcell. 1999. Recent advances in breeding for drought and aluminum resistance in soybean. In: Proceedings of the sixth World Soybean Research Conference, Chicago, IL. Ed: Kauffman, H. pp. 106-125. Superior Printing, Champaign, IL.

Cattivelli, L., F. Rizza, F.-W. Badeck, E. Mazzucotelli, A.M. Mastrangelo, E. Francia, C. Marè, A. Tondelli, and A.M. Stanca. 2008. Drought tolerance improvement in crop plants: an integrated view from breeding to genomics. Field Crops Res. 105:1-14.

Ceccarelli, S., E. Acevedo, and S. Grando. 1991. Breeding for yield stability in unpredictable environments: single traits, interaction between traits, and architecture of genotypes. Euphytica 56:169-185.

Chandler, J., and D. Bartels. 1999. Plant dessication In: Plant responses to environmental stresses: from phytohormones to genome reorganization. Ed: Lerner, H.R. New York, USA: Marcel Dekker. pp 575-590.

Charlson, D.V., S. Bhatnagar, C.A. King, J.D. Ray, C.H. Sneller, T.E. Carter, and L.C. Purcell. 2009. Polygenic inheritance of canopy wilting in soybean [*Glycine max* (L.) Merr.]. Theor. Appl. Genet. 119:587-594.

Chen, D., K. Neumann, S. Friedel, B. Kilian, M. Chen, T. Altmann, and C. Klukas. 2014. Dissecting the phenotypic components of crop plant growth and drought responses based on high-throughput image analysis. Plant Cell. 26:4636-4655.

Chen, J., S.X. Chang, and A.O. Anyia. 2011. The physiology and stability of leaf carbon isotope discrimination as a measure of water-use efficiency in barley on the Canadian Prairies. J. Agron. Crop. Sci. 197:1-11.

Chen, J., S.X. Chang, and O.A. Anthony. 2012. Quantitative trait loci for water-use efficiency in barley (*Hordeum vulgare* L.) measured by carbon isotope discrimination under rain-fed conditions on the Canadian Prairies. Theor. Appl. Genet.125:71-90.

Chung, G., and R.J. Singh. 2008. Broadening the genetic base of soybean: a multidisciplinary approach. Crit. Rev. Plant Sci. 27:295-341.

Collard, B.C.Y, and C.J. Mackill. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil. Trans. R. Soc. B. 363:557-572.

Collard, B.C.Y., M.Z.Z. Jahufer, J.B. Brouwer, and E.C.K. Pang. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142:169-196.

Condon, A.G., R.A. Richards, G.J. Rebetzke, and G.D. Farquhar. 2004. Breeding for high water use efficiency. J. Exp. Bot. 55:2447-2460.

Cregan, P.B., T. Jarvik, A.L. Bush, R.C. Shoemaker, K.G. Lark, V.C. Concibido, X. Delannay, J.E. Specht, and P.B. Cregan. 1999. An integrated genetic linkage map of the soybean. Crop Sci. 39:1464-1490.

Cui, Z., T.E. Carter Jr, J.W. Burton, and R. Wells. 2001. Phenotypic diversity of modern Chinese and North American soybean cultivars. Crop Sci. 41:1954-1967.

Davis, W.J., and T.A. Mansfield. 1983. The role of abscisic acid in drought avoidance. In: Abscisic Acid. Ed: Addicott, F. T. Praeger, New York. pp 237-68.

Delannay, X., D.M. Rogers, and R.G. Palmer. 1983. Relative genetic contributions among ancestral lines to North America soybean cultivars. Crop Sci. 23:944-949.

Dhanapal, A.P., J.D. Ray, S.K. Singh, V. Hoyos-Villegas, J.R. Smith, L.C. Purcell, A. King, and F.B. Fritschi. 2015b. Genome-wide association analysis of diverse soybean genotypes reveals novel markers for nitrogen traits. Plant Genome 8:1-15

Dhanapal, A.P., J.D. Ray, S.K. Singh, V. Hoyos-Villegas, J.R. Smith, L.C. Purcell, A. King, P.B. Cregan, Q. Song Q, and F.B. Fritschi. 2015a. Genome wide association study (GWAS) of carbon isotope ratio ($\delta^{13}C$) in diverse soybean [*Glycine max* (L.) Merr.] genotypes. Theor. Appl. Genet. 28:73-91.

Dogan, E., H. Kirnak, and O. Copur. 2007. Deficit irrigations during soybean reproductive stages and CROPGRO-soybean simulations under semi-arid climatic conditions. Field Crops Res. 103:154-159.

Donatelli, M., G.L. Hammer, and R.L. Vanderlip. 1992. Genotype and water limitation effects on phenology, growth, and transpiration efficiency in grain sorghum. Crop Sci. 21:781-786.

Dong, Y.S., B.C. Zhuang, L.M. Zhao, H. Sun, and M.Y. He. 2001. The genetic diversity of annual wild soybeans grown in China. Theor. Appl. Genet. 103:98-103.

Doughton, J.A., P.G. Saffigna, I. Vallis, and R.J. Mayer. 1995. Nitrogen fixation in chickpea. II. Comparison of ^{15}N enrichment and ^{15}N natural abundance methods for estimating nitrogen fixation. Aust. J. Agric. Res. 45:225-236.

Du, W., M. Wang, S. Fu, and D. Yu. 2009. Mapping QTL for seed yield and drought susceptibility index in soybean (*Glycine max* L.) across different environments. J. Genet. Genomics 36:721-731.

Ehdaie, B., A.E. Hall, G.D. Farquhar, H.T. Nguyen, and J.D. Waines. 1991. Water use efficiency and carbon isotope discrimination in wheat. Crop Sci. 31:1282-1288.

Ehdaie, B., and J.G. Waines. 1993. Variation in water-use efficiency and its components in wheat. I. Well-watered pot experiment. Crop Sci. 33:294-299.

Farquhar, G.D., J.R. Ehleringer, and K.T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Biol. 40: 503-537.

Farquhar, G.D., M.H. O'Leary, and J.A. Berry. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Funct. Plant Biol. 9:121-137.

Farquhar, G.D., and R.A. Richards. 1984. Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11:539-552.

Fischer, R.A., D. Rees, K.D. Sayre, Z.M. Lu, A.G. Condon, and A.L. Saavedra. 1998. Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. Crop Sci. 38:1467-1475.

Frederick, J.R., C.R. Camp, and P.J. Bauer. 2001. Drought-stress effects on branch and main stem seed yield and yield components of determinate soybean. Crop Sci. 41:759-763.

Garay, A.F., and W.W. Wilhelm. 1982. Root system characteristics of two soybean isolines undergoing water stress conditions. Agron. J. 75:973-977.

Garner, W.W., and H.A. Allard. 1920. Effect of the relative length of the day and night and other factors of the environment on growth and reproduction in plants. J. Agric. Res. 18:553-606.

Gates, D.M. 1968. Transpiration and leaf temperature. Annu. Rev. Plant Physiol. 19:211-238.

Gerhards, M., M. Schlerf, U. Rascher, T. Udelhoven, R. Juszczak, G. Alberti, F. Miglietta, and Y. Inoue. 2018. Analysis of airborne optical and thermal imagery for detection of water stress symptoms. Remote Sens. 10:1139.

Giller, K.E. 2001. Nitrogen fixation in tropical cropping systems, 2 edn. CABI, New York, USA

Gizlice, Z., T.E. Carter Jr., and J.W. Burton. 1994. Genetic base for North American public soybean cultivars released between 1947 and 1988. Crop Sci. 34:1143-1151.

Gizlice, Z., T.E. Carter Jr., T.M. Gerig, and J.W. Burton. 1996. Genetic diversity patterns in North American public soybean cultivars based on coefficient of parentage. Crop Sci. 36:753-765.

Gleick, P.H. 2003. Water use. Annu. Rev. Environ. Resourc. 28:275-314.

Graham, P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. Can. J. Microbiol. 38:475-484.

Heatherly, L.G., and C.D. Elmore. 1986. Irrigation and planting date effects on soybean grown on clay soil. Agron. J. 78:576-580.

Heatherly, L.G. and R.W. Elmore. 2004. Managing inputs for peak production. In: Soybean: Improvement, Production, and Uses. Eds: Specht J, and R. Boerma. 3rd ed. Agronomy Monograph 16. American Society of Agronomy, Madison, Wisconsin. Pp. 451-536.

Hermann, F. J. 1962. A revision of the genus Glycine and its immediate allies. United States Department of Agriculture, Agricultural Research Service. Technical Bulletin No. 1268, pp 82.

Hubick, K., and G.D. Farquhar. 1989. Carbon isotope discrimination and the ratio of carbon gained to water lost in barley cultivars. Plant Cell Environ. 12:795-804.

Hufstetler, E.V., H.R. Boerma, T.E. Carter, and H.J. Earl. 2007. Genotypic variation for three physiological traits affecting drought tolerance in soybean. Crop. Sci. 47:25-35.

Hwang, S., C.A. King, P. Chen, J.D. Ray, P.B. Cregan, T.E. Carter Jr, Z. Li, H. Abdel-Haleem, K.W. Matson, W. Schapaugh Jr, and L.C. Purcell. 2016. Meta-analysis to refine map position and reduce confidence intervals for delayed canopy wilting QTLs in soybean. Mol. Breed. 36:91.

Hwang, S., C.A. King, M.K. Davies, J.D. Ray, P.B. Cregan, and L.C. Purcell. 2013. QTL analysis of shoot ureide and nitrogen concentrations in soybean [*Glycine max* (L.) Merr.]. Crop Sci. 53:2421-2433.

Hwang, S., C.A. King, J.D. Ray, P.B. Cregan, P. Chen, T.E. Carter Jr, Z. Li, H. Abdel-Haleem, K.W. Matson, W. Schapaugh Jr, and L.C. Purcell. 2015. Confirmation of delayed canopy wilting QTLs from multiple soybean mapping populations. Theor. Appl. Genet. 128:2047-2065.

Hwang, S., J.D. Ray, P.B. Cregan, A. King, M.K. Davies, and L.C. Purcell. 2014. Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* L. [Merr.]). Euphytica 195:419-434.

Hymowitz, T., and J.R. Harlan. 1983. Introduction of soybean to North America by Samuel Bowen in 1765. Econ. Bot. 37:371-379.

Hyten, D.L., S.B. Cannon, Q. Song, N. Weeks, E.W. Fickus, R.C Shoemaker, J.E Specht, A.D Farmer, G.D May, and P.B Cregan. 2010. High-throughput SNP discovery through deep resequencing of a reduced representation library to anchor and orient scaffolds in the soybean whole genome sequence. BMC Genomics 11:38.

Hyten, D.L., Q.J. Song, Y.L. Zhu, I.Y. Choi, R.L. Nelson, J.M. Costa, J.E. Specht, R.C. Shoemaker, and P.B. Cregan. 2006. Impacts of genetic bottlenecks on soybean genome diversity. Proc. Natl. Acad. Sci. 103:16666-16671.

Ismail, A.M., and A.E. Hall. 1992. Correlation between water use efficiency and carbon isotope discrimination in diverse cowpea genotypes and isogenic lines. Crop Sci. 32:7-12.

Jackson, R.D., S.B. Idso, R.J. Reginato, and P.J. Pinter. 1981. Canopy temperature as a crop water stress indicator. Water Resour. Res. 17:1133-1138.

Jensen, E., and H. Hauggaard-Nielsen. 2003. How can increased use of biological N_2 fixation in agriculture benefit the environment? Plant Soil 252:177-186.

Johnson, D.A., and L.M. Bassett. 1991. Carbon isotope discrimination and water use efficiency in four cold season grasses. Crop Sci. 31:157-162.

Jones, H.G. 2004. Application of thermal imaging and infrared sensing in plant physiology and ecophysiology. Adv. Bot. Res. 41:107-163

Jones, H.G., R. Serraj, B.R. Loveys, L.Z. Xiong, A. Wheaton, and A.H. Price. 2009. Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. Funct. Plant Biol. 36:978-989.

Joshi, T., B. Valliyodan, J.H. Wu, S.H. Lee, D. Xu, and H.T. Nguyen. 2013. Genomic differences between cultivated soybean, *G. max* and its wild relative *G. soja*. BMC Genomics 14 (Suppl):S5.

Kaler, A.S., A.P. Dhanapal, J.D. Ray, C.A. King, F.B. Fritschi, and L.C. Purcell. 2017b. Genome-wide association mapping of carbon isotope and oxygen isotope ratios in diverse soybean genotypes. Crop Sci. 57:3085-3100.

Kaler, A.S., J.D. Ray, W.T. Schapaugh, R.A. Antonio, C.A. King, E.E. Gbur, and L.C. Purcell 2018a. Association mapping identifies loci for canopy temperature under drought in diverse soybean genotypes. Euphytica 214:135.

Kaler, A.S., J.D. Ray, W.T. Schapaugh, M.K. Davies, C.A. King, and L.C. Purcell. 2018b. Association mapping identifies loci for canopy coverage in diverse soybean genotypes. Mol. Breed. 38:50.

Kaler, A.S., J.D. Ray, W.T. Schapaugh, C.A. King, and L.C. Purcell. 2017a. Genome-wide association mapping of canopy wilting in diverse soybean genotypes. Theor. Appl. Genet. 130:2203-2217.

Kashiwagi, J., L. Krishnamurthy, H.D. Upadhyaya, H. Krishna, S. Chandra, V. Vadez, and R. Serraj. 2005. Genetic variability of drought-avoidance root traits in the mini-core germplasm collection of chickpea (*Cicer arietinum* L.).Euphytica146:213-222.

Kaya, M.D., G. Okai, M. Atak, Y. Cikili, and O. Kolsarici. 2006. Seed treatments to overcome salt and drought stress during germination in Sunflower (*Helianthus annus* L.). Eur. J. Agron. 24:291-95.

Kearsey, M.J., and V. Hyne. 1994. QTL analysis: a simple marker regression approach. Theor. Appl. Genet. 89:698-702.

Khush, G.S. 1999. Green revolution: preparing for the 21st century. Genome 42:646-65.

Kim, H.K., E.J. van Oosterom, M. Dingkuhn, D. Luquet, and G.L. Hammer. 2010b. Regulation of tillering in sorghum: environmental effects. Ann. Bot. 106:57-67.

Kim, M.Y., S. Lee, K. Van, T.H. Kim, S.C. Jeong, I.Y. Choi, D.S. Kim, Y.S. Lee, et al., 2010a . Whole-genome sequencing and intensive analysis of the undomesticated soybean (*Glycine soja* Sieb. and Zucc.) genome. Proc. Natl. Acad. Sci. 107: 22032-22037.

Knight, H., and M. Knight. 2001. Abiotic stress signaling pathways: specificity and cross-talk. Trends Plant Sci. 6:262-267.

Knipling, E.B. 1970. Physical and physiological basis for the reflectance of visible and near-infrared radiation from vegetation. Remote Sens. Environ. 1(3):155-159.

Koester R.P., J.A. Skoneczka, T.R. Cary, B.W Diers, and E.A. Ainsworth. 2014. Historical gains in soybean (*Glycine max* Merr.) seed yield are driven by linear increases in light interception, energy conversion, and partitioning efficiencies. J. Exp. Bot. 65:3311-3321.

Kohl, D.H., and G.B. Shearer. 1981. The use of soils lightly enriched in ^{15}N to screen for N_2-fixing activity. Plant Soil 60:487-489.

Kulkarni, M., R. Soolanayakanahally; S. Ogawa, Y. Uga, M.G. Selvaraj, and S. Kagale. 2017. Drought response in wheat: key genes and regulatory mechanisms controlling root system architecture and transpiration efficiency. Front. Chem. 5:106.

Lafitte, H.R., and B. Courtois. 2002. Interpreting cultivar × environment interactions for yield in upland rice. Assigning value to drought adaptive traits. Crop Sci. 42:1409-1420.

Lam, H.-M., X. Xu, X. Liu, W.B. Chen, G.H. Yang, F.L. Wong, M.W. Li, W.M. He, N. Qin, B. Wang, J. Li, M. Jian, J.A. Wang, G. Shao, J. Wang, S.S.M. Sun, and G.Y. Zhang. 2010. Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. Nature Genet. 42(12):1053-1059.

Lawlor, D.W., and G. Cornic. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ. 25:275-294.

Letolle, R. 1980. Nitrogen-15 in the natural environment. In: Handbook of Environmental Isotope Geochemistry. Eds: Fritz, P., and J.C. Fontes. Elsevier, Amsterdam, pp 407-433.

Li, Y., R. Guan, Z. Liu, Y. Ma, L. Wang, L. Li, F. Lin, W. Luan, P. Chen, Z. Yan, Y. Guan, L. Zhu, X. Ning, M.J.M. Smulders, W. Li, R. Piao, Y. Cui, Z. Yu, M. Guan, R. Chang, A. Hou, A. Shi, B. Zhang, S. Zhu, and L. Qiu. 2008. Genetic structure and diversity of cultivated soybean (*Glycine max* (L.) Merr.) land races in China. Theor. Appl. Genet. 117:857-871.

Liu, H., G. Zou, G. Liu, S. Hu, M. Li, X. Yu, H. Mei, and L. Luo. 2005. Correlation analysis and QTL identification for canopy temperature, leaf water potential and spikelet fertility in rice under contrasting moisture regimes. Chin. Sci. Bull. 50:317-326.

Ludlow, M.M., and R.C. Muchow. 1990. A critical evaluation of traits for improving crop yields in water-limited environments. Adv. Agron. 43:107-153.

Manavalan, L.P., S.K. Guttikonda, L.P. Tran, and H.T. Nguyen. 2009. Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol. 50:1260-1276.

Márquez-García, B., D. Shaw, J.W. Cooper, B. Karpinska, M.D. Quain, E.M. Makgopa, K. Kunert, and C.H. Foyer. 2015 Redox markers for drought-induced nodule senescence, a process occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine max*). Ann. Bot. 116(4):497-510.

Martínez J., G. Egea, J. Agüera, and M. Pérez-Ruiz. 2016. A cost-effective canopy temperature measurement system for precision agriculture: a case study on sugar beet. PRECIS. AGRIC. 18 (1):95-110.

Mastrodomenico, A.T., and L.C. Purcell. 2012. Soybean nitrogen fixation and nitrogen remobilization during reproductive development. Crop Sci. 52:1281-1289.

McCouch, S.R., and R.W. Doerge. 1995. QTL mapping in rice. Trends Genet. 11:482-487.

McWilliam, J. 1989. The dimensions of drought. In: Drought Resistance in Cereals. Ed: Baker, F. CAB International, Wallingford, UK. pp1-11.

McWilliams, D.A., D.R. Berglund, and G.J. Endres. 1999. Soybean Growth and Management Quick Guide. NDSU Extension Service. North Dakota State University, Fargo, North Dakota 58105.

Merlot, S., A-C. Mustilli, B. Genty, H. North, V. Lefebvre, B. Sotta, A. Vavasseur, and J. Giraudat. 2002. Use of infrared thermal imaging to isolate Arabidopsis mutants defective in stomatal regulation. Plant J. 30:601-609.

Mian, M.A.R., D.A. Ashley, and H.R. Boerma. 1998. An additional QTL for water use efficiency in soybean. Crop Sci. 38:390-393.

Mian, M.A.R., M.A. Mailey, D.A. Ashley, R. Wells, T.E. Carter, W.A. Parrot, and H.B. Boerma. 1996. Molecular markers associated with water use efficiency and leaf ash in soybean. Crop Sci. 36:1252-1257

Mohammadi, V., M.R. Qannadha, A.A. Zali, and B. Yazdi-Saamadi. 2004. Effects of post anthesis heat stress on head traits of wheat. Interl. J. Agri. Biol. 6:42-44.

Monclus, R., E. Dreyer, M. Villar, F.M. Delmotte, D. Delay, J-M. Petit, C. Barbaroux, D. Le Thiec, C. Bréchet, and F. Brignolas. 2006. Impact of drought on productivity and water use efficiency in 29 genotypes of *Populus deltoides* × *Populus nigra*. New Phytol. 169:765-777.

Monteros, M.J., G. Lee, A.M. Missaoui, T.E. Carter, and H.R. Boerma. 2006. Identification and confirmation of QTL conditioning drought tolerance in Nepalese soybean. The 11th Biennial Conference on the Molecular and Cellular Biology of the Soybean, Abstract PI471938. August 5-8, Lincoln, NE.

Nezhadahmadi, A., Z.H. Prodhan, and G. Faruq. 2013. Drought Tolerance in Wheat. Sci. World J. http://dx.doi.org/10.1155/2013/610721.

Nguyen, H.T., R.C. Babu, and A. Blum. 1997. Breeding for drought resistance in rice: physiology and molecular genetics considerations. Crop Sci. 37:1426-1434.

Olivares-Villegas, J.J., P.R. Matthew, and K.M. Glenn. 2007. Drought-adaptive attributes in the Seri/Babax hexaploid wheat population. Functional Plant Biology. 34(3):189-203.

O'Shaughnessy, S.A., S.R. Evett, P.D. Colaizzi, and T.A. Howell. 2011. Using radiation thermometry to evaluate crop water stress in soybean and cotton. Agric. Water Management. 98:1523-1535.

Passioura, J.B. 1996. Drought and drought tolerance. Plant. Growth. Regul. 20:79-83.

Pathan, M.S., J.D. Lee, J.G. Shannon, and H.T. Nguyen. 2007. Recent advances in breeding for drought and salt stress tolerance in soybean. In. Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops. Ed: Jenks, M.A., P.M. Hasegawa, and S.M. Jain. pp. 739-773. Springer, New York.

Paterson, A.H. 1996. Making genetic maps. In: Genome Mapping in Plants. Ed: Paterson, A.H. Pp. 23-39. R.G. Landes Company, San Diego, California; Academic Press, Austin, Texas.

Peoples. M.B., A.W. Faizah, B. Rerkasem, and D.F. Herridge. 1989. Methods for evaluating nitrogen fixation by nodulated legumes in the field. ACIAR Monograph No. 11, pp 76.

Pimentel D., and T. Patzek. 2008. Ethanol Production Using Corn, Switchgrass and Wood; Biodiesel Production Using Soybean. In: Biofuels, Solar and Wind as Renewable Energy Systems. Ed: Pimentel, D. Springer, Dordrecht.

Poland, J., J. Endelman, J. Dawson, J. Rutkoski, S. Wu, Y. Manes, S. Dreisigacker, J. Crossa, H. Sánchez-Villeda, M. Sorrells, J.-L. Jannink. 2012. Genomic selection in wheat breeding using genotyping- by-sequencing. Plant Genome 5:103-113.

Polania, J.A., C. Poschenrieder, S. Beebe, and I.M. Rao. 2016. Effective use of water and increased dry matter partitioned to grain contribute to yield of common bean improved for drought resistance. Front. Plant Sci. 7:660.

Prasad, P.V.V., S.R. Pisipati, Z. Ristic, U. Bukovnik, and A.K. Fritz. 2008. Impact of night time temperature on physiology and growth of spring wheat. Crop Sci. 48:2372-80.

Purcell, L.C. 2009. Physiological responses of N_2 fixation to drought and selecting genotypes for improved N_2 fixation. In: Nitrogen Fixation in Crop Production. Eds: Krishnan, H.B. and D.W. Emerich. Agronomy Monograph. 52. ASA CSSA SSSA, Madison, WI. pp. 211-238.

Purcell, L.C., M. DeSilva, C.A. King, and W.H. Kim. 1997. Biomass accumulation and allocation in soybean associated with genotypic differences in tolerance of nitrogen fixation to water deficits. Plant Soil 196:101-113.

Quisenberry, J.E., and B.L. McMichael. 1991. Genetic variation among cotton germplasm for water-use efficiency. Environ. Exp. Bot. 31:433-60.

Rafalski, J.A. 2010. Association genetics in crop improvement. Curr. Opin. Plant Biol. 13:174-180.

Rebetzke, G.J., A.R. Rattey, G.D. Farquhar, R.A. Richards, and A.G. Condon. 2013. Genomic regions for canopy temperature and their genetic association with stomatal conductance and grain yield in wheat. Funct. Plant Biol. 40:14-33.

Reddy, A.R., K.V. Chaitanya, and M. Vivekanandan. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Plant Physiol.161:1189-1202.

Reynolds, M.P., and R. Tuberosa. 2008. Translational research impacting on crop productivity in drought-prone environments. Curr. Opin. Plant Biol. 11:171-179.

Ribaut, J.M., C. Jiang, D. Gonzalez-de- Leon, G.O. Edmeades, and D.A. Hoisington. 1997. Identification of quantitative trait loci under drought conditions in tropical maize. 2. Yield components and marker assisted selection strategies. Theor. Appl. Genet. 94:887-896.

Ribaut, l. M., and D.A. Hoisington. 1998. Marker assisted selection: New tools and strategies. Trends Plant Sci. 3:236-239.

Richards, R., G. Rebetzke, A. Condon, and A. van Herwaarden. 2002. Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. Crop Sci. 42: 111-121.

Sadeghipour, O., and S. Abbasi. 2012. Soybean response to drought and seed inoculation. World Appl. Sci. J. 17:55-60

Sairam, R.K., D.S. Siiukla, and D.C. Saxena. 1997. Stress induced injury and antioxidant enzymes in relation to drought tolerance in wheat genotypes. Biol. Plant. 40(3):357-364.

Salado-Navarro, L. R., Sinclair, T. R., and Hinson, K. 1993. Changes in yield and seed growth traits in soybean cultivars released in the Southern USA from 1945 to 1983. Crop Sci. 33:1204-1209.

Santesteban, L.G., S.F. Di Gennaro, A. Herrero-Langreo, C. Miranda, J.B. Royo, and A. Matese. 2017. High-resolution UAV-based thermal imaging to estimate the instantaneous and seasonal variability of plant water status within a vineyard. Agric. Water Manage. 183:49-59.

Saranga, Y., I. Flash, A.H. Paterson, and D. Yakir. 1999. Carbon isotope ratio in cotton varies with growth stage and plant organ. Plant Sci. 142:47-56.

Schmutz, J., S.B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D.L. Hyten, Q. Song, J.J. Thelen, J. Cheng, D. Xu, U. Hellsten, G.D. May, Y. Yu, T. Sakurai, T. Umezawa, M.K. Bhattacharyya, D. Sandhu, B. Valliyodan, E. Lindquist, M. Peto, D. Grant, S. Shu, D. Goodstein, K. Barry, M. Futrell-Griggs, B. Abernathy, J. Du, Z. Tian, L. Zhu, N. Gill, T. Joshi, M. Libault, A. Sethuraman, X.C. Zhang, K. Shinozaki, H.T. Nguyen, R.A. Wing, P. Cregan, J. Specht, J. Grimwood, D. Rokhsar, G. Stacey, R.C. Shoemaker, and S.A. Jackson. 2010. Genome sequence of the palaeopolyploid soybean. Nature 463:178-183.

Serraj, R., T.R. Sinclair, and L.C. Purcell. 1999a. Symbiotic N_2 fixation response to drought. J. Exp. Bot. 50:143-155.

Serraj, R., V. Vadez, R.F. Denison, and T.R. Sinclair. 1999b. Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiol. 119:289-296.

Serraj, R., and T.R. Sinclair. 1997. Variation among soybean cultivars in nitrogen fixation response to drought. Agron. J. 89:963-969.

Schachtman, D.P., and J.Q. Goodger. 2008. Chemical root to shoot signaling under drought. Trends Plant Sci. 13:281-287.

Shearer, G., and D. Kohl. 1986. N_2 fixation in field settings: estimations based on natural ^{15}N abundance. Funct. Plant Biol. 13:699-756.

Shinozaki, K., K. Yamaguchi-Shinozaki. 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr. Opin Plant Biol. 3:217-223.

Shinozaki, K., K. Yamaguchi-Shinozaki, and M. Seki. 2003. Regulatory network of gene expression in the drought and cold stress responses. Curr. Opin. Plant Biol. 6:410-417.

Sinclair, T.R. 1986. Water and nitrogen limitations in soybean grain production. I. Model development. Field Crops Research 15:125-145.

Sinclair, T.R., and R. Serraj. 1995. Legume nitrogen fixation and drought. Nature 378:344.

Sirault, X.R., N. Fettell, A.G. Condon, and G.J. Rebetzke. 2004. Does leaf rolling slow water use and maintain wheat leaf area in a terminal drought? Eds: Black, C.K., J.F. Panozzo, and G.F. Rebetzke. Proc 54 Australian Cereal Chemistry Conference and 11th Wheat Breeders Assembly, Canberra. pp 52-55.

Slafer, G.A., J.L. Araus, C. Royo, and L.F. Garcia Del Moral. 2005. Promising ecophysiological traits for genetic improvement of cereal yields in Mediterranean environments. Ann. Appl. Biol. 146:61-70.

Sloane, R.J., R.P. Patterson, and T.E. Carter Jr. 1990. Field drought tolerance of a soybean plant introduction. Crop Sci. 30:118-123.

Smith, S., and I. De Smet. 2012. Root system architecture: insights from Arabidopsis and cereal crops. Philos. Trans. R. Soc. Lond. B Biol. Sci. 367:1441-1452.

Song. Q.J., L.F. Marek, R.C. Shoemaker, K.G. Lark, V.C. Concibido. X. Delannay, J.E Specht, and P.B. Cregan. 2004. Anew integrated genetic linkage map of the soybean. Theor. Appl. Genet. 109(1):122-128.

Song, Q., D.L. Hyten, G. Jia, C.V. Quigley, E.W. Fickus, R.L. Nelson, and P.B. Cregan. 2013. Development and evaluation of SoySNP50K, a high-density genotyping array for soybean. PLoS ONE 8(1):e54985.

Specht, J.E., K. Chase, M. Macrander, G.L. Graef, J. Chung, J.P. Markwell, M. Germann, J.H. Orf, and K.G. Lark. 2001. Soybean response to water: A QTL analysis of drought tolerance. Crop Sci. 41:493-509.

Specht, J.E., D.J. Hume, and S.V. Kumudnini. 1999. Soybean yield potential-a genetic and physiological perspective. Crop Sci. 39:1560-1570.

Steketee, C.J., T.R. Sinclair, M. Riar, W.T. Schapaugh, and Z. Li. 2019. Unraveling the genetic architecture for carbon and nitrogen related traits and leaf hydraulic conductance in soybean using genome-wide association analyses. BMC Genomics 20:811.

Steketee, C.J., W.T. Schapaugh, T.E. Carter Jr, and Z. Li. 2020. Genome-wide association analyses reveal genomic regions controlling canopy wilting in soybean. G3 10:1413-1425.

Strodtman, K.N., and D.W. Emerich. 2009. Nodule metabolism. In: Nitrogen fixation in crop production. Eds: Emerich, D.W., and H.B. Krishnan. Crop Science Society of America, Madison, pp 95-124.

Subbarao, G.V., C. Johansen, A.E. Slinkard, R.C.N. Rao, N.P. Saxena, and Y.S. Chauhan. 1995. Strategies and scope for improving drought resistance in grain legumes, Crit. Rev. Plant Sci. 14:469-523.

Sueedipour, S., and F. Moradi. 2011. Effects of drought at the post-anthesis stage on remobilization of carbon reserves and some physiological changes in the flag leaf of two wheat cultivars differing in drought resistance. J. Agric. Sci. 3:81-92.

Tanksley, S.D. 1993. Mapping polygenes. Annu. Rev. Genet. 27:205-233.

Tanner, C.B. 1963. Plant temperatures. Agron. J. 55:210-211.

Teulat, B., O. Merah, X. Sirault, C. Borries, R. Waugh, and D. This. 2002. QTLs for grain carbon isotope discrimination in field grown barley. Theor. Appl. Genet. 106:118-126.

Tuberosa, R., B.S. Gill, and S.A. Quarrie. 2002. Cereal genomics: ushering in a brave new world. Plant Mol. Biol. 48:445-449.

Turner, N.C., G.C. Wright, and K.H.M. Siddique. 2001. Adaptation of grain legumes (pulses) to water-limited environments. Adv. Agron. 71:193-231.

USDA. 2013. Soybean-irrigated, crop acreage, yield, and production, by county. 2012-2013. United States Department of Agriculture, National Agricultural Statistics Service, Delta Regional Office: Arkansas. http://www.nass.usda.gov/Statistics_by_State/Arkansas/Publications/County_Estimates/13_AR_soybean_irrigated.pdf (accessed 17 Sep. 2015).

USDA. 2014. Soybean-non irrigated, crop acreage, yield, and production, by county. 2013-2014. United States Department of Agriculture, National Agricultural Statistics Service, Delta Regional Office: Arkansas. http://www.nass.usda.gov/Statistics_by_State/Arkansas/Publications/County Estimates/14_AR_soybean_nonirrigated.pdf (accessed 17 Sep. 2015)

Van den Boogaard, R., D. Alewijnse, E.J. Veneklaas, and H. Lambers. 1997. Growth and water-use efficiency of 10 *Triticum aestivum* cultivars at different water availability in relation to allocation of biomass. Plant Cell Environ. 20:200-210.

Verma, V., P. Ravindran, and P.P. Kumar. 2016. Plant hormone mediated regulation of stress responses. BMC Plant Biol. 16:86.

Wright, G. 1996. Review of ACIAR selection for water use efficiency in legumes project recommends further research. ACIAR Food Legume Newslett. 2-3.

Wright, G.C., R.C. Nageswara, and G.D. Farquhar. 1994. Water-use efficiency and carbon isotope discrimination in peanut under water deficit conditions. Crop Sci. 34:92-97.

Xu, Y., D. This, R.C. Pausch, W.M. Vonhof, J.R. Coburn, J.P. Comstock, and S.R. McCouch. 2009. Leaf-level water use efficiency determined by carbon isotope discrimination in rice

seedlings: genetic variation associated with population structure and QTL mapping. Theor. Appl. Genet. 118:1065-1081.

Zhang, J., H.G. Zheng, A. Aarti, G. Pantuwan, T.T. Nguyen, J.N. Tripathy, A.K. Sarial, S. Robin, R.C. Babu, B.D. Nguyen, S. Sarkarung, A. Blum, and H.T. Nguyen. 2001. Locating genomic regions associated with components of drought resistance in rice: comparative mapping within and across species. Theor. Appl. Genet. 103(1):19-29.

Zhang, Z., J. Bian, W. Han, Q. Fu, S. Chen, and T. Cui. 2018. Cotton moisture stress diagnosis based on canopy temperature characteristics calculated from UAV thermal infrared image. Nongye Gongcheng Xuebao/Tran. Chin. Soc. Agri. Eng. 34(15):77-84.

Zia, S., K. Sophrer, W. Du, W. Spreer, G. Romano, H. Xiongkui, and J. Müller. 2011. Monitoring physiological responses to water stress in two maize varieties by infrared thermography. Int. J. Agric. Biol. Eng. 4(3):7-15

CHAPTER II

Identification of Quantitative Trait Loci for Carbon Isotope Ratio ($\delta^{13}C$) in a Recombinant Inbred Population of Soybean

Abstract

Drought is a major limitation to soybean yield, and the frequency of drought stress is likely to increase under future climatic scenarios. Water use efficiency (WUE) is associated with drought tolerance, and carbon isotope ratio ($\delta^{13}C$) is positively correlated with WUE. In this study, 196 F_6-derived recombinant inbred lines from a cross of PI 416997 (high WUE) × PI 567201D (low WUE) were evaluated in four environments to identify genomic regions associated with $\delta^{13}C$. There were positive correlations of $\delta^{13}C$ values between different environments ($0.67 \leq r \leq 0.78$). Genotype, environment, and genotype × environment interactions had significant effects on $\delta^{13}C$. Narrow sense heritability of $\delta^{13}C$ was 90% when estimated across environments. There was a total of 16 QTLs on seven chromosomes with individual QTLs explaining between 2.5 to 29.9% of the phenotypic variation and with additive effects ranging from 0.07 to 0.22‰. These 16 QTLs likely identified eight loci based on their overlapping confidence intervals. Of these eight loci, two loci on chromosome 20 (Gm20) were detected in at least three environments and were considered as stable QTLs. Additive QTLs on Gm20 showed epistatic interactions with 10 QTLs present across nine chromosomes. Five QTLs were identified across environments and showed significant QTL × environment interactions. These findings demonstrate that additive QTLs and QTL × QTL interactions play significant roles in genetic control of the $\delta^{13}C$ trait. Markers flanking identified QTLs may facilitate marker assisted selection to accumulate desirable QTLs to improve WUE and drought tolerance in soybean.

Introduction

Water deficit is a major abiotic stress that limits soybean (*Glycine max* (L.) Merr.) production and yield in many regions of world. The demand for agricultural water is expected to increase in the future due to predicted climatic change that may result in the occurrence of various crop stresses (Knox et al. 2018). Development of cultivars with increased drought tolerance may reduce irrigation requirements and help to improve crop performance under water deficit conditions (Polania et al. 2016). Under drought conditions, direct selection for yield is difficult due to low heritability and significant interaction of genotypes with the environment (Ceccarelli et al. 1991). A large number of morpho-physiological traits have been proposed as indirect selection criteria for genetic improvement of drought tolerance (Ludlow and Muchow 1990; Purcell and Specht 2004). Among these, water use efficiency (WUE) (defined as the ratio of biomass production to water transpired) was proposed as a genetic selection tool for improving adaptation in drought-prone environments.

Although WUE is recognized as an important trait for drought-prone environments, phenotyping for WUE at the field level is time-consuming, difficult, and expensive (Chen et al. 2011). Carbon isotope ratio ($\delta^{13}C$), through its positive relationship with WUE, has been proposed as a selection criterion for improved WUE (Farquhar and Richards 1984). In C_3 plants, differences in $\delta^{13}C$ among genotypes are due to the variation in the intercellular CO_2 concentration (C_i) in the leaves that is regulated by carboxylation capacity of the enzyme Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and by stomatal conductance (Brugnoli and Farquhar 2000; Farquhar et al. 1989). Therefore, $\delta^{13}C$ is negatively correlated with the ratio of intercellular to atmospheric CO_2 concentrations (Ci/Ca) and positively related with WUE (Farquhar et al. 1982).

In C_4 plants, the relationship between $\delta^{13}C$ and WUE is more complicated as carbon isotope discrimination occurs at both carboxylation phases (in mesophyll and bundle sheath cells); therefore, $\delta^{13}C$ in C_4 plants depends on the ratio of intercellular to atmospheric CO_2 concentrations and CO_2 leaking from the bundle sheath cells back to the mesophyll (Farquhar 1983). In contrast to C_3 plants, the correlation between $\delta^{13}C$ and Ci/Ca in C_4 plants is positive, negative or zero depending on extent of leakiness (Cernusak et al. 2013; Henderson et al. 1992; Hubick et al. 1990; Sandquist and Ehleringer 1995). Therefore, relationship between $\delta^{13}C$ and WUE in C_4 plants is not as strong as in C_3 plants.

Selection for high $\delta^{13}C$ may provide a useful method for indirect selection of high WUE due to its substantial genetic variance, high heritability, and small genotype by environment interaction (G×E) in water-limited environments (Hall et al. 1990; Kaler et al. 2017, 2018; Richards et al. 1999, Voltas et al. 1999). Several greenhouse and field experiments have shown genetic variation in $\delta^{13}C$ and close associations of both $\Delta^{13}C$ (carbon isotope discrimination; negatively correlated with WUE) or $\delta^{13}C$ with WUE in many crop species including barley (*Hordeum vulgare*) (Çağırgan et al. 2005), bread wheat (*Triticum aestivum*) (Condon et al. 1987; Ehdaie et al. 1991; Read et al. 1991), cotton (*Gossypium hirsutum*) (Brugnoli et al. 1988), cowpea (*Vigna unguiculata*) (Hall et al. 1990), durum wheat (*Triticum durum*) (Araus et al. 1998), maize (*Zea mays*) (Gresset et al. 2014; Monneveux et al. 2007; Twohey et al. 2019), peanut (*Arachis hypogaea*) (Hubick et al. 1986), sorghum (*Sorghum bicolor*) (Henderson et al. 1998; Hubick et al.1990), and soybean (White et al. 1996). These properties of $\delta^{13}C$ make it an attractive surrogate for WUE in research and breeding programs (Farquhar and Richards 1984; Xu et al. 2008).

Linkage mapping is a statistical procedure to identify the genomic regions or quantitative trait loci (QTLs) associated with a trait in a segregating population across multiple environments. In addition, QTL × environment interactions also affect the expression of identified QTLs and detection of QTLs that are stable across different environments (Campbell et al. 2003). In soybean, genomic regions associated with WUE have been identified using an F_4-derived population developed from a cross between Young (PI 508266) and PI 416937 (Mian et al. 1996) and from an F_2-derived population from a cross of S100 × Tokyo (Mian et al. 1998).

Several QTLs associated with $\delta^{13}C$ or $\Delta^{13}C$ have been identified in tomato (*Solanum lycopersicum*) (Martin et al. 1989), barley (Teulat et al. 2002), pasture legume (*Stylosanthes scabra*) (Thumma et al. 2001), rice (*Oryza sativa*) (Ishimaru et al. 2001; Price et al. 2002; Xu et al. 2009), cotton (*Gossypium* spp.) (Saranga et al. 2001), *Brassica oleracea* (Hall et al. 2005), maritime pine (*Pinus pinaster*) (Brendel et al. 2002), maize (Avramova et al. 2019; Gresset et al. 2014), wheat (*Triticum* spp.) (Peleg et al. 2009), and soybean (Bazzer et al. 2019; Dhanapal et al. 2015; Kaler et al. 2017; Specht et al. 2001). Genome wide association mapping studies on a panel of 373 diverse soybean accessions identified 39 (Dhanapal et al. 2015) and 54 SNPs (Kaler et al. 2017) associated with $\delta^{13}C$.

In this study, a high-density genetic map was developed for 196 F_6-derived recombinant inbred lines (RILs) developed from a cross between PI 416997 × PI 567201D. Plant introductions, PI 416997 and PI 567201D belong to maturity group 4 and were originally collected from Japan and Georgia, respectively (www.ars-grin.gov). PI 416997 was selected for high $\delta^{13}C$ while PI 567201D was selected for low $\delta^{13}C$ based on data from a multi-environment study conducted previously (Dhanapal et al. 2015) and because PI 416997 had higher genomic estimated breeding values for $\delta^{13}C$ than PI 567201D (Kaler et al. 2018). Our objectives in the

present research were to: (1) identify the genomic regions associated with $\delta^{13}C$ within and across environments; (2) confirm identified QTLs for $\delta^{13}C$ with the genomic regions identified in previous mapping studies; and (3) identify potential candidate genes associated with $\delta^{13}C$ underlying detected QTLs.

Materials and Methods

Field Experiments

A population of 196 F_6-derived RILs was developed from a cross between PI 416997 (high $\delta^{13}C$ and WUE) × PI 567201D (low $\delta^{13}C$ and WUE). Each of the 196 RILs was developed by single-plant descent from a single random F_2 plant and each F_2 plant produced only one RIL. Hence, this RIL population represents a population of random F_2 plants. Along with both parents, five check cultivars (AG4632, Dillon, Maverick, Osage, and Pella 86) were grown with the RILs at Stoneville, MS (33.42° N, 90.90° W) in 2016 and 2017, at Bradford Research Center near Columbia, MO (38.95° N, 92.33° W) in 2017, and at Main Arkansas Agricultural Research Center, Fayetteville, AR (36.05° N, 94.15° W) in 2017. At Stoneville, the soil was a Bosket very fine sandy loam (fine-loamy, mixed, active, thermic, Mollic Hapludalfs) in 2016 and a Dundee silty clay loam (Dundee fine-silty, mixed, active, thermic, Typic Endoaqualfs) in 2017. At Columbia, the soil was a Mexico silt loam (fine, smectitic, mesic Vertic Epiaqualf), and at Fayetteville, the soil was a Captina silt loam (fine-silty, siliceous, active, mesic Typic Fragiudult).

For both years at Stoneville, plots consisted of one row, 66 cm apart and 2.74 m in length. At Columbia, 3.05 m long single row plots were spaced 76 cm apart. At Fayetteville, plots consisted of two rows, 6 m in length with 45 cm spacing between rows. At each experimental site, treatments (genotypes) were analyzed as a randomized complete block design with two replications.

All seeds were treated with Apron Maxx RTA and Moly (Syngenta, Greenboro, NC) prior to planting at Stoneville and Columbia in 2017, and with IleVo (Bayer CropScience, Research Triangle Park, NC) and CruiserMaxx (Syngenta, Greenboro, NC) at Fayetteville.

Sowing occurred on 6 May 2016 and 16 May 2017 at Stoneville, 14 May 2017 at Columbia, and 10 June 2017 at Fayetteville. At Stoneville in both years, furrow irrigation was applied as needed, while experiments were conducted under rainfed conditions at Columbia. At Fayetteville, irrigation was applied with a sprinkler irrigation system as required. Insecticides and herbicides were applied as needed at all environments. Rainfall data were collected online from the Southern Regional Climate Center (https://www.srcc.lsu.edu/station_search) for Stoneville in 2016 and 2017, from Missouri Historical Agricultural Weather Database (http://agebb.missouri.edu/weather/history/index.asp?station_prefix=bfd) for Columbia in 2017, and from the University of Arkansas Weather Station for Fayetteville in 2017.

Phenotypic Evaluations

Between the R1 (beginning bloom) and R2 (full bloom) developmental stages (Fehr and Caviness 1977), the aboveground portion of four random plants from each plot was harvested on 29 June 2016 and 21 June 2017 (Stoneville), and 21 July 2017 (Columbia and Fayetteville). Plants were selected at this time to be consistent with the methodology of Dhanapal et al. (2015) and because small differences in maturity were assumed to have little or no physiologically impact relative to differences among genotypes that would be evident during seedfill. After drying at 60° C until weight was constant, samples were coarse ground to pass a 6 mm sieve using a Wiley Mill (Thomas Model 4 Wiley® Mill, Thomas Scientific, NJ USA). A subsample of the coarse-ground samples was finely-ground to pass a 1 mm sieve. About 0.4 to 0.5 g of the finely ground sample was transferred to a 15 mL tube (part # 2252-PC-30; SPEX CertiPrep, Inc., NJ USA) along with two 9.52 mm diameter stainless steel balls (440C Stainless Steel Ball, Tolerance/Grade: 100, Abbott Ball Company, Inc., CT USA) and ground to a fine powder using a Geno Grinder (SPEX CertiPrep, Inc., NJ USA) at 1,500 rpm for 10 min. About 3-5 mg of the

powdered sample was packed into tin capsules and arranged in 96-well plates (Costech Analytical Technologies Inc., CA USA). The $\delta^{13}C$ isotope analysis was conducted at the University of California-Davis Stable Isotope Facility (https://stableisotopefacility.ucdavis.edu/) using an elemental analyzer interfaced with a continuous flow isotope ratio mass spectrometer. Data from the stable isotope facility were received as $\delta^{13}C$ (‰) and were expressed relative to the international standard of the $^{13}C/^{12}C$ ratio of Vienna PeeDee Belemnite (V-PDB) as follows:

$$\delta^{13}C = \frac{R_{sample}}{(R_{std} - 1)} * 1000$$

where, R_{sample} and R_{std} are the isotope ratios of the sample and standard, respectively. Additional methodological information is available from the Stable Isotope facility website, http://stableisotopefacility.ucdavis.edu/13cand15n.html.

Statistical Analysis

Combinations of location and year were considered as individual environments and were designated as ST16, ST17, CO17, and FAY17 for Stoneville 2016, Stoneville 2017, Columbia 2017, and Fayetteville 2017, respectively. The data from each environment were subjected to descriptive statistics and Pearson correlation analysis using the PROC UNIVARIATE and PROC CORR procedures (α = 0.05) of SAS version 9.4 (SAS, Institute 2013), respectively. Overall analysis of variance (ANOVA) was performed using the PROC MIXED procedure (α = 0.05) of SAS version 9.4 (SAS Institute, 2013) by using the data collected from all environments. Genotype and environment were considered as fixed effects, and replication within environment was considered a random effect according to the model proposed by Bondari (2003).

$$Y_{ijk} = \mu + G_i + E_j + GE_{ij} + B_{jk} + \varepsilon_{ijk}$$

where, Y_{ijk} is phenotypic value of the i^{th} genotype in the j^{th} environment in the k^{th} replication, μ is the mean, G_i is the effect of the i^{th} genotype, E_j is the effect of the j^{th} environment, GE_{ij} is the

interaction of the i^{th} genotype with the j^{th} environment, B_{jk} is the effect of the k^{th} replication in the j^{th} environment and ε_{ijk} is the error effect. ANOVA was also performed for individual environments (ST16, ST17, CO17, and FAY17) to check the significant effect of genotypes (RILs).

The variance components were estimated using the PROC VARCOMP procedure of SAS 9.4 with the restricted maximum likelihood estimation (REML) method. Heritability (h^2) was calculated as follows (Holland et al. 2003):

Across environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{GE}^2}{E}\right) + \left(\frac{\sigma_e^2}{rE}\right)}$$

Within environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_e^2}{r}\right)}$$

where, σ_G^2 is the genotypic variance, σ_{GE}^2 is the genotype × environment interaction variance, σ_e^2 is the residual error variance, E is the number of environments, and r is the number of replications. Because RILs were F_6-derived, there was minimal heterozygosity within lines and σ_G^2 is therefore composed almost entirely of additive variance, with only negligible dominance variance and additive × dominance variance. Therefore, this heritability should be considered a narrow sense estimate. To minimize the effects of environmental variation, the best linear unbiased prediction (BLUP) values for each individual environment and across environments were estimated by using the PROC MIXED procedure. For calculation of BLUP values for individual environments, all factors were considered as random effects, and for calculation of BLUP values across environments, environment was considered a fixed effect, while genotypes and replications were considered as random effects (Littell et al. 1996; Piepho et al. 2008). QTL analysis was performed using BLUP values.

GBS Library Construction, Genotyping and SNP Calling

Leaf samples were collected from one replication of the field experiment conducted at Stoneville in 2017. Genomic DNA was isolated by using a Maxwell 16™ automated DNA isolation machine (Promega, Madison, WI 53711, USA) following the manufacturer's protocol for leaf tissue after being lyophilized in a Model 2400 freeze dryer (The Freeze Dry Company, Nisswa, MN 56468, USA). Genotype-By-Sequencing (GBS, Elshire et al. 2011) analysis was conducted by LGC Genomics GmbH (Berlin, Germany) following their established protocols (https://www.biosearchtech.com/) for normalized GBS (nGBS) with MsII and Illumina NextSeq (2×150 bp) sequencing. LGC Genomics bioinformaticists processed sequences for quality and aligned sequences against the "Williams 82" soybean reference genome assembly 1 (https://www.soybase.org/). Sequencing reads were separated for each sample using the in-line barcode sequences. The reads were processed to remove the barcode and adapter sequences. The Burrows-Wheeler Aligner (BWA v.0.7.12; http://bio-bwa.sourceforge.net/) software was used for alignment (Li and Durbin 2009), and SNPs were called using Freebayes v.1.92-16 (Garrison and Marth 2012).

Marker Quality Control

Markers that were monomorphic, had more than 15% missing data, that were heterozygous, and that did not follow a 1:1 segregation ratio (chi-square *P*-value $\leq$ 0.01) were removed, leaving 3,234 SNPs (3,221+13 scaffold) for further analysis. Missing marker data were imputed using a LD-kNNi method, which is based on a k-nearest neighbor-genotype (Money et al. 2015) implemented in TASSEL software (https://www.maizegenetics.net/tassel). The filtered and imputed 3,234 polymorphic SNPs were used for construction of the linkage map.

Construction of Linkage Map

Initially, 3,234 polymorphic SNPs were used for the construction of the linkage map. Binning of markers based on their segregation pattern resulted in the elimination of redundant and low-quality markers (approximately 766 SNPs). The final genetic map was constructed using 2,468 polymorphic markers with MAP functionality of QTL IciMapping v4.1 software (Meng et al. 2015). MAP functionality has three steps: grouping, ordering, and rippling. Grouping of binned markers was performed using logarithm of the odds (LOD) threshold values ≥ 4.5 and resulted in 23 linkage groups representing the 20 chromosomes of soybean. Linkage groups (LGs) were assigned to different chromosomes by using the genomic position of SNP markers determined during the SNP calling. Linkage groups belonging to the same chromosome were merged together, and LGs with less than five SNPs or unlinked LGs were not used in further genetic map construction. The RECORD (Recombination Counting and ORDering) algorithm was used to order 2,466 SNP markers within LGs over 20 chromosomes. Recombination frequencies between markers were converted into centiMorgans by using the Kosambi mapping function. To identify the final order of markers within each linkage group, fine tuning of marker order was performed by the sum of adjacent recombination fractions with a window size of 5 cM as a rippling criterion. Marker order was adjusted or reversed within a LG or rippled again to get the shortest linkage map length. Finally, a genetic map was constructed using Prism software (https://www.graphpad.com/) to show the marker density among chromosomes.

QTL Analysis

The BIP functionality of QTL IciMapping v4.1 software (http://www.isbreeding.net/) was used to conduct QTL analysis. Inclusive Composite Interval Mapping of Additive (ICIM-

ADD) and Epistatic QTL (ICIM-EPI) functions were used as mapping methods to detect putative additive QTLs and QTL × QTL interactions, respectively. The mapping parameters were 1.0 cM scanning steps and a probability of 0.01 in stepwise regression for additive QTL identification. Significant LOD thresholds to declare QTLs were determined by 1000 permutation tests using a Type I error set at $P < 0.05$. For the detection of epistasis, scanning steps of 5 cM, a probability of 0.0001 in stepwise regression, and a LOD threshold of 5.0 or more were used to declare significant epistasis. Identification of QTLs across environments and QTL × environment interactions were evaluated by using MET (Multi-Environment Trials) functionalities of IciMapping software by using individual values of $\delta^{13}C$ for each RIL from each environment. The whole genome was scanned with walking speed of 1.0 cM, a probability of 0.001 in stepwise regression, and a LOD threshold was calculated by 1000 permutations test using a Type I error set at $P < 0.05$ for claiming significant additive QTL × environment (AE) interactions. The MET functionality uses multi-environment data to identify QTL × environment interactions and detects additional QTLs that may not be identified in analysis by BIP functionality.

A QTL with a LOD score ≥ 3 and that explained phenotypic variation (R^2) of approximately 10% or more in two or more environments was considered a major QTL. A QTL detected in at least two of the four environments (ST16, ST17, CO17, and FAY17) was defined as a stable QTL (Cao et al. 2019; Li et al. 2015). Prism software (https://www.graphpad.com/) was used for the graphical representation of QTLs on the soybean chromosomes.

Identification of Putative Candidate Genes and their Functions

Glycine max genome sequence (Williams 82 assembly 1) in Soybase (https://www.soybase.org/) was scanned for potential genes present between markers flanking

identified QTLs in each environment. Only those genes that had a biological connection with abiotic stresses were considered putative candidate genes.

Results

Total cumulative rainfall was calculated from planting to sampling date for each individual environment (ST16, ST17, CO17, and FAY17) (Figure 2_1). ST16 and CO17 received more rainfall than ST17 and particularly FAY17. All environments except CO17 received irrigation as needed, but there were 17 days prior to plant sampling for CO17 when there was only a total of 6 mm of rainfall. This resulted in moderate water deficit for CO17 at the time of plant sampling whereas soil moisture at all other environments was considered adequate.

The $\delta^{13}C$ values among the RILs extended beyond that of both parents (transgressive segregation) in all environments except ST17 (Figure 2_2, Table 2_1), indicating that alleles with positive (favorable) and negative effects were derived from both parents. A favorable allele was defined as an allele that increased $\delta^{13}C$ values (increased WUE). Values of $\delta^{13}C$ had a range of 2.38‰ (ST16), 2.44‰ (ST17), 2.36‰ (CO17), and 2.85‰ (FAY17) with RIL means of -29.61‰ (ST16), -29.22‰ (ST17), -28.32‰ (CO17), and -28.40‰ (FAY17). A broad range of $\delta^{13}C$ indicated considerable phenotypic variation for this trait in all environments and its quantitative inheritance.

The parents, PI 416997 and PI 567201D, were significantly different for $\delta^{13}C$ in ST16 ($P \leq 0.10$), ST17 ($P \leq 0.05$), and CO17 ($P \leq 0.05$) as PI 416997 had greater $\delta^{13}C$ values (high WUE) compared to PI 567201D (low WUE). The $\delta^{13}C$ value of parents, PI 416997 and PI 567201D, was similar for FAY17 and parents were not significantly different. Among check cultivars (AG4632, Dillon, Maverick, Osage, and Pella 86), Pella 86 had low $\delta^{13}C$, close to the $\delta^{13}C$ value of PI 567201D (data not shown). Other checks (AG4632, Dillon, Maverick, and Osage) had moderate $\delta^{13}C$ values lying between the $\delta^{13}C$ of the parents.

A significant positive correlation ($P < 0.001$) was observed between all environments for $\delta^{13}C$ of RILs, ranging from 0.67 to 0.78 (Table 2_2). Analysis of variance (ANOVA) across environments showed that $\delta^{13}C$ was significantly affected by genotype (G), environment (E), and the interaction of genotype with environment (G × E) (Table 2_3). ANOVA performed by environment showed a significant effect ($P < 0.05$) of genotypes in all environments (Table 3). Narrow sense heritability of $\delta^{13}C$ on an entry-mean basis over all environments was 90% (Table 2_3). For individual environments, heritability was 89% (ST16), 64% (ST17), 80% (CO17), and 78% (FAY17).

Genetic Map Construction

The genetic map constructed using 2,466 polymorphic SNPs had a total length of 3,836 cM with an average marker density of 0.64 markers per cM. The number of markers per chromosome ranged from 38 on Gm12 to 321 on Gm18. Approximately 90% of the markers had a distance of 3 cM or less between adjacent markers. Several gaps > 20 cM between markers were present on different chromosomes with the largest gaps often appearing near the centromere (Figure 2_3) where recombination is less frequent. The presence of large gaps resulted in a larger linkage map size than previously reported maps for soybean (Abdel-Haleem et al. 2011; Charlson et al. 2009; Cui et al. 2008; Du et al. 2009; Hwang et al. 2013, 2015). Figure 2_3 shows the physical location of the SNPs on soybean chromosomes based on their genomic sequence location.

QTL Analysis

A total of 16 additive QTLs associated with $\delta^{13}C$ were identified in the four environments (ST16, ST17, CO17, and FAY17) using BIP functionality of ICIM mapping (Figure 2_3, Table 2_4). These QTLs were distributed on seven chromosomes (Gm06, Gm07, Gm10, Gm11, Gm15,

Gm17, and Gm20) with additive effects that ranged from 0.07 to 0.22‰ and individually explained 2.50 to 29.88% of the phenotypic variation. The additive effect is defined as one-half of the difference between the average effects of parental alleles (PI 416997 and PI 567201D). A positive additive effect indicates that the favorable allele (allele that increases the $\delta^{13}C$ value) was contributed by PI 416997, whereas a negative additive effect indicates that the favorable allele was contributed by PI 567201D. The additive effects (0.07 to -0.22‰) of identified QTLs were positive and negative, indicating that the favorable alleles were contributed by both parents. Of these 16 QTLs, eight QTLs were detected for ST16, two QTLs for ST17, five QTLs for CO17, and one QTL for FAY17. Due to their overlapping confidence intervals, these QTLs identified in different environments denoted eight putative loci associated with $\delta^{13}C$ distributed on seven chromosomes (Figure 2_3, Table 2_4). The average distance between the markers flanking a QTL was 0.9 MB and had a range of 0.04 MB to 3 MB.

Locus 7 (Gm20 at 108 cM, Table 2_4) was stable and consistently appeared in all environments, explaining 4.58 to 29.88% of the phenotypic variation and additive effects that ranged from -0.08 to -0.22‰. Locus 8 (Gm20 at 121-125 cM, Table 4) was observed in three environments (ST16, ST17, and CO17) and across environments (AE; Table 2_5). The favorable alleles for both these loci on Gm20 were from PI 567201D. Locus 1 (Gm06 at 61-67 cM, Table 2_4) was identified in ST16, CO17, and AE (56 cM, Table 2_5). Locus 3 (Gm10 at 94 cM) was identified in CO17 and AE (Tables 2_4 and 2_5). Similarly, Locus 6 (Gm17 at 96-102 cM) was identified in ST16, CO17, and AE (Tables 2_4 and 2_5). The favorable alleles for the Loci 1, 3, and 6 were from PI 416997, and the other loci derived favorable alleles from PI 567201D.

QTL × Environment Interaction for $\delta^{13}C$

MET functionality (Table 2_5) was used to identify QTL interactions with different environments. Across environments, QTL analysis by MET functionality identified a total of five QTLs that were present on Gm06 (56 cM), Gm10 (93 cM), Gm17 (97 cM), and two QTLs on Gm20 (108 and 122 cM) with additive effects that ranged from 0.05 to 0.13‰ and individually explained 3.80 to 23.89% of the phenotypic variation (Table 2_5). These QTLs on Gm06, Gm10, Gm17, and Gm20 were also identified as additive QTLs by BIP functionality, which were designated as Loci 1, 3, 6, 7, and 8, respectively (Figure 2_3, Table 2_4). The total phenotypic variation (PVE), phenotypic variation explained by additive effects (PVE (A)), and phenotypic variation explained by additive × environment effects (PVE (A × E)) along with their LOD scores are presented in Table 2_5. The QTLs present at Loci 1, 3, 6, and 7 showed relatively small QTL × environment interactions as indicated by the high LOD score for additive effects (LOD (A)) and low LOD score for the additive × environment effects (LOD (A×E)). In contrast, Locus 8 on Gm20 had a strong QTL × environment interaction as indicated by a low LOD score (4.08) for additive effects (LOD (A)) compared to the LOD score (4.63) for the additive × environment effects (LOD (A×E)). In addition, the phenotypic variation explained by additive effects (PVE (A)) for Loci 1, 3, 6, and 7 was considerably greater than the additive × environment effects (PVE (A × E)) and showed the stability for these four QTLs across environments.

QTL × QTL Interaction

The polygenic nature of $\delta^{13}C$ may result in identification of a large number of QTLs with smaller effects and epistasis between different loci. Numerous interactions were identified between loci for $\delta^{13}C$ in individual environments (ST16, ST17, CO17, and FAY17) (Table 2_6).

A QTL × QTL interaction with a threshold LOD score greater than 5.0 was used to declare a significant epistatic interaction (Cao et al. 2019; Li et al. 2015). Loci present on Gm02, Gm03, Gm04, Gm05, Gm06, Gm09, Gm11, Gm17, and Gm19 showed QTL × QTL interactions with the additive QTLs present on Gm20 (Loci 7 and 8, Table 2_4 and 2_6). Among all QTL × QTL interactions, one or more loci on Gm20 (between 42-45 million base pairs) were involved. QTLs on Gm03, Gm05, Gm06, Gm 09, and Gm19 had significant interactions in three or more environments with the identified additive QTLs on Gm20 located between 42 and 45 million base pairs. Epistatic loci present on Gm20 were also identified as additive QTLs (Loci 7 and 8, Table 2_4) but epistatic loci on other chromosomes were not identified as additive QTLs. The phenotypic variation explained by identified epistatic interactions ranged from 3 to 4%. The favorable alleles for identified QTL × QTL interactions were contributed by both parents.

Discussion

The broad range of phenotypic variation for $\delta^{13}C$ found in all environments indicates that $\delta^{13}C$ has quantitative inheritance and could be useful for QTL analysis. We found that the parental genotype PI 416997 had statistically greater $\delta^{13}C$ than PI 567201D in ST16, ST17, and CO17. Our results agree with previous experiments indicating that PI 416997 had higher $\delta^{13}C$ (high WUE) than PI 567201D (low WUE) based on the genomic estimated breeding values (GEBVs) (Dhanapal et al. 2015, Kaler et al. 2018).

The $\delta^{13}C$ of parents and RIL means were overall greater in CO17 as compared to other environments (ST16, ST17, and FAY17) (Table 1). The greater $\delta^{13}C$ for CO17 is consistent with this environment being the only rainfed environment and for which there were 17 days prior to sampling with a total of only 6mm of rainfall (Figure 2_1). Previous research has found that WUE increases under water deficit due to the partial stomatal closure (Bloch et al. 2006; Condon et al. 2002). Environmental factors such as temperature, radiation, vapor pressure deficit, and soil water availability affect WUE (Hopkins 1999) and results in the differential response of genotypes to environmental conditions. The greater total cumulative rainfall and constant irrigation application in ST16 compared to other environments was associated with low $\delta^{13}C$ values of parents and RILs in ST16.

Presence of significant positive correlations of $\delta^{13}C$ among different environments (Table 2_2) indicates the stability of $\delta^{13}C$ across environments although there were significant genotype and G × E interaction effects ($P < 0.001$) (Table 2_3). Kaler et al. (2018) found high repeatability (0.79 to 0.94) of $\delta^{13}C$ in soybean across environments. The heritability of $\delta^{13}C$ within environments ranged from 64 to 89%, which is moderate to high compared with other physiological traits associated with drought tolerance such as root score ($h^2 = 39\%$, Pantalone et

al. 1996), canopy wilting (h^2=60%, Abdel-Haleem et al. 2012; h^2 =46%, Charlson et al. 2009; h^2 =58-78%, Hwang et al. 2015), and shoot ureides (h^2 =73%, Hwang et al. 2013). Similarly, high heritability of $\delta^{13}C$ was reported in soybean (h^2= 85%, Bazzer et al. 2019; h^2 =80%, Specht et al. 2001). High heritability of $\delta^{13}C$ indicates strong genetic control and that the direct selection of genotypes based on $\delta^{13}C$ (indirect measure of WUE) for drought tolerance could be effective in soybean breeding.

Among the identified eight putative loci, three loci (Locus 1, 2, and 6; Table 2_4) derived the favorable allele from PI 416997, and five loci (Locus 3, 4, 5, 7, and 8) received the favorable alleles from PI 567201D (Table 2_4). The presence of alternative alleles at multiple loci inherited from both parents in the RILs resulted in transgressive segregants (Tanksley 1993). That is, specific RILs exceeded the parental values for both high and low $\delta^{13}C$, and these extreme lines may be further utilized in understanding the physiology of $\delta^{13}C$ and WUE.

Among these eight loci, Locus 7 was found in all environments and when averaged across environments (Tables 2_4 and 2_5). This locus explained approximately 10% of the phenotypic variation in three of environments and was considered a major and stable QTL. Locus 8 appeared in three environments and when averaged across environments. Locus 1 and Locus 6 appeared in two individual environments and when averaged across environments (Table 2_4 and 2_5). These loci (1, 6, and 8) were also considered stable QTLs, although they explained less phenotypic variation than Locus 7. The favorable alleles for these stable loci were from PI 416997 (Loci 1 and 6) and PI 567201D (Loci 7 and 8). The remaining Loci (Loci 2, 3, 4, and 5) were found in individual environments (Table 2_4). Inconsistent detection of QTLs among environments indicates that there may be differential expression of genes that are

environment specific (Campbell et al. 2003; Crossa et al. 1999; Veldboom and Lee 1996), resulting in QTL × environment interactions.

Across environments, QTL analysis identified five QTLs (on Gm06 at 56 cM, Gm10 at 93 cM, Gm17 at 97 cM, and Gm20 at 108 and 122 cM) with QTL × environment interactions (Table 2_5). Although all interactions were significant, the additive × environment effect was comparatively small as indicated by their low LOD (A×E) score. Less phenotypic variation was explained by these interactions except for the QTL on Gm20 at 122 cM, which had high QTL × environment interaction (PVE (A × E) among all identified interactions. Low QTL × environment interaction of most of the QTLs across environments indicates that these QTLs were stable across environments, and that selection will be effective for genotypes with high $\delta^{13}C$ (or WUE). These QTLs also had additive QTL effects in specific environments. The QTL with environment interactions located on Gm20 at 108 cM explained the highest phenotypic variation (23.89%) and had a high LOD score (Table 2_5); this QTL was also identified as a major and stable additive QTL (Table 2_4). Loci 7 and 8 on Gm20 had more additive effects and explained high phenotypic variation in individual environments and across environment compared to all other identified loci; the favorable alleles for these loci were from PI 567201D. It is noteworthy that the low WUE parent (PI 567201D) contributed favorable alleles on Gm20, increasing $\delta^{13}C$ for specific RILs.

Besides the additive main effects and G × E interactions, QTL × QTL interactions between different genomic regions were detected, indicating the complex nature of $\delta^{13}C$. Epistasis between QTLs contributes significantly to the genetic variance of many agronomic traits (Ma et al. 2005; Rebetzke et al. 2007; Reif et al. 2011; Zhang et al. 2008). Epistasis was found between the additive QTLs on Gm20 with the genomic regions that were not identified as

additive QTLs. It is possible that two QTLs without additive effects can result in significant epistasis (Carlborg and Haley 2004). An advantage of ICIM mapping is that it can identify the epistasis between QTLs regardless of whether those QTLs have any additive effects. We used conservative criteria to determine epistasis (LOD score > 5.0 and R^2 > 0.03). One of the QTLs involved in the most QTL × QTL interactions was present on Gm20 at 105-125 cM (Table 2_6). It may be that the genomic regions on Gm20 play an important role in controlling $\delta^{13}C$. The presence of genetic effects of additive and additive × additive epistasis plays a significant role in controlling complex traits (Jiang et al. 2011; Zhao et al. 2005), and also appears to be the case for $\delta^{13}C$.

Co-localization of QTL for $\delta^{13}C$ with QTLs for other Agronomic Traits

Based on the physical positions of the SNP markers, the QTLs identified in the present study were compared using Soybase (http://www.soybase.org/) with QTLs for other traits mapped in previous studies. Locus 1 (Gm06), Locus 2 (Gm07), and Locus 5 (Gm15) were previously identified as associated with $\delta^{13}C$ in genome wide association studies (GWAS) using 373 diverse soybean accessions (Dhanapal et al. 2015; Kaler et al. 2017). Locus 3 (Gm10) coincided with the genomic regions previously reported for aluminum tolerance (Korir et al. 2011), low leaf hydraulic conductance (Carpentieri-Pipolo et al. 2011), and flood tolerance (Githiri et al. 2006) QTLs. Presence of Al^{3+} in soil limits root growth and the absorption of water and nutrients from soil, ultimately decreasing soybean yield (Foy 1984; Ma and Furukawa 2003). Low leaf hydraulic conductance increases transpiration efficiency during drought stress (Sadok and Sinclair 2010). A QTL associated with WUE (Kumar and Lal 2015) was also identified in the same genomic region of Locus 3. The presence of low hydraulic conductance QTLs and WUE in the same genomic regions indicates a potential pleiotropic effect. The co-localization of

genomic regions associated with several stress tolerances indicates that this genomic region may have multiple genes that undergo differential gene expression under stress.

Locus 6 (Gm17) overlapped with the QTLs associated with canopy wilting that were detected in multiple mapping populations (Abdel-Haleem et al. 2012; Charlson et al. 2009; Hwang et al. 2015, 2016). Likewise, Locus 6 overlapped with a QTL for drought index (Du et al. 2009) (measure of yield under drought stress) along with canopy wilting QTL. This $\delta^{13}C$ locus also co-localized with a significant SNP associated with $\delta^{13}C$ and oxygen isotope ratio ($\delta^{18}O$) identified by GWAS of diverse soybean accessions (Kaler et al. 2017). Transpiration and stomatal conductance are closely associated with $\delta^{18}O$ (Bindumadhava et al. 1999; Dhanapal et al. 2015; Kaler et al. 2017; Sheshshayee et al. 2005).

The loci on Gm20 (Loci 7 and 8) identified in the present study coincide with genomic regions associated with $\delta^{13}C$ QTLs identified by GWAS (Kaler et al. 2017). These loci on Gm20 also overlapped with $\delta^{13}C$ QTLs identified in a bi-parental population developed from KS4895 and Jackson (Bazzer et al. 2019). A QTL associated with WUE (Kumar and Lal 2015) and drought index (Du et al. 2009) were identified in this same genomic region on Gm20. Co-localization of QTLs associated with different traits in the same genomic interval may be due to the presence of different genes controlling those traits or those traits may be directly or indirectly co-related (Prioul et al. 1997). The major stable QTL identified on Gm20 (108 cM) in this study may be valuable in marker-assisted selection (MAS) to improve drought tolerance in soybean and in confirmation of QTLs identified by GWAS.

Candidate genes were identified that underlie the position of identified QTLs that may directly or indirectly be related to physiological mechanisms associated with drought tolerance. For example, the gene AP2-1 (*Glyma.06g08990*) encodes a dehydration responsive protein, and

gene BT093886.1 (*Glyma.06g08910*) regulates the aquaporin protein function underlying Locus 1 (Gm06). Further, the gene BT097722.1 (*Glyma.17g13350*) on Gm17 (Locus 6) is involved in production of dehydration induced proteins, and BT098823.1 (*Glyma.06g14000*) on Gm06 (Locus 1) produces a heat shock protein that is up regulated during stress conditions and helps maintain membrane stability. The presence of potential candidate genes in identified QTL regions may support the association of the identified QTLs to WUE or $\delta^{13}C$ and help in validating the accuracy of the QTL identification. Further studies are needed for fine resolution of the identified QTLs and use of flanking markers of QTLs for marker assisted selection.

Conclusions

A high-density genetic map of 196 recombinant inbred lines was used to dissect the inheritance of $\delta^{13}C$ in soybean. A total of 16 additive QTLs denoting eight putative loci were identified in four environments. Of these, two loci on Gm20 at 108 cM and 121-125 cM were stable across different environments. The favorable alleles for both of these QTLs were from PI 567201D, the parent with low $\delta^{13}C$ (low WUE). Out of eight putative loci, three loci inherited their favorable alleles from PI 416997. Several QTLs were detected in individual environments indicating the presence of QTL × environment interactions. Detection of additive QTLs, QTL × environment interactions, and QTL × QTL interactions indicated the complex inheritance of the $\delta^{13}C$ trait. Moderate-to-high narrow sense heritability estimates indicate that progress can be made through traditional plant breeding for increasing WUE in future cultivars. Additionally, identified QTLs may be useful in MAS. Combined traditional and MAS approaches will ensure more rapid progress. The findings from this study provide useful information on the genetic basis of WUE and may be helpful in the genetic improvement of yield potential in drought-prone environments.

Table 2_1. Phenotypic data of $\delta^{13}C$ (‰) for parents (PI 416997 and PI 567201D) and recombinant inbred lines (RILs) evaluated at Stoneville, MS in 2016 (ST16) and in 2017 (ST17), Columbia, MO in 2017 (CO17), and Fayetteville, AR in 2017 (FAY17).

Descriptive statistics	ST16	ST17	CO17	FAY17
PI 416997	-29.24	-28.39	-27.81	-27.94
PI 567201D	-30.08	-30.47	-28.75	-27.99
RILs mean	-29.61	-29.22	-28.32	-28.40
Range	2.38	2.44	2.36	2.85
Std. deviation	0.42	0.44	0.42	0.63
Variance	0.17	0.19	0.18	0.40
Skewness	0.20	0.97	1.06	1.18
Kurtosis	1.31	1.21	1.04	0.73
Coefficient of variation (%)[†]	1.41	1.51	1.49	2.43

[†] Absolute value of coefficient of variation

Table 2_2. Pearson correlation coefficients between $\delta^{13}C$ of RILs derived from PI 416997 × PI 567201D at Stoneville, MS in 2016 (ST16) and in 2017 (ST17), Columbia, MO in 2017 (CO17), and Fayetteville, AR in 2017 (FAY17).

	ST16	ST17	CO17
ST17	0.69^{***}		
CO17	0.74^{***}	0.67^{***}	
FAY17	0.76^{***}	0.72^{***}	0.78^{***}

*** Significant at the 0.001 probability level.

Table 2_3. Analysis of variance and heritability (h^2) of $\delta^{13}C$ across environment and for individual environment.

Analysis	Genotype (G)	Environment (E)	G × E	h^2 (%)
Across E	***	***	***	90
By E				
ST16	***	-	-	89
ST17	***	-	-	64
CO17	***	-	-	80
FAY17	***	-	-	78

G, RILs; E, environment; G×E, RILs × environment interaction.
*, **, ***, ns, significant at 0.05, 0.01, 0.001 probability levels, or non-significant, respectively.
ST16, ST17, CO17, and FAY17 represent the experiments at Stoneville in 2016, at Stoneville in 2017, at Columbia in 2017, and at Fayetteville in 2017, respectively.

Table 2_4. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ detected in four environments (ST16, ST17, CO17, and FAY17) in the RIL population derived from the cross of PI 416997 and PI 567201D using ICIM mapping.

Locus[##]	Chrom.[†]	Env[‡]	Position (cM)[§]	Flanking markers[¶]	LOD[#]	R^{2}[††]	Add[‡‡]	Favorable allele[§§]
1	Gm06	ST16	67	S_006_011_083_603--S_006_011_720_632	3.37	2.50	0.07	PI416997
		CO17	61	S_006_009_849_532--S_006_010_818_547	4.76	6.63	0.09	PI416997
2	Gm07	ST16	26	S_007_008_123_545--S_007_008_253_555	21.81	20.05	0.21	PI416997
		ST16	38	S_007_010_062_239--S_007_010_105_271	4.93	3.60	0.09	PI416997
3	Gm10	CO17	94	S_010_039_228_834--S_010_041_413_938	5.24	9.22	-0.10	PI567201D
4	Gm11	ST16	19	S_011_002_798_050--S_011_005_908_494	3.61	2.98	-0.08	PI567201D
5	Gm15	ST16	16	S_015_001_550_336--S_015_002_934_524	4.49	3.34	-0.08	PI567201D
6	Gm17	ST16	96	S_017_008_851_004--S_017_007_961_686	3.94	2.93	0.08	PI416997
		CO17	102	S_017_007_961_686--S_017_010_605_971	3.99	6.75	0.09	PI416997
7	Gm20	ST16	108	S_020_042_507_730 --S_020_042_835_316	5.97	4.58	-0.10	PI567201D
		ST17	108	S_020_042_507_730 --S_020_042_835_316	4.58	11.22	-0.08	PI567201D
		CO17	108	S_020_042_507_730 --S_020_042_835_316	7.28	10.33	-0.11	PI567201D
		FAY17	108	S_020_042_507_730 --S_020_042_835_316	13.13	29.88	-0.22	PI567201D
8	Gm20	ST16	122	S_020_044_532_207--S_020_044_686_226	10.05	7.88	-0.13	PI567201D

Table 2_4. (Cont.)

Locus[##]	Chrom.[†]	Env[‡]	Position (cM)[§]	Flanking markers[ϙ]	LOD[#]	R^{2}[††]	Add[‡‡]	Favorable allele[§§]
8	Gm20	ST17	121	S_020_042_835_366 -- S_020_044_532_207	6.51	17.04	-0.10	PI567201D
		CO17	125	S_020_044_865_885-- S_020_044_973_367	3.61	4.78	-0.07	PI567201D

[##] Closely spaced putative QTL falling within the same flanking markers were consider as one locus.
[†] *Glycine max* chromosome on which putative QTL was identified.
[‡] Environment in which a significant QTL was identified.
[§] Position of QTL in centiMorgans on *Glycine max* chromosome
[ϙ] SNPs identified in the mapping analysis as flanking the putative QTL.
[#] Log-likelihood at QTL peak position
[††] Phenotypic variation explained by putative QTL
[‡‡] Additive effect explained by the QTL
[§§] Allele that increases $\delta^{13}C$ value

Table 2_5. QTLs identified across environments for $\delta^{13}C$ and QTL × environment interaction detected in four environments using MET functionality.

Locus[##]	Chrom.[†]	Position (cM)[§]	Marker interval [ǀ]	LOD	LOD (A)[‡]	LOD (A × E)[#]	PVE[††]	PVE (A)[‡‡]	PVE (A × E)[§§]	Add [ǀǀ]
1	Gm06	56	S_006_006_069_381--S_006_009_849_532	6.68	5.93	0.75	5.00	4.68	0.32	0.06
3	Gm10	93	S_010_039_228_834--S_010_041_413_938	5.50	4.43	1.08	3.80	3.43	0.37	-0.05
6	Gm17	97	S_017_007_961_686--S_017_010_605_971	7.62	6.79	0.83	5.31	5.23	0.08	0.06
7	Gm20	108	S_020_042_507_730--S_020_042_835_316	25.12	24.83	0.29	23.89	21.28	2.61	-0.13
8	Gm20	122	S_020_044_532_207--S_020_044_686_226	8.70	4.08	4.63	5.45	3.17	2.28	-0.05

[##]Locus number was assigned same as to locus number used for QTLs identified by BIP functionality of ICIM Mapping in Table 2_4

[†] *Glycine max* chromosome on which putative QTL was identified

[§] Position of QTL in centiMorgans on *Glycine max* chromosome

[ǀ] Markers flanking identified QTL on specific chromosome

[‡] LOD score for additive effects

[#] LOD score for additive by environment effects

[††] Total phenotypic variance explained by QTL × environment interaction

[‡‡] Phenotypic variance explained by additive effects

[§§] Phenotypic variance explained by additive by environments effects.

[ǀǀ] Additive effect explained by the QTL

Table 2_6. Epistatic QTLs identified for $\delta^{13}C$ in RIL population derived from cross of PI 416997 × PI 567201D by the ICIM-EPI method implemented in QTL IciMapping.

Chrom. 1[†]	Env.[‡]	Pos. 1[§]	Chrom. 2[¶]	Pos. 2[#]	Add-by-Add[††]
Gm02	ST16	38,198,173 - 44,554,449	Gm20	42,835,366 - 44,532,207	0.20
	ST17				-0.15
	FAY17			44,865,885 - 44,973,367	-0.23
Gm03	FAY17	8,323,204 - 20,069,692	Gm20	44,865,885 - 44,973,367	0.27
	ST16	20,069,801 - 28,771,952		42,835,366 - 44,532,207	0.19
	ST17				0.14
	CO17				0.16
Gm04	FAY17	34,130,710 - 47,240,282	Gm20	42,835,366 - 44,532,207	-0.24
Gm05	ST16	41,542,487 - 36,175,591	Gm20	42,182,496 - 42,095,689	0.15
	ST17			42,835,366 - 44,532,207	0.13
	CO17				0.15
	FAY17				0.21
Gm06	ST16	18,147,076 - 49,721,339	Gm20	42,835,366 - 44,532,207	-0.16
	ST17				0.15
	CO17				0.17
	FAY17				0.21
Gm09	ST16	20,208,823 - 39,516,946	Gm20	42,835,366 - 44,532,207	0.18
	CO17				0.18
	FAY17				0.25
	ST17	44,429,663 - 43,615,936			0.15
Gm11	ST16	10,996,050 - 12,268,323	Gm20	42,182,496 - 42,095,689	0.18

Table 2_6. (Cont.)

Chrom. 1[†]	Env.[‡]	Pos. 1[§]	Chrom. 2[ǁ]	Pos. 2[#]	Add-by-Add[††]
Gm11	FAY17	10,996,050 - 12,268,323	Gm20	42,835,366 - 44,532,207	-0.25
Gm17	ST16	897,750 - 2,482,975	Gm20	42,835,366 - 44,532,207	-0.15
	FAY17			44,865,885 - 44,973,367	-0.21
Gm19	ST16	22,947,398 - 22,051,112	Gm20	42,835,366 - 44,532,207	-0.15
	ST17				-0.15
	CO17				-0.18
	FAY17				-0.23

Glycine max chromosome on which first QTL involved in epistasis was identified
[‡] Environment in which a significant QTL × QTL interaction was identified
[§] Position of flanking markers of first QTL in epistasis on specific chromosome
[ǁ] *Glycine max* chromosome on which second QTL involved in epistasis was identified
[#] Position of flanking markers of second QTL in epistasis on specific chromosome
[††]Epistatic effect between two QTLs

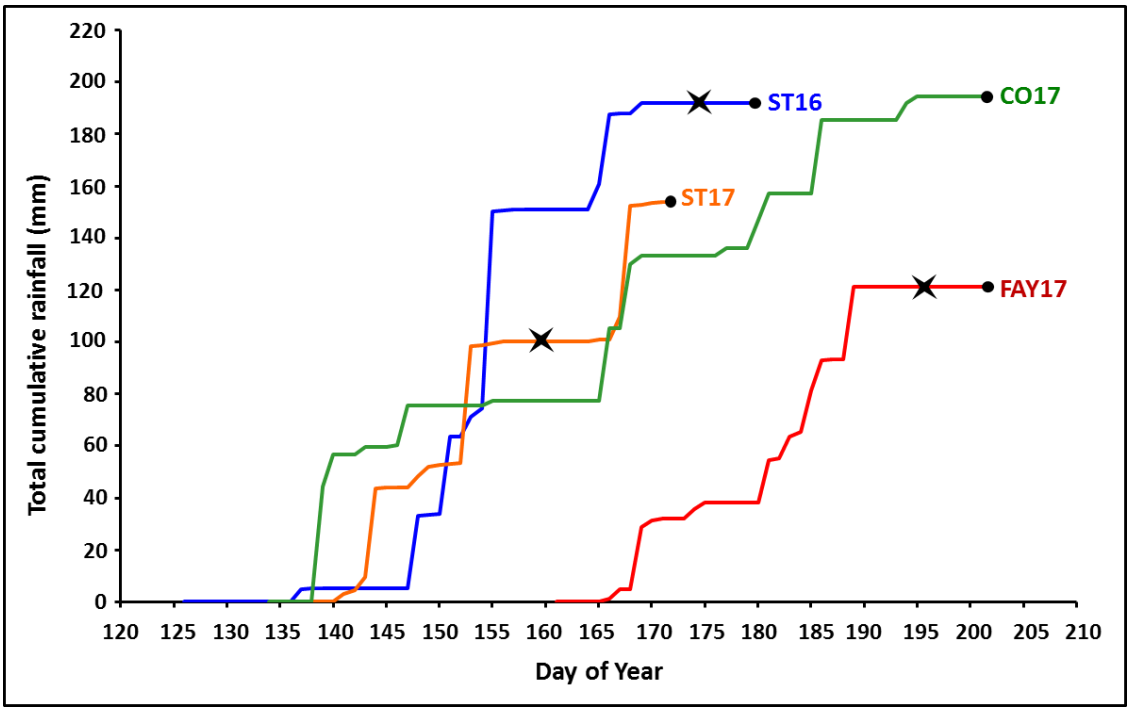

Figure 2_1. Total cumulative rainfall (mm) for the four environments (ST16, ST17, CO17, and FAY17). At ST16, ST17, and FAY17 irrigation was applied (stars indicate the time of irrigation) as needed whereas CO17 was rainfed. Sampling dates for $\delta^{13}C$ for each environment are indicated by a filled circle.

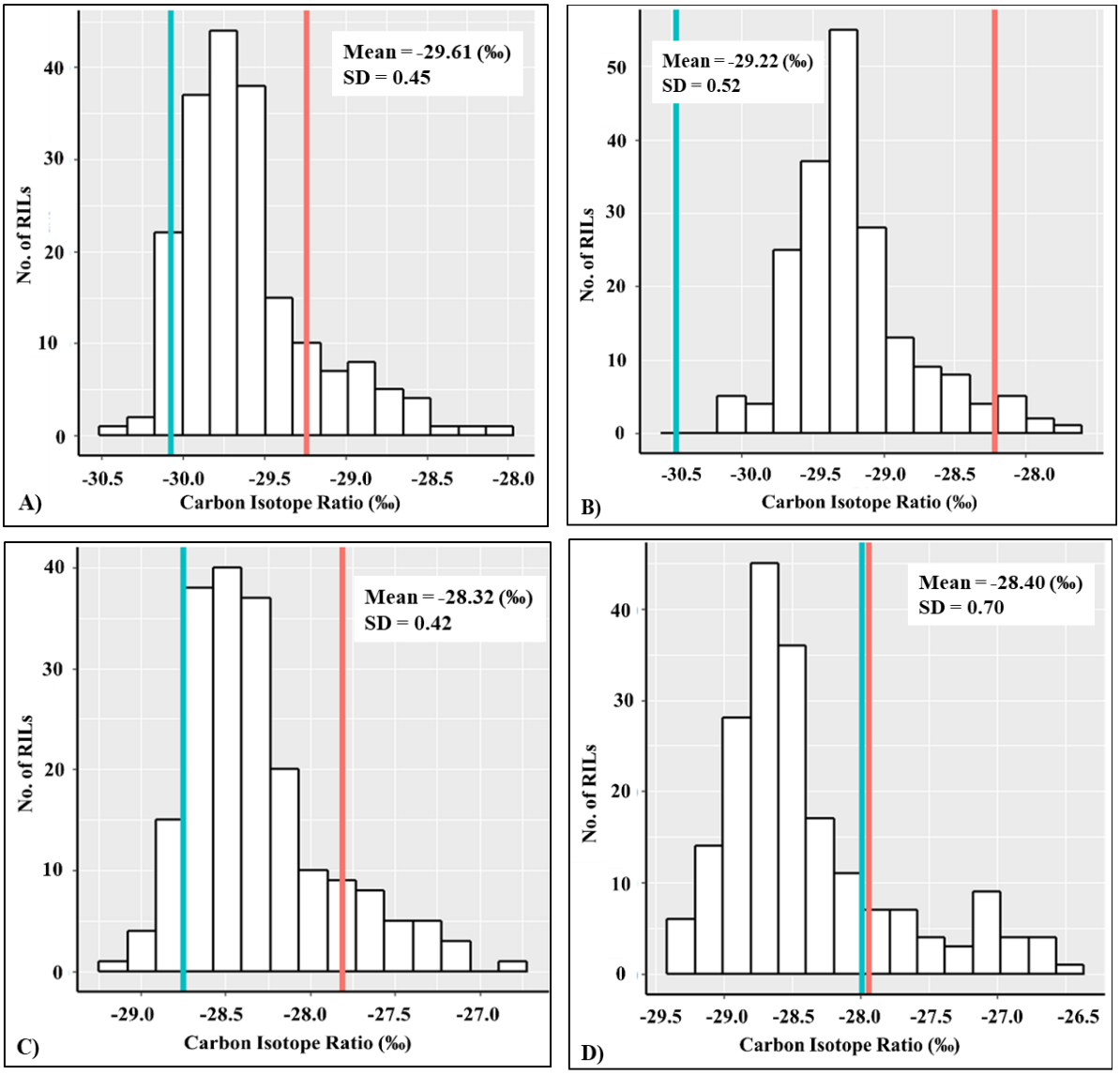

Figure 2_2. Frequency distribution showed broad range of $\delta^{13}C$ within each environment. Vertical lines indicate the mean of the parental genotypes PI 416997 (Red) × PI 567201D (Blue). Mean of RILs and Standard deviation (SD) were shown on the top corner of each histogram. A, B, C, and D indicated the environment ST16, ST17, CO17, and FAY17, respectively.

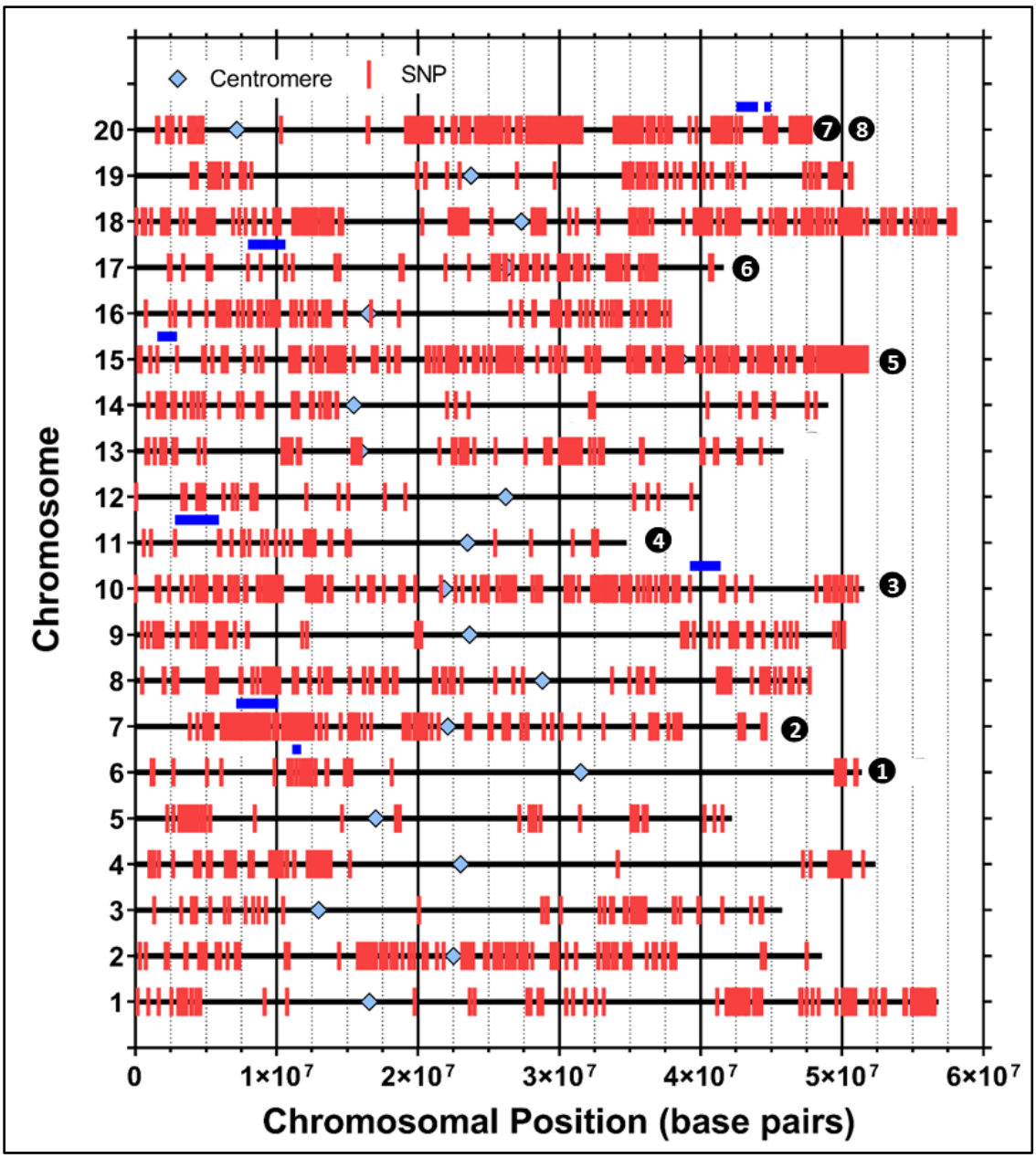

Figure 2_3. Physical position of SNPs on soybean chromosomes and position of loci associated with $\delta^{13}C$ identified by ICIM mapping. The physical positions of SNP markers indicated in base pairs are shown on the x-axis and the y-axis represents chromosome number. The solid blue diamond represents the centromere location. The numbers in the black circles represent the locus numbers on a specific chromosome. The QTL positions for individual loci are designated by a blue bar above the respective chromosome. The length of the blue bar represents the distance between flanking markers.

References

Abdel-Haleem H, Carter TE Jr, Purcell LC, King CA, Ries LL, Chen P, Schapaugh W Jr, Sinclair TR, Boerma HR (2012) Mapping of quantitative trait loci for canopy wilting trait in soybean (*Glycine max* L. Merr). Theor Appl Genet 125:837-846

Abdel-Haleem H, Lee G, Boerma RH (2011) Identification of QTL for increased fibrous roots in soybean. Theor Appl Genet 122:935-946

Araus JL, Amaro T, Casadesús J, Asbati A, Nachit MM (1998) Relationships between ash content, carbon isotope discrimination and yield in durum wheat. Aust J Plant Physiol 25:835-842

Avramova V, Meziane A, Bauer E, Blankenagel S, Eggels S, Gresset S, Grill E, Niculaes C, Ouzunova M, Poppenberger B, Presterl T, Rozhon W, Welcker C, Yang Z, Tardieu F, Schön C (2019) Carbon isotope composition, water use efficiency, and drought sensitivity are controlled by a common genomic segment in maize. Theor Appl Genet 132:53-63

Bazzer SK, Kaler AS, King CA, Ray JD, Hwang S, Purcell LC (2019) Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean. Crop Sci https://doi.org/10.1002/csc2.20240

Bindumadhava H, Sheshshayee S, Devendra R, Prasad TG, Udayakumar M (1999) Oxygen (18O) isotopic enrichment in the leaves as a potential surrogate for transpiration and stomatal conductance. Curr Sci 76:1427-1428

Bloch D, Hoffmann CM, Märländer B (2006) Impact of water supply on photosynthesis, water use and carbon isotope discrimination of sugar beet genotypes. Eur J Agron 24:218-225

Bondari K (2003) Statistical analysis of genotype × environment interaction in agricultural research. In: Paper SD15, SESUG: The Proceedings of the South East SAS Users Group, St. Pete Beach, FL

Brendel O, Pot D, Plomion C, Rozenberg P, Guehl JM (2002) Genetic parameters and QTL analysis of delta C-13 and ring width in maritime pine. Plant Cell Environ 25:945-953

Brugnoli E, Farquhar GD (2000) Photosynthetic fractionation of carbon isotopes. In: Leegood RC, Sharkey TD, von Caemmerer S (ed) Photosynthesis: Physiology and Metabolism. Kluwer Academic Publishers, Dordrecht, pp 399-434

Brugnoli E, Hubick KT, Caemmerer SV, Farquhar GD (1988) Correlation between the carbon isotope discrimination in leaf starch and sugars of C3 plants and the ratio of intercellular and atmospheric partial pressures of carbon dioxide. Plant Physiol 88:1418-1424

Çag˘irgan M, Özbas MO¸ Heng LK, Afza R (2005) Genotypic variability for carbon isotope discrimination in the mutant and improved lines of barley. Isotopes Environ Health Stud 41:229-235

Campbell BT, Baenziger PS, Gill KS, Eskridge KM, Budak H, Erayman M, Dweikat I, Yen Y (2003) Identification of QTL and environmental interactions associated with agronomic traits on chromosome 3A of wheat. Crop Sci 43:1493-1505

Cao Z, Guo Y, Yang Q, He Y, Fetouh MI, Warner RM, Deng Z (2019) Genome-wide identification of quantitative trait loci for important plant and flower traits in petunia using a high-density linkage map and an interspecific recombinant inbred population derived from *Petunia integrifolia* and *P. axillaris*. Hortic Res 6:27

Carlborg O, Haley CS (2004) Epistasis: too often neglected in complex trait studies? Nat Rev Genet 5:618-625

Carpentieri-Pipolo V, Pipolo AE, Abdel-haleem H, Boerma HR, Sinclair TR (2011) Identification of QTLs associated with limited leaf hydraulic conductance in soybean. Euphytica 186:679-686

Ceccarelli S, Acevedo E, Grando S (1991) Breeding for yield stability in unpredictable environments: single traits, interaction between traits, and architecture of genotypes. Euphytica 56:169-185

Cernusak LA, Ubierna N, Winter K, Holtum JA, Marshall JD, Farquhar GD (2013) Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. New Phytol 200:950-965

Charlson DV, Bhatnagar S, King CA, Ray JD, Sneller CH, Carter TE, Purcell LC (2009) Polygenic inheritance of canopy wilting in soybean [*Glycine max* (L.) Merr.]. Theor Appl Genet 119:587-594

Chen H, He H, Zou Y, Chen W, Yu R, Liu X, Yang Y, Gao YM, Xu JL, Fan LM, Li Y, Li ZK, Deng XW (2011) Development and application of a set of breeder-friendly SNP markers for genetic analyses and molecular breeding of Rice (*Oryza sativa* L.). Theor Appl Genet 6:869-879

Condon AG, Richards RA, Farquhar GD (1987) Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown wheat. Crop Sci 27:996-1001

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2002) Improving intrinsic water-use efficiency and crop yield. Crop Sci 42:122-131

Crossa, J, Vargas M, van Eeuwijk FA, Jiang C, Edmeades GO, Hoisington D (1999) Interpreting genotype environment interaction in tropical maize using linked molecular markers and environmental covariables. Theor Appl Genet 99:611-625

Cui S, He X, Fu S, Meng Q, Gai J, Yu D (2008) Genetic dissection of the relationship of apparent biological yield and apparent harvest index with seed yield and yield related traits in soybean. Aust J Agric Res 59:86-93

Dhanapal AP, Ray JD, Singh SK, Hoyos-Villegas V, Smith JR, Purcell LC, King CA, Cregan PB, Song Q, Fritschi FB (2015) Genome wide association study (GWAS) of carbon isotope ratio ($\delta^{13}C$) in diverse soybean [*Glycine max* (L.) Merr.] genotypes. Theor Appl Genet 28:73-91

Du W, Wang M, Fu S, Yu D (2009) Mapping QTL for seed yield and drought susceptibility index in soybean (*Glycine max* L.) across different environments. J Genet Genomics 36:721-731

Ehdaie B, Hall AE, Farquhar GD, Nguyen HT, Waines JD (1991) Water use efficiency and carbon isotope discrimination in wheat. Crop Sci 31:1282-1288.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE 6:e19379

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Biol 40: 503-537

Farquhar GD, O'Leary MH, Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Funct Plant Biol 9:121-137

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol 11:539-552

Farquhar GD (1983) On the nature of carbon isotope discrimination in C4 species. Aust J Plant Physiol 10:205-226

Fehr WR, Caviness CE (1977) Stages of soybean development. Spec. Rep. 80. Iowa State Univ. Coop. Ext. Serv., Ames, IA

Foy CD (1984) Physiological effects of hydrogen, aluminum and manganese toxicities in acid soil. In: Pearson RW, Adams F (ed) Soil acidity and liming. American Society of Agronomy, Wisconsin, pp 57-97

Garrison E, Marth G (2012) Haplotype-based variant detection from short-read sequencing. Preprint at https://arxiv.org/abs/1207.3907.

Githiri SM, Watanabe S, Harada K, Takahashi R (2006) QTL analysis of flooding tolerance in soybean at an early vegetative growth stage. Plant Breed 125:613-618

Gresset S, Westermeier P, Rademacher S, Ouzunova M, Presterl T, Westhoff P, Schön CC (2014) Stable carbon isotope discrimination is under genetic control in the C_4 species maize with several genomic regions influencing trait expression. Plant Physiol 164:131-143

Hall AE, Mutters RG, Hubick KT, Farquhar GD (1990) Genotypic differences in carbon isotope discrimination by cowpea under wet and dry field conditions. Crop Sci 30:300-305.

Hall NM, Griffiths H, Corlett JA, Jones HG, Lynn J, King GJ (2005) Relationships between water-use traits and photosynthesis in *Brassica oleracea* resolved by quantitative genetic analysis. Plant Breed 124:557-564

Henderson SA, von Caemmerer S, Farquhar GD (1992) Short-term measurements of carbon isotope discrimination in several C4 species. Aust J Plant Physiol 19:263-0285

Henderson SA, von Caemmerer S, Farquhar GD, Wade L, Hammer H (1998) Correlation between carbon isotope discrimination and transpiration efficiency in lines of the C_4 species *Sorghum bicolor* in the glasshouse and the field. Aust J Plant Physiol 25:111-123

Holland JB, Nyquist WE, Cervantes-Martinez CT (2003) Estimating and interpreting heritability for plant breeding: an update. Plant Breed Rev 22:9-111

Hopkins WG (1999) Introduction to plant physiology. 2nd Ed. John Wiley & Sons, Inc., New York, NY

Hubick KT, Farquhar GD, Shorter R (1986) Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. Aust J Plant Physiol 13:803-816

Hubick KT, Hammer GL, Farquhar GD, Wade LJ, von Caemmerer S, Henderson SA (1990) Carbon isotope discrimination varies genetically in C4 species. Plant Physiol 92:534-537

Hwang S, King CA, Chen P, Ray JD, Cregan PB, Carter TE Jr, Li Z, Abdel-Haleem H, Matson KW, Schapaugh W Jr, Purcell LC (2016) Meta-analysis to refine map position and reduce confidence intervals for delayed canopy wilting QTLs in soybean. Mol Breed 36:91

Hwang S, King CA, Davies MK, Ray JD, Cregan PB, Purcell LC (2013) QTL analysis of shoot ureide and nitrogen concentrations in soybean [*Glycine max* (L.) Merr.]. Crop Sci 53:2421-2433

Hwang S, King CA, Ray JD, Cregan PB, Chen P, Carter TE Jr, Li Z, Abdel-Haleem H, Matson KW, Schapaugh W Jr, Purcell LC (2015) Confirmation of delayed canopy wilting QTLs from multiple soybean mapping populations. Theor Appl Genet 128:2047-2065

Ishimaru K, Yano M, Aoki N, Ono K. Hirose T, Lin SY, Monna L, Sasaki T, Ohsugi R (2001) Toward the mapping of physiological and agronomic characters on a rice function map: QTL analysis and comparison between QTLs and expressed sequence tags. Theor Appl Genet 102:793-800

Jiang ZF, Zhang BB, Teng WL, Han YP, Zhao X, Sun DS, Zhan ZC, Li WB (2011) Impact of epistasis and QTL × environmental interaction on the oil filling rate of soybean seed at different developmental stages. Euphytica 177(3):431-442

Kaler AS, Bazzer SK, Sanz-Saez A, Ray JD, Fritschi FB, Purcell LC (2018) Carbon isotope ratio fractionation among plant tissues of soybean. Plant Phenome J 1:180002

Kaler AS, Dhanapal AP, Ray JD, King CA, Fritschi FB, Purcell LC (2017) Genome-wide association mapping of carbon isotope and oxygen isotope ratios in diverse soybean genotypes. Crop Sci 57:3085-3100

Knox JW, Haro D, Hess T, Morris J (2018) Forecasting Changes in Agricultural Irrigation Demand to Support a Regional Integrated Water Resources Management Strategy. 10.1016/bs.apmp.2018.08.003

Korir P, Qi B, Wang Y, Zhao T, Yu D, Chen S, Gai J (2011) A study on relative importance of additive, epistasis and unmapped QTL for aluminum tolerance at seedling stage in soybean. Plant Breed 130:551-562

Kumar M, Lal SK (2015) Molecular analysis of soybean varying in water use efficiency using SSRs markers. J Environ Biol 36:1011-1016

Li C, Bai G, Carver BF, Chao S, Wang Z (2015) Mapping quantitative trait loci for plant adaptation and morphology traits in wheat using single nucleotide polymorphisms. Euphytica 208:229-312

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 25:1754-1760

Littell RC, Milliken GA, Stroup WW, Wolfinger RD (1996) SAS system for mixed models. SAS Inst. Inc., Cary, NC

Ludlow MM, Muchow RC (1990) A critical evaluation of traits for improving crop yields in water-limited environments. Adv Agron 43:107-153

Ma JF, Furukawa J (2003) Recent progress in the research of external Al detoxification in higher plants: a mini review. J Inorg Biochem 97:46-51

Ma W, Appels R, Bekes F, Larroque O, Morell MK, Gale KR (2005) Genetic characterization of dough rheological properties in a wheat doubled haploid population: additive genetic effects and epistatic interactions. Theor Appl Genet 111:410-422

Martin B, Nienhuis J, King G, Schaefer A (1989) Restriction fragment length polymorphisms associated with water use efficiency in tomato. Science 243:1725-1728

Meng L, Li H, Zhang L, Wang J (2015) QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. Crop J 3:269-283

Mian MAR, Ashley DA, Boerma HR (1998) An additional QTL for water use efficiency in soybean. Crop Sci 38:390-393

Mian MAR, Mailey MA, Ashley DA, Wells R, Carter TE, Parrot WA, Boerma HB (1996) Molecular markers associated with water use efficiency and leaf ash in soybean. Crop Sci 36:1252-1257

Money D, Gardner K, Migicovsky Z, Schwaninger H, Zhong GY, Myles S (2015) LinkImpute: Fast and accurate genotype imputation for non-model organisms. G3 5:2383-2390

Monneveux P, Sheshshayee MS, Akhter J, Ribaut JM (2007) Using carbon isotope discrimination to select maize (*Zea mays* L.) inbred lines and hybrids for drought tolerance. Plant Sci 173:390-396

Pantalone VR, Burton JW, Carter TE (1996) Soybean fibrous root heritability and genotypic correlations with agronomic and seed quality traits. Crop Sci 36:1120-1125

Peleg Z, Fahima T, Krugman T, Abbo S, Yakir D, Korol AB, Saranga Y (2009) Genomic dissection of drought resistance in durum wheat × wild emmer wheat recombinant inbred line population. Plant Cell Environ 32:758-779

Piepho HP, Möhring J, Melchinger AE, Büchse A. (2008) BLUP for phenotypic selection in plant breeding and variety testing. Euphytica 161:209-228

Polania JA, Poschenrieder C, Beebe S, Rao IM (2016) Effective use of water and increased dry matter partitioned to grain contribute to yield of common bean improved for drought resistance. Front Plant Sci 7:660

Price AH, Cairns JE, Horton P, Jones HG, Griffiths H (2002) Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. J Exp Bot 53:989-1004

Prioul JL, Quarrie S, Causse M, de Vienne D (1997) Dissecting complex physiological functions through the use of molecular quantitative genetics. J Exp Bot 48:1151-1163

Purcell LC, Specht JE (2004) Physiological traits for ameliorating drought stress. In: Boerma HR, JE Specht (eds) Soybeans: Improvement, Production, and Uses. American Society of America, Madison, WI, pp 569-620

Read JJ, Johnson DA, Asay KH, Tienzen C (1991) Carbon isotope discrimination, gas exchange and WUE in crested wheat grass clones. Crop Sci 31:1203-1208

Rebetzke GJ, Ellis MH, Bonnett DG, Richards RA (2007) Molecular mapping of genes for coleoptile growth in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 114:1173-1183

Reif JC, Maurer HP, Korzun V, Ebmeyer E, Miedaner T, Wurschum T (2011) Mapping QTL with main and epistatic effects underlying grain yield and heading time in soft winter wheat. Theor Appl Genet 123:283-292

Richards RA, Rebetzke GJ, Condon AG, Van Herwaarden AF, Duggan BL, Munnas R (1999) Targeting physiological traits to overcome environmental limitations and raise the yield potential of dryland crops. In: 11th Aust Plant Breed Conf. Proc. Adelaide. 19-23 April 1999. Vol 1. pp 27-33

Sadok W, Sinclair TR (2010) Transpiration response of 'slow wilting' and commercial soybean (*Glycine max* (L.) Merr.) genotypes to three aquaporin inhibitors under high evaporative demand. J Exp Bot 61:821-829

Sandquist DR, Ehleringer JR (1995) Carbon isotope discrimination in the C_4 shrub *Atriplex confertifolia* along a salinity gradient. West N Am Nat 55:135-141

Saranga Y, Menz M, Jiang C, Wright RJ, Yakir D, Paterson AH (2001) Genomic dissection of genotype × environment interactions conferring adaptation of cotton to arid conditions. Genome Res 11:1988-1995

SAS institute (2013) The SAS System window version 9.4. SAS Institute Inc., Cary, NC

Sheshshayee MS, Bindumadhava H, Ramesh R, Prasad TG, Lakshminarayana MR, Udayakumar M (2005) Oxygen isotope enrichment ($\Delta^{18}O$) as a measure of time-averaged transpiration rate. *J Exp Bot* 56:3033-3039

Specht JE, Chase K, Macrander M, Graef GL, Chung J, Markwell JP, Germann M, Orf JH, Lark KG (2001) Soybean response to water-a QTL analysis of drought tolerance. Crop Sci 41:493-509

Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27:205-233

Teulat B, Merah O, Sirault X, Borries C, Waugh R, This D (2002) QTLs for grain carbon isotope discrimination in field grown barley. Theor Appl Genet 106:118-126

Thumma BA, Naidu BP, Chandra A, Cameron DF, Bahnisch LM, Liu C (2001) Identification of causal relationships among traits related to drought resistance in *Stylosanthes scabra* using QTL analysis. J Exp Bot 52:203-214

Twohey III RJ, Roberts LM, Studer AJ (2019) Leaf stable carbon isotope composition reflects transpiration efficiency in Zea mays. Plant J 97:475-484

Veldboom LR, Lee M (1996) Genetic mapping of quantitative trait loci in maize in stress and nonstress environments: I. Grain yield and yield components. Crop Sci 36:1310-1319

Voltas J, Romagossa I, Lafarga A, Armesto AP, Sombrero A, Araus JL (1999) Genotype by environment interaction for grain yield and carbon isotope discrimination of barley in Mediterranean Spain. Aust J Agr Res 50:1263-1271

White DS, Bell MJ, Wright GC (1996) The potential to use carbon isotope discrimination as a selection tool to improve water use efficiency in soybean. In: Proceedings of the 8th Australian agronomy conference, Toowoomba, Qld, pp 728

Xu X, Martin B, Comstock JP, Vision TJ, Tauer CG, Zhao B, Pausch RC, Steven K (2008) Fine mapping a QTL for carbon isotope composition in tomato. Theor Appl Genet 117:221-233

Xu Y, This D, Pausch RC, Vonhof WM, Coburn JR, Comstock JP, McCouch SR (2009) Leaf-level water use efficiency determined by carbon isotope discrimination in rice seedlings: genetic variation associated with population structure and QTL mapping. Theor Appl Genet 118:1065-1081

Zhang KP, Tian JC, Zhao L, Wang SS (2008) Mapping QTL with epistatic effects and QTL × environment interactions for plant height using a doubled haploid population in cultivated wheat. J Genet Genomics 35:119-127

Zhao J, Becker HC, Zhang DQ, Zhang YF, Ecke W (2005) Oil content in a European × Chinese rapeseed population: QTL with additive and epistatic effects and their genotype-environment interactions. Crop Sci 45(1):51-59

CHAPTER III

Mapping Quantitative Trait Loci (QTL) for Plant Nitrogen isotope Ratio ($\delta^{15}N$) in Soybean

Abstract

Soybean (*Glycine max* (L.) Merr.) meets a large portion of its nitrogen (N) need via biological N_2 fixation, which is highly sensitive to drought stress. Nitrogen isotope ratios between ^{15}N and ^{14}N ($\delta^{15}N$) can be used as a metric for relative differences among soybean genotypes for N_2 fixation, as $\delta^{15}N$ is negatively associated with N_2 fixation. This study evaluated the genetic basis of $\delta^{15}N$ using a mapping population of 196 F_6-derived recombinant inbred lines (RILs) developed from a cross between PI 416997 and PI 567201D that was assessed in multiple environments. There was a wide range of $\delta^{15}N$ in all environments and narrow-sense heritability for $\delta^{15}N$ was 35% when estimated across environments. Analysis of variance of $\delta^{15}N$ showed significant effects of genotype and environment, whereas the genotype × environment interaction was not significant ($P<0.05$). Inclusive composite interval mapping for individual environments identified 10 additive QTLs on seven chromosomes with additive effects ranging from 0.02 to 0.13‰ and that individually explained phenotypic variation from 1.72 to 9.34%. In total, eight QTL × environment interactions were found, and several genomic regions were involved in QTL × QTL interactions that were not identified as additive QTLs. These identified QTLs were co-localized with genomic regions associated with N_2 fixation and other physiological traits identified in previous studies. A search for candidate genes resulted in detection of genes for nodulation and N-metabolism underlying many additive and epistatic QTLs. These identified regions may serve as potential targets for enhancing N_2 fixation in soybean.

Introduction

Soybean (*Glycine max (L.*) Merr.) is one of the most important leguminous crops grown and consumed worldwide due to its high protein (~40%) and oil (~18-19%) concentrations. Soybean establishes symbiotic associations with *Bradyrhizobium japonicum* (Strodtman and Emerich 2009) which reduces atmospheric N_2 to ammonia and provides N to the plant. This association decreases the requirement for N fertilizers for soybean and other leguminous crops and improves soil fertility (Giller 2001; Jensen and Hauggaard-Nielsen 2003). In soils with little available soil N, symbiotic N_2 fixation can meet up to 85-90% of the soybean N requirement (Mastrodomenico and Purcell 2012).

Symbiotic N_2 fixation is sensitive to various abiotic stresses including drought, flooding, soil salinity, soil acidity, mineral deficiency or toxicity, and low/high temperature (Ramaekers et al. 2013). Water deficit conditions negatively impact N_2 fixation in soybean by reducing nodulation and nitrogenase activity, which ultimately decreases soybean yield (Márquez-García et al. 2015; Serraj et al. 1999a). Also, it has been reported that N_2 fixation is more sensitive to water deficit than photosynthesis under both controlled and field conditions (Adams et al. 2016; Djekoun and Planchon 1991; Durand et al. 1987; Kuo and Boersma 1971; Sinclair et al. 1987). Proposed mechanisms for decreased N_2 fixation during water deficit conditions include carbon shortage, oxygen limitation, and feedback inhibition by products of N_2 fixation (Purcell 2009; Serraj et al. 1999b).

Methods for quantifying N_2 fixation include the N-difference method (Weaver and Danso 1994), acetylene reduction assay (ARA) (Hardy et al. 1968), ^{15}N enrichment (Fried and Broeshart 1975; Fried and Middleboe 1977), ^{15}N natural abundance (Shearer and Kohl 1986), and relative abundance of ureides (Serraj et al. 1999b; Sinclair and Serraj 1995). Each method

has specific advantages over others, but easy, rapid, inexpensive, and quantitative methods for estimation of N_2 fixation under both controlled and field conditions are still needed.

Among the various methods for estimatingN_2 fixation, ^{15}N natural abundance ($\delta^{15}N$) is frequently used to quantify the fraction of N derived from the atmosphere (NDFA) in large scale field experiments and to serve as an index of N_2 fixation (Andrews and Lea 2013; Barrie et al. 1995; Letolle 1980). This method compares the abundance of the ^{15}N isotope in plant tissue, the atmosphere, and the soil environment with respect to the ^{14}N isotope. The atmosphere has a lower concentration of ^{15}N compared to the soil due to the N transformations in soil. The difference in ^{15}N and ^{14}N concentration between soil and atmosphere is expressed in terms of parts per thousand (‰) and is referred to as the N isotope ratio ($\delta^{15}N$) (Peoples et al. 1989). N_2 fixation dilutes the ^{15}N in plants actively fixing N_2 as compared to plants that depend on mineral N as a N source (Doughton et al. 1995; Shearer and Kohl 1986). A low $\delta^{15}N$ value is a favorable trait for selection because it indicates greater dilution of ^{15}N by biological N_2 fixation.

The percentage of NDFA from $\delta^{15}N$ (Kohl and Shearer 1981) is calculated according to the equation below:

$$\% \, NDFA = \frac{\delta^{15}N_{ref} - \delta^{15}N_{samp}}{\delta^{15}N_{ref} - \delta^{15}N_0} * 100$$

where $\delta^{15}N_{ref}$ is the composition of a plant totally dependent on soil N (non-nodulating genotype), $\delta^{15}N_{samp}$ is the composition of the individual samples, and $\delta^{15}N_o$ (-1.30 for soybean, Bergersen et al. 1989) is the $\delta^{15}N$ from a plant totally dependent on N_2 fixation. The reference genotype in this equation reduces the error/noise caused by soil N variability in calculating % NDFA. However, the $\delta^{15}N$ of the reference genotype is often relatively uniform across a field (Peoples et al. 2002), indicating that in the absence of a reference crop, $\delta^{15}N$ can be used directly to estimate the amount of N fixed by genotypes via N_2 fixation (Steketee et al. 2019).

The difference among genotypes for N_2 fixation under normal and stress conditions may help identify genomic regions controlling N_2 fixation under water deficit conditions. Quantitative trait loci (QTLs) mapping is the molecular approach used to understand the genetic architecture of many physiological and agronomical traits. Recent advances in high throughput genotyping and phenotyping platforms have revolutionized the dissection of the genetic basis of quantitative traits like N_2 fixation and will accelerate development of soybean lines with enhanced N_2 fixation.

Several studies have mapped QTLs for N_2 fixation or related traits in soybean (www.soybase.org). Tanya et al. (2005) used a population of 136 F_2-derived recombinant inbred lines (RILs) to identify a total of nine QTLs for nodule number per plant, nodule fresh and dry weight per plant, and acetylene reduction activity (ARA). Nicolás et al. (2006) identified two genomic regions associated with nodule number and nodule dry weight. Santos et al. (2013) studied the genetic control of nodule number and individual nodule weight and confirmed a QTL for nodule number identified previously by Nicolás et al. (2006). Hwang et al. (2014) were the first to map QTLs for nodule number, nodule size, and nodule weight in field experiments.

Dhanapal et al. (2015b) used association mapping on a diverse panel of 374 maturity group 4 accessions to identify QTLs for NDFA and N concentration. This analysis identified 17 and 19 SNPs significantly associated with NDFA and N concentration, respectively. Steketee et al. (2019) used association mapping for $\delta^{15}N$ using a panel of 211 diverse soybean accessions and found 23 and 26 SNPs associated with $\delta^{15}N$ and N concentration, respectively. To date, more than 70 QTLs for N_2 fixation or traits directly or indirectly linked with N_2 fixation have been mapped on all 20 chromosomes of soybean (www.soybase.org).

In the present study, a high-density genetic linkage map was constructed using 196 F_6-derived RILs developed from PI 416997 × PI 567201D. The parents of this population were originally chosen because they were extremes for the ratio between ^{13}C and ^{12}C (Bazzer et al. 2020), which serves as a surrogate measure of water use efficiency (Farquhar and Richards 1984). Although the parents were not selected for $\delta^{15}N$ or N_2 fixation, their RILs segregated for $\delta^{15}N$. Therefore, the main objectives of our study were to identify additive QTLs for $\delta^{15}N$, epistatic QTLs, and QTL × environment interactions. Further characterization of genes underlying the QTLs identified in this study will help to understand the biological mechanisms regulating N_2 fixation in soybean and the genetic basis of N_2 fixation.

Material and Methods

Development of RIL Population

The cross between PI 416997 and PI 567201D was made at Stoneville, MS in 2011. The F_1 generation was grown during the winter of 2011-2012 at the Tropical Agricultural Research Station at Isabela, Puerto Rico. The F_2 generation was grown in Stoneville in 2012, where over 200 individual F_2 plants were harvested without selection. Leaf tissue was harvested from each tagged F_2 plant for DNA extraction and genotyping of the population. The $F_{2:3}$ and $F_{4:5}$ generations were grown in Homestead, FL during the winters of 2012-2013 and 2013-2014, respectively, harvesting one random plant from each single-plant-derived row in each nursery. The $F_{3:4}$, $F_{5:6}$, and $F_{6:7}$ generations were grown in Stoneville, with the former two generations being advanced by single-plant descent in 2013 and 2014, respectively, and the latter generation being bulk harvested in 2015 to create bulked F_6-derived lines for phenotyping.

Field Trials

A mapping population consisting of 196 F_6-derived RILs generated from a cross between PI 416997 × PI 567201D was used to identify the genomic regions associated with $\delta^{15}N$. The RIL population and parents were evaluated in four environments: at Stoneville, MS (33.42° N, 90.90° W) on a Bosket very fine sandy loam soil (fine-loamy, mixed, active, thermic, Mollic Hapludalfs) in 2016 and on a Dundee silty clay loam soil (Dundee fine-silty, mixed, active, thermic, Typic Endoaqualfs) in 2017, at the Milo J Shult Arkansas Agricultural Research Center, Fayetteville, AR (36.05° N, 94.15° W) on a Captina silt loam soil (fine-silty, siliceous, active, mesic Typic Fragiudult) in 2017, and at the Bradford Research Center near Columbia, MO (38.95° N, 92.33° W) on a Mexico silt loam soil (fine, smectitic, mesic Vertic Epiaqualf) in 2017. The combinations of locations and years were considered as individual environments and

designated as ST16 (Stoneville in 2016), ST17 (Stoneville in 2017), FAY17 (Fayetteville in 2017), and CO17 (Columbia in 2017). Plantings occurred on 6 May 2016 at ST16 and 16 May 2017 at ST17 in one-row plots (0.66 m wide by 2.74 m long), 10 June 2017 at FAY17 in two rows plots (0.45 m wide by 6 m long), and 14 May 2017 at CO17 in single row plots (0.76 m wide by 3.05 m long). At each environment, the experimental design was a randomized complete block design with two replications. Experiments were irrigated as needed. Recommended practices were followed for insect and weed control.

Data Collection

Shoot biomass of four random plants was sampled from each plot between beginning bloom (R1) and the full bloom (R2) stages (Fehr and Caviness 1977) on 29 June 2016 at ST16, 21 June 2017 at ST17, and 21 July 2017 at FAY17 and CO17. Biomass samples were dried at 60°C and coarse ground with a Wiley Mill (Thomas Model 4 Wiley® Mill, Thomas Scientific, NJ USA). Subsamples were finely-ground to pass a 1 mm sieve, and then ground to a fine powder with a Geno Grinder (SPEX CertiPrep, Inc., NJ USA) as described by Bazzer et al. (2020). About 3-5 mg of the powdered sample was weighed into tin capsules, for δ^{15}N isotope analysis which was conducted at the University of California-Davis Stable Isotope Facility (https://stableisotopefacility.ucdavis.edu/) using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer. Data from the stable isotope facility were expressed as δ^{15}N in per mil (‰) and determined according to the equation below:

$$\delta^{15}N = \frac{R_{sample}}{(R_{air} - 1)} * 1000$$

where, R_{sample} and R_{air} are the isotope ratios ($^{15}N/^{14}N$) of the sample and air, respectively. For more information refer to the Stable Isotope facility website, http://stableisotopefacility.ucdavis.edu/13cand15n.html.

Statistical Analysis

Descriptive statistics of $\delta^{15}N$ for each environment and correlation coefficients between different environments for $\delta^{15}N$ were calculated with SAS version 9.4 (SAS, Institute 2013). The difference between parents for $\delta^{15}N$ in different environments was determined using a *t*-test. Analysis of variance (ANOVA) was conducted using SAS version 9.4 (SAS Institute, 2013) with the PROC MIXED procedure (α=0.05). Genotype and environment were considered as fixed effects and replication nested within environment was considered as a random effect (Bondari 2003). Heritability (h^2, Holland et al. 2003) of $\delta^{15}N$ for each environment and averaged across environments was computed using the PROC VARCOMP procedure of SAS 9.4 based on the following formula:

Across environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{GE}^2}{e}\right) + \left(\frac{\sigma_e^2}{re}\right)}$$

Within environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_e^2}{r}\right)}$$

where, σ_G^2, σ_{GE}^2, and σ_e^2 are the genotypic variance, genotypic × environment interaction variance, and residual error variance, respectively, e is the number of environments, and r is the number of replications within environment. This heritability should be considered a narrow sense estimate, as F_6-derived RILs have a minimal level of heterozygosity within lines. Hence, most of the genotypic variance is composed of additive variance, with negligible variance due to dominance effects and its interaction with additive effects. The best linear unbiased prediction (BLUP) values for each individual environment and across environments were calculated using a mixed model to reduce environmental variance. All factors were considered as random effects in the case of individual environments. For calculation of BLUP values averaged across environments, environment was considered a fixed effect and genotypes and replications were considered as

random effects (Littell et al. 1996; Piepho et al. 2008). QTL analysis was conducted using BLUP values for individual environments and across environments.

Selection of lines with Extreme Values for $\delta^{13}C$ and $\delta^{15}N$

As described previously (Bazzer et al. 2020), this population was also evaluated for carbon isotope ratio ($\delta^{13}C$) (a proxy measurement for water use efficiency, WUE) as the parents were different in their $\delta^{13}C$ values based on phenotypic values and genomic estimated breeding values (GEBVs) (Dhanapal et al. 2015a; Kaler et al. 2017). The linear regression between $\delta^{13}C$ and $\delta^{15}N$ was performed using phenotypic values from each individual environment and averaged across environments. The biplots were divided into four quadrants using median values of $\delta^{13}C$ and $\delta^{15}N$ to select the lines that were extremes for both traits.

Genotyping-by-Sequencing and Construction of Linkage Map

Detailed information on genotyping and linkage map construction of this population were provided by Bazzer et al. (2020), which are summarized below. The 196 RILs, together with their two parents, were sequenced and genotyped using genotype-by-sequencing (GBS). DNA was isolated from lyophilized leaf tissue of the 196 RILs and both parents, and GBS library construction was conducted at LGC Genomics GmbH (Berlin, Germany). The sequence reads were mapped to the 'Williams 82' soybean reference genome (assembly 1). Markers with more than 15% missing data, markers that were heterozygous, or did not follow a 1:1 segregation ratio pattern (chi-square *P*-value ≤ 0.01) were removed, resulting in a total of 3,234 polymorphic markers. Missing marker data were imputed using a LD-kNNi method which was implemented in TASSEL software (https://www.maizegenetics.net/tassel).

The filtered and imputed 3,234 polymorphic SNPs were used initially for construction of the linkage map. The MAP functional module of IciMapping software v4.1 (Meng et al. 2015)

was used for the genetic linkage map construction using 2,468 out of 3,234 polymorphic markers after dropping redundant and low-quality markers. Genetic linkage groups, marker order and distances between markers were determined as described previously (Bazzer et al. 2020). Linkage groups were numbered as soybean chromosomes according to the genomic position of SNPs on the reference genome. Finally using 2,466 SNPs, a map with 20 linkage groups was constructed that corresponded to the 20 soybean chromosomes. The SNP markers information along with their position (in base pairs and cM) on specific chromosomes were reported by Bazzer et al. (2020).

QTL Analysis

The BLUP values calculated for each individual environment and averaged across environments were used for QTL analysis. QTL mapping was performed using the QTL IciMapping v4.1 software (http://www.isbreeding.net/) through BIP and MET functional modules. A BIP module of inclusive composite interval mapping (ICIM) was used to detect the additive QTLs within and across environments. QTL × QTL interactions were identified by using the Inclusive Composite Interval Mapping of Epistatic QTL (ICIM-EPI) function. The LOD threshold to declare significant additive QTLs and interactions between QTLs was calculated using 1,000 iteration permutation tests with a genome-wide significance level of 0.05 (Doerge and Churchill 1996; Li et al. 2007). The mapping parameters were 1.0 cM walking speed with *P*-value inclusion threshold of 0.01 for ICIM-ADD and 5 cM walking speed with a *P*-value inclusion threshold of 0.0001 for ICIM-EPI. Multi-Environment Traits (MET) module was used for detection of QTL × environment interactions using $\delta^{15}N$ BLUP data from all four environments. The missing phenotypic values were calculated by using the 'mean replacement' method. The specific parameters for detecting QTL× environment interactions were 1.0 cM

walking speed and a probability of 0.01 in stepwise regression. Finally, the position of SNPs on different chromosomes and the position of identified QTLs on the genetic map were drawn using Prism software (https://www.graphpad.com/).

Identification of Putative Candidate Genes

The search for putative candidate genes related to nodulation and N-metabolism underlying the genomic regions associated with $\delta^{15}N$ identified in the present study was performed using the genome browser option (William 82 assembly 1) of Soybase (www.soybase.org). Genes between flanking markers and up to ±1MB outside of the confidence interval for flanking markers were considered as potential candidate genes. Additionally, the position of 54 soybean genes (28 nodulin + 24 regulatory genes) associated with nodulation or biological N_2 fixation (Schmutz et al. 2010) were compared to the genomic regions of the $\delta^{15}N$ QTLs identified in the present research.

Results

Phenotypic Evaluation

The phenotypic values of parents and descriptive statistics of the RIL population are presented in Table 3_1. The parent PI 416997 had lower $\delta^{15}N$ values than PI 567201D in all environments, but parents were not significantly ($P<0.05$) different in any single environment. However, ANOVA across environments did indicate that $\delta^{15}N$ for PI 416997 was significantly ($P<0.01$) lower than PI 567201D (data not shown). There was wide segregation in the RIL population for $\delta^{15}N$ (Figure 3_1, Table 3_1) as indicated by $\delta^{15}N$ ranges of 3.96‰ in ST16, 4.08‰ in ST17, 3.43‰ in FAY17, and 4.55‰ in CO17. The frequency distribution of $\delta^{15}N$ was normal in all environments except CO17, as indicated by Shapiro-Wilk test (data not shown, Shapiro and Wilk 1965) and absolute values of skewness and kurtosis (less than 1.0, Table 3_1). Transgressive segregants exceeding both parents were observed, which indicates that favorable alleles for $\delta^{15}N$ were distributed between both parents. A significant positive correlation ($P<0.05$) was found between ST16 and ST17 ($r=0.15$), ST17 and FAY17 ($r=0.15$), and ST17 and CO17 ($r=0.14$) (data not shown).

Analysis of variance (ANOVA) averaged across environments showed significant ($P<0.05$) effects of genotype (G) and environment (E), whereas the interaction of genotype and environment (G × E) was not significant (Table 3_2). The narrow sense heritability of $\delta^{15}N$ averaged across environments was 35%. Estimates of narrow sense heritability for $\delta^{15}N$ within environments were 8% (ST16), 13% (ST17), 27% (FAY17), and 24% (CO17). Overall, low narrow sense heritability estimates across and within environments indicate that environmental effects play a major role in the expression of this trait and that phenotypic selection for this trait

may only be successful using replicated trials of homogeneous lines across multiple environments.

The RIL population used in the present study was also evaluated for $\delta^{13}C$ because the parents, PI 416997 and PI 567201D, also differed in WUE (Bazzer et al. 2020). Although no significant correlation was found between these traits, regression analysis was performed between $\delta^{15}N$ and $\delta^{13}C$ phenotypic values for within and across environments to identify extremes among RILs for different combinations of $\delta^{15}N$ and $\delta^{13}C$. The selected five RILs for high relative N_2 fixation and high WUE in individual environments and averaged across environments are listed in Table 3_3. The lines RIL.14, RIL.25, RIL.75, and RIL.135 were identified in at least two individual environments and across environments as having a favorable combination (low $\delta^{15}N$ and high $\delta^{13}C$). Similarly, lines with the unfavorable combination of high $\delta^{15}N$ and low $\delta^{13}C$ values were identified in individual environments and averaged across environments. The lines RIL.24, RIL.127, and RIL.161 were identified in at least two individual environments and RIL.112 appeared across environments in addition to individual environments.

QTL Analysis

The linkage map was 3,836 cM with individual linkage groups varying between 116 to 409 cM, based on the construction using 2,466 SNP markers (data not shown, Bazzer et al. 2020). QTL analysis conducted using $\delta^{15}N$ BLUP values from individual environments identified a total of 10 additive QTLs within environments (Figure 3_2, Table 3_4), which were distributed on seven chromosomes (Gm01, Gm04, Gm07, Gm08, Gm10, Gm13, and Gm17). Of these QTLs, there were four QTLs in ST16, two QTLs in ST17, and four QTLs in Fay17. No QTLs were identified in CO17. The phenotypic variation explained by individual QTLs (denoted as R^2), their additive effect, and parent contributing favorable alleles are presented in Table 3_4.

These QTLs individually accounted for 1.72 to 9.34% of the phenotypic variation and had additive effects ranging from 0.02 to 0.13‰. The QTL present on Gm04 at 49,247,258 bp detected in ST17 had a high R^2 value (9.34) as compared to other QTLs.

QTL analysis by using the BLUP values averaged across environment (AE) by the ICIM-ADD mapping method identified eight QTLs (Figure 3_2, Table 3_4). These QTLs were present on Gm01 (2), Gm04 (1), Gm07 (1), Gm13 (1), Gm14 (1), and Gm15 (2) and had additive effects that ranged from 0.04 to 0.12‰ that explained individually 1.83 to 14.39 % of the phenotypic variation (Table 3_4). The QTLs on Gm01, Gm04, Gm07, and Gm13 appeared in both individual environments and across environments. The QTLs on remaining chromosomes were detected only in single environments or only across environments. An allele decreasing $\delta^{15}N$ values was considered as the favorable allele, and the favorable allele for these QTLs was equally distributed between parents (PI 416997 and PI 567201D). When considering overlapping confidence intervals, there were 13 loci detected within and across environments (Figure 3_2, Table 3_4).

QTL × Environment and QTL × QTL Interactions Analysis

The interactions between QTL × environment and QTL × QTL play important roles in the genetic control of quantitative traits (Rebetzke et al. 2007; Reif et al. 2011). Eight QTLs present on Gm01 (2), Gm04, Gm07, Gm08 (2), Gm10, and Gm13 showed significant QTL × environment interactions as identified by MET functionality (Table 3_5). This interaction explained phenotypic variation that ranged from 1.17 to 28.25% and with additive effects from 0.01 to 0.03‰ (Table 3_5). Phenotypic variation due to additive × environment effects (PVE (A × E)) was greater than additive effects (PVE (A)) and the LOD score of additive effects (LOD (A)) was less than the LOD score for additive × environments effects (LOD (A × E)) for most of

these QTLs, indicating that these QTLs had strong interaction with environments. The QTL on Gm01 (3,032,794 bp) had a greater LOD score and PVE for additive effect than additive × environment effect (Table 3_5), indicating the stability of this QTL across environments.

QTL × QTL interactions were detected using the Epistatic QTL (ICIM-EPI) method of BIP functional module for $\delta^{15}N$ values from individual environments. Epistatic interactions between different genomic regions were detected in ST16 and FAY17 and across environments (AE) (Table 3_6). The phenotypic variation explained by these interactions ranged from 3.53 to 7.78 %, with the LOD score of these interactions being greater or equal to 3.5. The QTLs involved in epistasis were not identified as additive QTLs. No epistasis was detected in ST17 or CO17.

Identification of Putative Candidate Genes

Of 13 additive loci, five loci (loci 1, 3, 6, 9, and 12) fell in the genomic regions carrying published soybean nodulation genes reported by Schmutz et al. (2010) (Figure 3_2) that are directly involved in nodulation through production of nodulin proteins, nodulation signaling proteins, and different regulatory proteins. For example, *Glyma.01g03470* (Locus 1), *Glyma.04g43090* (Locus 3), *Glyma.08g05370* (Locus 6), *Glyma.14g05690* (Locus 9), and *Glyma.17g08110* (Locus 12) genes (Schmutz et al. 2010) are involved directly in the process of nodulation. The nodulation genes, *Glyma.11g06740, Glyma.13g40400,* and *Glyma.15g05010* were in the genomic regions of epistatic QTLs present on Gm11, Gm13, and Gm15, respectively.

Discussion

A prerequisite for genetic improvement of N_2 fixation is adequate genetic variability for the trait, and understanding the genetic basis of this variability using a dense genetic map would be helpful for implementing the most appropriate strategies in a soybean breeding programs. In this study, we investigated the variability in $\delta^{15}N$ as a proxy for biological N_2 fixation using a population of RILs. In previous studies, NDFA was used for estimation of N_2 fixation in soybean and other legumes (Dhanapal et al. 2015b; Heilig et al. 2017; Ramaekers et al. 2013). In the present study, $\delta^{15}N$ values were directly used as an estimate of N_2 fixation, as a non-nodulating/reference genotype was not planted with the experimental material in order to calculate NDFA values. Steketee et al. (2019) also used $\delta^{15}N$ values in mapping N_2 fixation in a GWAS panel for soybean. Our results found a significant difference ($P<0.01$) between the parents when combined over environments, with PI 416997 having lower $\delta^{15}N$ values than PI 567201D. The low $\delta^{15}N$ value of PI 416997 indicates that the proportion of N from N_2 fixation was greater for PI 416997 compared to PI 567201D.

The RILs had a wide phenotypic range for $\delta^{15}N$ in all environments (ranged 3.43 to 4.55‰) (Table 3_1), but the specific range of $\delta^{15}N$ in soybean is not well defined (Dhanapal et al. 2015b; Ludidi et al. 2007). The presence of transgressive segregants indicates that selection of lines for both low and high $\delta^{15}N$ (along with low and high $\delta^{13}C$) values would be possible. Biplot analysis of $\delta^{15}N$ and $\delta^{13}C$ identified five RILs with favorable (low $\delta^{15}N$ and high $\delta^{13}C$) and unfavorable (high $\delta^{15}N$ and low $\delta^{13}C$) phenotypic combinations for $\delta^{13}C$ and $\delta^{15}N$ (Table 3_3). For the favorable combination of $\delta^{15}N$ and $\delta^{13}C$, RIL.25 and RIL.135 were among the five top RILs in three of the four environments as well as across environments. Comparison of lines with

contrasting $\delta^{15}N$ and $\delta^{13}C$ signatures may be important in characterizing the physiological and interactions between of N_2 fixation and WUE.

Narrow sense heritability of $\delta^{15}N$ across environments was 35% and for individual environments ranged from 8 to 27% in this study. Steketee et al. (2019) reported low broad sense heritability of $\delta^{15}N$ (H=17%) in an association study conducted using a diverse panel of soybean accessions. Similarly, Dhanapal et al. (2015b) found that broad sense heritability of NDFA was low (H=21%) in a GWAS panel. In previous research, several physiological traits linked with N_2 fixation were used for studying the genetic basis of N_2 fixation (Hwang et al. 2013; Ray et al. 2015; Santos et al. 2013; Vieira et al. 2006). Narrow sense heritability was 18% (Vieira et al. 2006) and 33% (Santos et al. 2013) for nodule number, and 27% for weight per nodule (Santos et al. 2013). Broad sense heritability ranged from 33% (Ray et al. 2015) to 73% (Hwang et al. 2013) for shoot ureides and 59% for shoot N concentration (Hwang et al. 2013). Therefore, heritability of traits related to N_2 fixation generally appear to be low to moderate, which is consistent with strong influence of environmental conditions on N_2 fixation (Mastrodomenico and Purcell 2012; Ramaekers et al. 2013; Serraj et al. 1999a; Sinclair et al. 1987).

BLUP values of $\delta^{15}N$ were used in the QTL analysis as it increases the accuracy of detection of QTLs by reducing the impact of environment. QTL analysis for $\delta^{15}N$ by individual environment identified 10 QTLs present on seven chromosomes (Figure 3_2, Table 3_4). No common QTLs were detected in two or more environments, but five QTLs were detected in specific environments that were also found across environments (Table 3_4). In addition to identified additive QTLs by individual environment analysis, one QTL on Gm14 and two QTLs on Gm15 were detected in QTL analysis across environments (Table 3_4). When considering

QTLs detected in individual environments and across environments and overlapping confidence intervals, a total of 13 loci (Figure 3_2, Table 3_4) were identified.

Of the eight QTLs that had significant QTL × environment interaction, six were also detected in individual environment analysis (Table 3_5). Although we did not find any QTLs common among individual environments, the detection of additive QTLs in joint-environment analysis by MET functionality increases the confidence of detection of these QTLs. The low phenotypic variation explained by the additive effect (PVE (A)) compared with the additive × environment effect (PVE (A×E)) indicates a large effect of environment for all QTLs except for the QTL on Gm01 (3,032,794 bp) (Table 3_5). The phenotypic variation explained by these additive QTLs is small (R^2 <10%), which indicates the complex nature of biological N_2 fixation (Santos et al. 2013).

In this study, eight epistatic interactions explained 41% of the phenotypic variation present on different chromosomes (Table 3_6). Further, these epistatic QTLs were not identified as additive QTLs (Table 3_4). An epistatic QTL present on Gm05 was detected in both ST16 and FAY17, but this QTL interacted with different epistatic QTLs in ST16 (Gm06) and FAY17 (Gm02). Also, an epistatic QTL present on Gm03 that was detected in both FAY17 and AE interacted with the QTLs present on Gm15 (FAY17) and Gm14 (AE) (Table 3_6). Our results indicate that additive QTLs, QTL × environment interactions, and epistasis were important factors influencing the variations in $\delta^{15}N$ in soybean.

The presence of QTLs associated with N_2 fixation and other N-related physiological traits in the genomic regions of identified $\delta^{15}N$ loci were screened in Soybase (www.soybase.org). Loci 3, 7, and 12 co-localized with previously identified QTLs for nodule-related traits such as nodule size (Hwang et al. 2013), nodule number (Shi et al. 2018), and nodule weight (Hwang et

al. 2013; Shi et al. 2018). Locus 4 and Locus 10 coincided with ureide QTLs (Ray et al. 2015), and Locus 1 with a shoot N QTL (Dhanapal et al. 2015b) identified in a GWAS panel. Also, Loci 1, 7, and 12 co-localized with QTLs for $\delta^{15}N$ identified in an association study (Steketee et al. 2019). Among these loci, Loci 1, 3, and 12 were located in candidate genes involved in the nodulation process. Gene *N36 (Glyma.01g03470),* underlying Locus 1, is an early nodulin gene involved in initiation of nodule development (Kouchi and Hata 1993). This gene also plays an important role in translocation of photosynthate into nodule tissue. *Glyma.04g43090*, underlying Locus 3, encodes for a nodulation signaling proteins (NSP2), which are Nod-factor activated transcriptional factors required for nodulation initiation (Murakami et al. 2006). Similarly, the *N315* gene (*Glyma.17g08110*, underlying Locus 12) is expressed at the time of nodule emergence and plays a unique role in nodule formation (Kouchi and Hata 1993).

The $\delta^{15}N$ QTLs at Loci 4 and 12 also overlapped with the genomic regions associated with $\delta^{13}C$ identified in the same population (Bazzer et al. 2020). Additionally, Loci 1, 3, 8, and 12 coincided with $\delta^{13}C$ QTLs identified in GWAS mapping (Dhanapal et al. 2015a; Kaler et al. 2017). The greater $\delta^{13}C$ may indirectly lead to high N_2 fixation under drought due to a greater supply of carbohydrates to nodules. The co-localized QTLs with genomic regions providing drought tolerance may also increase N_2 fixation through reducing drought sensitivity of N_2 fixation.

Similarly, epistatic QTLs (except QTLs present on Gm02) coincided with QTLs reported for nodule related traits (Hwang et al. 2013; Nicolás et al. 2006; Santos et al. 2013; Shi et al. 2018), ureide concentration (Ray et al. 2015), NDFA (Dhanapal et al. 2015b), and $\delta^{15}N$ (Stekettee et al. 2019). Epistatic QTLs on Gm11, Gm13, and Gm15 were in the genomic regions carrying nodulation genes *Glyma.11g06740*, *Glyma.13g40400*, and *Glyma.15g05010,*

respectively (Schmutz et al. 2010). Epistatic QTLs present on Gm06, Gm11, Gm15, and Gm 17 coincided with additive QTLs for $\delta^{13}C$ (Bazzer et al. 2020). In addition, epistatic QTLs also co-localized with $\delta^{13}C$ QTLs found in various studies (Bazzer et al. 2019; Dhanapal et al. 2015a; Kaler et al. 2017).

The co-localization of identified additive and epistatic $\delta^{15}N$ QTLs with N_2 fixation and WUE related traits supports the evidence that genetic links exist between these traits in soybean. This is the first study on QTLs analysis for $\delta^{15}N$ using a biparental population in soybean. The QTLs identified in this study as being associated with N_2 fixation are supported by the presence of genes directly involved in nodulation in soybean. These QTLs could be of great interest to breeders for developing soybean varieties with higher yields through optimization of N_2 fixation

Conclusion

In the present study, a mapping population of 196 F_6-derived RILs was evaluated in multiple environments to understand the genetic basis of $\delta^{15}N$. A wide range of $\delta^{15}N$ values were observed in all environments and narrow sense heritability of $\delta^{15}N$ was low, indicating significant effects of environment on $\delta^{15}N$. Transgressive segregants for $\delta^{15}N$ were observed among the RILs, indicating that it is possible to create, from the specific parents used, extreme genotypes with lower and higher $\delta^{15}N$ values than observed in either parent. Both parents contributed to the higher and lower values observed. The extreme genotypes created in this population may be useful in future studies to better assess the physiological mechanisms of N_2 fixation. QTL analysis by environment identified 10 additive QTLs present on seven chromosomes that individually explained less than 10% of the observed phenotypic variation. Considering QTLs identified across environments, along with individual environments, there were 13 loci for $\delta^{15}N$ based on their overlapping confidence intervals. A lack of consistency of QTL detection was found as QTLs identified in an individual environment did not overlap with QTLs in any of the other environments. Co-localization of $\delta^{15}N$ QTLs with QTLs for important agronomic and physiological traits related to N_2 fixation, and the presence of reported nodulation genes associated with these QTLs, increases the likelihood that the newly identified regions are associated with N_2 fixation. Further studies are needed for fine mapping these QTLs to understand their expression and to determine how they interact with putative candidate genes.

Table 3_1. Phenotypic variation for $\delta^{15}N$ (‰) in the parents (PI 416997 and PI 567201D) and RIL population grown in four environments: Stoneville in 2016 (ST16), Stoneville in 2017 (ST17), Fayetteville in 2017 (FAY17), and Columbia in 2017 (CO17).

Environment	Parent		RIL Population			
	PI 416997	PI 567201D	Mean±SD	Range	Skewness	Kurtosis
ST16	1.99	2.78	2.79±0.70	3.96	0.17	0.01
ST17	3.15	3.65	4.20±0.84	4.08	0.19	-0.11
FAY17	2.25	4.41	3.01±0.68	3.43	0.14	-0.35
CO17	1.07	3.12	2.02±0.88	4.55	0.45	0.06

Table 3_2. Analysis of variance (ANOVA) for $\delta^{15}N$ in the RIL population along with parents evaluated in four environments (ST16, ST17, FAY17, and CO17).

Effect	DF	*P*-value	h^2 (%)
Genotypes (G)	195	<0.0001	35
Environments (E)	3	0.016	
G × E	585	0.267	

h^2, Narrow sense heritability

Table 3_3. RILs with high $\delta^{13}C$ and low $\delta^{15}N$ phenotypic values or RILs with low $\delta^{13}C$ and high $\delta^{15}N$ phenotypic values in individual environments (ST16: Stoneville in 2016, ST17: Stoneville in 2017, FAY17: Fayetteville in 2017, and CO17: Columbia in 2017) and across environments (AE). Values in parentheses are the phenotypic values of $\delta^{13}C$ and $\delta^{15}N$ for that RIL. Bold values indicate the RILs appeared within multiple individual environments as well as across environments.

ST16	ST17	CO17	FAY17	AE
RILs with high $\delta^{13}C$ (>WUE and > N_2 fixation)				
RIL.14 (-28.85, 1.89)	RIL.25 (-27.70, 3.39)	RIL.14 (-27.54, 0.25)	RIL.25 (-26.56, 2.43)	**RIL.14 (-28.02, 2.02)**
RIL.25 (-28.38, 2.18)	RIL.75 (-28.02, 3.31)	RIL.42 (-27.27, 1.02)	RIL.69 (-26.93, 1.98)	**RIL.25 (-27.34, 2.55)**
RIL.132 (-29.02, 1.79)	RIL.120 (-28.02, 3.63)	RIL.75 (-27.17, 1.00)	RIL.85 (-26.94, 1.93)	**RIL.75 (-27.65, 2.49)**
RIL.193 (-28.75, 2.13)	RIL.135 (-27.99, 3.03)	RIL.85 (-27.44, 1.50)	RIL.135 (-26.69, 2.52)	**RIL.135 (-27.59, 2.47)**
RIL.209 (-28.87, 2.30)	RIL.204 (-28.10, 3.19)	RIL.135 (-27.18, 1.13)	RIL.151 (-26.92, 1.71)	RIL.204 (-27.72, 2.86)
RILs with low $\delta^{13}C$ and high $\delta^{15}N$ (< WUE and < N_2 fixation)				
RIL.12 (-29.96, 3.77)	RIL.24 (-30.14, 6.25)	RIL.15 (-28.66, 3.25)	RIL.92 (-29.06, 3.73)	**RIL.112 (-29.18, 3.87)**
RIL.24 (-30.19, 3.19)	RIL.50 (-29.97, 4.91)	RIL.112 (-28.89, 3.55)	RIL.127 (-29.29, 4.23)	RIL.154 (-29.17, 3.81)
RIL.86 (-30.17, 3.59)	RIL.52 (-29.77, 5.09)	RIL.127 (-28.61, 3.60)	RIL.129 (-28.99, 3.79)	RIL.177 (-29.12, 3.81)
RIL.112 (-30.14, 3.28)	RIL.110 (-29.86, 4.76)	RIL.195 (-28.55, 3.97)	RIL.161 (-29.06, 3.76)	RIL.203 (-29.03, 4.24)
RIL.114 (-30.02, 4.32)	RIL.161 (-29.86, 5.77)	RIL.205 (-28.51, 4.80)	RIL.213 (-29.03, 3.82)	RIL.205 (-29.10, 4.01)

Table 3_4. Quantitative trait loci (QTLs) associated with $\delta^{15}N$ detected in individual environments (ST16, ST17, FAY17, and CO17) and across environment (AE) in the RIL population of PI 416997 and PI 567201D using BIP functional module of ICIM mapping.

Locus[a]	Chrom.[b]	Env[c]	Nearest SNP position (bp)[d]	LOD[e]	R^{2f}	Add[g]	Favorable allele[h]
1	Gm01	ST16	3,284,926	10.56	3.14	-0.08	PI567201D
		AE		18.36	7.93	-0.09	
2	Gm01	ST16	3,955,325	6.08	1.72	0.06	PI416997
		AE	3,605,601	12.57	5.16	0.07	
3	Gm04	ST17	49,247,258	6.25	9.34	0.06	PI416997
		AE	49,618,681	4.87	1.83	0.04	
4	Gm07	FAY17	15,382,101	7.15	6.80	-0.03	PI567201D
		AE	15,546,393	8.31	3.21	-0.05	
5	Gm08	ST16	2,681,851	24.21	9.10	-0.13	PI567201D
6	Gm08	ST16	2,960,542	18.44	6.26	0.11	PI416997
7	Gm10	FAY17	41,413,995	5.11	4.71	0.02	PI416997
8	Gm13	FAY17	27,584,266	5.98	6.88	0.03	PI416997
		AE	30,216,959	28.57	14.39	0.12	
9	Gm14	AE	4,448,348	4.37	1.63	-0.04	PI567201D
10	Gm15	AE	17,072,416	6.17	2.35	-0.05	PI567201D
11	Gm15	AE	23,341,546	12.63	5.22	0.07	PI416997
12	Gm17	ST17	7,961,686	4.29	7.64	-0.06	PI567201D
13	Gm17	FAY17	40,829,268	3.40	3.02	-0.02	PI567201D

[a] Closely spaced putative QTL falling within the same flanking markers were consider as one locus
[b] *Glycine max* chromosome on which putative QTL is present
[c] Environment in which a significant QTL was identified

[d] Nearest marker position in base pairs (bp) on specific chromosome
[e] Log-likelihood at QTL peak position
[f] Phenotypic variation explained by putative QTL
[g] Additive effect of the QTL
[h] Allele that decreases $\delta^{15}N$ value

Table 3_5. QTLs showing QTL × environment interaction in four environments detected using MET functional module of ICIM mapping.

Locus[a]	Chrom.[b]	Nearest SNP position[c]	LOD[d]	LOD (A)[e]	LOD (A × E)[f]	PVE[g]	PVE (A)[h]	PVE (A × E)[i]	Add[j]
1	Gm01	3,032,794	13.17	11.42	1.75	12.25	6.43	5.83	-0.03
2	Gm01	3,955,325	6.18	2.11	4.07	5.75	1.21	4.53	0.01
3	Gm04	49,247,258	6.86	4.38	2.49	7.11	2.46	4.65	0.02
4	Gm07	15,382,101	7.25	0.90	6.35	1.40	0.50	0.90	-0.01
5	Gm08	2,681,851	24.21	13.82	10.38	28.25	8.06	20.19	-0.03
6	Gm08	2,960,542	18.44	7.87	10.57	19.68	4.45	15.23	0.03
7	Gm10	41,413,995	7.28	1.97	5.31	2.94	1.11	1.82	0.01
-[*]	Gm13	27,584,266	6.03	0.49	5.54	1.17	0.25	0.92	0.01

[a] Locus number assigned is the same as the locus number used for QTLs identified by BIP functionality of ICIM Mapping in Table 4
[*] New loci, not identified earlier in ICIM-ADD analysis
[b] *Glycine max* chromosome on which putative QTL is present
[c] Nearest marker position in base pairs (bp) on specific chromosome
[d] Total LOD score for QTL × environment interaction
[e] LOD score for additive effects
[f] LOD score for additive by environment effects
[g] Total phenotypic variance explained by QTL × environment interaction
[h] Phenotypic variance explained by additive effects
[i] Phenotypic variance explained by additive by environments effects.
[j] Additive effect explained by the QTL

Table 3_6. Epistatic QTLs identified for $\delta^{15}N$ in the RIL population of PI 416997 × PI 567201D by the ICIM-EPI method of BIP functional module.

Env.[a]	Chrom. 1[b]	Pos. 1[c]	Chrom. 2[d]	Pos. 2[e]	PVE[f]	Add×Add[g]
ST16	1	52,051,133--52,386,863	15	1,550,336--2,934,524	4.13	0.06
	5	41,542,487--36,175,591	6	12,165,433--12,361,723	7.78	0.08
	12	4,858,604--6,236,974	12	7,201,220--7,201,391	4.45	-0.08
	13	35,909,641--40,053,812	16	33,339,297--33,694,437	6.08	-0.07
FAY17	3	43,610,628--44,233,282	15	7,684,532--8,529,280	3.53	-0.03
	5	41,542,487--36,175,591	2	4,726,469--5,730,965	4.44	0.03
AE	3	41,518,436--43,565,653	14	7,259,215--8,645,366	4.60	0.05
	6	14,794,344--18,148,587	11	585,930--1,088,794	6.17	-0.07

[a] Environment in which a significant QTL × QTL interaction was identified
[b] *Glycine max* chromosome on which first QTL involved in epistasis present
[c] Position of flanking markers of first QTL in epistasis on specific chromosome
[d] *Glycine max* chromosome on which second QTL involved in epistasis present
[e] Position of flanking markers of second QTL in epistasis on specific chromosome
[f] Phenotypic variation explained by epistasis
[g] Additive by additive epistatic effect

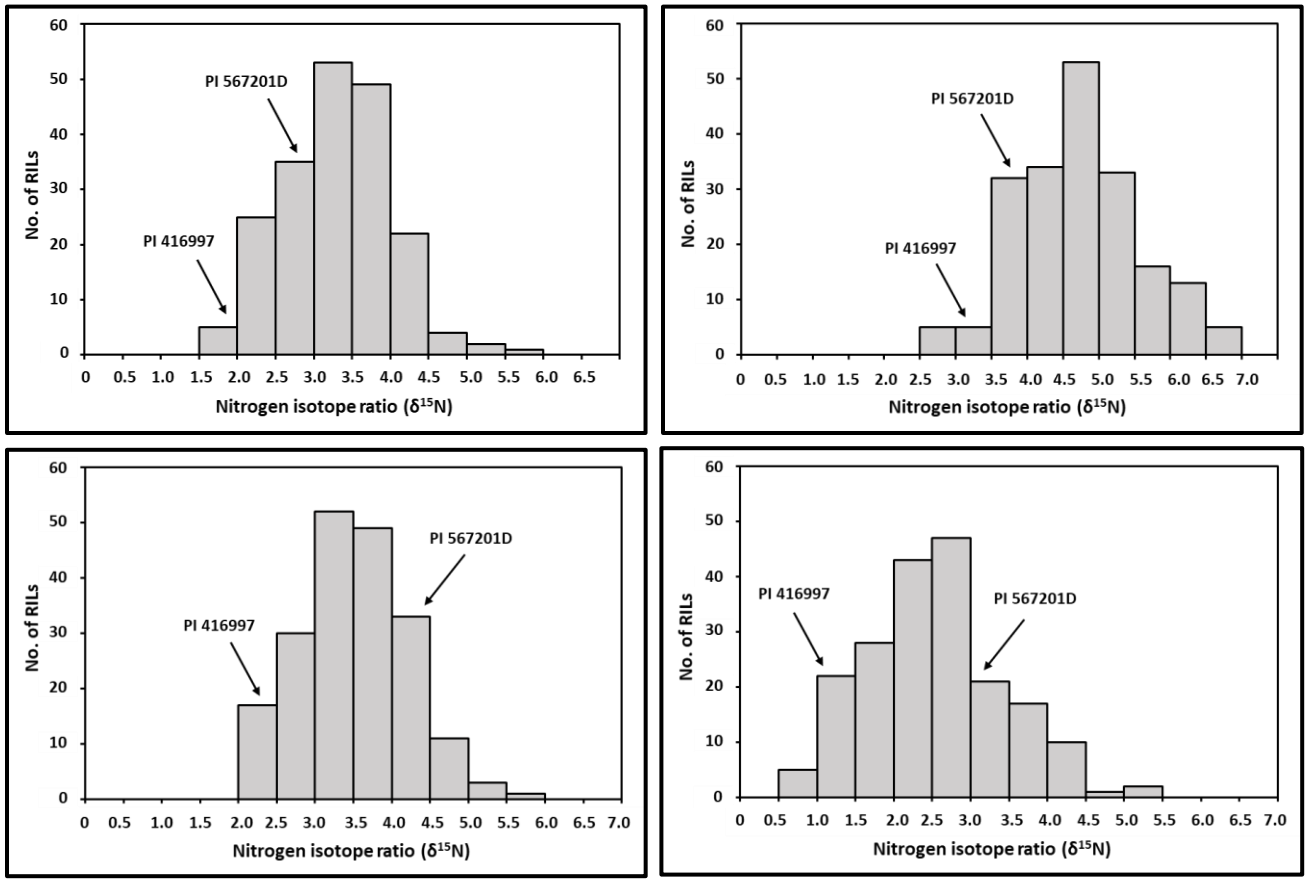

Figure 3_1. Distribution of $\delta^{15}N$ among recombinant inbred lines and parental genotypes at Stoneville, MS in 2016 (a), Stoneville, MS in 2017 (b), Fayetteville, AR in 2017 (c), and Columbia, MO in 2017 (d).

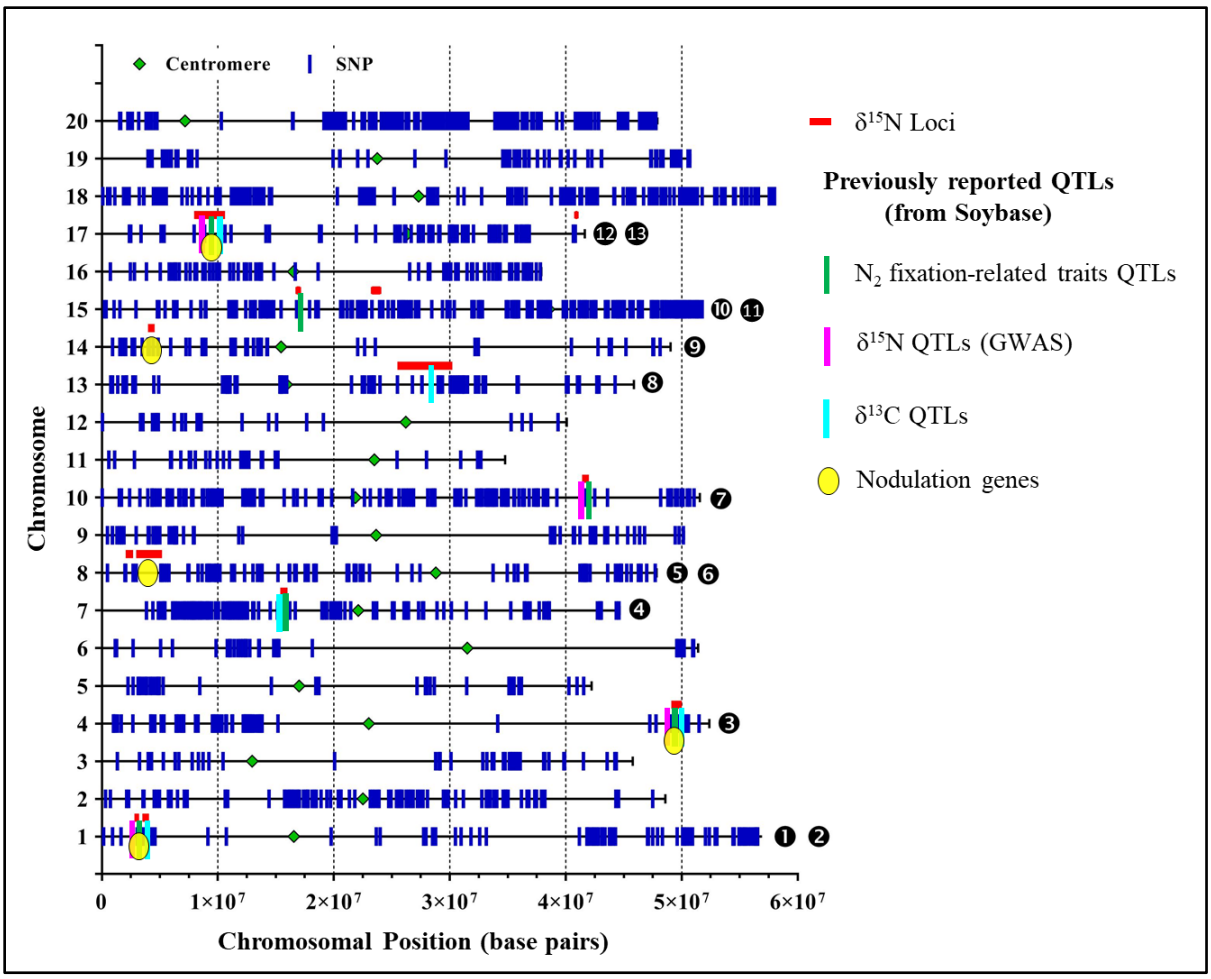

Figure 3_2. Physical position of SNPs on soybean chromosomes and position of loci (horizontal red bars) associated with $\delta^{15}N$ identified by ICIM mapping for additive QTLs. The numbers in the black circles represent the loci number on a specific chromosome. Green, pink, and light-blue vertical bars indicate QTLs found at the same positions in previous studies, and yellow circles indicate the position of nodulation genes.

References

Adams MA, Turnbull TL, Sprent JI, Buchmann N (2016) Legumes are different: leaf nitrogen, photosynthesis, and water use efficiency. P Natl Acad Sci 113:4098-4103

Andrews M, Lea PJ (2013) Our nitrogen "footprint": the need for increased crop nitrogen use efficiency. Ann Appl Biol 163:165-169

Barrie A, Debney S, Workman CT, Pullan C (1995) Recent developments in high productivity stable isotope analysis. In: International symposium on nuclear and related techniques in soil-plant studies for sustainable agricultural and environmental preservation. Vienna (Austria), 17-21 Oct 1994. IAEA TECDOC Vienna, Austria, pp 29-61

Bazzer SK, Kaler AS, Ray JD, Smith JR, Fritschi FB, Purcell LC (2020) Identification of quantitative trait loci for carbon isotope ratio ($\delta^{13}C$) in a recombinant inbred population of soybean. Theor Appl Genet 122:1-15, https://doi.org/10.1007/s00122-020-03586-0

Bazzer SK, Kaler AS, King CA, Ray JD, Hwang S, Purcell LC (2019) Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean. Crop Sci https://doi.org/10.1002/csc2.20240

Bergersen FT, Brockwell J, Gault RR, Morthorpe L, Peoples MB, Turner GL (1989) Effects of available soil nitrogen and rates of inoculation on nitrogen fixation by irrigated soybeans and evaluation of delta 15N methods for measurement. Aust J Agric Res 40:763-780

Bondari K (2003) Statistical analysis of genotype × environment interaction in agricultural research. In: Paper SD15, SESUG: The Proceedings of the South East SAS Users Group, St. Pete Beach, FL

Dhanapal AP, Ray JD, Singh SK, Hoyos-Villegas V, Smith JR, Purcell LC, King A, Cregan PB, Song Q, Fritschi FB (2015a) Genome-wide association study (GWAS) of carbon isotope ratio ($\delta^{13}C$) in diverse soybean [*Glycine max* (L.) Merr.] genotypes. Theor Appl Genet 128:73-91

Dhanapal AP, Ray JD, Singh SK, Hoyos-Villegas V, Smith JR, Purcell LC, King A, Fritschi FB (2015b) Genome-wide association analysis of diverse soybean genotypes reveals novel markers for nitrogen traits. Plant Genome 8:1-15

Djekoun A, Planchon C (1991) Water status effect on dinitrogen fixation and photosynthesis in soybean. Agron J 83:316-322

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics 142: 285-294

Doughton JA, Saffigna PG, Vallis I, Mayer RJ (1995) Nitrogen fixation in chickpea. II. Comparison of 15N enrichment and 15N natural abundance methods for estimating nitrogen fixation. Aust J Agric Res 45:225-236

Durand JL, Sheehy JE, Minchin FR (1987) Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. J Exp Bot 38:311-321

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust J Plant Physiol 11:539-552

Fehr WR, Caviness CE (1977) Stages of soybean development. Spec. Rep. 80. Iowa State Univ. Coop. Ext. Serv., Ames, IA

Fried M, Broeshart H (1975) An independent measurement of the amount of nitrogen fixed by legume crops. Plant Soil 43:707-711

Fried M, Middelboe V (1977) Measurement of amount of nitrogen fixed by a legume crop. Plant and Soil 47:713-715

Giller KE (2001) Nitrogen fixation in tropical cropping systems, 2 edn. CABI, New York, USA

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43:1185-1207

Holland JB, Nyquist WE, Cervantes-Martinez CT (2003) Estimating and interpreting heritability for plant breeding: an update. Plant Breed Rev 22:9-112

Hwang S, King CA, Davies MK, Ray JD, Cregan PB, Purcell LC (2013) QTL analysis of shoot ureide and nitrogen concentrations in soybean [*Glycine max* (L.) Merr.]. Crop Sci 53:2421-2433

Hwang S, Ray JD, Cregan PB, King A, Davies MK, Purcell LC (2014) Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* L. [Merr.]). Euphytica 195:419-434

Heilig JA, Beaver JS, Wright EM, Song Q, Kelly JD (2017) QTL Analysis of symbiotic nitrogen fixation in a black bean population. Crop Sci 57:118-129

Jensen E, Hauggaard-Nielsen H (2003) How can increased use of biological N_2 fixation in agriculture benefit the environment? Plant Soil 252:177-186

Kaler AS, Dhanapal AP, Ray JD, King CA, Fritschi FB, Purcell LC (2017) Genome-wide association mapping of carbon isotope and oxygen isotope ratios in diverse soybean genotypes. Crop Sci 57:1-16

Kohl DH, Shearer GB (1981) The use of soils lightly enriched in ^{15}N to screen for N_2-fixing activity. Plant Soil 60:487-489

Kouchi H, Hata S (1993) Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol Gen Genet 238:106-119

Kuo T, Boersma L (1971) Soil water suction and root temperature effects on nitrogen fixation in soybeans. Agron J 63:901-904

Letolle R (1980) Nitrogen-15 in the natural environment. In: Fritz P, Fontes JC (eds) Handbook of Environmental Isotope Geochemistry. Elsevier, Amsterdam, pp 407-433

Li HH, Ye GY, Wang JK (2007) A modified algorithm for the improvement of composite interval mapping. Genetics 175:361-374

Littell RC, Milliken GA, Stroup WW, Wolfinger RD (1996) SAS system for mixed models. SAS Inst. Inc., Cary, NC

Ludidi NN, Pellny TK, Kiddle G, Dutilleul C, Groten K, Van Heerden PD, Dut S, Powers SJ, Römer P, Foyer CH (2007) Genetic variation in pea (*Pisum sativum* L.) demonstrates the importance of root but not shoot C/N ratios in the control of plant morphology and reveals a unique relationship between shoot length and nodulation intensity. Plant Cell Environ 10:1256-1268

Márquez-García B, Shaw D, Cooper JW, Karpinska B, Quain MD, Makgopa EM, Kunert K, Foyer CH (2015) Redox markers for drought-induced nodule senescence, a process occurring after drought-induced senescence of the lowest leaves in soybean (*Glycine max*). Ann Bot 116(4):497-510

Mastrodomenico AT, Purcell LC (2012) Soybean nitrogen fixation and nitrogen remobilization during reproductive development. Crop Sci 52:1281-1289

Meng L, Li H, Zhang L, Wang J (2015) QTL IciMapping: Integrated software for genetic linkage map construction and quantitative trait locus mapping in biparental populations. Crop J 3:269-283

Murakami Y, Miwa H, Imaizumi-Anraku H, Kouchi H, Downie JA, Kawaguchi M, Kawasaki S (2006) Positional cloning identifies *Lotus japonicus* NSP2, a putative transcription factor of the GRAS family, required for NIN and ENOD40 gene expression in nodule initiation. DNA Res 13:255-265

Nicolás MF, Hungria M, Arias CAA (2006) Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars. Field Crops Res 95:355-366

Peoples MB, Faizah AW, Rerkasem B, Herridge DF (1989) Methods for evaluating nitrogen fixation by nodulated legumes in the field. ACIAR Monograph No. 11, pp 76

Peoples MB, Boddy RM, Herridge DF (2002) Quantification of nitrogen fixation. In: Leigh GJ (ed) Nitrogen fixation at the millennium. Elsevier Science, Amsterdam, pp 357-389

Piepho HP, Möhring J, Melchinger AE, Büchse A (2008) BLUP for phenotypic selection in plant breeding and variety testing. Euphytica 161:209-228

Purcell LC (2009) Physiological responses of N2 fixation to drought and selecting genotypes for improved N2 fixation. In: Krishnan HB and Emerich DW (eds) Nitrogen fixation in crop production. Agron Monogr 52 ASA CSSA SSSA, Madison, WI, pp 211-238

Ramaekers L, Galeano CH, Garzon N, Vanderleyden J, Blair MW (2013) Identifying quantitative trait loci for symbiotic nitrogen fixation capacity and related traits in common bean. Mol Breed 31:163-180

Ray JD, Dhanapal AP, Singh SK, Hoyos-Villegas V, Smith JR, Purcell LC, King CA, Boykin D, Cregan PB, Song Q, Fritschi FB (2015) Genome-wide association study of ureide concentration in diverse maturity group IV soybean [*Glycine max* (L.) Merr.] accessions. G3 5(11):2391-2403

Rebetzke GJ, Ellis MH, Bonnett DG, Richards RA (2007) Molecular mapping of genes for coleoptile growth in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 114:1173-1183

Reif JC, Maurer HP, Korzun V, Ebmeyer E, Miedaner T, Wurschum T (2011) Mapping QTL with main and epistatic effects underlying grain yield and heading time in soft winter wheat. Theor Appl Genet 123:283-292

Santos MA, Geraldi IO, Garcia AAF, Bortolatto N, Schiavon A, Hungria M (2013) Mapping of QTLs associated with biological nitrogen fixation traits in soybean. Hereditas 150:17-25

SAS Institute. 2013. The SAS system for Windows. Release 9.4. SAS Inst., Cary, NC

Schmutz J, Cannon S, Schlueter J, Ma J, Mitros T, Nelson W, Hyten D, Song Q, Thelen, J, Cheng J, Xu D, Hellsten Uffe, May G, Yu Y, Sakurai T, Umezawa T, Bhattacharyya M, Sandhu D, Valliyodan B, Jackson S (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178-183

Serraj R, Sinclair TR, Purcell LC (1999a) Symbiotic N_2 fixation response to drought. J Exp Bot 50:143-155

Serraj R, Vadez V, Denison RF, Sinclair TR (1999b) Involvement of ureides in nitrogen fixation inhibition in soybean. Plant Physiol 119:289-296

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591-611

Shearer G, Kohl D (1986) N_2 fixation in field settings: estimations based on natural ^{15}N abundance. Funct Plant Biol 13:699-756

Shi X, Yan L, Yang CY, Yan WW, Moseley DO, Wang T, Liu BQ, Di R, Chen PY, Zhang MC (2018) Identification of a major quantitative trait locus underlying salt tolerance in 'Jidou 12' soybean cultivar. BMC Res Notes 11:95

Sinclair TR, Serraj R (1995) Legume nitrogen fixation and drought. Nature 378:344

Sinclair TR, Muchow RC, Bennett JM, Hammond LC (1987) Relative sensitivity of nitrogen and biomass accumulation to drought in field-grown soybean. Agron J 79:986-991

Steketee CJ, Sinclair TR, Riar M, Schapaugh WT, Li Z (2019) Unraveling the genetic architecture for carbon and nitrogen related traits and leaf hydraulic conductance in soybean using genome-wide association analyses. BMC Genomics 20:811

Strodtman KN, Emerich DW (2009) Nodule metabolism. In: Emerich DW, Krishnan HB (eds) Nitrogen fixation in crop production. Crop Science Society of America, Madison, pp 95-124

Tanya P, Srinives P, Toojinda T, Vanavichit A, Lee S-H (2005) Identification of SSR markers associated with N_2 fixation components in soybean [*Glycine max* (L.) Merr.]. Korean J Genet 27:351-359

Vieira A, de Oliveira A, Soares T, Schuster I, Piovesan N, Martinez C, de Barros E, Moreira M (2006) Use of the QTL approach to the study of soybean trait relationships in two populations of recombinant inbred lines at the F7 and F8 generations. Braz J Plant Physiol 18:281-290

Weaver RW, Danso SKA (1994) Dinitrogen fixation. In: Weaver RW, Angle S, Bottomley P, Bezdicek D, Tabatabai A, Wollum A (eds) Methods of soil analysis. Part 2, Microbiological and biochemical properties. Soil Science Society of America, Madison, WI, pp 1019-1045

CHAPTER IV

Mapping and Confirmation of Quantitative Trait Loci (QTLs) Associated with Carbon Isotope Ratio ($\delta^{13}C$) in Soybean.

Abstract

Insufficient moisture availability often limits soybean [*Glycine max* (L.) Merr.] yield. Carbon isotope ratio ($\delta^{13}C$) provides an integrated measure of water use efficieny in C_3 plants due to its substantial genetic variance, high heritability, and small genotype × environment interaction (G×E). The objective of this study was to identify quantitative trait loci (QTLs) associated with $\delta^{13}C$ using a recombinant inbred line population derived from a cross between KS4895 and Jackson. The field experiment was conducted in five environments to evaluate $\delta^{13}C$ under rainfed and irrigated conditions. Analysis of variance of $\delta^{13}C$ averaged over environment and irrigation treatment showed significant effects of genotype (G), environment (E), and G × E interactions. Heritability of $\delta^{13}C$ in different environments and irrigation treatments ranged from 66 to 79%. Averaged over environments and irrigation treatments heritability was 83%. A total of 24 QTLs associated with $\delta^{13}C$ were identified and clustered in nine genomic regions on seven chromosomes. The QTL clusters on Gm05 (1), Gm06 (2) and Gm20 (1) were detected across different environments and irrigation regimes. Collectively, these four QTL clusters accounted for 55% of the phenotypic variation in $\delta^{13}C$. The QTLs on Gm06 and Gm20 also showed additive × additive epistasis that contributed approximately 4.2% to the total phenotypic variation. Several identified $\delta^{13}C$ QTLs overlapped with QTLs associated with other physiological traits related to plant water status, biological nitrogen fixation, and plant morphology. The identified genomic regions may be an important resource in genomic selection studies to improve drought tolerance in soybean.

Introduction

Soybean [*Glycine max* (L.) Merr.] production and yield stability are often limited by drought because most of the soybean hectarage in the Inited States is grown in areas of erratic rainfall (Dogan et al., 2007). The nature of drought is complex and is caused by insufficient rainfall to replenish the soil moisture supply to the point that plant growth, development and yield are impacted. Yield improvements in rainfed environments could be achieved by identifying secondary traits contributing to drought tolerance. Traits associated with plant water status and drought tolerance include: carbon isotope ratio (Bai and Purcell, 2018a; Dhanapal et al., 2015; Kaler et al., 2017a, 2018a), canopy wilting (Charlson et al., 2009; Hwang et al., 2015b; Kaler et al., 2017b; Sloane et al., 1990), canopy temperature (Bai and Purcell, 2018b; Blum, 1988; Kaler et al., 2018b; Ludlow and Muchow, 1990; Pinto et al., 2010), canopy coverage (Kaler et al., 2018c), leaf relative water content (Babu et al., 2003), and rooting depth (Purcell and Specht, 2004).

Water use efficiency (WUE) is an important physiological trait associated with drought tolerance and is defined as the amount of dry matter produced per unit of water transpired. Thus, WUE is an important yield-determining factor due to its positive association with total biomass yield (Chen et al., 2011; Passioura, 1996; Wright, 1996). Direct measurement of WUE depends either on extensive leaf gas-exchange data or long-term measures of plant water consumption and biomass production (Manavalan et al., 2009).

To avoid the difficulty of measuring WUE of field grown plants, Farquhar et al. (1982) and Farquhar and Richards (1984) proposed the use of differences in carbon isotopes of ^{13}C and ^{12}C in plant tissues. The carbon isotope ratio between $^{13}CO_2$ and $^{12}CO_2$ ($\delta^{13}C$) reflects the isotopic fractionation that occurs mainly at the initial carboxylation step in the photosynthetic

CO_2 fixation that is catalyzed by Rubisco (Xu et al., 2009). In this process, plants discriminate against the heavier isotope of carbon (^{13}C) over the lighter isotope of carbon (^{12}C) during carbon fixation. The extent of this carbon isotope discrimination is related to the ratio of internal to external concentration of CO_2 (C_i/C_a), which is further controlled by stomatal conductance and photosynthetic capacity (Brugnoli and Farquhar, 2000; Farquhar et al., 1989).

Therefore, $\delta^{13}C$ is negatively correlated with C_i/C_a and has a positive relationship with WUE (Ehdaie et al., 1991; Farquhar and Richards, 1984; Johnson and Bassett, 1991). Carbon isotope composition can also be expressed as carbon isotope discrimination ($\Delta^{13}C$). While $\delta^{13}C$ is positively related to WUE, $\Delta^{13}C$ is negatively related to WUE (Condon et al., 1987; Farquhar and Richards, 1984). Several greenhouse and field experiments have documented close associations of both $\Delta^{13}C$ and $\delta^{13}C$ with WUE in many crop species including barley (*Hordeum vulgare*) (Çagˇirgan et al., 2005), bread wheat (*Triticum aestivum*) (Condon et al., 1987; Read et al., 1991; Ehdaie et al., 1991), cotton (*Gossypium hirsutum*) (Brugnoli et al., 1988), cowpea (*Vigna unguiculata*) (Hall et al., 1990), durum wheat (*Triticum durum*) (Araus et al., 1998), peanut (*Arachis hypogaea*) (Hubick et al., 1986), and soybean (White et al., 1996). The major advantage of using $\delta^{13}C$ or $\Delta^{13}C$ in selection is its substantial genetic variance, high heritability, and small genotype by environment interaction (G×E) in dryland areas (Hall et al., 1990; Kaler et al., 2017a, 2018a; Merah et al., 2001; Richards et al., 1999).

Identification of quantitative trait loci (QTLs) associated with $\delta^{13}C$ (or $\Delta^{13}C$) in replicated, segregating populations under different environments allows the variance to be partitioned into genotype and environment components together with an estimate of the number of loci controlling the trait (Kearsey and Hyne, 1994). The first QTLs identified for $\Delta^{13}C$ were reported in tomato (*Solanum lycopersicum*) by Martin et al. (1989), and subsequently QTLs for

$\Delta^{13}C$ were reported in *Arabidopsis thaliana* (Hausmann et al., 2005; Juenger et al., 2005), barley (Diab et al., 2004; Ellis et al., 1997, 2002; Teulat et al., 2002), cotton (Saranga et al., 2001), rice (*Oryza sativa*) (Laza et al., 2006; Takai et al., 2006; This et al., 2010; Xu et al., 2009), soybean (Dhanapal et al., 2015; Kaler et al., 2017a; Specht et al., 2001), and wheat (Peleg et al., 2009; Rebetzke et al., 2008).

Mian et al. (1996) identified soybean QTLs associated with WUE and leaf ash using a F_4-derived population developed from a cross between Young (PI 508266) and PI 416937. A total of four and six independent QTLs were associated with WUE and leaf ash, respectively. Mian et al. (1998) identified two independent markers associated with WUE in a F_2- derived population from a cross of S100 × Tokyo. Dhanapal et al. (2015) and Kaler et al. (2017a) used genome-wide association mapping (GWAM) to identify single nucleotide polymorphism (SNPs) associated with $\delta^{13}C$ on a panel of 373 diverse soybean accessions. Genome-wide association analysis identified 39 SNPs associated with $\delta^{13}C$, which likely tagged 21 different loci (Dhanapal et al., 2015). Subsequently Kaler et al. (2017a) identified 54 SNPs for $\delta^{13}C$ that likely tagged 46 loci.

To improve drought tolerance in soybean, more studies are needed to identify and confirm QTLs associated with drought tolerance, to map new QTL(s)/ gene(s), and to determine gene action under drought. High-density genetic maps and confirmed QTLs/genes, which are screened across the various environments and across genetic backgrounds, are the most important criteria for developing drought-resistant soybean (Manavalan et al., 2009). The next steps are to confirm QTLs identified previously in different genetic backgrounds, in different environments, and to evaluate the efficacy of identified QTLs in conferring drought tolerant/resistant genotypes.

In the present research, recombinant inbred lines (RILs) derived from a cross between 'KS4895' (PI 595081) (Schapaugh and Dille, 1998) with 'Jackson' (PI 548657) (Johnson HW, 1958) were used to: identify the genomic regions associated with the $\delta^{13}C$ under rainfed (RF) and irrigated (IRR) conditions, identify the common QTL regions across environments, confirm QTLs identified previously by GWAM, and to find potential markers associated with these identified genomic regions which can be used to improve WUE in soybean.

Materials and Methods

Field Experiments

A population of 168 F_5- derived RILs (Hwang et al., 2015b) from a cross between KS4895 and Jackson was used to identify the genomic regions (QTLs) associated with $\delta^{13}C$. KS4895 is a Maturity Group (MG) IV cultivar that was developed by the Kansas Agricultural Experiment Station, and Jackson is a MG VII cultivar developed by the North Carolina Agricultural Experiment Station and the USDA-ARS. Beginning at the F_2 generation, individual plants were selected to have a maturity similar to that of 'Hutcheson' (PI 518664, Buss et al., 1988), which has a relative maturity of 5.4. The full range of maturity differences of plants at the F_2 generation was approximately 20 d, but after selecting for similar maturity at each generation the F_5-derived RILs had a maturity range of approximately 3 d with an average relative maturity of 5.5. These two genotypes were chosen as parents because previous research reported that Jackson was more tolerant than KS4895 with regards to N_2 fixation in response to drought (King and Purcell, 2001, 2006).

Field experiments evaluating the 168 RILs along with both parents were conducted under rainfed (RF) and irrigated (IRR) conditions at the Arkansas Rice Research and Extension Center near Stuttgart, AR (34.50° N, 91.55° W) in 2012 and 2013 (ST12, ST13, respectively) on a Crowley silt loam (Fine, montmorillonitic, thermic Typic Albaqualfs), and at the Northeast Research and Extension Center near Keiser, AR (35.06° N, 90.80° W) in 2013 (KS13) on a Sharkey silty clay soil (Very-fine, smectitic, thermic Chromic Epiaquerts). Similar experiments were conducted under RF conditions in 2017 at the Pine Tree Research Station near Colt, AR (35.11° N, 90.91° W) (PT17) on a Calloway silt loam soil (Fine, montmorillonitic, thermic Typic Albaqualfs) and at the Southeast Branch Research Station near Rohwer, AR (33.80° N, 91.28°

W) (RH17) on a Sharkey silty clay soil (Very-fine, smectitic, thermic Chromic Epiaquerts). Planting dates were 4 June 2012 and 31 May 2013 at Stuttgart, 26 June 2013 at Keiser, 9 June 2017 at Pine Tree and 8 June 2017 at Rohwer. At Stuttgart, there were two rows per plot that were 76 cm apart and 4.5 m long in both years. Plots at Keiser consisted of two rows that were 96 cm apart and 5.2 m in length. At Pine Tree, plots consisted of nine rows spaced 18 cm apart that were 4.26 m in length, whereas at Rohwer, there were nine-row plots spaced 15 cm apart that were 3.96 m long.

Plant samples for $\delta^{13}C$ estimation were harvested 80 d after planting in 2012 and 89 d after planting in 2013 at Stuttgart, 71 d after planting in 2013 at Keiser, 60 d after planting in 2017 at Pine Tree, and 70 d after planting in 2017 at Rohwer. Rainfed and IRR experiments at ST12, ST13 and KS13 were conducted in side by side fields. Experiments were furrow irrigated at Stuttgart and irrigated with an overhead, lateral-move sprinkler at Keiser when the estimated soil-moisture deficit exceeded 37 and 50 mm, respectively (Purcell et al., 2007). Irrigation was terminated in RF blocks when plants reached the full bloom (R2) growth stage (Fehr and Caviness, 1977) but continued until late full seed (R6) growth stage in the IRR blocks. Maximum temperature, minimum temperature, and rainfall were collected online from Southern Regional Climate Center (https://www.srcc.lsu.edu/station_search) using Climate Data Portal for each experimental site.

Phenotypic Evaluations

Between beginning seedfill and mid-seedfill, the aboveground portion of four random plants from each plot was harvested. The plant samples were completely dried at 60° C and then coarse ground to pass a 6 mm sieve using a Wiley Mill (Thomas Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ). A subsample of the coarse-ground samples was finely ground to

pass a 1 mm sieve. After thoroughly mixing the finely ground samples, about 500 mg was transferred to a 15 ml tube (part # 2252-PC-30; SPEX CertiPrep, Metuchen, NJ) and two 9.52-mm diameter stainless steel balls (440C Stainless Steel Ball, Tolerance/Grade: 100, Abbott Ball Company, West Hartford, CT) were placed inside the tube. Each sample was ground to a fine powder using a Geno Grinder (SPEX CertiPrep, Metuchen, NJ) for 10 min at 1,500 rpm. Thereafter, about 3–5 mg of powdered sample was carefully packed in tin capsules and arranged in 96-well plates (Costech Analytical Technologies, Vaalencia, CA). The $\delta^{13}C$ isotope analysis was performed at the University of California–Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/13cand15n.html) using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer. Data from the stable isotope facility were received as $\delta^{13}C$ (‰) and were expressed relative to the international standard of the $^{13}C/^{12}C$ ratio Vienna PeeDee Belemnite (V-PDB) as:

$$\delta^{13}C = \frac{R_{sample}}{(R_{std} - 1)} * 1000$$

where, R_{sample} and R_{std} are the isotope ratios of the sample and standard, respectively.

Statistical Analysis

At ST12, ST13, and at KS13, the experimental design was a randomized complete block with two replications and two irrigation conditions, that is, RF and IRR, whereas at PT17 and RH17, the experiments were conducted only under RF conditions with two replications. Combinations of location and year were considered as separate environments. Descriptive statistics and Pearson correlation analysis for $\delta^{13}C$ for each environment under different irrigation conditions were calculated using the PROC UNIVARIATE and PROC CORR procedures (α = .05) of SAS version 9.4 (SAS, Institute 2013), respectively. Tests of homogeneity of variance (Levene's test, Levene, 1960) were performed by environment and by

treatment before pooling of $\delta^{13}C$ data across environments and treatments. The $\delta^{13}C$ data collected in PT17 and RH17 were not included in the overall analysis of variance because the experiments were only conducted under RF conditions. Analysis of variance (ANOVA) was performed using the PROC MIXED procedure (α = .05) of SAS version 9.4 (SAS Institute, 2013). In the overall ANOVA, all factors were considered fixed except replication within environment × irrigation treatment (Table 3). For ANOVA of individual environments (KS13, ST12, and ST13) and of different irrigation treatments (RF and IRR), genotype, environment, treatment, genotype × environment, and genotype × treatment effects were fixed and replication within irrigation treatment and replication within environment were considered as random effects. The variance components were estimated using the PROC VARCOMP procedure of SAS 9.4 with the restricted maximum likelihood estimation (REML) method. Heritability (h^2) was calculated as follows:

Within each environment: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{GT}^2}{t}\right) + \left(\frac{\sigma_e^2}{rt}\right)}$$

Within each treatment: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{GE}^2}{E}\right) + \left(\frac{\sigma_e^2}{rE}\right)}$$

Over environments and treatments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{G\times T(E)}^2}{t}\right) + \left(\frac{\sigma_{G\times E}^2}{E}\right) + \left(\frac{\sigma_e^2}{rtE}\right)}$$

where σ_G^2 is the genotypic variance, σ_{GT}^2 is the genotype × treatment interaction variance, σ_{GE}^2 is the genotype × environment interaction variance, σ_e^2 is the residual error variance, t is the number of treatments, E is the number of environments, and r is the number of replications. As F_5- derived RILs were used in present study, σ_G^2 is mostly composed of additive variance and additive × additive epistasis variance with negligible variance associated with dominance, additive × dominance epistasis, and dominance × dominance epistasis. Therefore, this

heritability should be considered as narrow sense heritability. To reduce the environmental variation, the best linear unbiased prediction (BLUP) for each environment under RF and IRR conditions, across environments, and across irrigation conditions were estimated using the PROC MIXED procedure. For calculation of BLUP values, all factors were considered as random effects. The QTL analysis was performed using BLUP values.

QTL Analysis

A genetic map of 2,089 cM that was previously constructed using 548 polymorphic markers (37 asimple sequence repeats and 511 SNPs) (Hwang et al., 2015b) was used to identify the genomic regions associated with the variation of $\delta^{13}C$ in different environments and irrigation conditions. The QTL analysis was performed using WinQTL Cartographer version 2.5 (Wang et al., 2007). Single marker analysis (SMA) was used to identify genetic markers associated with the $\delta^{13}C$. Markers significant at $P < .05$ identified by SMA were used as cofactors in composite interval mapping (CIM) to control the genetic background noise (Zeng, 1994). The CIM analysis was performed using Model 6 of the Zmapqtl program module (Zeng, 1994) with forward and backward stepwise regression ($\alpha = .05$) to detect QTLs and estimate the magnitude of their effects (Jansen and Stam, 1994). The genome was scanned with walking speed of 1 cM along the chromosome with a window size of 1 cM. A permutation test (1000 times) at a significance level of $P = .05$ was used to determine the genome-wide likelihood ratio test (LRT) threshold (Churchill and Doerge, 1994). A minimum logarithm of odds (LOD) declination value of 2 was used to declare two peaks as separate QTLs (if both peaks exceeded the LOD threshold value).

Multiple interval mapping (MIM) was performed using the stepwise model procedure of Kao et al. (1999) in which QTLs identified by CIM were used in an initial MIM selection model of markers. The pre-selected model was optimized to find the maximum likelihood position of

QTLs, searching for new QTLs and epistasis between identified QTLs using the "optimize QTL positions", "search for new QTL", and "QTL interaction" options. Settings for MIM included a genome walk speed and window size of 1 cM with Bayesian information criterion (BIC), $c(n) = 3*In(n)$ for fitting the best QTL model.

The QTL Network v.2.0 (Yang et al., 2008) was also used to determine the QTL × QTL interactions and their interaction with environments. The QTL Network v.2.0 software uses the mixed-model based CIM method. In the one- dimensional genome scan, a significant marker interval was identified by a marker pair selection (Piepho and Gauch, 2001). All possible significant epistasis between marker intervals were identified by two-dimension genome scans. Permutation tests were conducted 1,000 times to determine a critical *F*-value. The QTL effects were estimated by using the Monte Carlo Markov Chain (MCMC) method (Yang et al., 2007) with Gibbs sampler iterations of 20,000. A genome-wide threshold value of .05 was used for the selection of the best model based on *F*-statistic values. Mapchart (Voorrips, 2002) was used for the graphical representation of QTLs on the genetic linkage map according to the data from WinQTLCartographer.

Results

The monthly average maximum and minimum temperature from planting to plant sampling indicated that ST12 was warmer than other environments (data not shown). Temperatures at PT17 and RH17 were 1 to 2.5°C cooler compared to other environments at the time of plant sampling. Overall averaged minimum temperatures ranged from 20 to 22 °C and maximum temperatures ranged from 30 to 34 °C during the growing season. All environments differed in total cumulative rainfall (Figure 4_1). Rainfall at ST13 was the least whereas RH17 received the most rainfall from the period of planting to sample harvesting. The experiments at ST12 and ST13 had relatively little rainfall throughout the season (Figure 4_1). Although total rainfall was high for KS13, there was a period of 24 d prior to sampling without rainfall. At RH17, rainfall throughout the season minimized drought stress prior to plant sampling. At PT17, there was relatively high rainfall, but there was a period of 12 d prior to plant sampling without rainfall.

Descriptive statistics of $\delta^{13}C$ under RF and IRR conditions within environments (Table 4_1) indicated a significant range of phenotypic variation. Under RF conditions, $\delta^{13}C$ had a range of 1.22‰ when averaged over environments, whereas under IRR conditions, $\delta^{13}C$ had a range of 1.68‰ when averaged over environments (Figure 4_2). In all cases, $\delta^{13}C$ within environment, averaged over RILs, was lower (more negative) for the IRR than the RF conditions, which is consistent with greater WUE under drought conditions.

The average $\delta^{13}C$ values of the two parents at ST12_RF, ST12_IRR, ST13_RF, and ST13_IRR environments indicated that mid-parent values were close to the population mean (Table 4_1). In contrast, mid-parent values were less than population mean in KS13_RF, KS13_IRR, and PT17_RF and greater than the population mean in RH17_RF. Values of $\delta^{13}C$ for

KS4895 tended to be greater than Jackson in all environments and treatments except for ST12_IRR (Table 4_1). When data were analyzed by environment over treatment, $\delta^{13}C$ of KS4895 was greater than Jackson for ST13 ($P \leq .001$) and KS13 ($P \leq .01$) (data not shown). Similarly, when data were analyzed by treatment over environments, $\delta^{13}C$ of KS4895 was significantly greater than Jackson in IRR ($P \leq 0.05$) and RF ($P \leq .01$) conditions (Figure 4_2). Importantly, the $\delta^{13}C$ values among the RILs extended beyond that of the parents in all environments under both irrigation conditions (Table 4_1), indicating the possibility of multi-genetic inheritance and transgressive segregation (Rieseberg et al., 1999).

The $\delta^{13}C$ values followed a normal distribution in all environments under RF and IRR conditions as indicated by the Shapiro-Wilk test ($P > .05$, Table 4_1). There was a significant positive correlation ($P \leq .01$) of $\delta^{13}C$ of RILs between all environments and irrigation conditions ($.27 \leq r \leq .65$) except for ST12_RF and PT17_RF in which the correlation ($r = .13$) was not significant (Table 4_2). The homogeneity of variance analysis showed that there was no significant difference ($P < .05$) of variance among different environments and treatments.

Data collected from ST12, ST13, and from KS13 under RF and IRR conditions were used in an overall analysis of variance (Table 4_3). There was a significant effect ($P < .05$) of genotype (G), environment (E), and the interaction of G × E on $\delta^{13}C$, whereas the main effect of irrigation treatment within environment and its interaction with G was non-significant. Analysis of variance by irrigation treatment showed the significant effect of G and E under both irrigation conditions, but G × E was only significant under RF conditions. Analysis of variance by E indicated a significant effect of G in all cases, but the effect of treatment was only significant in ST13. Data collected from Pine Tree (PT17_RF) and Rohwer (RH17_RF) were not included in either the overall analysis of variance or the analysis of variance by environment because $\delta^{13}C$

data were collected only under RF conditions. Analysis of variance for each irrigation condition within an environment indicated a significant effect ($P < .05$) of G in all cases.

Heritability of $\delta^{13}C$ on an entry-mean basis combined across all environments and both irrigation treatments was 83% (Table 4_3). Heritability under RF and IRR conditions over environments was 72 and 79%, respectively. For individual environments averaged over irrigation treatments, heritability was 66 (KS13), 77 (ST12), and 78% (ST13). Heritability for different irrigation treatment and environment combinations ranged from 35 (PT17_RF) to 70% (ST13_RF).

Among locations there were large differences in the number of rows per plot (from 2 to 9) and row spacing (15 - 96 cm) that could potentially contribute to the G × E effects observed (Table 4_3). However, we could find no discernable patterns in RIL means of $\delta^{13}C$ among locations that were associated with row spacing, but this remains a possibility that would require further experimentation to determine. Given the relatively high heritability of $\delta^{13}C$ across environments ($h^2 = 83\%$) and the large differences among environments with regards to soil characteristics, rainfall/irrigation distribution, and temperature, it is not likely that row spacing had a major effect on $\delta^{13}C$. Additionally, Kaler et al. (2018a) evaluated soybean $\delta^{13}C$ in four environments with row spacing ranging between 19- and 76-cm. Although they found significant main effects of environment and genotype on $\delta^{13}C$, they did not find a significant G × E effect, indicating that genotypes responded similarly with different row spacing.

QTL Analysis

Table 4 describes the QTLs identified by CIM for $\delta^{13}C$ in each environment (ST12, ST13, and KS13), under RF and IRR conditions, and over environments and irrigation treatments (AEI). Significant markers ($P < .05$) identified by SMA were located near the maximum likely

QTL positions. Multiple significant markers ($P < .01$) were found on different positions within each chromosome by SMA. The marker explaining the largest phenotypic variation (R^2) on the specific position of each chromosome was chosen as the representative QTL for that chromosomal regions. Although PT17 and RH17 were only conducted under RF conditions, the data from these environments were included in the QTL analysis to identify the QTLs associated with $\delta^{13}C$ across a wider range of environments.

QTL Analysis by Environment:

For the analysis averaged over irrigation treatments by individual environments (KS13, ST12, and ST13), there were a total of eight QTLs identified on Gm04 (1), Gm06 (4), Gm18 (1), and Gm20 (2) with R^2 values ranging from .07 to .24 and with additive effects that ranged from 0.05 to 0.13‰ (Table 4_1; Figure 4_3). Additive QTL effects were calculated as one-half of the difference between the average effects of parental alleles (KS4895 and Jackson). Of these eight QTLs, two favorable QTLs were from KS13 (Gm06 and Gm18), three favorable QTLs were from ST12 (Gm04, Gm06, and Gm20), and three favorable QTLs were from ST13 (Gm06 (2) and Gm20 (1)). The QTL on Gm06 positioned at 247.3 cM (47,413,332 bp) from ST12 had an overlapping confidence interval with QTLs from ST13 (248.3 cM) and KS13 (248.3 cM), and had R^2 values ranging from .12 to .18 and additive effects ranging from 0.07 to 0.10‰. The QTL on Gm20 from ST12 positioned at 95 cM (41,983,096 bp) was also found in ST13 and accounted for the highest phenotypic variation (R^2 = .18 and .24) and the highest additive effect (0.10 to 0.13 ‰). Using the MIM model, one QTL on Gm05 at 232.9 cM (37,322,242 bp) was detected in ST13 that was not detected by CIM with a R^2 value of .05 (Table 4_5). Favorable parental alleles were defined as ones that increased $\delta^{13}C$ (increase WUE) at a QTL position. The favorable allele for the QTLs present on Gm06 were from KS4895 except for the QTL at 204.8

cM (14,849,154 bp) identified in ST13, which was from Jackson. The QTLs positioned at 78 cM (9,299,958 bp) on Gm04 and at 125.2 cM (5,8976,307 bp) on Gm18 appeared only in ST12 and KS13, respectively. Favorable alleles for QTLs found on Gm04, Gm05, Gm18, and Gm20 were from Jackson. There was no significant interaction among QTLs in MIM models, but significant ($P < .05$) interactions were identified by QTL Network between the QTLs present on Gm18 and Gm20 for KS13 and QTLs present on Gm06 and Gm20 for ST13.

QTL Analysis by Irrigation Treatment:

When analysis was performed by treatment averaged over environment using the CIM model, five QTLs were detected under RF conditions and four were found under IRR conditions (Table 4_4, Figure 4_3). QTLs detected under RF were present on Gm05 (1), Gm06 (3), and Gm20 (1) with R^2 values ranging from .06 to .25 and additive effects from 0.05 to 0.10‰. The QTL present on Gm05 at 232.9 cM (37,322,242 bp) was only detected under RF conditions. The three QTLs found on Gm06 at 206.3 cM (14,849,154 bp), 248.3 cM (47,413,332 bp), and 253.4 cM (47,823,144 bp), collectively explained 53% of the phenotypic variation, and the favorable allele for two of these QTLs (248.3 and 253.4 cM) were from KS4895. Jackson contributed the favorable allele for the QTL present on Gm20 (95 cM) with an additive effect of 0.07‰. Out of the five QTLs identified by the CIM model, only two QTLs were detected with MIM on Gm06 at 246.3 cM (47,413,332 bp) and Gm20 at 95.0 cM (41,983,096 bp; Table 4_5). QTL Network detected an additive × additive interaction between above QTLs identified by MIM with $R^2 = .08$.

The QTL analysis was also performed separately for $\delta^{13}C$ data collected from RH17 and PT17 under RF conditions (Table 4_4). Three QTLs were identified by the CIM model in PT17_RF on Gm06, Gm12, and Gm19 with R^2 values that ranged from 0.07 to 0.13 and additive

effects from 0.03 to 0.05‰ (Table 4_4). The QTLs on Gm06, Gm12, and Gm19 positioned at 246.3 cM (47,413,332 bp), 35.2 cM (6,621,791 bp), and 102.7 cM (44,658,800 bp), respectively, had favorable alleles that were from KS4895. No QTLs were identified for RH17.

For the four putative QTLs identified with the CIM model for the IRR treatment (Table 4_4; Figure 4_3), three QTLs on Gm06 positioned at 241.8 cM (43,167,030 bp), 247.3 cM (47,413,332 bp), and 267.4 cM (47,413,332 bp), together explained 34% of the phenotypic variation. For these three QTLs, the KS4895 allele increased the $\delta^{13}C$ and had an additive effect that ranged from 0.07 to 0.09‰. Of these QTLs on Gm06, the QTL at 247.3 cM was also detected with the MIM model in addition to a QTL on Gm05 at 231.9 cM (37,322,242 bp) (R^2 = .05 and additive effect of 0.06‰; Table 4_5). The QTL present on Gm20 at 95 cM (41,983,096 bp) was detected by both CIM and MIM models and had the favorable allele from Jackson. No significant QTL × QTL interactions were identified in the MIM model. However, an additive × additive epistasis was found between the QTL present on Gm06 and Gm20 by QTL Network, which had an epistatic effect of 0.05‰ with a R^2 = .05.

QTL Analysis over Environment and Irrigation Treatment (AEI):

When analysis was performed by using $\delta^{13}C$ data averaged over all environments (excluding data from PT17 and RH17) and from both irrigation conditions, one QTL was found on Gm05 and Gm20, and two on Gm 06 (Table 4_4; Figure 4_3). Thus, these QTLs were stable over environments and over irrigation conditions. The QTL present on Gm05 (positioned at 229.9 cM (37,322,242 bp) explained 9% of the phenotypic variation and had an additive effect of 0.07‰. The two QTLs present on Gm06 at 206.3 (14,849,154 bp) and 247.3 cM (47,413,332 bp) collectively accounted for 14% of the phenotypic variation, whereas the QTL on Gm20 (at 95 cM (41,983,096 bp) had an additive effect of 0.11‰ and explained the highest phenotypic

variation (23%) among identified QTLs. For three of these QTLs (Gm05, Gm06, and Gm20), the favorable allele was from Jackson, while the QTL on Gm06 (247.3 cM), the favorable allele was from KS4895. The QTLs on Gm06 (247.3 cM) and Gm20 (95 cM) had a significant interaction ($P < .00001$) with an effect of -0.06‰.

Discussion

The present study mapped $\delta^{13}C$ in a population derived from KS4895 × Jackson under RF and IRR conditions across different environments. A broad range of $\delta^{13}C$ within each environment indicated a wide phenotypic range, which is the first requisite for QTL analysis. Within environment and irrigation treatment, $\delta^{13}C$ values averaged over RILs were lower for the IRR than the RF conditions. Similarly, in cotton, there was high $\delta^{13}C$ values in water deficit treatment as compared to well-watered conditions, indicating greater WUE (Saranga et al., 1999; Yakir et al., 1990). Under water deficit conditions, there is partial closure of stomata, which may lead to an increase in WUE (Specht et al., 2001).

The parents of this population were originally selected because they differed in their response of N_2 fixation to drought with KS4895 being affected considerably more by drought than was Jackson (King and Purcell, 2001, 2006). It is interesting to note, however, that KS4895 generally had greater $\delta^{13}C$ than Jackson (Table 4_1) that was significant for both IRR ($P \leq .05$) and RF ($P \leq .01$) treatments when analyzed over environments (Figure 4_2). Surprisingly, these results are consistent with KS4895 having greater water use efficiency than Jackson despite having N_2 fixation more sensitive to drought than Jackson (King and Purcell, 2001, 2006). Nevertheless, there was transgressive segregation among RILs with favorable alleles at five of the nine QTLs for $\delta^{13}C$ being derived from Jackson (Tables 4_4, 4_5).

The significant positive correlation for $\delta^{13}C$ between different environments and irrigation conditions ranged from $r = .27$ to .65 (Table 4_2). Likewise, correlations between RF and IRR conditions from different environments were positively related ($r = .49$ to .65), which is consistent with non-significant interactions of genotype × treatment for all the environments. The strong correlation between RF and IRR environments and a lack of genotype and environment

interaction was previously reported for many crops (Ashok et al., 1999; Impa et al., 2005; Ismail and Hall, 1992; Kaler et al., 2017a, 2018a).

The heritability of $\delta^{13}C$ averaged over irrigation and environments (83%) is similar to broad-sense heritability (H = 0.76, Kaler et al., 2017a; H = 0.76, Hall et al., 1990; H = 0.81, Hubick et al., 1988; H = 0.68, Stiller et al., 2005) and narrow-sense heritability (h^2 = 0.80, Specht et al., 2001; h^2 = 0.37-0.91, Rebetzke et al., 2008) for $\delta^{13}C$ reported previously. The heritability of $\delta^{13}C$ suggests that selection on the basis of $\delta^{13}C$ could be effective for improving WUE under drought conditions.

In soybean, there are 11 known genes controlling maturity and flowering date (Cober et al., 2010; Kong et al., 2014; Li et al., 2017; Ray et al., 1995). Because this population was developed to have a narrow range of maturity (~3 d), the full range of potential segregants was not evaluated. Therefore, genes associated with $\delta^{13}C$ and linked with maturity and flowering-date would not likely be identified in the present research. However, by having similar maturity among the RILs, QTLs associated with $\delta^{13}C$ would not be confounded with large differences in developmental stages at the time of plant sampling that may have occurred had the full range of segregants been used. Others have noted the advantages of selecting for a relatively narrow soybean maturity range for mapping physiological traits (Abdel-Haleem et al., 2012; Charlson et al., 2009; Hwang et al., 2013, 2014, 2015a, 2015b).

The QTL analysis identified a total of 24 QTLs on seven chromosomes (Gm04, Gm05, Gm06, Gm12, Gm18, Gm19, and Gm20) when $\delta^{13}C$ data were analyzed by environment, by irrigation, and over environment and irrigation treatment (Table 4_4; Figure 4_3), indicating polygenic inheritance of $\delta^{13}C$. Considering the overlapping confidence intervals, we conclude that there were nine genomic loci on seven chromosomes (Gm04, Gm05, Gm06, Gm12, Gm18,

Gm19, and Gm20) associated with $\delta^{13}C$ (Figure 4_3). On Gm06, the QTLs present between 197.0 and 220.0 cM were considered as one locus (Locus 3; Table 4_4). The nine QTLs (KS13, ST12, ST13, PT17_RF, IRR [2], RF [2], and AEI) present between 232.0 and 263.0 cM on Gm06 (Locus 4) had over lapping confidence intervals with one or more QTLs in this region and were also considered as one locus. There is the possibility of more than one locus at this position that would require fine mapping to resolve. Of these nine loci, Loci 2 (Gm05), 3 (Gm06), 4 (Gm06), and 9 (Gm20) were identified consistently in multiple environments and both irrigation conditions, which explained phenotypic variation from 9 to 23%. A QTL on Gm06 (Locus 4) and on Gm20 (Locus 9) collectively accounted for most of the phenotypic variation and had the largest additive effect (Table 4_4) with the favorable alleles coming from Jackson. The nearest marker linked with these QTLs are candidates for marker assisted selection in future breeding efforts to improve WUE.

The 93705 KS4895 × Jackson population (93705KJ), which consisted of 97 RILs, was developed earlier to study the inheritance of traits associated with nitrogen fixation (Hwang et al., 2013; Hwang et al., 2014) as both parents differed in nitrogen fixation. To study the inheritance of other physiological responses associated with drought, crosses between parents were made in 2008 to increase the population size; the resulting population, consisting of 168 RILs, was designated as 08705 KS4895 × Jackson population and was used in the present study. Hwang et al. (2015b) described the 08705 KS4895 × Jackson population as a confirmation population of the 93705 KJ population, which was mapped for canopy wilting QTLs. It was of interest to consider if wilting and QTLs for other traits co-segregated with $\delta^{13}C$ QTLs. To confirm the identified QTLs with previously reported QTLs, the identified $\delta^{13}C$ QTLs were aligned with the soybean linkage map in Soybase (http://soybase.org/). For this analysis, we

included QTLs associated with other traits within the 95% confidence interval and ± 5 cM beyond the identified $\delta^{13}C$ QTL boundary in this study. We found numerous QTLs from previous reports for traits related to abiotic stresses, plant morphology and development, seed composition and nitrogen accumulation that overlapped with $\delta^{13}C$ QTLs identified in the present research (Table 4_6).

The QTL on Gm04 (Locus 1) at 78 cM overlapped with the canopy wilting QTL identified by Abdel-Haleem et al. (2012) in a population derived from Benning (PI 595645) × PI 416937. This QTL was also close to a $\delta^{13}C$ QTL found in a GWAM study using a diverse panel of soybean accessions (Kaler et al., 2017a). At the genomic location of the QTL cluster on Gm05 (Locus 2), there were QTLs reported for oxygen isotope ratio ($\delta^{18}O$; Kaler et al., 2017a), low hydraulic conductance (Carpentieri-Pipolo et al., 2011), drought index (Du et al., 2009a), and canopy wilting (Hwang et al., 2016; Kaler et al., 2017b). Leaf hydraulic conductance and $\delta^{18}O$ are traits associated with transpiration. Low hydraulic conductance QTLs identified by Carpentieri-Pipolo et al. (2011) in a Benning and PI 416937 population, may conserve soil water by limiting hydraulic conductance leading to increase WUE and slow canopy wilting. The genomic region on Gm05 associated with low hydraulic conductance (Carpentieri-Pipolo et al., 2011) and slow canopy wilting (Kaler et al., 2017b) were consistent and stable across different environments and irrigation conditions in this study.

The QTL on Gm06 (Locus 3, 197.0-220.0 cM) was associated with $\Delta^{13}C$ in RILs developed from a cross of Minsoy × Noir 1 (Specht et al., 2001). Using the 93705 KS4895 × Jackson population, Hwang et al. (2014) identified QTLs associated with nodule number that mapped to this position on Gm06 and with the favorable allele coming from KS4895, as was similar for the $\delta^{13}C$ QTL. Likewise, Hwang et al. (2015b) located a QTL for canopy wilting at

the same genomic region (Locus 4, 232.0-263.0 cM) on Gm06 in the 93705 KS4895 × Jackson population. Both QTLs (canopy wilting and $\delta^{13}C$) had the same marker, ss107921293, as the nearest marker but carried the favorable allele from different parents. Significant associations of SNPs with canopy wilting (Kaler et al., 2017b), canopy temperature (Kaler et al., 2018b), and canopy coverage (Kaler et al., 2018c) were identified from GWAM within the confidence interval of this $\delta^{13}C$ QTL. The canopy related traits may function to conserve soil moisture (Jones et al., 1981; Valliyodan and Nguyen, 2006), resulting in increased WUE.

The $\delta^{13}C$ QTL on Gm18 (Locus 7, 125.2 cM) coincided with a significant SNP associated with $\delta^{13}C$ identified by GWAM of diverse soybean accessions, which found that this QTL was stable across several environments (Kaler et al., 2017a; Dhanapal et al., 2015). Similarly, the $\delta^{13}C$ on Gm18 co-segregated with a wilting QTL identified by GWAM (Kaler et al., 2017b).

The QTL on Gm19 (Locus 8, 102.7 cM) appeared only in the PT17 environment under RF conditions. This QTL co-localized with QTLs reported previously for WUE (Mian et al., 1998), $\Delta^{13}C$ (Specht et al., 2001), oxygen isotope ratio (Kaler et al., 2017a), and canopy wilting (Kaler et al., 2017b). For this same genomic region, Hwang et al. (2015b) reported a QTL for canopy wilting from four different populations.

The QTL cluster on Gm20 (Locus 9, 95.0 cM) co-localized with markers associated with $\delta^{13}C$ and canopy wilting from previous GWAM (Kaler et al., 2017a, 2017b). Du et al. (2009a) also identified a QTL for drought susceptibility index (measurement of yield under drought conditions) located within the confidence interval for the $\delta^{13}C$ on Gm20. Previously reported QTLs for canopy width (Mian et al., 1998) and root morphology (Abdel-Haleem et al., 2011;

Hwang et al., 2014, Liang et al., 2014) were also co-localized with the identified QTLs on Gm20.

The *Glycine max* genome sequence (Williams 82 assembly 1) in the Soybase database (https://www.soybase.org/) was scanned for potential genes present between flanking markers of identified QTLs. Around 106 potential candidate genes were identified in the flanking-marker regions of nine loci associated with $\delta^{13}C$. Most of the candidate genes either have unknown functions or have annotated functions related to regulation and signaling through various transcription factors. Of the 106 candidate genes we considered, only two genes had functions that we could relate to carbon assimilation, drought response, or WUE. In the genomic region of locus 1, there was a DREB3 gene (*Glyma.04g11290* – Williams 82 assembly 1, *Glyma.04g103900* – Williams 82 assembly 2) that encodes for a dehydration responsive element binding protein. In the genomic region of locus 7, there was a BT098288.1 gene (*Glyma.18g49410* – Williams 82 assembly 1, *Glyma.18g259500* – Williams 82 assembly 2) that encodes an aquaporin protein, which may have roles in water transport and transpiration. Further comprehensive research is needed to narrow down the regulatory genes that are directly or indirectly involved in pathways associated with WUE and drought tolerance.

The QTLs on Gm05 (~230.0 cM), Gm06 (~248.0 cM), and Gm20 (~95.0 cM) were identified in all environments, irrigation conditions, and combined environment and irrigation treatment analysis. These QTLs also had large allelic effects (Table 4_4). The stability of these genomic regions would make them attractive for use in marker-assisted selection for $\delta^{13}C$ under RF and IRR conditions. Several of these identified QTLs coincided with previously reported QTLs associated with $\delta^{13}C$, canopy wilting, canopy temperature, and canopy coverage and indicated multiple mechanisms associated with tolerance to soil moisture deficit. Further

research will be required for fine mapping of identified QTLs and expression analysis of underlying genes at putative identified QTLs to understand the complex quantitative nature of drought tolerance.

Table 4_1. Descriptive statistics of $\delta^{13}C$ (‰) of parents and recombinant inbred lines (RILs) evaluated at Keiser, AR in 2013 (KS13) and Stuttgart, AR in 2012 and 2013 (ST12 and ST13) under rainfed (RF) and irrigated (IRR) conditions, and at Pine Tree (PT17) and Rohwer (RH17) in 2017 under RF conditions.

Descriptive statistics	**KS13_RF**	**KS13_IRR**	**ST12_RF**	**ST12_IRR**	**ST13_RF**	**ST13_IRR**	**PT17_RF**	**RH17_IRR**
KS4895	-28.40	-28.46	-28.45	-28.74	-28.45	-28.38	-27.87	-27.88
Jackson	-29.02	-28.95	-28.62	-28.57	-29.00	-29.24	-28.42	-28.13
Parents mean	-28.71	28.71	-28.54	-28.65	-28.73	-28.81	-28.15	-28.00
RILs mean	-28.13	-28.28	-28.49	-28.59	-28.68	-28.87	-27.87	-28.18
Range	1.64	1.68	1.97	2.34	1.69	1.97	2.15	1.60
Std. deviation	0.30	0.33	0.37	0.36	0.31	0.35	0.37	0.32
Variance	0.09	0.11	0.14	0.13	0.09	0.12	0.14	0.10
Skewness	0.18	-0.15	0.16	0.30	0.25	0.26	-0.05	0.19
Kurtosis	0.18	-0.27	-0.05	0.10	0.05	0.25	-0.15	0.02
Shapiro-Wilk test (*P*-values)	0.42ns[a]	0.73ns	0.54ns	0.13ns	0.38ns	0.59ns	0.24ns	0.45ns
Coefficient of variation (%)[b]	1.05	1.15	1.31	1.26	1.08	1.20	1.33	1.13

[a]ns not significant at the .05 probability level.
[b] Absolute value of coefficient of variation.

Table 4_2. Pearson correlation coefficients between $\delta^{13}C$ of RILs derived from KS4895 x Jackson in different environments including Stuttgart in 2012 (ST12) and 2013 (ST13), Keiser in 2013 (KS13), Pine Tree in 2017 (PT17), and Rohwer in 2017 (RH17) under rainfed (RF) and irrigated (IRR) conditions. Correlation coefficients were calculated using RIL means (n=166-168).

	KS13_RF	KS13_IRR	ST12_RF	ST12_IRR	ST13_RF	ST13_IRR	PT17_RF
KS13_IRR	.49***						
ST12_RF	.35***	.47***					
ST12_IRR	.51***	.53***	.62***				
ST13_RF	.59***	.54***	.47***	.63***			
ST13_IRR	.49***	.48***	.44***	.64***	.65***		
PT17_RF	.27**	.32***	.13ns[a]	.38***	.41***	.37***	
RH17_RF	.43***	.44***	.33***	.44***	.58***	.56***	.32***

** Significant at the .01 probability level.

*** Significant at the .001 probability level.

[a]ns, not significant ($P > .05$)

Table 4_3. Analysis of variance and heritability (h^2) of $\delta^{13}C$ over environment and irrigation treatment, by environments (KS13, ST12, and ST13), by irrigation treatment (rainfed, RF; irrigated, IRR) and for individual environments within irrigation treatment.

Analysis	Genotype (G)	Environment (E)	Irrigation treatment (T(E))	G × E	G × T[E][a]	h^2 (%)
Combined over E and T	***	***	ns	***	ns	83
By T						
RF[b]	***	*	-	***	-	72
IRR	***	***	-	ns	-	79
By E						
KS13	***	-	ns	-	ns	66
ST12	***	-	ns	-	ns	77
ST13	***	-	*	-	ns	78
By E and T						
KS13_RF	***	-	-	-	-	52
KS13_IRR	***	-	-	-	-	53
ST12_RF	***	-	-	-	-	67
ST12_IRR	***	-	-	-	-	66
ST13_RF	***	-	-	-	-	70
ST13_IRR	***	-	-	-	-	64
PT17_RF	**	-	-	-	-	35
RH17_RF	***	-	-	-	-	48

G, RILs; E, environment; T, irrigation treatments; G*E, RILs × environment interaction; G*T(E), RILs and treatment interaction within environment.

[a] For analysis by E, this represents G × T.

[b] RF, rainfed; IRR, irrigation.

*, **, *** Significant at the .05, .01, .001 probability level, repectively; ns, nonsignificant.

Table 4_4. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ identified by composite interval mapping (CIM) using $\delta^{13}C$ BLUP values by environments (ST12, ST13, and KS13) averaged over irrigation treatment, by irrigation treatment (RF and IRR) averaged over environments, and averaged over both environments and irrigation treatments (AEI).

Locus	Chrom-osome	Nearest marker	Nearest marker position[a] (bp)	QTL position (cM)	Env/Trt[b]	LOD[c]	QTL effect[d] (‰)	R^{2e}	Favorable allele[f]	FM at 95% confidence interval[g]
1	Gm04	ss107923464	9,299,958	78.0	ST12	4.40	-0.07	.07	Jackson	ss107923464-ss107920293
2	Gm05	Sat_267	37,322,242	232.9	RF	3.67	-0.05	.07	Jackson	ss107921710-ss107923047
				229.9	AEI	4.00	-0.07	.09	Jackson	ss107921710-ss107923047
3	Gm06	ss107921293	14,849,154	204.8	ST13	4.00	-0.07	.08	Jackson	ss107913501-ss107917110
				206.3	RF	3.37	-0.05	.06	Jackson	ss107913501-ss107917110
				206.3	AEI	3.38	-0.05	.05	Jackson	ss107913501-ss107917110
4	Gm06	ss107928665	43,167,030	241.8	IRR	6.02	0.09	.14	KS4895	ss107928665-ss107914184
		ss107914184	47,413,332	248.3	KS13	6.50	0.07	.14	KS4895	ss107914184-ss107919937
				247.3	ST12	6.70	0.09	.12	KS4895	ss107914184-ss107913752
				248.3	ST13	9.10	0.10	.18	KS4895	ss107928665-ss107913752
				248.3	RF	11.45	0.09	.22	KS4895	ss107914184-ss107913752
				247.3	IRR	5.96	0.08	.11	KS4895	ss107914184-ss107919937
				246.3	PT17_ RF[h]	4.36	0.04	.09	KS4895	ss107928665-ss107913752
				247.3	AEI	8.61	0.09	.16	KS4895	ss107914184-ss107913752
		ss107913752	47,823,144	253.4	RF	10.67	0.10	.25	KS4895	ss107913752-ss107919937
5	Gm06	ss107912626	49,974,718	267.4	IRR	4.53	0.07	.09	KS4895	ss107912626-ss107918790

Table 4_4. (Cont.)

Locus	Chrom-osome	Nearest marker	Nearest marker position[†] (bp)	QTL position (cM)	Env/Trt[‡]	LOD[§]	QTL effect[¶] (‰)	$R^{2\#}$	Favorable allele[††]	FM at 95% confidence interval[‡‡]
6	Gm12	ss107924439	6,621,791	35.2	PT17_ RF[h]	3.10	0.03	.07	KS4895	ss107913327-ss107913253
7	Gm18	ss107912608	5,8976,307	125.2	KS13	4.50	-0.05	.09	Jackson	ss107919928-ss107917416
8	Gm19	ss107912574	44,658,800	102.7	PT17_ RF[h]	3.92	0.05	.13	KS4895	ss107912574-ss107929955
9	Gm20	ss107920249	41,983,096	95.0	ST13	9.80	0.10	.18	Jackson	ss107920249-ss107927898
				95.0	ST12	12.70	-0.13	.24	Jackson	ss107913931-ss107927898
				95.0	RF	7.90	-0.07	.15	Jackson	ss107913931-ss107927898
				95.0	IRR	9.04	-0.10	.19	Jackson	ss107913931-ss107927898
				95.0	AEI	12.77	-0.11	.23	Jackson	ss107913931-ss107927898

[a] SNP position or simple sequence repeat start position in base pairs (bp) according to Soybase (www.soybase.org).
[b] Environment (KS13, ST12, and ST13) or Treatment (RF and IRR) or averaged over environments and irrigation treatments (AEI).
[c] LOD is the log-likelihood threshold score at which significant QTLs were declared.
[d] QTL effect is equal to half of difference between the average effects of two parental alleles (Jackson and KS4895)
[e] The proportion of phenotypic variation explained by specific QTL.
[f] Allele that increases $\delta^{13}C$ value at QTL position.
[g] Flanking markers indicates the markers present near or at 95% confidence interval of the maximum likely QTL positions. The LOD values with ±2 declination was used to estimate the 95% confidence interval.
[h] PT17_RF denotes experiments at Pine Tree conducted in 2017 under rainfed conditions

Table 4_5. Quantitative trait loci (QTLs) associated with $\delta^{13}C$ identified by multiple interval mapping (MIM) using $\delta^{13}C$ data by environments (ST12, ST13, and KS13) averaged over irrigation treatment, by irrigation treatment (rainfed, RF; irrigated, IRR) averaged over environments, and averaged over both environments and irrigation treatments (AEI) in a KS4895 × Jackson population.

Locus[a]	Chrom-osome	Nearest marker	Nearest marker position[b] (bp)	QTL position (cM)	Env/Trt[c]	LOD[d]	QTL effect[e] (‰)	R^{2f}	Favorable allele[g]	FM at 95% confidence interval[h]
1	Gm04	ss107923464	9,299,958	77.4	ST12	3.85	-0.06	.08	Jackson	ss107923464-ss107920293
2	Gm05	Sat_267	37,322,242	232.9	ST13	3.71	-0.06	.05	Jackson	ss107921710-ss107923047
				232.9	IRR	3.08	-0.06	.05	Jackson	ss107921710-ss107923047
				231.0	AEI	4.37	-0.07	.07	Jackson	ss107921710-ss107923047
3	Gm06	ss107921293	14,849,154	203.8	ST13	3.32	-0.06	.05	Jackson	ss107913501-ss107921293
				204.8	AEI	3.27	-0.05	.04	Jackson	ss107913501-ss107921293
4	Gm06	ss107914184	47,413,332	247.3	ST12	7.66	0.10	.14	KS4895	ss107914184-ss107913752
				243.8	ST13	9.81	0.11	.20	KS4895	ss107928665-ss107914184
				246.3	RF	9.03	0.08	.17	KS4895	ss107914184-ss107913752
				247.3	IRR	7.20	0.09	.14	KS4895	ss107914184-ss107913752
				247.3	AEI	12.66	0.11	.21	KS4895	ss107914184-ss107913752
		ss107913752	47,823,144	252.5	KS13	4.27	0.06	.12	KS4895	ss107913752-ss107919937
7	Gm18	ss107912608	5,8976,307	126.0	KS13	4.12	-0.05	.10	Jackson	ss107919928-ss107917416
9	Gm20	ss107920249	41,983,096	95.0	ST12	11.98	-0.12	.23	Jackson	ss107913931-ss107927898
				95.0	ST13	10.49	-0.11	.20	Jackson	ss107913931-ss107927898

Table 4_5. (Cont.)

Locus[##]	Chrom-osome	Nearest marker	Nearest marker position[†] (bp)	QTL position (cM)	Env/Trt[‡]	LOD[§]	QTL effect[¶] (‰)	$R^{2\#}$	Favorable allele[††]	FM at 95% confidence interval[‡‡]
				95.0	RF	9.57	-0.08	.19	Jackson	ss107913931-ss107927898
				95.0	IRR	12.00	-0.11	.24	Jackson	ss107913931-ss107927898
				95.0	AEI	12.31	-0.10	.23	Jackson	ss107920249-ss107927898

[a] Locus number is assigned as according to locus identified in composite interval mapping (CIM).
[b] Single nucleotide polymorphism position or simple sequence repeat start position in base pairs (bp) according to soybase (www.soybase.org).
[c] Environment (KS13, ST12, and ST13) or Treatment (RF and IRR) or averaged over environments and irrigation treatments (AEI).
[d] LOD is the log-likelihood threshold score at which significant quantitative trait loci (QTLs) were declared.
[e] QTL effect is equal to half of difference between the average effects of two parental alleles (Jackson and KS4895)
[f] The proportion of phenotypic variation explained by specific QTL.
[g] Allele that increases $\delta^{13}C$ value at QTL position.
[h] Flanking markers indicates the markers present near or at 95% confidence interval of the maximum likely QTL positions. The LOD values with ±2 declination was used to estimate the 95% confidence interval.

Table 4_6. Reported genomic regions associated with other traits overlapping with the quantitative trait loci positions for the $\delta^{13}C$ identified in the KS4895 × Jackson population.

Chromosome	Locus	Traits overlapping chromosomal regions associated with $\delta^{13}C$ from different mapping studies
Gm04	1	$\delta^{13}C$ (Kaler et al., 2017a), Canopy wilting (Abdel-Haleem et al., 2012), Seed protein (Orf et al., 1999), Seed glycinin plus beta conglycinin (Ma et al., 2016), and Isoflavone (Ma et al., 2016)
Gm05	2	Oxygen isotope ratio ($\delta^{18}O$) (Kaler et al., 2017a), Low hydraulic conductance (Carpentieri-Pipolo et al., 2011), Drought susceptibility index (Du et al., 2009a), Canopy wilting (Hwang et al., 2016, Kaler et al. 2017b), Canopy coverage (Kaler et al., 2018c), Plant height (Chen et al., 2007), Node number (Chen et al., 2007), Flood tolerance (Carpentieri-Pipolo et al., 2011), Seed yield (Li et al., 2008), and Seed oil (Orf et al., 1999).
Gm06	3	$\Delta^{13}C$ (Specht et al., 2001), Internode length (Alcivar et al., 2007), Node number (Moongkanna et al., 2011), Nodule number (Hwang et al., 2014), Nodule size (Hwang et al., 2014), Root nodule (Shi et al., 2018), Seed protein (Csanadi et al., 2001, Lu et al., 2013), and Leaflet area (Mian et al., 1998)
Gm06	4	Canopy wilting (Hwang et al., 2015b), Canopy width (Mian et al., 1998), Flood tolerance (Githiri et al., 2006), Aluminum tolerance (Sharma et al., 2010), Internode length (Alcivar et al., 2007), Node number (Gai et al., 2007), Primary root length (Brensha et al. 2012), Seed protein (Hyten et al., 2004), and Seed yield (Gai et al., 2007)
Gm12	6	Total growth duration (Qi et al., 2014), Isoflavone (Gutierrez-Gonzalez et al., 2009), Reproductive stage length (Li et al., 2008), Seed protein (Liang et al., 2010), and Seed sucrose (Kim et al., 2006)
Gm18	7	$\delta^{13}C$ (Kaler et al., 2017a), Canopy wilting (Kaler et al., 2017b), Flood tolerance (Van Toai et al., 2001), Isoflavone (Yoshikawa et al., 2010), Seed oil (Lee et al., 1996), Seed protein (Brummer et al., 1997, Mao et al., 2013), and Reproductive stage length (Cheng et al., 2011)

Table 4_6. (Cont.)

Chromosome	Locus	Traits overlapping chromosomal regions associated with $\delta^{13}C$ from different mapping studies
Gm19	8	Water use efficiency (Mian et al., 1998), $\Delta^{13}C$ (Specht et al., 2001), Canopy wilting (Kaler et al., 2017b, Hwang et al., 2015b), Seed oil (Qi et al., 2011), Seed weight (Pathan et al., 2013), and Reproductive stage length (Orf et al., 1999)
Gm20	9	$\delta^{13}C$ (Kaler et al., 2017a), Canopy wilting (Kaler et al., 2017b), Drought susceptibility index (Du et al., 2009a), Canopy width (Mian et al., 1998), Nodule number (Hwang et al., 2014), Root morphology (Abdel-Haleem et al., 2011), Seed yield (Du et al., 2009b), and Seed oil (Specht et al., 2001)

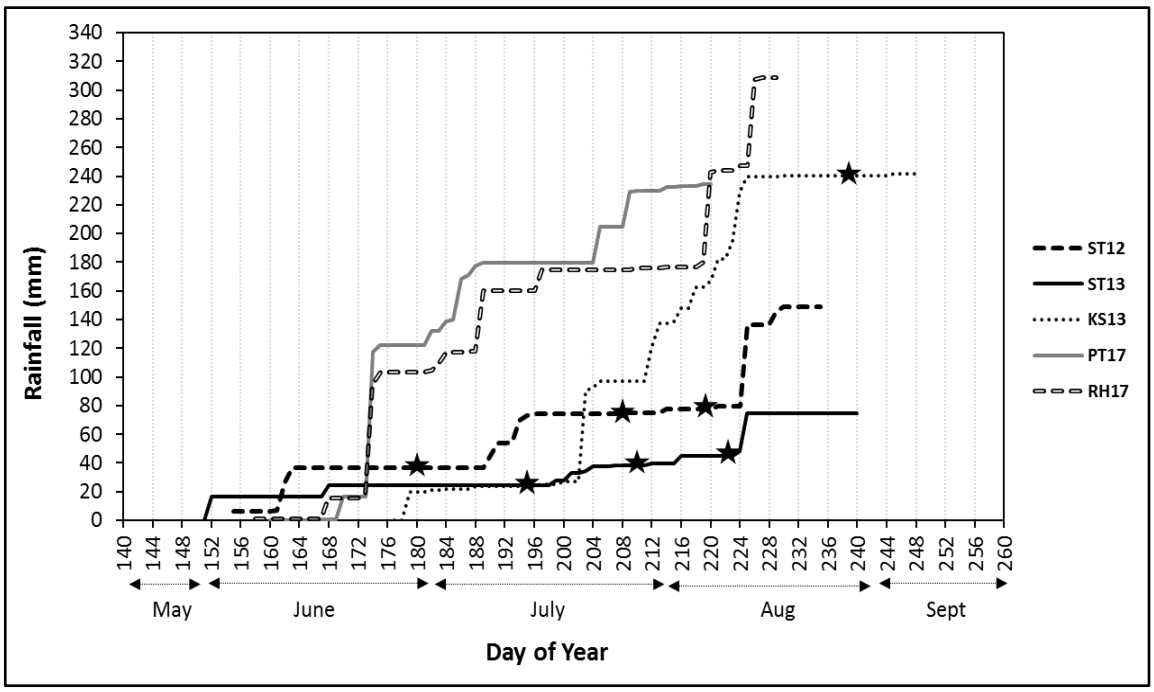

Figure 4_1. Cumulative rainfall (mm) starting from planting to sampling period for the five environments (Stuttgart in 2012 (ST12) and in 2013 (ST13), Keiser in 2013 (KS13), Pine Tree in 2017 (PT17), and Rohwer in 2017 (RH17)). Stars indicate dates of irrigation for the irrigated treatment.

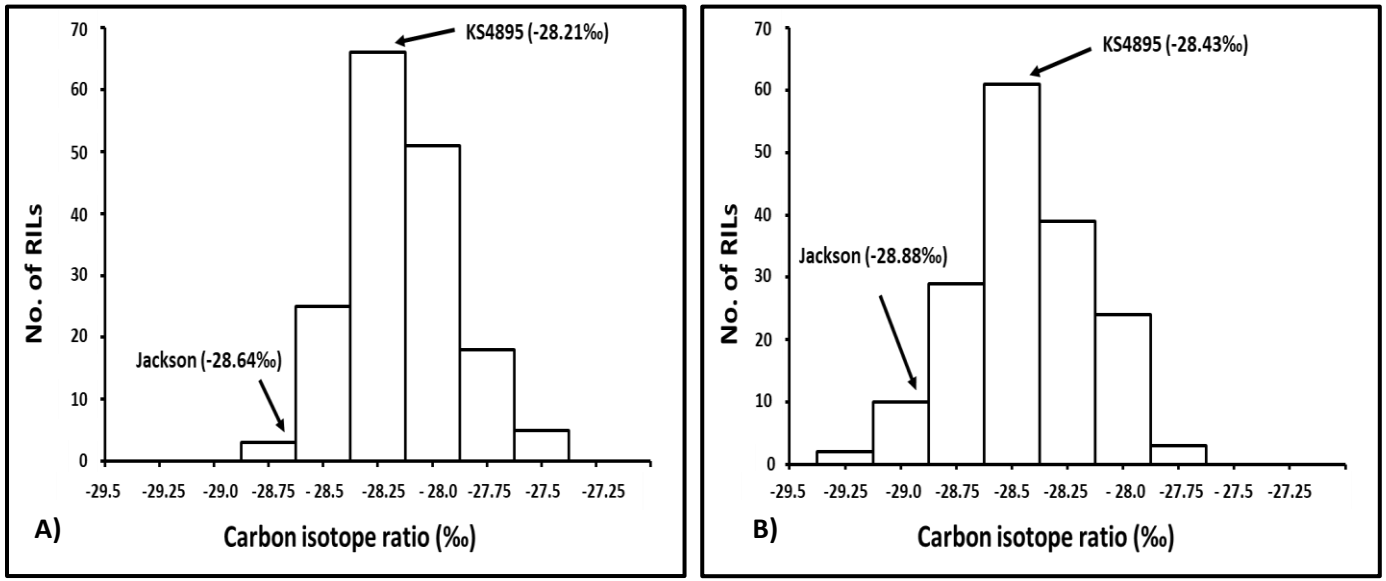

Figure 4_2. Frequency distribution of δ13C (‰) averaged over environments for rainfed (A) and irrigated conditions (B) in recombinant inbred lines (RILs) derived from a cross between KS4895 and Jackson. Values of δ13C for KS4895 were greater than for Jackson in both rainfed ($P \leq .01$) and irrigated ($P \leq .05$) treatments.

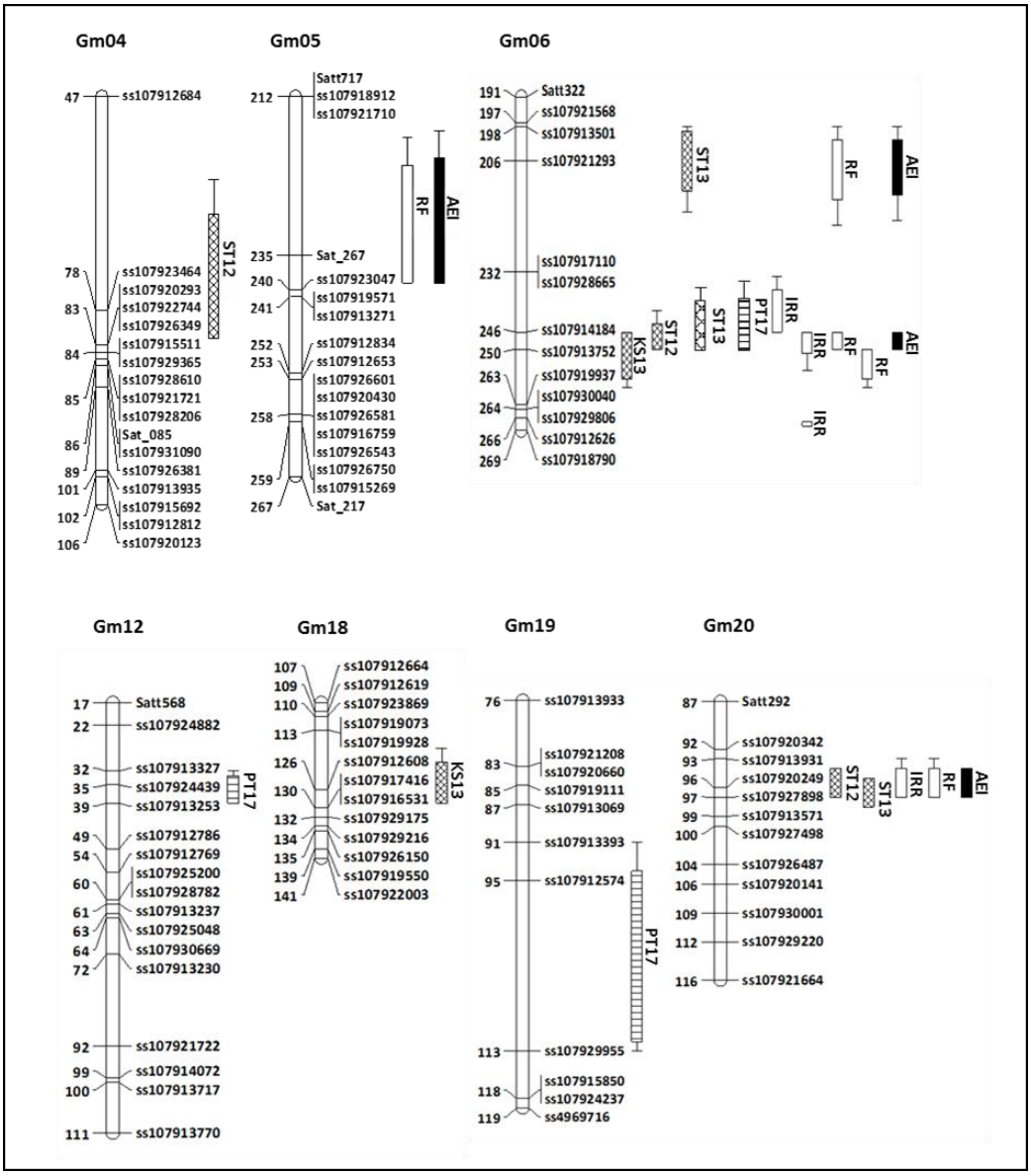

Figure 4_3. Position of quantative trait loci associated with $\delta^{13}C$ based on composite interval mapping (CIM) in the KS4895 × Jackson population.

†Cross-hatched bar indicates the QTLs identified in different environments (Keiser in 2013 (KS13), Stuttgart in 2012 and 2013 (ST12, ST13), open bar indicates the QTLs were identified in different irrigation conditions (Rainfed, RF and Irrigated, IRR), and solid bars indicate the QTLs were identified when averaged over environments and irrigation conditions (AEI). Bars with horizontal lines indicates the QTLs were identified in the Pine Tree environment in 2017 (PT17) under rainfed conditions. The length of the QTL bar indicates the LOD-1 confidence interval and error bar extended to the LOD-2 confidence interval based on the maximum likelihood value.

References

Abdel-Haleem, H., G.J. Lee, and R.H. Boerma. 2011. Identification of QTL for increased fibrous roots in soybean. Theor. Appl. Genet. 122:935-946.

Abdel-Haleem, H., T.E. Carter Jr, L.C. Purcell, C.A. King, L.L. Ries, P. Chen, W. Schapaugh Jr, T.R. Sinclair, and H.R. Boerma. 2012. Mapping of quantitative trait loci for canopy wilting trait in soybean (*Glycine max* L. Merr). Theor. Appl. Genet. 125:837-846.

Alcivar, A., J. Jacobson, J. Rainho, K. Meksem, D.A. Lightfoot, and M.A. Kassem, 2007. Genetic analysis of soybean plant height, hypocotyl and internode lengths. J. Food Agric. Environ. Sci. 1:40-50.

Araus, J.L., T. Amaro, J. Casadesús, A. Asbati, and M.M. Nachit. 1998. Relationships between ash content, carbon isotope discrimination and yield in durum wheat. Aust. J. Plant Physiol. 25:835-842.

Ashok, I.S.A.H., T.G. Prasad, M.U. Kumar, R.C.N. Rao, and G.C. Wright. 1999. Variation in transpiration efficiency and carbon isotope discrimination in cowpea. Aust. J. Plant Physiol. 26:503-510.

Babu, R.C., B.D. Nguyen, V. Chamarerk, P. Shanmugasundaram, P. Chezhian, P. Jeyaprakash, S. K. Ganesh, A. Palchamy, S. Sadasivam, S. Sarkarung, L. J. Wade, and H.T. Nguyen. 2003. Genetic analysis of drought resistance in rice by molecular markers association between secondary traits and field performance. Crop Sci. 43:1457-1469.

Bai, H., and L. C. Purcell. 2018a. Response of carbon isotope discrimination and oxygen isotope composition to mild drought in slow- and fast-wilting soybean genotypes. J. Crop Improv. 32:239-253.

Bai, H., and L. C. Purcell. 2018b. Aerial canopy temperature differences between fast- and slow-wilting soybean genotypes. J. Agron. Crop Sci. 2018. 204:243-251.

Blum, A. 1988. Drought resistance. In: plant breeding for stress environment. Pp 43-76. CRC Press, Boca Raton, Florida, USA.

Brensha, W., S.K. Kantartzi, K. Meksem, R.L. Grier, A. Barakat, D.A. Lightfoot, and A. Kassem. 2012. Genetic analysis of root and shoot traits in the 'Essex' by 'Forrest' recombinant inbred line (RIL) population of soybean [*Glycine max* (L.) Merr.]. J. Plant Genome Sci. 1:1-9.

Brugnoli, E., and G.D. Farquhar. 2000. Photosynthetic fractionation of carbon isotopes. In: Photosynthesis: Physiology and Metabolism. Ed: Leegood, R.C., T.D. Sharkey, and S. von Caemmerer. Pp 399-434. Kluwer Academic Publishers, Dordrecht.

Brugnoli, E., K.T. Hubick, S.V. Caemmerer, and G.D. Farquhar. 1988. Correlation between the carbon isotope discrimination in leaf starch and sugars of C3 plants and the ratio of

intercellular and atmospheric partial pressures of carbon dioxide. Plant Physiol. 88:1418-1424.

Brummer, E.C., G.L. Graef, J. Orf, J.R. Wilcox, and R.C. Shoemaker. 1997. Mapping QTL for seed protein and oil content in eight soybean populations. Crop Sci. 37:370-378.

Buss, G.R., H.M. Camper, Jr., and C.W. Roane. 1988. Registration of 'Hutcheson' soybean. Crop Sci. 28:1024-1025.

Çag˘irgan, M., M.O. Özbas¸ L.K. Heng, and R. Afza. 2005. Genotypic variability for carbon isotope discrimination in the mutant and improved lines of barley. Isotopes Environ. Health Stud. 41:229-235.

Carpentieri-Pipolo, V., A.E. Pipolo, H. Abdel-haleem, H.R. Boerma, and T.R. Sinclair. 2011. Identification of QTLs associated with limited leaf hydraulic conductance in soybean. Euphytica. 186:679-686.

Charlson, D.V., S. Bhatnagar, C.A. King, J.D. Ray, C.H. Sneller, T.E. Carter, and L.C. Purcell. 2009. Polygenic inheritance of canopy wilting in soybean [*Glycine max* (L.) Merr.]. Theor. Appl. Genet. 119:587-594.

Chen, J., S.X. Chang, and A.O. Anyia. 2011. The physiology and stability of leaf carbon isotope discrimination as a measure of water use efficiency in barley on the Canadian Prairies. J. Agron. Crop. Sci. 197:1-11.

Chen, Q.S., Z.C. Zhang, C.Y. Liu, D.W. Xin, D.P. Shan, H.M. Qiu, and C.Y. Shan. 2007. QTL analysis of major agronomic traits in soybean. Agric. Sci China. 6(4):399-405.

Cheng, L., Y. Wang, C. Zhang, C. Wu, J. Xu, H. Zhu, J. Leng, Y. Bai, R. Guan, W. Hou, L. Zhang, and T. Han. 2011. Genetic analysis and QTL detection of reproductive period and post-flowering photoperiod responses in soybean. Theor. Appl. Genet. 123:421-429.

Churchill, G.A., and R.W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. Genetics. 138(3):963-971.

Cober, E.R., S.J. Molnar, M. Charette, and H.D. Voldeng. 2010. A new locus for early maturity in soybean. Crop Sci. 50:524–527. doi:10.2135/cropsci2009.04.0174

Condon, A.G., R.A. Richards, and G.D. Farquhar. 1987. Carbon isotope discrimination is positively correlated with grain yield and dry matter production in field-grown wheat. Crop Sci. 27:996-1001.

Csanadi, G., J. Vollmann, G. Stift, and T. Lelley. 2001. Seed quality QTLs identified in molecular map of early maturing soybean. Theor. Appl. Genet. 103:912-919.

Dhanapal, A.P., J.D. Ray, S.K. Singh, V. Hoyos-Villegas, J.R. Smith, L.C. Purcell, C.A King, P.B. Cregan, Q. Song, and F.B. Fritschi. 2015. Genome wide association study (GWAS)

of carbon isotope ratio ($\delta^{13}C$) in diverse soybean [*Glycine max* (L.) Merr.] genotypes. Theor. Appl. Genet.128:73-91.

Diab, A.A., B. Teulat-Merah, D. This, N.Z. Ozturk, D. Benscher, and M.E. Sorrells. 2004. Identification of drought-inducible genes and differentially expressed sequence tags in barley. Theor. Appl. Genet. 109:1417-1425.

Dogan, E., H. Kirnak, and O. Copur. 2007. Deficit irrigations during soybean reproductive stages and CROPGRO-soybean simulations under semi-arid climatic conditions. Field Crops Res. 103:154-159.

Du, W., D. Yu, and S. Fu. 2009b. Detection of quantitative trait loci for yield and drought tolerance traits in soybean using a recombinant inbred line population. J. Integr. Plant Biol. 51:868-878.

Du, W., M. Wang, S. Fu, and D. Yu. 2009a. Mapping QTLs for seed yield and drought susceptibility index in soybean (*Glycine max* L.) across different environments. J. Genet. Genomics. 36:721-731.

Ehdaie, B., A.E. Hall, G.D. Farquhar, H.T. Nguyen, and J.D. Waines. 1991. Water use efficiency and carbon isotope discrimination in wheat. Crop Sci. 31:1282-1288.

Ellis, R.P., B.P. Forster, D.C. Gordon, L.L. Handley, R.P. Keith, P. Lawrence, R. Meyer, W. Powell, D. Robinson, C.M. Scrimgeour, G. Young, and W.T. Thomas. 2002. Phenotype/ genotype associations for yield and salt tolerance in a barley mapping population segregating for two dwarfing genes. J. Exp. Bot. 53:1163-1176.

Ellis, R.P., B.P. Forster, R. Waugh, N. Bonar, L.L. Handley, D. Robinson, D.C. Gordon, and W. Powell. 1997. Mapping physiological traits in barley. New Phytologist. 137:149-157.

Farquhar, G.D., and R.A. Richards. 1984. Isotopic composition of plant carbon correlates with water use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11:539-552.

Farquhar, G.D., J.R. Ehleringer, and K.T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Biol. 40:503-537.

Farquhar, G.D., M.H. O'Leary, and J.A. Berry. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9:121-137.

Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean development. Spec. Rep. 80. Iowa State Univ. Coop. Ext. Serv., Ames, IA.

Gai J., Y. Wang, X. Wu, and S. Chen. 2007. A comparative study on segregation analysis and QTL mapping of quantitative traits in plants- with a case in soybean. Front. Agric. China. 1:1-7.

Githiri, S.M., S. Watanabe, K. Harada, and R. Takahashi. 2006. QTL analysis of flooding tolerance in soybean at an early vegetative growth stage. Plant Breed. 125:613-618.

Gutierrez-Gonzalez, J.J., X. Wu, J. Zhang, J.D. Lee, M. Ellersieck, G.J. Shannon, O. Yu, H.T. Nguyen, and D.A. Sleper. 2009. Genetic control of soybean seed isoflavone content: importance of statistical model and epistasis in complex traits, Theor. Appl. Genet. 119:1069-1083.

Hall, A.E., R.G. Mutters, K.T. Hubick, and G.D. Farquhar. 1990. Genotypic differences in carbon isotope discrimination by cowpea under wet and dry field conditions. Crop Sci. 30:300-305.

Hausmann, N.J., T.E. Juenger, S. Sen, K. Stowe, T.E. Dawson, and E.L. Simms. 2005. Quantitative trait loci affecting $\delta^{13}C$ and response to differential water availability in Arabidopsis thaliana. Evolution; Internaltional Journal of Organic Evolution. 59:81-96.

Hubick, K.T., G.D. Farquhar, and R. Shorter. 1986. Correlation between water-use efficiency and carbon isotope discrimination in diverse peanut (*Arachis*) germplasm. Aust. J. Plant Physiol. 13:803-816.

Hubick, K.T., R. Shorter, and G.D. Farquhar. 1988. Heritability and genotype × environment interactions of carbon isotope discrimination and transpiration efficiency in peanut (*Arachis hypogaea* L.). Aust. J. Plant. Physiol. 15:799-813.

Hwang, S., C.A. King, M.K. Davies, D.V. Charlson, J.D. Ray, P.B. Cregan, C.H. Sneller, P. Chen, T.E. Carter, Jr., and L.C. Purcell. 2015a. Registration of the KS4895 x Jackson mapping population (AR93705). J. Plant Reg. 9:266-271.

Hwang, S., C.A. King, M.K. Davies, J.D. Ray, P.B. Cregan, and L.C. Purcell. 2013. QTL analysis of shoot ureide and nitrogen concentrations in soybean [Glycine max (L.) Merr.]. Crop Sci. 53:2421-2433. Hwang, S., C.A. King, J.D. Ray, P.B. Cregan, P. Chen, T.E.J. Carter Jr, Z. Li, H. Abdel-Haleem, K.W. Matson, W. Schapaugh Jr, and L.C. Purcell. 2015b. Confirmation of delayed canopy wilting QTLs from multiple soybean mapping populations. Theor. Appl. Genet. 128: 2047-2065.

Hwang, S., C.A. King, P. Chen, J.D. Ray, P.B. Cregan, T.E. Carter Jr, Z. Li, H. Abdel-Haleem, K.W. Matson, W. Schapaugh Jr, and L.C. Purcell. 2016. Meta-analysis to refine map position and reduce confidence intervals for delayed canopy wilting QTLs in soybean. Mol. Breed. 36:91.

Hwang, S., J.D. Ray, P.B. Cregan, C.A. King, M.K. Davies, and L.C. Purcell. 2014. Genetics and mapping of quantitative traits for nodule number, weight, and size in soybean (*Glycine max* (L) Merr). Euphytica. 195:419-434.

Hyten, D.L., V.R. Pantalone, Pantalone, C.E. Sams, A.M. Saxton, D. Landau-Ellis, T.R. Stefaniak, M.E. Schmidt. 2004. Seed quality QTL in a prominent soybean population. Theor. Appl. Genet. 109:552-552.

Impa, S.M., S. Nadaradjan, P. Boominathan, G. Shashidhar, H. Bindumadhava, and M.S. Sheshshayee. 2005. Carbon isotope discrimination accurately reflects variability in WUE measured at a whole plant level in rice. Crop Sci. 45:2517-2522.

Ismail, A.M., and Hall, A.E. 1992. Correlation between water use efficiency and carbon isotope discrimination in diverse cowpea genotypes and isogenic lines. Crop Sci. 32:7-12.

Jansen, R.C., and P. Stam. 1994. High resolution of quantitative traits into multiple loci via interval mapping. Genetics. 136:1447-1455.

Johnson, D.A., and L.M. Bassett. 1991. Carbon isotope discrimination and water use efficiency in four cold season grasses. Crop Sci. 31:157-162.

Johnson, H.W. 1958. Registration of soybean varieties. VI. J. Agron. 11:690-691.

Jones, M.M., N.C. Turner, and C.B. Osmond. 1981. Mechanisms of drought resistance. In: The physiology and biochemistry of drought resistance in plants. Ed: Paleg, L.G., and D. Aspinall. Pp-15-37. Academic Press, Australia.

Juenger, T.E., J.K. McKay, N. Hausmann, J.J.B. Keurentjes, S. Sen, K.A. Stowe, T.E. Dawson, E.L. Simms, and J.H. Richards. 2005. Identification and characterization of QTL underlying whole plant physiology in *Arabidopsis thaliana*: $\delta^{13}C$, stomatal conductance and transpiration efficiency. Plant Cell Environ. 28:697-708.

Kaler, A. S., A.P. Dhanapal, J.D. Ray, C.A. King, F.B. Fritschi, and L.C. Purcell. 2017a. Genome-wide association mapping of carbon isotope and oxygen isotope ratios in diverse soybean genotypes. Crop Sci. 57:3085-3100.

Kaler, A. S., J. D. Ray, W.T. Schapaugh, M.K. Davies, C. A. King, and L. C. Purcell. 2018c. Association mapping identifies loci for canopy coverage in diverse soybean genotypes. Mol. Breed. 38:50.

Kaler, A. S., J.D. Ray, W.T. Schapaugh, C.A. King, and L.C. Purcell. 2017b. Genome-wide association mapping of canopy wilting in diverse soybean genotypes. Theor. Appl. Genet. 130:2203-2217.

Kaler, A. S., J.D. Ray, W.T. Schapaugh, R.A. Antonio, C.A. King, E.E. Gbur, and L.C. Purcell. 2018b. Association mapping identifies loci for canopy temperature under drought in diverse soybean genotypes. Euphytica. 214:135.

Kaler, A.S., S.K. Bazzer, A. Sanz-Saez, J.D. Ray, F.B. Fritschi, and L.C. Purcell. 2018a. Carbon isotope ratio fractionation among plant tissues of soybean. Plant Phenome J. 1:180002.

Kao, C.H., Z.B. Zeng, and R.D. Teasdale. 1999. Multiple interval mapping for quantitative trait loci. Genetics. 152:1203-1216.

Kearsey, M.J., and V. Hyne. 1994. QTL analysis: a simple marker regression approach. Theor. Appl. Genet. 89:698-702.

Kim, H.K., S.T. Kang, and K.W. Oh. 2006. Mapping of putative quantitative trait loci controlling the total oligosaccharide and sucrose content of *Glycine max* seeds. J. Plant Res. 119:533-538.

King, C.A., and L.C. Purcell. 2001. Water and carbon allocation to soybean nodules differing in size and the importance to nitrogen fixation response to water deficit. Crop Sci. 41:1099 1107.

King, C.A., and L.C. Purcell. 2006. Genotypic variation for shoot N concentration and response to water deficits in soybean. Crop Sci. 46:2396-2402.

Kong, F., H. Nan, D. Cao, Y. Li, F. Wu, J. Wang et al. 2014. A new dominant gene conditions early flowering and maturity in soybean. Crop Sci. 54:2529–2535. doi:10.2135/cropsci2014.03.0228

Laza, M.R., M. Kondo, O. Ideta, E. Barlaan, and T. Imbe. 2006. Identification of quantitative trait loci for 13C and productivity in irrigated lowland rice. Crop Sci. 46:763-773.

Lee, S.H., M.A. Bailey, M.A. Mian, T.E. Carter, E.R. Shipe, D.A. Ashley, W. Parrott, R.S. Hussey, and H.R. Boerma. 1996. RFLP loci associated with soybean seed protein and oil content across populations and locations. Theor. Appl. Genet. 93: 649-657.

Levene, H. 1960. Robust testes for equality of variances. In: Contributions to Probability and Statistics. Ed: Olkin, I . Pp 278-292. Stanford University Press, Palo Alto, CA.

Li, X., C. Fang, M. Xu, F. Zhang, S. Lu, H. Nan, T. Su, S. Li, X. Zhao, L. Kong, X. Yuan, B. Liu, J. Abe, E.R. Cober, and F. Kong. 2017. Quantitative trait locus mapping of soybean maturity gene *E6*. Crop Sci. Crop Sci. 57:2547–2554. doi: 10.2135/cropsci2017.02.0106

Li, D., T.W. Pfeiffer, and P.L. Cornelius. 2008. Soybean QTL for yield and yield components associated with *Glycine soja* alleles. Crop Sci. 48:571-581.

Liang, H., Y. Yu, H. Yang, L. Xu, W. Dong, H. Du, and H. Zhang. 2014. Inheritance and QTL mapping of related root traits in soybean at the seedling stage. Theor. Appl. Genet. 127:2127-2137.

Liang, H.Z., Y.L. Yu, S.F. Wang, Y. Lian, T.F. Wang, Y.L. Wei, P.T. Gong, X.Y. Liu, X.J. Fang, and M.C. Zhang. 2010. QTL mapping of isoflavone, oil and protein contents in soybean (Glycine max L. Merr.). Agric. Sci. China. 9:1108-1116.

Lu, W., Z. Wen, H. Li, D. Yuan, J. Li, H. Zhang, Z. Huang, S. Cui and W. Du. 2013. Identification of the quantitative trait loci (QTL) underlying water soluble protein content in soybean. Theor. Appl. Genet. 126:425-433.

Ludlow, M.M., and R.C. Muchow. 1990. A critical evaluation of traits for improving crop yields in water-limited environments. Adv. Agron. 43:107-153.

Ma, Y., G. Kan, X. Zhang, Y. Wang, W. Zhang, H. Du, and D. Yu. 2016. Quantitative trait Loci (QTL) mapping for glycinin and β-conglycinin contents in soybean (*Glycine max* L. Merr.). J. Agric. Food Chem. 64:3473-3483.

Manavalan, L.P., S.K. Guttikonda, L.P. Tran, and H.T. Nguyen. 2009. Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol. 50:1260-1276.

Mao, T., Z. Jiang, Y. Han, W. Teng, X. Zhao, and W. Li. 2013. Identification of quantitative trait loci underlying seed protein and oil contents of soybean across multi-genetic backgrounds and environments. Plant Breed. 132:630-641.

Martin, B., and J. Nienhuis, G King, and A Schaefer. 1989. Restriction fragment length polymorphisms associated with water use efficiency in tomato. Science. 243:1725-1728.

Merah, O., E. Deléens, I. Souyris, M. Nachit, and P. Monneveux. 2001. Stability of carbon isotope discrimination and grain yield in durum wheat. Crop Sci. 41:677-681.

Mian, M.A.R., D.A. Ashley, and H.R. Boerma. 1998. An additional QTL for water use efficiency in soybean. Crop Sci. 38:390-393.

Mian, M.A.R., M.A. Mailey, D.A. Ashley, R. Wells, T.E. Carter, W.A. Parrot, and H.B. Boerma. 1996. Molecular markers associated with water use efficiency and leaf ash in soybean. Crop Sci. 36:1252-1257.

Moongkanna, J., S. Nakasathien, W.P. Novitzky, P. Kwanyuen, P. Sinchaisri, and P. Srinives. 2011. SSR markers linking to seed traits and total oil content in soybean. Thai J. Agric. Sci. 44:233-241.

Orf, J.H., K. Chase, T. Jarvik, L.M. Mansur, P.B. Cregan, F.R. Adler, and K.G. Lark. 1999. Genetics of soybean agronomic traits: I. Comparison of three related recombinant inbred populations. Crop Sci. 39:1642-1651.

Passioura, J.B. 1996. Drought and drought tolerance. Plant. Growth. Regul. 20:79-83.

Pathan, S.M., T. Vuong, K. Clark, J. Lee, J.G. Shannon, C.A. Roberts, M.R. Ellersieck, J.W. Burton, P.B. Cregan, D.L. Hyten, H.T. Nguyen, and D.A. Sleper. 2013. Genetic Mapping and Confirmation of Quantitative Trait Loci for Seed Protein and Oil Contents and Seed Weight in Soybean. Crop Sci. 53:765-774

Peleg, Z., T. Fahima, T. Krugman, S. Abbo, D. Yakir, A.B. Korol, and Y. Saranga. 2009. Genomic dissection of drought resistance in durum wheat × wild emmer wheat recombinant inbreed line population. Plant Cell Environ. 32:758-779.

Piepho, H.P., and H.G. Gauch. 2001. Marker pair selection for mapping quantitative trait loci. Genetics. 157:433-444.

Pinto, R.S., M.P. Reynolds, K.L. Mathew, C.L. McIntyre, J. Jose, and S.C. Chapman. 2010. Heat and drought adaptive QTL in a wheat population designed to minimize confounding agronomic effects. Theor. Appl. Genet. 121:1001-1021.

Purcell, L. C., J.T. Edwards, and K.R. Brye. 2007. Soybean yield and biomass responses to cumulative transpiration: Questioning widely held beliefs. Field Crop Res. 101:10-18.

Purcell, L.C., and J. Specht. 2004. Physiological traits for ameliorating drought stress. In: Soybeans: Improvement, Production, and Uses. Eds: H.R. Boerma and J.E. Specht. Madison, WI: ASA, CSSA, SSSA. Pp 569-620.

Qi, X., M.W. Li, M. Xie, X. Liu, M. Ni, G. Shao, C. Song, Y.A. Kay-Yuen, Y. Tao, F.L. Wong, S. Isobe, C.F. Wong, K.S. Wong, C. Xu, C. Li, Y. Wang, R. Guan, F. Sun, G. Fan, Z. Xiao, F. Zhou, T.H. Phang, X. Liu, S.W. Tong, T.F. Chan, S.M. Yiu, S. Tabata, J. Wang, X. Xu, and H.M. Lam. 2014. Identification of a novel salt tolerance gene in wild soybean by whole-genome sequencing. Nat Commun. 5:4340.

Qi, Z., Q. Wu, X. Han, Y. Sun, X. Du, C. Liu, H.Jiang, G. Hu, and Q. Chen. 2011. Soybean oil content QTL mapping and integrating with meta-analysis method for mining genes. Euphytica. 179:499-514.

Ray, J.D., K. Hinson, J.E.B. Mankono, and M.F. Malo. 1995. Genetic control of a long-juvenile trait in soybean. Crop Sci. 35:1001–1006. doi:10.2135/cropsci1995.0011183X003500040012x

Read, J.J., D.A. Johnson, K.H. Asay, and C. Tienzen. 1991. Carbon isotope discrimination, gas exchange and WUE in crested wheat grass clones. Crop Sci. 31:1203-1208.

Rebetzke, G.J., A.G. Condon, G.D. Farquhar, R. Appels, and R.A. Richards. 2008. Quantitative trait loci for carbon isotope discrimination are repeatable across environments and wheat mapping populations. Theor. Appl. Genet.118:123-137.

Richards, R.A., G.J. Rebetzke, A.G. Condon, A.F. Van Herwaarden, B.L. Duggan, and R. Munnas. 1999. Targeting physiological traits to overcome environmental limitations and raise the yield potential of dryland crops. In: 11th Aust Plant Breed Conf. Proc. Adelaide. 19-23 April 1999. Vol 1. pp 27-33.

Rieseberg, L., M. Archer, and R. Wayne. 1999. Transgressive segregation, adaptation and speciation. Heredity. 83:363–372.

Saranga, Y., I. Flash, A.H. Paterson, and D Yakir. 1999. Carbon isotope ratio in cotton varies with growth stage and plant organ. Plant Sci. 142:47-56.

Saranga, Y., M. Menz, C. Jiang, R.J. Wright, D. Yakir, and A.H. Paterson. 2001. Genomic dissection of genotype × environment interactions conferring adaptation of cotton to arid conditions. Genome Res. 11:1988-1995.

SAS Institute. 2013. The SAS System for Windows. Version 9.4. SAS Inst. Inc., Cary, NC.

Schapaugh, W.T., and R.E. Dille. 1998. Registration of 'KS4895' soybean. Crop Sci. 38:892.

Sharma, A.D., H. Sharma, and D. Lightfoot. 2010. The genetic control of tolerance to aluminum toxicity in the 'Essex' by 'Forrest' recombinant inbred line population. Theor. Appl. Genet. 122:687-694.

Shi, X., L.X. Yan, C. Yang, W. Yan, D.O. Moseley, T.W. Wang, B. Liu, R. Di, P. Chen, and M. Zhang. 2018. Identification of a major quantitative trait locus underlying salt tolerance in 'Jidou 12' soybean cultivar. BMC Res. Notes. 11:95.

Sloane, R., R. Patterson, and T. Carter Jr. 1990. Field drought tolerance of a soybean plant introduction. Crop. Sci. 30:118-123.

Specht, J.E., K. Chase, M. Macrander, G.L. Graef, J. Chung, J.P. Markwell, M. Germann, J.H. Orf, and K.G. Lark. 2001. Soybean response to water-a QTL analysis of drought tolerance. Crop Sci. 41:493-509.

Stiller, W.N., J.J. Read, G.A. Constable, and P.E. Reid. 2005. Selection for water use efficiency traits in a cotton breeding program: cultivar differences. Crop Sci. 45:1107-1113.

Takai, T., Y. Fukuta, A. Sugimoto, T. Shiraiwa, and T. Horie. 2006. Mapping of QTLs controlling carbon isotope discrimination in the photosynthetic system using recombinant inbred lines derived from a cross between two different rice (*Oryza sativa* L.) cultivars. Plant Prod. Sci. 9:271-280.

Teulat, B., O. Merah, X. Sirault, C. Borries, R. Waugh, and D. This. 2002. QTLs for grain carbon isotope discrimination in field grown barley. Theor. Appl. Genet. 106:118-126.

This, D., J. Comstock, B. Courtois, Y. Xu, N. Ahmadi, W.M. Vonhof, C. Fleet, T. Setter, and S. McCouch. 2010. Genetic analysis of water use efficiency in rice (*Oryza sativa* L.) at the leaf level. Rice. 3:72-86.

Valliyodan, B., and H.T. Nguyen. 2006. Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr. Opin. Plant Biol. 9:189-195.

VanToai, T.T., S.K. St Martin, K. Chase, G. Boru, V. Schnipke, A.F. Schmitthenner, and K.G. Lark. 2001. Identification of a QTL associated with tolerance of soybean to soil waterlogging. Crop Sci. 41:1247-1252.

Voorrips, R.E. 2002. MapChart: software for the graphical presentation of linkage maps and QTLs. J. Hered. 93:77-78.

Wang, S., C.J. Basten, and Z.B. Zeng. 2007. Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, North Carolina. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm.

White, D.S., M.J. Bell, and G.C. Wright. 1996. The potential to use carbon isotope discrimination as a selection tool to improve water use efficiency in soybean. Proc. of the 8th Australian agronomy conference, Toowoomba.

Wright, G. 1996. Review of ACIAR selection for water use efficiency in legumes project recommends further research. ACIAR Food Legume Newslett. 1996:2-3.

Xu Y., D. This, R.C. Pausch, W.M. Vonhof, J.R. Coburn, J.P. Comstock, and S.R. McCouch. 2009. Leaf-level water use efficiency determined by carbon isotope discrimination in rice seedlings: genetic variation associated with population structure and QTL mapping. Theor. Appl. Genet. 118:1065-1081.

Yakir, D., M.J. De Niro, and J.E. Ephrath. 1990. Effect of water stress on oxygen, hydrogen and carbon isotope ratios in two species of cotton plants. Plant Cell Environ. 13:949-955.

Yang, J., C. Hu, H. Hu, R. Yu, Z. Xia, X. Ye, and J. Zhu. 2008. QTL Network: mapping and visualizing genetic architecture of complex traits in experimental populations. Bioinformatics 24:721-723.

Yang, J., J. Zhu, and R.W. Williams. 2007. Mapping the genetic architecture of complex traits in experimental populations. Bioinformatics. 23:1527-1536.

Yoshikawa, T., Y. Okumoto, &D. Ogata, T. Sayama, M. Teraishi, M. Terai, T. Toda, K. Yamada, K. Yagasaki, N. Yamada, T. Tsukiyama, T. Yamada, and Y. Tanisaka. 2010. Transgressive segregation of isoflavone contents under the control of four QTLs in a cross between distantly related soybean varieties. Breeding Science. 60:243-254.

Zeng, Z.B. 1994. Precision mapping of quantitative trait loci. Genetics. 136:1457-1468.

CHAPTER V

Identification of Quantitative Trait Loci Associated with Canopy Temperature in Soybean

Abstract

A consistent risk for soybean (Glycine max L.) production is the impact of drought on growth and yield. Canopy temperature (CT) is an indirect measure of transpiration rate and stomatal conductance and may be valuable in distinguishing differences among genotypes in response to drought. The objective of this study was to map quantitative trait loci (QTLs) associated with CT using thermal infrared imaging in a population of recombinant inbred lines (RIL) developed from a cross between KS4895 and Jackson. Heritability of CT was 35% when estimated across environments. QTL analysis identified 11 loci for CT distributed on eight chromosomes that individually explained between 4.6 to 12.3% of the phenotypic variation. The locus on Gm11 was identified in two individual environments and across environments and explained the highest proportion of phenotypic variation (9.3% to 11.5%) in CT. Several of these CT loci coincided with the genomic regions from previous studies associated with canopy wilting, canopy temperature, water use efficiency, and other morpho-physiological traits related with drought tolerance. Candidate genes with biological function related to transpiration, root development, and signal transduction underlie these putative CT loci. These genomic regions may be important resources in soybean breeding programs to improve tolerance to drought.

Introduction

Soybean (Glycine max (L.) Merr.) is one of the most important crops grown in the US on an area of 30.8 million hectares with a production of 96.8 million metric tons and that contributes around 28% to global production (www.soystats.com). The sustainability of soybean production is threatened by climate changes such as increased temperature, erratic precipitation, and variable weather patterns. Among these factors, drought is one of the major constraints limiting yield potential in legumes and other cereal crops. Various field studies had reported that drought stress leads to the reduction of 5 to 50% of soybean yield[1,2]. Thus, there is need for development of cultivars with drought tolerance to cope with adverse climatic conditions and to improve crop performance. Selection for high yield is difficult under drought stress conditions due to its quantitative nature and because of a high interaction of genotype with the environment[3]. Therefore, it is important to identify specific physiological traits that may improve the crop performance and yield under water deficit conditions.

Canopy temperature can be used as surrogate measurement of plant water balance/relations and is an important physiological trait associated with drought tolerance[4-10]. Canopy temperature is closely associated with transpiration rate and stomatal conductance in many crops[8,11]. Due to evaporative cooling, transpiration is negatively correlated with canopy temperature[12]. Under optimum moisture conditions, increased vapor pressure deficit increases evaporative demand resulting in higher transpiration rate and a decrease in canopy temperature provided that stomatal conductance does not change. However, decreased transpiration rate and stomatal conductance under water deficit conditions limits evaporative cooling and leads to increased canopy temperature[13-15]. The genotypes with a cooler canopy under water deficit

condition continue transpiration at a relatively high rate, presumably due to a larger store of soil available water compared to genotypes with higher canopy temperature[16-18].

In bread wheat (*Triticum aestivum*) and durum wheat (*Triticum durum*), there is a significant correlation between cooler canopy temperature high yield[19,20,10]. Likewise, there were significant genetic gains in wheat yield when direct selection was made based on a cooler canopy[21,22,18]. A significant correlation between canopy temperature and transpiration was found in sugar beet (*Beta vulgaris*), rice (*Oryza sativa*), and potatoes (*Solanum tuberosum*)[23]. Cooler canopy (or canopy temperature depression) was positively correlated with grain yield in rice[24], sugarcane (*Saccharum officinarum*)[25,5], and chickpea (*Cicer arietinum*)[26,27], pearl millet (*Penniserum americanum*)[28], and soybean[29] under water deficit conditions. Canopy temperature depression (CTD) is defined as deviation of plant canopy temperature from the air temperature. Bai and Purcell[4] found that slow wilting genotypes under drought had a cooler canopy than fast wilting genotypes and that a cooler canopy was positively associated with grain yield.

Manual phenotyping/measurement of transpiration and stomatal conductance to detect canopy temperatures differences is difficult and tedious. Therefore, selection criteria for genotypes with cooler canopy must be rapid, relatively simple, and allow the screening of large number of field plots in a short period of time[30,31]. The advent of high throughput phenotyping platforms has led to rapid, accurate, and non-destructive monitoring of whole-plant responses and differences in stomatal behavior to water stress[32-35]. Unmanned aerial systems (UAS) provide an efficient phenotyping platform to evaluate a large number of experimental fields for precise, quantitative assessment of CT in segregating mapping populations, and allowing a comparison among genotypes for CT differences[8]. Thermal infrared imaging from a UAS has

become an important tool in early growth stages to detect drought stress, improve water use efficiency (WUE), and precisely manage irrigation[36,37,7,38,39].

Combining thermal infrared imaging with genetic mapping may help in understanding the genetic architecture of drought tolerance[40-44]. Mapping studies for CT have been reported in wheat[11], rice[45], and maize (*Zea mays*)[46]. In soybean, genome wide association mapping (GWAM) and linkage mapping studies have dissected the genetic basic of several morpho-physiological traits such as canopy wilting[47-51], carbon isotope ratio ($\delta^{13}C$)[52-55], oxygen isotope ratio ($\delta^{15}O$)[55], and canopy coverage[56]. The first GWAM study for CT in soybean was conducted using a diverse panel of 345 maturity group IV accessions[9]. Association analysis identified 34 loci associated with CT. However, to date, there has been no report of linkage mapping using thermal infrared imaging to study the genetic basis of CT in soybean.

Thus, the present study aimed to identify the genomic regions associated with CT using a mapping population of 168 recombinant inbred lines (RILs) developed from a cross between KS4895 and Jackson. The objectives of this study were to (i) identify QTLs associated with CT (ii) confirm identified CT QTLs with previously mapped QTLs associated with drought tolerance; and (iii) search for putative candidate genes underlying these QTLs.

Material and Methods

One hundred and sixty-eight F_5-derived recombinant inbred lines (RILs) generated from a cross between KS4895 (PI 595081)[57] and Jackson (PI 548657)[58] were used to identify genomic regions associated with CT. KS4895 is a maturity group (MG) IV cultivar developed by the Kansas Agricultural Experiment Station, and Jackson is a MG VII cultivar developed by the North Carolina Agricultural Experiment Station and the USDA-ARS. This RIL population was previously used to study the genetic control of canopy wilting[49,50] and $\delta^{13}C$[53].

The RIL population along with parents were evaluated for CT at the Pine Tree Research Station, AR (35.11° N, 90.91° W) on a Calloway silt loam soil (fine, montmorillonitic, thermic Typic Albaqualfs) and Rohwer Research Station, AR (33.80° N, 91.28° W) on a Sharkey silty clay soil (very-fine, smectitic, thermic Chromic Epiaquerts) in three consecutive years (2017-2019). Each location by year combination was treated as an individual environment and designated as PT17 (Pine Tree 2017), RH17 (Rohwer 2017), PT18 (Pine Tree 2018), RH18 (Rohwer 2018), PT19 (Pine Tree 2019), and RH19 (Rohwer 2019). A randomized complete block experimental design with two replications was employed at each environment. The details of planting date, CT measurement date, and weather data on the CT measurement day are presented in Table 1. The RILs were planted in nine-row plots that were 4.0-4.5 m long with 0.15-0.18 m spacing between rows. The average minimum temperature, maximum temperature, and rainfall for Pine Tree and Rohwer in 2017, 2018, and 2019 were collected from Southern Regional Climate Center (www.srcc.lsu.edu/station_search) using Climate Data Portal. An irrigation scheduling program[59] was used to estimate soil moisture deficit. Vapor pressure deficit (VPD) for each environment was estimated from daily maximum and minimum temperatures, assuming that water vapor pressure was saturated at the minimum temperature[60,61]. The

experiments were rainfed for all environments, and experimental fields were managed using recommended practices.

Phenotypic Evaluation:

Canopy temperature was determined by using aerial infrared image analysis. A drone (DJI Phantom 4 Pro, www.dji.com/phantom-4-pro) equipped with an infrared (IR) camera (Model: FLIR Tau 2, www.flir.com) with a sensor size of 640 × 512 pixels, 25 mm focal length, sensitivity of 0.05°C, and an accuracy of ±5%, was flown at a height of 120 meters above the ground at full canopy coverage. A digital video recorder recorded the video stream from the camera. The IR camera is not calibrated. That is, values of CT from the IR range from 0 (cool) to 255 (hot) and cover a range of 12.5°C, but the specific temperature of the canopy is not determined. Herein, we report values directly from the IR camera as a measure of CT.

The IR images were extracted from the video file using VLC video player (www.videolan.org), and 6 to 17 images representing each plot multiple times were selected manually. Selected IR images were processed using FieldAnalyzer software (www.turfanalyzer.com/field-analyzer) to extract CT values from the average values of 400 to 2000 pixels from the center portion of the IR image of each plot and was used as a measure of CT. There were multiple CT values of each plot extracted from multiple selected images. The final CT values used for analysis was the average CT values determined from analyzing multiple images and after removing values that were more than ±2 standard errors from the mean.

Statistical Analysis

The phenotypic analysis of CT was performed using SAS v.9.2 software (SAS Institute, 2013). The normality of CT in an individual environment was checked using a Q-Q plot of residuals and the Shapiro-Wilk test[62]. The presence of statistical differences between parents for

CT was estimated using a *t*-test. Pearson's correlation analysis between environments and analysis of variance (ANOVA) averaged over environments were performed using PROC CORR (α=0.05) and PROC MIXED procedures, respectively. Heritability was estimated from the variance components calculated with restricted maximum likelihood (REML) method of VARCOMP procedure. Narrow sense heritability (h^2) was calculated using the following formula[63]:

Across environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_{GE}^2}{E}\right) + \left(\frac{\sigma_e^2}{RE}\right)}$$

Within environments: $$h^2 = \frac{\sigma_G^2}{\sigma_G^2 + \left(\frac{\sigma_e^2}{R}\right)}$$

where σ_G^2, σ_{GE}^2, σ_e^2 are genotypic variance, genotype-by-environment variance, and error variance, respectively, and *E* and *R* are the number of environments and replications, respectively. Because F_5-derived RILs were used in this research, σ_G^2 was composed entirely of additive variance and additive × additive epistasis variance, with negligible variance associated with other components of dominance variance. As the result, this heritability should be considered as narrow sense heritability. BLUPs (best linear unbiased predictions) were calculated using PROC MIXED procedure for individual environments and averaged across environments, considering all factors in the model as random. Environment was considered a fixed effect in the combined data analysis. QTL analysis of CT was performed using BLUP values to reduce the environmental variations.

QTL Analysis

The genetic map for the KS4895 × Jackson mapping population was previously described by Hwang et al.[49] and used for QTL analysis in the present study. Briefly, the linkage map consists of 37 simple-sequence repeat (SSR) and 511 single-nucleotide polymorphism (SNP)

markers with a map size of 2089 cM. The BLUP estimates calculated for individual environments and averaged across environments were used for QTL analysis. The QTL analysis was performed with WinQTL Cartographer v.2.5[64] using composite interval mapping (CIM) and multiple interval mapping (MIM) methods. CIM was performed using Model 6 (standard model) of the *Zmapqtl* program module[65]. Markers as co-factors to control background noise were selected with forward and backward stepwise regression methods with a walk speed and window size of 1 cM. A significant LOD (log of odds) threshold score was determined by a permutation test with 1,000 runs and with a genome wide type I error of 5%[66]. The most likely position of QTLs and an estimate of the magnitude of their additive effects were determined using the CIM method[67]. The confidence interval for putative QTL positions was determined by one-LOD drop on either side of the LOD peak.

Multiple interval mapping (MIM) is a stepwise model procedure[68] in which presence of significant QTLs and QTL × QTL interactions are detected using QTLs identified in the CIM method as an initial MIM selection model. This pre-selected MIM model was optimized for identified QTLs, search for new QTLs, and QTL × QTL interactions by using the 'optimize QTLs positions', 'search for new QTLs', and 'QTL interactions' options, respectively. The MIM model was determined with the minimum Bayesian information criterion (BIC), $c(n) = In(n)$, and with genome walk speed and window size of 1 cM. The criterion used to declare coincident QTLs between environments was based on at least a 10 cM overlap in QTL intervals on the linkage map. In this study, a QTL that explain more than 10% of phenotypic variation was considered a major QTL.

Candidate Genes Identification

The genomic regions underlying the putative QTLs for CT identified in each environment and across environments were searched for candidate genes using the genome browser of Soybase (www.soybase.com). The candidate genes falling within ±1MB from the nearest marker of putative QTLs were selected according to the *Glyma1.1* assembly in Soybase (www.soybase.org) with consideration for those genes having biological function related to transpiration, canopy temperature, rooting, and plant water relations.

Results

Phenotypic Evaluation

CT measurement dates, weather data, estimated VPD, and soil moisture deficit for all environments are presented in Table 1. From all the CT measurements, CT data were extracted from the flight made on the date/day when canopy was completely closed and had the most intense water deficit conditions. The maximum temperature ranged from 29°C (PT17) to 37°C (PT19) and minimum temperature ranged from 15°C (PT17) to 25°C (RH17) on the day of CT measurements (Table 5_1). The estimated VPD was ≥ 2.3 kPa on day of CT measurements in all environments except for RH18 in which VPD=1.6 kPa. There had been no rainfall from 4 days to 13 days prior to CT measurements. The estimated soil moisture deficit ranged from 49 mm to more than 75 mm on the day of CT measurements (Table 5_1). Irrigation is recommended if estimated soil moisture deficit exceeds 37 mm for silt loam soils present at Pine Tree and 50 mm for silt clay soils present at Rohwer[59], indicating drought-stress conditions on the day of CT measurements.

The CT values had a wide range in all environments with RIL means that ranged from 50 to 64 (Figure 5_1, Table 5_2). Jackson had lower CT values than KS4895 in all environments except RH17 (Table 5_2), and parents were significantly (P<0.05) different in PT17, RH19, and across environments (AE) (Table 5_2). Averaged over environments, IR values were 12 units less for Jackson than KS4895, which indicate a CT that was approximately 0.59°C cooler for Jackson. The distributions of CT values were approximately normal (P<0.001) except for PT18 and RH18 which were slightly skewed left as indicated by the Shapiro and Wilk test (data not shown, Figure 5_1). There were weakly significant correlations for CT between environments for PT17 and PT18 (r = 0.18*), PT17 and RH19 (r = 0.26**), RH17 and RH19 (r = 0.15*), PT19

and RH18 (r = -0.16*), and PT19 and RH19 (r = 0.49***). Across environments, ANOVA indicated that there was a significant effect (P<0.05) of RILs, environment, and RILs × environment interaction on CT, indicating that CT among RILs was affected differently by environmental conditions (data not shown). The narrow sense heritability of CT for individual environments was 9% (PT17), 11% (PT18), 51% (PT19), 22% (RH17), 7% (RH18), and 62% (RH19) and was 35% when estimated across environments (Table 5_1).

QTL Analysis

Analysis of QTLs associated with CT in individual environments identified seven QTLs present on five chromosomes using the CIM method. Of these seven QTLs, one QTL was identified in RH17, PT18, and RH18 and two QTLs were identified in PT19 and RH19. No QTLs were detected in PT17. These QTLs had additive effects that ranged from -0.08 to -1.17 and explained 5.7% to 12.3% of the phenotypic variation (Table 5_3). The QTLs identified in PT18 (1), PT19 (2), RH18 (1), and RH19 (1) were also identified by the MIM method. Two additional QTLs present on Gm13 (at 30,174,729 bp) in RH18 and on Gm16 (at 26,399,875 bp) in PT19 were identified by the MIM method but were not identified by the CIM method (Table 5_3).

Across environment (AE) QTL analysis of CT identified five QTLs present on Gm02 (1), Gm11 (1), and Gm18 (3) with additive effects ranging from -0.39 to -0.51 and explaining phenotypic variation from 5.3% to 9.3%. Three out of five AE QTLs were common between CIM and MIM methods, and one new QTL on Gm15 (at 52,582,411 bp) was identified only by the MIM method (Table 5_3). The QTL present on Gm11 (at 10,319,200 bp) was common among PT19, RH19, and AE. The QTL on Gm18 (at 55,000,978 bp) was identified in RH17 and AE, and the QTL on Gm18 (at 60,441,713 bp) was common for RH19 and AE. All other QTLs

were environmentally specific (Table 5_3). The favorable alleles for deceasing CT at all the QTLs were from KS4895 except for the QTLs on Gm10 and Gm16. QTL × QTL interactions were identified between the QTLs present on Gm11 and Gm16 in PT19 by the MIM method. This interaction explained 5.7% of the phenotypic variation with the favorable allele contributed by KS4895

Candidate Gene Identification

There were more than 1200 candidate genes present within ±1MB of the nearest markers for putative QTLs with a range from 75 to 208 genes for individual QTLs. Out of these 1200 genes, those having biological function related to stomatal complex morphogenesis, regulation of stomatal movement, response to water deprivation, response to abscisic acid (ABA), ABA mediated signaling pathway, ABA transport, root hair elongation, root hair cell differentiation, primary and adventitious root development, root morphogenesis, water transport, response to osmotic and oxidative stress, signal transduction, and response to different hormones stimulus were considered to play a potential role in controlling CT in response to different soil moisture conditions.

Discussion

The present study investigated the genetic control of CT in a population derived from a cross between KS4895 and Jackson, which was evaluated across six environments. Under replete soil moisture and aerial environmental conditions, plants continue to transpire through open stomata. In contrast, as soil moisture becomes limiting, plants close stomata as a preventive mechanism, transpiration decreases, and there is an increase in CT[26]. Those genotypes that have access to soil moisture continue transpiration during drought stress, resulting in a cooler canopy. High soil moisture deficit and VPD at the time of CT measurements resulted in drought stress in all environments (Table 5_1). The PT19 and RH19 environments had greater soil moisture deficit and VPD, resulting in greater differences among RILs and higher heritability of CT as compared to other environments.

For all environments there was transgressive segregation among RILs with lower and greater CT than the parents, indicating the distribution of favorable alleles for cooler canopy temperature in both parents. CT is highly influenced by environmental conditions (soil moisture availability, vapor pressure deficit, air temperature) and plant morphology (canopy and root architecture conditions)[69,29], resulting in significant genotype × environment interactions. While the h^2 of CT was 35% when estimated across six environments, and the range of h^2 among environments was from 7% to 62% (Table 5_1). The two environments in which h^2 was >50% were noticed for a severe soil moisture deficit (>75mm) and maximum temperatures ≥ 36 °C (Table 5_1). Kaler et al.[9] reported a broad sense heritability (H) of 19% for CT in a diverse panel of soybean accessions evaluated in different environments using GWAM. Likewise, low to moderate broad sense heritability of CT/CTD was reported in wheat (H=16% to 38%)[70-72], in sugarcane (H=9% to 44%)[73], and in rice (H=21% to 30%)[74]. The low heritability of CT/CTD in

soybean and other crops indicated that this trait is highly influenced by environmental conditions[75].

The complexity of CT in soybean is further indicated by detection of multiple QTLs by CIM and MIM methods. The QTL analysis for CT using CIM and MIM methods identified a total of nine QTLs in individual environments and six QTLs when data were averaged across environments (Table 5_3). Most of these QTLs were environmentally specific except the QTLs present on Gm11 (at 10,319,200 bp) and Gm18 (at 55,000,978 bp and 60,441,713 bp), which were common in at least one individual environment and across environments (Table 5_3). The QTLs present on Gm11 explained phenotypic variation more than 10% (or ranged from 9.3% to 11.5%) and is considered as a major QTL (Table 5_3). The markers linked with these QTLs have potential utility using marker assisted selection or genomic selection in a breeding program. The inconsistency of QTLs among environments might be due to different environmental factors such as field moisture status, soil temperature and depth, solar radiation, and VPD. Although QTLs were generally environmentally specific, most of these QTLs were detected by both CIM and MIM methods, which increases the confidence of these results. Of particular interest are environmental conditions that could improve consistency and increase heritability of CT. More research is needed to increase the utility of markers linked with QTLs identified in specific environments in selecting genotypes with a cooler canopy and to determine environmental conditions that optimize heritability.

Considering the overlapping confidence interval of QTLs identified in individual environments and across environments, we found 11 loci on eight chromosomes (Table 5_3, Figure 5_2). Even though Jackson tended to have lower CT among environments (Table 5_2), there were nine favorable loci from KS4895 and two favorable loci from Jackson. Hwang et al.[49]

found that KS4895 contributed the favorable alleles for slow canopy wilting for most canopy wilting QTLs identified in multiple biparental populations. Bai and Purcell[4] found a positive correlation ($0.37 < r < 0.62$) between cooler CT and slow canopy wilting. There is a possibility that KS4895 has both slow canopy wilting and cooler canopy temperature alleles at these genomic loci in the KS4895 × Jackson population.

For comparative analysis of CT loci with QTLs associated with plant water relations and drought tolerance related traits, the putative CT loci were aligned on the soybean linkage map in Soybase (www.soybase.com). The QTLs previously mapped within the 95% confidence interval of putative QTLs in this study were considered to be present in same genomic regions. The CT Loci 1 and 2 (on Gm02) coincide with a canopy wilting QTL found in a population derived from KS4895 and Jackson (09705KJ population) that is different from the population used in the present research and in a population derived from Benning and PI 416937[49]. The favorable alleles for slow canopy wilting at this locus are from KS4895 and PI 416937, consistent with the present research that the KS4895 allele is associated with cool CT. These loci localized in a genomic cluster found in meta-analysis of canopy wilting QTLs using multiple biparental populations[50]. The loci were also mapped in an association panel in the genomic regions associated with canopy wilting[51], canopy coverage[56], and $\delta^{13}C$[55]. The co-localization of these drought-related QTLs with CT indicates the strong relationship among transpiration, WUE, canopy wilting, and CT.

Locus 3 (on Gm03) and Locus 4 (on Gm10) coincide with QTLs for CT[9] and canopy wilting[51], respectively, that were identified in GWAM studies. Locus 5 (on Gm11) maps to the same genomic region associated with CT[9], canopy wilting[51], and canopy coverage[56] identified in GWAM analysis conducted using a diverse panel of soybean accessions. Locus 5 also coincides

with the QTLs for canopy wilting identified in population with KS4895 as a parent[49]; the favorable alleles for slow canopy wilting in both these populations were from KS4895 as they were for CT in the present study.

Locus 6 (on Gm13) overlaps with $\delta^{13}C$ found by GWAM[55]. Nguyen et al.[76] mapped a QTL for root area and root length at the same genomic location in a population derived from Tachinagaha × Iyodaizu. Rooting depth and area are drought avoidance mechanisms that may increase water availability[77]. The coincidence of CT and root morphology QTLs may point to a root system that extracts water from deeper soil horizons and results in cooler canopy during drought. In wheat, CT QTLs have been linked with rooting traits that allow extraction of more water from soil under drought[78].

Locus 7 (on Gm15) and Locus 8 (on Gm16) coincide with canopy wilting[51] and canopy coverage[56] in GWAM studies. In addition, Locus 8 also overlaps with $\delta^{13}C$[55]. Earlier canopy coverage helps to decrease the water loss by soil evaporation relative to transpiration and improve WUE[79]. Locus 9 (on Gm18 at 17,568,794 bp) coincides with the WUE QTLs mapped in a Young × PI416937 population with the 'Young' allele increasing WUE[80]. Locus 9 and Locus 10 (on Gm18 at 55,000,978 bp) also fall in the genomic region harboring QTLs for CT[9], canopy wilting[51], $\delta^{13}C$[54,55] identified in GWAM studies. In addition, Locus 10 overlaps with the oxygen isotope ratio[55], which is a proxy for measurement of transpiration and is associated with stomatal conductance[81].

Locus 11 (on Gm18 at 60,441,713 bp) coincides with $\delta^{13}C$ identified in the same population as the present research[53]. The favorable allele for $\delta^{13}C$ at this locus was from Jackson, while KS4895 provided the favorable allele for CT at this locus. The coincidence of $\delta^{13}C$ and CT QTLs illustrates a shared genetic relationship between these two physiological traits. The co-

localization of putative CT loci with QTLs associated with other morpho-physiological traits such as WUE, canopy wilting, canopy coverage, and transpiration may be a pleiotropic effect of the same genes controlling these traits or that the genes are spaced closely together on specific chromosomes.

The candidate gene search underlying putative CT loci identified genes with functions related to plant water relations, root morphology, and transpiration. These include genes involved in stomatal complex morphogenesis, regulation of stomatal movement, response to water deprivation, response to abscisic acid (ABA), ABA mediated signaling pathway, ABA transport, root hair elongation, root hair cell differentiation, response to oxidative stress, and signal transduction.

The upregulation of root morphology (lateral root formation, root hair elongation, root development response to ABA) related genes during drought may result in extracting residual soil moisture that maintains primary growth and developmental processes. In wheat, deeper root development in response to drought stress resulted in a cooler canopy and an increase in yield[20,82]. Aquaporin-related genes were also found in underlying CT loci and these membrane proteins allow movement of water throughout plant in response to stress[83]. The co-localization of CT loci with QTLs associated with drought tolerance related traits and with underlying candidate genes with biological function related to transpiration, stomatal conductance, and plant water relations increases the probability that putative CT loci are associated with variation in CT in the present research. Additional research is needed to confirm the canopy temperature QTLs in different populations and in different environments to increase the efficiency of these genomic regions in selecting genotypes with a cooler canopy and with drought tolerance.

Conclusion

In the present study, we identified genomic regions associated with CT in a recombinant inbred population derived from KS4895 and Jackson that was phenotyped in six different environments. These results represent the first QTLs for CT identified in soybean using a biparental population. The population segregated for CT in all environments and was used for QTLs analysis. The heritability of CT was relatively low as compared to other morpho-physiological traits due to greater influence of different environmental factors. Eleven genomic loci present on eight chromosomes for CT were identified across several environments. The CT locus present on Gm11 explained phenotypic variation more than 10% and was considered as major QTL. The favorable allele for cooler canopy for most of the loci were from KS4895, which were also coincident with canopy wilting QTLs identified in multiple biparental populations by Hwang et al.[49]. The identified CT QTLs coincided QTLs associated with drought tolerance related traits mapped in previous studies and genomic regions underlying these putative CT have the genes with biological function related to transpiration, stomatal conductance, and plant water relations. More research is needed to confirm these QTLs in different genetic backgrounds and in multiple/different environments to evaluate the efficiency of these QTLs to use in soybean breeding program for selecting genotypes with a cooler canopy and drought tolerance.

Table 5_1. Planting date and weather data including maximum temperature (MaxT), minimum temperature (MinT), No. of days without rainfall, estimated vapor pressure deficit (VPD), and estimated soil moisture deficit at the time of canopy temperature measurements for the field experiments conducted at Pine Tree (PT) and Rohwer (RH) in 2017, 2018, and 2019.

Env.	Planting date	CT recording date	MaxT (°C)	MinT (°C)	No. of Days	VPD (kPa)	Soil moist. deficit (mm)	h^{2b} (%)
PT17	9 June 2017	25 Aug 2017	29	15	8	2.3	49	9
RH17	8 June 2017	21 July 2017	34	25	4	2.3	50	22
PT18	7 June 2018	25 July 2018	33	19	8	2.9	>75	11
RH18	31 May 2018	19 July 2018	31	24	11	1.6	71	7
PT19	31 May 2019	10 Sept 2019	37	22	13	3.5	>75	51
RH19	12 June 2019	9 Sept 2019	36	21	13	3.3	>75	62
AE[a]	-	-	33	21	57	2.6	66	35

[a] AE denote averaged across environments.

[b] h^2, Narrow sense heritability

Table 5_2. A summary statistic of canopy temperature (CT) in the parents (KS4895 and Jackson) and RILs (n=168) population of KS4895 and Jackson evaluated at Pine Tree and Rohwer in 2017, 2018 and 2019.

Trait	Env.[a]	Parental means		RILs population	
		KS4895	Jackson	Mean	Range
CT	PT17	70*	59	63	45
	RH17	57	67	60	29
	PT18	71	54	58	37
	RH18	59	47	50	24
	PT19	71	63	64	39
	RH19	86*	53	63	36
	AE[b]	69*	57	60	35

[a] Environments: Prefixes PT and RH denotes Pine Tree and Rohwer, respectively followed by 17 (2017), 18 (2018), and 19 (2019) for years.

[b] AE denote averaged across environments.

* Indicated significant difference ($P \leq 0.05$) between parents

Table 5_3. The QTLs associated with canopy temperature identified by composite interval mapping (CIM) and multiple interval mapping (MIM) in a RIL population of KS4895 and Jackson which were evaluated at Pine Tree and Rohwer in 2017, 2018, and 2019.

Locus	Chrom.[a]	Env.[b]	Position (bp)[c]	Nearest marker[d]	Additive effect[e]	R^{2f}	Favorable allele[g]	FM at 95% CI[h]	Method
1	Gm02	PT18	2,145,083	ss107919808	-0.27	12.3	KS4895	ss107919971-ss107912545	CIM, MIM
2		AE	3,111,654	ss107912545	-0.42	6.3	KS4895	ss107919808-ss107913715	CIM, MIM
3	Gm03	RH18	3,827,362	ss107929820	-0.08	6.5	KS4895	ss107913533-ss107912527	CIM, MIM
4	Gm10	PT19	2,445,123	ss107921662	1.19	8.5	Jackson	ss107921662-ss107930841	CIM, MIM
5	Gm11	PT19	10,319,200	ss107919087	-1.28	11.5	KS4895	ss107919087-ss107913812	CIM, MIM
		RH19	10,319,200	ss107919087	-1.47	10.8	KS4895	Satt197-ss107927406	CIM, MIM
		AE	10,319,200	ss107919087	-0.51	9.3	KS4895	Satt197-ss107927406	CIM, MIM
6	Gm13	RH18	30,174,729	ss107912665	-0.07	4.6	KS4895	ss107915606-ss107912922	MIM
7	Gm15	AE	50,582,411	ss107925861	-0.42	5.4	KS4895	ss107914616-Sat_376	MIM
8	Gm16	PT19	26,399,875	ss107927055	0.65	4.9	Jackson	ss107927440-ss107913908	MIM
9	Gm18	AE	17,568,794	ss107921048	-0.39	5.3	KS4895	ss107920369-ss107914987	CIM
10	Gm18	RH17	55,000,978	ss107913405	-0.27	5.7	KS4895	ss107919708-ss107921608	CIM
		AE	55,000,978	ss107913405	-0.40	5.4	KS4895	ss107919708-ss107913107	CIM
11	Gm18	RH19	60,441,713	ss107929216	-1.29	8.8	KS4895	ss107929175-ss107919550	CIM
		AE	60,441,713	ss107929216	-0.41	6.2	KS4895	ss107929175-ss107919550	CIM, MIM

[a] *Glycine max* chromosome on which QTL was present

[b] Environment in which specific QTL was identified. Prefixes PT and RH denotes Pine Tree and Rohwer, respectively followed by 17 (2017), 18 (2018), and 19 (2019) for years. AE denotes averaged across environments

[c] QTL position in base pairs on respective chromosomes according to Soybase (www.soybase.com)

[d] Nearest marker to the QTL
[e] Additive effect of the QTL
[f] Proportion (%) of phenotypic variation explained by specific QTL
[g] Allele that decreases CT values considered as favorable allele; Positive sign indicates that favorable alleles (decreasing CT) were from Jackson and negative sign indicates the KS4895 allele
[h] Flanking markers (FM) present near or at 95% confidence interval (CI) of the maximum likely QTL positions. The LOD values with ±1 declination was used to estimate the 95% confidence interval.

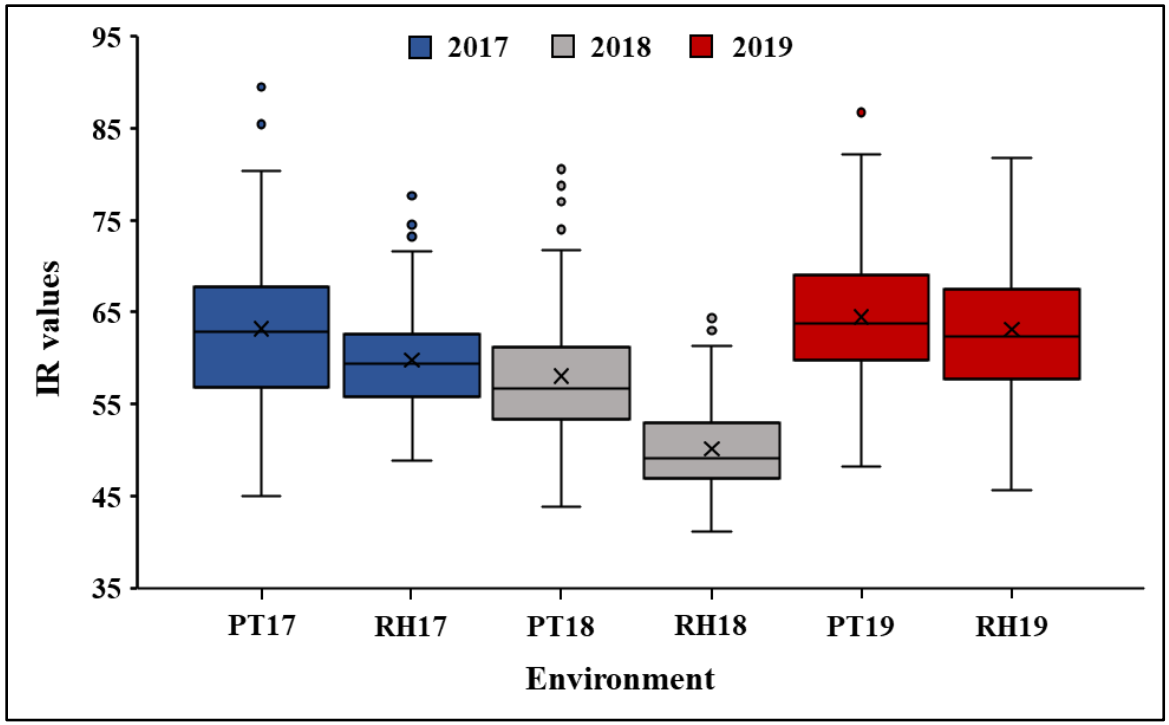

Figure 5_1. The box plots showed a broad range of canopy temperature in KS4895 × Jackon RIL population within each environment. Environment prefixes PT and RH denotes Pine Tree and Rohwer, respectively followed by 17 (2017), 18 (2018), and 19 (2019) for years. Box edges represent the upper and lower quartile with a median (bold line in the middle of box) and mean value (cross in the middle of box).

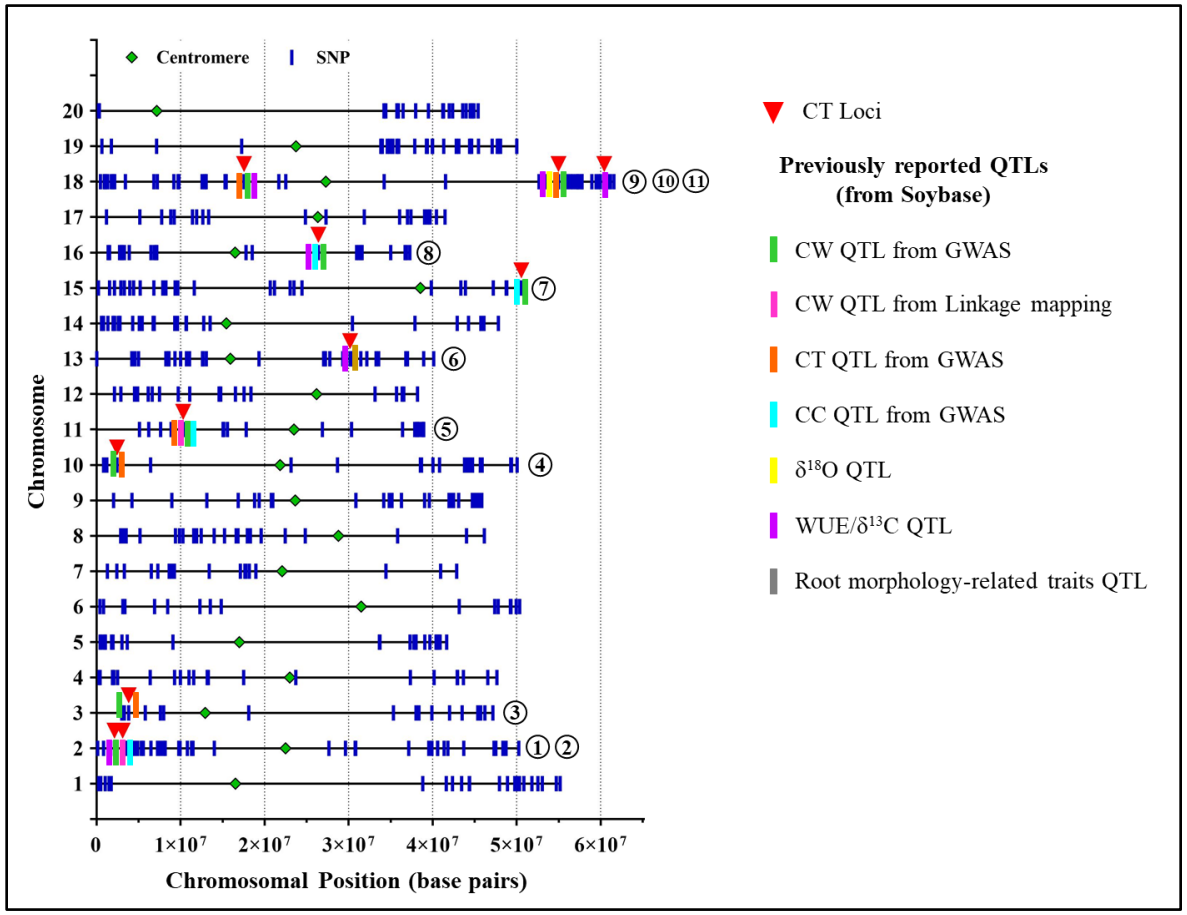

Figure 5_2. Physical position of SNPs on soybean chromosomes and position of loci (red downward triangle) associated with CT. The numbers in the black circles represent the loci number on a specific chromosome. Vertical colored bars (except blue) indicate the other QTLs found at the same positions in previous studies. CW, CT, CW denote canopy wilting, canopy temperature, and canopy wilting, respectively.

References

[1]Frederick, J. R., Camp, C. R. & Bauer, P. J. Drought-stress effects on branch and mainstem seed yield and yield components of determinate soybean. *Crop Sci.* **41**, 759-763 (2001).

[2]Sadeghipour, O. & Abbasi, S. Soybean response to drought and seed inoculation. *World Appl. Sci. J.* **17**, 55-60 (2012).

[3]Jackson, P., Robertson, M., Cooper, M. & Hammer, G. The role of physiological understanding in plant breeding; from a breeding perspective. *Field Crops Res.* **49**, 11-37 (1996).

[4]Bai, H. & Purcell, L. C. Aerial canopy temperature differences between fast- and slow-wilting soybean genotypes. *J. Agron. Crop Sci.* **204**, 243-251 (2018).

[5]Chapman, S.C. *et al*. Pheno-copter: a low-altitude, autonomous remote-sensing robotic helicopter for high-throughput field-based phenotyping. *Agronomy* **4**, 279-301 (2014).

[6]Cobb, J. N., Declerck, G., Greenberg, A., Clark, R. & McCouch, S. Next-generation phenotyping: Requirements and strategies for enhancing our understanding of genotype-phenotype relationships and its relevance to crop improvement. *Theor. Appl. Genet.* **126**, 867-887 (2013).

[7]Jackson, R. D., Idso, S. B, Reginato, R. J. & Pinter, P. J. Canopy temperature as a crop water stress indicator. *Water Resour. Res.* **17**, 1133-1138 (1981).

[8]Jones, H. G., Serraj, R., Loveys, B. R., Xiong, L. Z, Wheaton, A. & Price, A. H. Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. *Funct. Plant Biol.* **36**, 978-989 (2009).

[9]Kaler, A. S. *et al*. Association mapping identifies loci for canopy temperature under drought in diverse soybean genotypes. *Euphytica* **214**, 135 (2018a).

[10]Yousfi, S. *et al*. Comparative performance of remote sensing methods in assessing wheat performance under Mediterranean conditions. *Agr. Water. Manag.* **164**, 137-147 (2016).

[11]Rebetzke, G. J., Rattey, A. R., Farquhar, G. D., Richards, R. A. & Condon, A. G. Genomic regions for canopy temperature and their genetic association with stomatal conductance and grain yield in wheat. *Funct. Plant Biol.* **40**, 14-33 (2013).

[12]Inoue, Y., Kimball, B. A., Jackson, R. D., Pinter, P. J. & Reginato, R. J. Remote estimation of leaf transpiration rate and stomatal resistance based on infrared thermometry. *Agric. For. Meteorol.* **51**, 21-33 (1990).

[13]Gates, D. M. Transpiration and leaf temperature. *Annu. Rev. Plant Physiol.* **19**, 211-238 (1968).

[14]Jones, H. Remote detection of crop water stress and distinguishing it from other stresses. *Acta Hortic.* **922**, 23-34 (2010).

[15]Tanner, C. B. Plant temperatures. *Agron. J.* **55**, 210-211 (1963).

[16]Ludlow, M. M. & Muchow, R. C. A critical evaluation of traits for improving crop yields in water-limited environments. *Adv. Agron.* **43**, 107-153 (1990).

[17]Reynolds, M., Dreccer, F. & Trethowan, R. Drought-adaptive traits derived from wheat wild relatives and landraces. *J. Exp. Bot.* **58**, 177-186 (2007).

[18]Reynolds, M. P., Manes, Y., Izanloo, A. & Langridge, P. Phenotyping for physiological breeding and gene discovery in wheat. *Ann. Appl. Biol.* **155**, 309-320 (2009).

[19]Fischer, R. A. *et al*. Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. *Crop Sci.* **38**, 1467-1475 (1998).

[20]Lopes, M.S. & Reynolds, M. P. Partitioning of assimilates to deeper roots is associated with cooler canopies and increased yield under drought in wheat. *Funct. Plant. Biol.* **37**, 147-156 (2010).

[21]Brennan, J. P., Condon, A. G., Van Ginkel, M. & Reynolds, M. P. An economic assessment of the use of physiological selection for stomatal aperture-related traits in the CIMMYT wheat breeding programme. *J. Agr. Sci.* **145**, 187-194 (2007).

[22]Reynolds, M. P., Rajaram, S. & Sayre, K. D. Physiological and genetic changes of irrigated wheat in the post-green revolution period and approaches for meeting projected global demand. *Crop Sci.* **39**, 1611-1621 (1999).

[23]Fukuoka, M. Improvement of a method for measuring canopy temperature in field crops using an infrared thermograph. Dissertation, Hokkaido University, Sapporo, Japan (2005).

[24]Prince, S. J., Beena, R., Gomez, S.M., Senthivel, S. & Babu, R. C. Mapping consistent rice (*Oryza sativa* L.) yield qtls under drought stress in target rainfed environments. *Rice* **8**, 25 (2015).

[25]Basnayake, J., Jackson, P.A., Inman-Bamber, N.G. & Lakshmanan, P. Sugarcane for water-limited environments: variation in stomatal conductance and its genetic correlation with crop productivity. *J. Exp. Bot.* **66**, 3945-3958 (2015).

[26]Kashiwagi, J., Krishnamurthy, L., Upadhyaya, H. D. & Gaur, P. M. Rapid screening technique for canopy temperature status and its relevance to drought tolerance improvement in chickpea. *J. SAT Agric. Res.* **6**, 105-114 (2008).

[27]Purushothaman, R. *et al*. Association of mid-reproductive stage canopy temperature depression with the molecular markers and grain yields of chickpea (*Cicer arietinum* L.) germplasm under terminal drought. *Field Crops Res.* **174**, 1-11 (2015).

[28]Singh, P. & Kanemasu, E. T. Leaf and canopy temperatures of pearl millet genotypes under irrigated and nonirrigated conditions. *Agron J.* **75**, 497-501 (1983).

[29]Kumar, M. *et al*. Canopy temperature depression (CTD) and canopy greenness associated with variation in seed yield of soybean genotypes grown in semi-arid environment. *S. Afr. J. Bot.* **113**, 230-238 (2017).

[30]Araus, J. L., Slafer, G. A., Reynolds, M. P. & Royo, C. Plant breeding and drought in C3 cereals: what should we breed for? *Ann. Bot.* **89**, 925-940 (2002).

[31]Mitra, J. Genetics and genetic improvement of drought resistance in crop plants. *Curr. Sci.* **80**, 758-763 (2001).

[32]Berger, B., Parent, B. & Tester, M. High-throughput shoot imaging to study drought responses. *J. Exp. Bot.* **61**, 3519-3528 (2010).

[33]Chen, D. *et al*. Dissecting the phenotypic components of crop plant growth and drought responses based on high-throughput image analysis. *Plant Cell* **26**, 4636-4655 (2014).

[34]Golzarian, M. R. *et al*. Accurate inference of shoot biomass from high-throughput images of cereal plants. *Plant Methods* **7**, 2 (2011).

[35]Honsdorf, N., March, T. J., Berger, B., Tester, M. & Pillen, K. High throughput phenotyping to detect drought tolerance QTL in wild barley introgression lines. *PLoS One* **9**, e97047 (2014).

[36]Idso, S. B., Jackson, R. D., Pinter, P. J. Jr., Reginato, R. J. & Hatfield, J. L. Normalizing the stress-degree-day parameter for environmental variability. *Agric. Meteorol.* **24**, 45-55 (1981).

[37]Jackson, R. D., Reginato, R.J. & Idso, S. B. Wheat canopy temperature: a practical tool for evaluating water requirements. *Water Resour. Res.* **13**, 651-656 (1977).

[38]Jones, H. G. Application of thermal imaging and infrared sensing in plant physiology and ecophysiology. *Adv. Bot. Res.* **41**, 107-163 (2004).

[39]Mengistu, A., Tachibana, H., Epstein, A. H., Bidne, K. G. & Hatfield, J. D. Use of leaf temperature to measure the effect of brown stem rot and soil moisture stress and its relation to yields of soybeans. *Plant Disease* **71**, 632-634 (1987).

[40]Bac-Molenaar, J. A., Vreugdenhil, D., Granier, C. & Keurentjes, J. J. B. Genome-wide association mapping of growth dynamics detects time-specific and general quantitative trait loci. *J. Exp. Bot.* **66**, 5567-5580 (2015).

[41]Busemeyer, L. *et al*. Precision phenotyping of biomass accumulation in triticale reveals temporal genetic patterns of regulation. *Sci. Rep.* **3**, 2442 (2013).

[42]Moore, C. R., Johnson, L. S., Kwak, I. Y., Livny, M., Broman, K. W. & Spalding, E. P. High-throughput computer vision introduces the time axis to a quantitative trait map of a plant growth response. *Genetic*s **195**, 1077-1086 (2013).

[43]Slovak, R., Göschl, C., Su, X., Shimotani, K., Shiina, T. & Busch, W. A scalable open-source pipeline for large-scale root phenotyping of Arabidopsis. *Plant Cell* **26**, 2390-2403 (2014).

[44]Yang, W. *et al*. Combining high-throughput phenotyping and genome-wide association studies to reveal natural genetic variation in rice. *Nat. Commun.* **5**, 5087 (2014).

[45]Liu, H. *et al*. Correlation analysis and QTL identification for canopy temperature, leaf water potential and spikelet fertility in rice under contrasting moisture regimes. *Chin. Sci. Bull.* **50**, 317-326 (2005).

[46]Liu, Y., Subhash, C., Yan, J., Song, C., Zhao, J. & Li, J. Maize leaf temperature responses to drought: thermal imaging and quantitative trait loci (QTL) mapping. *Environ. Exper. Bot.* **71**, 158-165 (2011).

[47]Abdel-Haleem, H. *et al*. Mapping of quantitative trait loci for canopy wilting trait in soybean (*Glycine max* L. Merr). *Theor. Appl. Genet.* **125**, 837-846 (2012).

[48]Charlson, D. V. *et al*. Polygenic inheritance of canopy wilting in soybean [*Glycine max* (L.) Merr.]. *Theor. Appl. Genet.* **119**, 587-594 (2009).

[49]Hwang, S. *et al*. Confirmation of delayed canopy wilting QTLs from multiple soybean mapping populations. *Theor. Appl. Genet.* **128**, 2047-2065 (2015).

[50]Hwang, S. *et al*. Meta-analysis to refine map position and reduce confidence intervals for delayed canopy wilting QTLs in soybean. *Mol. Breed.* **36**, 91 (2016).

[51]Kaler, A. S., Ray, J. D., Schapaugh, W. T., King, C. A. & Purcell, L. C. Genome-wide association mapping of canopy wilting in diverse soybean genotypes. *Theor. Appl. Genet.* **130**, 2203-2217 (2017a).

[52]Bazzer, S. K., Kaler, A. S., Ray, J. D., Smith, J.R., Fritschi, F.B. & Purcell, L.C. Identification of quantitative trait loci for carbon isotope ratio ($\delta^{13}C$) in a recombinant inbred population of soybean. *Theor. Appl. Genet.* **133**, 2141-2155 (2020a).

[53]Bazzer, S. K., Kaler, A. S., King, C. A., Ray, J. D., Hwang, S. & Purcell, L. C. Mapping and confirmation of quantitative trait loci (QTLs) associated with carbon isotope ratio ($\delta^{13}C$) in soybean. *Crop Sci.* https://doi.org/10.1002/csc2.20240 (2020b).

[54]Dhanapal, A. P. *et al*. Genome-wide association study (GWAS) of carbon isotope ratio ($\delta^{13}C$) in diverse soybean [*Glycine max* (L.) Merr.] genotypes. *Theor. Appl. Genet.* 128:73-91 (2015).

[55]Kaler, A. S., Dhanapal, A. P., Ray, J. D., King, C. A., Fritschi, F. B. & Purcell, L. C. Genome-wide association mapping of carbon isotope and oxygen isotope ratios in diverse soybean genotypes. *Crop Sci*. **57**, 3085-3100 (2017b).

[56]Kaler, A. S., Ray, J. D., Schapaugh, W. T., Davies, M. K., King, C. A. & Purcell, L. C. Association mapping identifies loci for canopy coverage in diverse soybean genotypes. *Mol. Breed.* **38**, 50 (2018b).

[57]Schapaugh, W. T. & Dille, R. E. Registration of 'KS4895' soybean. *Crop Sci.* **38**, 892 (1998).

[58]Johnson, H. W. Registration of soybean varieties VI. *J. Agron.* **11**, 690-691 (1958).

[59]Purcell, L. C., Edwards, J. T. & Brye, K. R. Soybean yield and biomass responses to cumulative transpiration: questioning widely held beliefs. *Field Crop Res.* **101**, 10-18 (2007).

[60]Allen, R. G., Pereira, L. S., Raes, D. & Smith, M. Crop evapo-transpiration: Guidelines for computing crop water requirements. FAO Irrig. and Drainage Paper 56. FAO, Rome, Italy (1998).

[61]Purcell, L. C., Sinclair, T. R. & McNew, R. W. Drought avoidance assessment for summer annual crops using long-term weather data. *Agron. J.* **95**, 1566-1576 (2003).

[62]Shapiro, S. S. & Wilk, M. B. An Analysis of Variance Test for Normality (Complete Samples). *Biometrika* **52**, 591-611 (1965).

[63]Holland, J. B., Nyquist, W. E. & Cervantes-Martinez, C. T. Estimating and interpreting heritability for plant breeding: an update. *Plant Breed. Rev.* **22**, 9-112 (2003).

[64]Wang, S., Basten, C. J. & Zeng, Z. B. Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, North Carolina. (2007).

[65]Zeng, Z. B. Precision mapping of quantitative trait loci. *Genetics* **136**, 1457-1468 (1994).

[66]Doerge, R. W. & Churchill, G. A. Permutation tests for multiple loci affecting a quantitative character. *Genetics* **142**, 285-294 (1996).

[67]Jansen, R. C. & Stam, P. High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* **136**, 1447-1455 (1994).

[68]Kao, C. H., Zeng, Z. B. & Teasdale, R. D. Multiple interval mapping for quantitative trait loci. *Genetics* **152**, 1203-1216 (1999).

[69]Hopkins, W. G. Introduction to plant physiology. (2nd ed. John Wiley & Sons, New York, 1999).

[70]Gao, F. *et al*. Genome-wide linkage mapping of QTL for physiological traits in a Chinese wheat population using the 90K SNP array. *Euphytica* **209**, 789-804 (2016).

[71]Sukumaran, S., Dreisigacker, S., Lopes, M., Chavez, P. & Reynolds, M. P. Genome-wide association study for grain yield and related traits in an elite spring wheat population grown in temperate irrigated environments. *Theor. Appl. Genet.* **128**, 353-363 (2015).

[72]Tahmasebi, S., Heidari, B., Pakniyat, H. & McIntyre, C. L. Mapping QTLs associated with agronomic and physiological traits under terminal drought and heat stress conditions in wheat (*Triticum aestivum* L.). *Genome* **59**, 1-20 (2016).

[73]Natarajan, S., Basnayake, J., Wei, X. & Lakshmanan, P. High-throughput phenotyping of indirect traits for early-stage selection in sugarcane breeding. *Remote Sens.* **11**, 2952 (2019).

[74]Saikumar, S. *et al*. Grain yield responses to varied level of moisture stress at reproductive stage in an interspecific population derived from Swarna/*O. glaberrima* introgression line. *NJAS - Wagen J. Life Sci.* **78**, 111-122 (2016).

[75]Ripullone, F. *et al*. Environmental effects on oxygen isotope enrichment of leaf water in cotton leaves. *Plant Physiol.* **146**, 729-736 (2008).

[76]Nguyen, L.V. *et al*. Mapping quantitative trait loci for root development under hypoxia conditions in soybean (*Glycine max* L. Merr.). *Theor. Appl. Genet.* **130**, 743-755 (2017).

[77]Pantalone, V. R., Rebetzke, G. J., Burton, J. W., & Carter, T. E. Phenotypic evaluation of root traits in soybean and applicability to plant breeding. *Crop Sci.* **36**, 456-459 (1996).

[78]Pinto, R. S. & Reynolds, M. P. Common genetic basis for canopy temperature depression under heat and drought stress associated with optimized root distribution in bread wheat. *Theor. Appl. Genet.* **128**, 575-585 (2015).

[79]Purcell, L. C. & Specht, J. E. Physiological traits for ameliorating drought stress. In: *Soybeans: Improvement, production, and uses.* (eds. Boerma, H. R. & Specht, J. E.) 569-620 (American Society of America, Madison, WI, 2004).

[80]Mian, M. A. R. *et al*. Molecular markers associated with water use efficiency and leaf ash in soybean. *Crop Sci.* **36**, 1252-1257 (1996).

[81]Farquhar, G. D., Barbour, M. M. & Henry, B. K. Interpretation of oxygen isotope composition of leaf material In: *Stable isotopes: Integration of biological, ecological, and geochemical processes.* (ed. Griffiths, H.) 27-62 (BIOS Scientific Publishers, Oxford, 1998).

[82]Wasson, A. P., Rebetzke, G. J., Kirkegaard, J. A., Christopher, J., Richards, R. A., & Watt, M. Soil coring at multiple field environments can directly quantify variation in deep root traits to select wheat genotypes for breeding. *J. Exp. Bot.* **65**, 6231-6249 (2014).

[83]Kaldenhoff, R & Fischer, M. Aquaporins in plants. *Acta Physiol.* **187**, 169-176 (2006).

CHAPTER VI

Conclusion

Drought stress is one of the major factors limiting crop production worldwide. The frequency, duration, and severity of drought, which leads to a significant reduction in crop productivity. Demand for agricultural water is expected to increase in the future due to an increase in human population and the impact of climatic change. Thus, there is need for development of cultivars with drought tolerance to cope with adverse climatic conditions and to improve crop performance. The direct selection for high yield under water deficit conditions is difficult due to its quantitative nature, low heritability, and interaction with environmental factors. Therefore, selection of various morpho-physiological traits that contribute to drought tolerance may help improve yield in water limited environments. The identification of genomic regions associated with physiological traits and understanding the genetic basis of physiological traits associated with drought tolerance would enable breeders to develop high yielding cultivars with improved drought tolerance.

In this research, physiological traits in soybean were studied to identify quantitative trait loci (QTLs) associated these traits and to compare the identified QTLs with QTLs mapped in previous studies. These traits included carbon isotope ratio ($\delta^{13}C$, positively correlated with water use efficiency), nitrogen isotope ratio ($\delta^{15}N$, negatively correlated with N_2 fixation), and canopy temperature (CT, an index for transpiration and stomatal conductance). To accomplish the proposed objectives, two different biparental populations were used: (1) a population of 196 F_6-derived recombinant inbred lines (RILs) developed from cross between PI 416997 and PI 567201D and (2) a population of 168 F_5-derived RILs developed from a cross between KS4895 and Jackson. Linkage mapping was performed to identify the QTLs associated with drought tolerance related traits in these genetically different biparental populations.

The $\delta^{13}C$ and $\delta^{15}N$ experiments with the PI 416997 × PI 567201D population were conducted in four environments (Stoneville, MS in 2016, Stoneville, MS in 2017, Columbia, MO in 2017, and Fayetteville, AR in 2017). Plant samples were collected at flowering, dried, ground, and sent to UC Davis for $\delta^{13}C$ and $\delta^{15}N$ isotope analysis. These results determined that $\delta^{13}C$ has high narrow sense heritability (90%) and $\delta^{15}N$ has low narrow sense heritability (35%) when estimated across environments, indicating that environmental factors had a greater influence on $\delta^{15}N$ than on $\delta^{13}C$. Parent PI 416997 had higher $\delta^{13}C$ and lower $\delta^{15}N$ values than PI 567201D, indicating that PI 416997 had greater WUE and fixed more N_2 than did PI 567201D.

QTL analysis for $\delta^{13}C$ identified eight loci present on seven chromosomes with individual loci explaining between 2.5 and 29.9% of the phenotypic variation. Of these eight loci, two loci on chromosome Gm20 were detected in at least three environments and were considered as stable loci. QTL analysis for $\delta^{15}N$ identified 13 loci present on nine chromosomes and these loci explained the phenotypic variations ranging from 1.7 to 9.3%. QTL × environment interaction analysis indicated that $\delta^{15}N$ loci showed greater interactions with different environments than $\delta^{13}C$ loci. The loci present on chromosomes Gm10 and Gm17 were associated with both $\delta^{13}C$ and $\delta^{15}N$. The favorable alleles (allele that increases $\delta^{13}C$ and decreases $\delta^{15}N$ values) at these specific loci were derived from different parents, indicating that the same allele at this locus has opposite effect for these traits. The identified QTLs for $\delta^{13}C$ and $\delta^{15}N$ coincided with genomic regions associated with N_2 fixation and other physiological traits related to drought tolerance from previous mapping studies. A search for candidate genes resulted in detection of genes that may directly or indirectly be related to physiological mechanisms associated with drought tolerance, nodulation, and N-metabolism.

The CT measurements in the KS4895 × Jackson population were made in six environments including two locations (Pine Tree, AR and Rohwer, AR) over three years (2017, 2018, and 2019) using aerial thermal infrared imaging. There was large segregation of CT in the KS4895 × Jackson population in all environments. The narrow sense heritability of CT was low (35%) when averaged over environments, indicating the impact of environmental conditions on CT measurements. The QTL analysis identified 11 loci distributed on eight chromosomes that individually explained phenotypic variation ranging from 4.6 to 12.3%. Nine loci out of 11 derived the cooler canopy allele from KS4895. Identified CT loci co-localized with the QTLs associated with canopy wilting, canopy temperature, WUE, and other drought-tolerance-related traits that were mapped previously.

The KS4895 × Jackson RIL population was also evaluated for $\delta^{13}C$ in five environments (Stuttgart, AR in 2012, Stuttgart, AR in 2013, Keiser, AR in 2013, Pine Tree, AR in 2017, and Rohwer, AR in 2017) under irrigated (IRR) and rainfed (RF) conditions. The heritability of $\delta^{13}C$ was moderate to high and ranged from 66% to 79% in different environments and irrigation treatments. The QTL analysis for $\delta^{13}C$ identified nine loci on seven chromosomes. Out of the nine loci, four loci (on Gm05, Gm06, and Gm02) collectively accounted for 55% of the phenotypic variation in $\delta^{13}C$ and were considered stable loci as they were identified across environments and irrigation treatments. The identified $\delta^{13}C$ loci coincided with previously reported QTLs linked with physiological traits related with drought tolerance.

In this research, identified genomic loci for $\delta^{13}C$, $\delta^{15}N$, and CT co-localized with QTLs associated with drought tolerance related traits mapped in previous studies Candidate genes underlying these QTLs may directly or indirectly be related to physiological mechanisms of drought tolerance. The identified QTLs for $\delta^{13}C$, $\delta^{15}N$, and CT may be an important resource for

marker-assisted selection and genomic selection studies to improve drought tolerance in soybean. Further research will be required for fine mapping the identified QTLs and for expression analysis of underlying genes to understand the quantitative nature of drought tolerance. In addition, more research is needed to confirm these QTLs in different genetic backgrounds and in other environments to evaluate the efficacy of these QTLs.