COTTON AND MAIZE PRIMARY ROOT GROWTH RESPONSES TO WATER DEFICIT: COMPARATIVE PHYSIOLOGICAL AND METABOLIC ANALYSIS

A Dissertation

presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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JULY 2019

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

COTTON AND MAIZE PRIMARY ROOT GROWTH RESPONSES TO WATER DEFICIT: COMPARATIVE PHYSIOLOGICAL AND METABOLIC ANALYSIS

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a candidate for the degree of Doctor of Philosophy, Plant Insect and Microbial Sciences, and hereby certify that, in their opinion, it is worthy of acceptance.

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ACKNOWLEDGMENTS

First and foremost, I need to give my most appreciation to my advisors, Dr. Robert Sharp and Dr. Melvin Oliver. I thank them for providing an excellent environment for research and helping me develop my abilities as an independent-thinking researcher. They were generous with their time while discussing experiments, data interpretation and manuscripts. Besides the science, they were also paying much attention on my emotion. Every time when I'm facing difficulties and feel down, they would sense my feeling and encourage me. They are the first advisors or teachers I ever meet who put themselves in an equal position when talking with me. They always tell me about their difficulties and tough experiences in their student years, letting me know that PhD years are both the toughest and the most substantial time in my life and I should dedicate myself to achieve my goals as a real scientist in the future. Their guidance and encouragement were indispensable for my completion of my Ph.D. program.

I greatly appreciate my committee members, Drs. Felix Fritschi and Abraham Koo for their valuable suggestions and guidance. I particularly thank Dr. Fritschi for his expertise on plant nutrient and soil properties which helped me conquer problems I was facing when dealing with the field condition experiments. Thanks are also due to Dr. Koo for his invaluable input to the metabolism contents of my study with his expertise.

I highly appreciate the training and technical advice provided by Dr. John Boyer and his warm-hearted instructions in the water potential aspect in my study. As a pioneer of modern plant physiology, Dr. Boyer is so humble who gave a model of a real scientist. I

am also grateful to Drs. Jim Schoelz and Jeanne Mihail for instilling confidence in me to continue my graduate program and helping me with my writing.

I really appreciate the current and previous members in the Sharp and Oliver labs. I thank Drs. Elizabeth Hoyos-Miernyk and Priyamvada Voothuluru with my genuine appreciations for their selfless assistance and counsel to my experiments and writing.

I am grateful to Drs. Tyler Dowd, Laura Greeley, Rachel Mertz, Kara Riggs, Anderson Silva and Dante Smith as well as Nick Baert, Cheyenne Becker, Jim Elder, Kate Guill, Shannon King, Tyler McCubbin, Paul Sathi and Hallie Thompson for being wonderful collaborators and colleagues.

I appreciate Cotton Incorporated for funding my project and thank the Division of Plant Sciences, Interdisciplinary Plant Group, College of Agriculture, Food and Natural Resources and Graduate School for supporting my graduate program. I would like to thank the faculty, staff and fellow graduate students of the Division of Plant Sciences and Interdisciplinary Plant Group for providing a vibrant research and mentoring environment. I also thank Metabolon for the precise processing and analysis of my metabolomics data.

I would like to specially mention my friends, Dr. Yutian Feng, Yunshu Fan, Chen Wang, Yao Wang, Jinpeng Wang and Yiyun Xu for their friendship, understanding, hearing and supporting during this long period of my PhD years.

Finally, I would give the sincerest appreciations to my family. As a successful scientist, my father, Shaozhong Kang, is always teaching me how be a scientist and a person of

integrity. Despite his extremely busy schedules, he spent several summer and winter break times to visit me. Even not in the same research area, he is always giving me suggestions and information about plant science studies in China. My mother, Mei Zhu, supports me with her delicate thoughts on my feelings. Her meticulous care for me over these years helped me get over many tough times when facing difficulties in science and daily life. I also want to give my thanks to my grandparents and other family members. Their presence is the fountain of constant inspiration and happiness in my life.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
4CLL	4-coumarate—CoA ligase
4HNE	4-hydroxynonenal
5OPase	5-oxoprolinase
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxidase
ACS	1-aminocyclopropane-1-carboxylate synthase
AGAL	α-galactosidase
ALDH	1-pyrroline-5-carboxylate dehydrogenase
APN	Aminopeptidase N
APX	Ascorbate peroxidase
C4H	Trans-cinnamate 4-monooxygenase
CAT	Catalase
CCR	Cinnamoyl-CoA reductase
CGS	Cystathionine γ-synthase
COMT	Caffeic acid 3-O-methyltransferase
CSE	Caffeoylshikimate esterase
CYS	Cysteine synthase
DCMT	DNA (cytosine-5)-methyltransferase 3A
DHAR	Dehydroascorbate reductase
F5H	Ferulate-5-hydroxylase
FC	Fold change
G6PDH	Glucose-6-phosphate 1-dehydrogenase
GABA	γ-aminobutyrate
GABAT	4-aminobutyrate—pyruvate transaminase
GCS	γ-glutamylcysteine synthetase
GDH	Glutamate decarboxylase
GGCT	γ-glutamylcyclotransferase
GGT	γ-glutamyltranspeptidase / glutathione hydrolase

GLT	Glutamate synthase (NADH)
GPX	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Reduced glutathione
GSHS	Glutathione synthase
GSSG	Oxidized glutathione/glutathione disulfide
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HRP	Hydrogen peroxidase
IDH	Isocitrate dehydrogenase
INV	β-fructofuranosidase
LAP	Leucyl aminopeptidase
METE	5-methyltetrahydropteroyltriglutamate—homocysteine methyltransferase
MTA	5-methylthioadenosine
P5CS	δ-1-pyrroline-5-carboxylate synthetase
PAL	Phenylalanine ammonia-lyase
ROS	Reactive oxygen species
RS	Raffinose synthase
S+	Sulfur supplementation
S-	Without sulfur supplementation
SAHH	Adenosylhomocysteinase / S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SAMS	S-adenosylmethionine synthetase
SOD	Superoxide dismutases
SPDS	Spermidine synthase
SPP	Sucrose-6-phosphatase
SPS	Sucrose-phosphate synthase
SS	Sucrose synthase

SSADH	Succinate-semialdehyde dehydrogenase
WS	Water-stressed
WW	Well-watered
WW_D	Well-watered developmental control
WW_T	Well-watered temporal control
Ψ_w	Water potential

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ABSTRACT

Cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.) are important economic crops that can suffer significant yield loss under drought. Cotton and maize root systems have fundamentally different architectures, in addition to cotton being a dicotyledonous perennial plant while maize is a monocotyledonous annual plant. However, both species exhibit relative maintenance of elongation of the primary root at low water potentials compared with the shoot. Previous studies on the mechanisms of primary root growth maintenance were conducted mainly in maize but have not been conducted in cotton. The objective of this dissertation was to compare the metabolic responses to water deficit of the primary root growth zone in the two species to determine whether similarities or differences occur.

A series of experiments was conducted to establish the foundation for directly comparing the metabolic responses to water deficit of cotton and maize primary roots. A modified vermiculite-culture seedling system was developed to achieve stable growth rates for cotton roots under different water stress conditions. With this system, a cotton genotype

(cv. AU90810) was selected and the spatial pattern of cell elongation within the primary root growth zone was determined using a kinematic approach. These results showed that the cotton primary root exhibits a similar spatial growth pattern to a drought-tolerant maize line (cv. FR697) when compared at equivalent root tissue water potentials, allowing for direct comparisons of the metabolic responses to water deficit in the two species.

Experiments for comprehensive metabolite analysis of the responses to water stress in the primary root growth zone in both species were conducted. Commonalities were found primarily in the metabolites that function as osmolytes. However, there were significant differences that have important implications for the control of growth in water-stressed roots of the two species. Anti-oxidative mechanisms and sulfur metabolism exhibited the most striking differences that separate the responses of the two species to water deficit. In the water-stressed treatments, glutathione and sulfate decreased in abundance in cotton whereas they increased in abundance in maize, which indicated that sulfur limitation may occur in cotton roots under water-stressed conditions. Further investigations into the abundance of glutathione and hydrogen peroxide verified the quantitative differences in the glutathione response of the two species but showed relatively low hydrogen peroxide levels in cotton, suggesting that cotton may have alternative anti-oxidative mechanisms that are independent of glutathione. A transcriptomics study of the cotton primary root growth zone that was focused on sulfur metabolism and anti-oxidative mechanisms further confirmed the alterations of these metabolic pathways in response to water-deficit stress.

To test the hypothesis that water-stressed cotton roots are sulfur-limited, the effects of sulfur supplementation on root growth and plant-water relations in responses to water stress were examined in a simulated field condition, but the results demonstrated that growth limitation of the root system by exposure to water deficits was not relieved by sulfur supplementation.

Future studies will focus on investigating the roles of alternative anti-oxidative mechanisms as well as alterations in sulfur metabolism in cotton under water-deficit conditions, with the aim of generating novel strategies for cotton yield improvement under water-limited conditions.

Chapter 1

Literature Review

Introduction

Water deficit is one of the most important abiotic stresses affecting plant growth and crop productivity worldwide (Boyer, 1982). In recent years, climate change has caused a series of natural disasters world-wide including heat waves and droughts from shifting rainfall patterns, which enhances the seriousness of soil water-deficit stress on plant productivity (Boyer et al., 2013). Under such circumstances, the ability of a plant to survive water-deficit stress is critical. Therefore, research into how plants respond to exposure to soil water deficit is of great importance as it could lead to a solution of maintaining plant growth in a drier future climate and preventing the decline in crop productivity.

Plants have numerous responses to water deficit. The most visible response is the inhibition of plant growth in all plant organs, including roots (Huang & Eissenstat, 2000; Sekhon et al., 2010), shoots (El Soda et al., 2010), leaves (Zhang et al., 2004; Farooq et al., 2009), and stems (Specht et al., 2001; Wu et al., 2008). The inhibition of growth reduces both biomass and productivity for crop plants (Specht et al., 2001; Kamara et al., 2003; Monneveux et al., 2006; Dickin & Wright, 2008). At the cellular level, damaging molecules such as reactive oxygen species (ROS) accumulate upon exposure to water-deficit stress. Cellular homeostasis becomes unbalanced by ROS through oxidation of required cellular components, causing metabolic pathway disorder which limits the ability of the plant to grow and produce seed (Lee et al., 2009; Goodarzian Ghahfarokhi et al., 2015; Ahanger et al., 2017).

Among the plant organs that are affected by water deficit, the root is the first organ that emerges from the seed and comes in contact with the environment. Water and nutrients

are taken up by the root system and transported to the rest of the plant. Therefore, the establishment of the root system is essential to the establishment and ultimately the survival of the plant especially when the seedling is exposed to water-stressed conditions. This is also important in an agricultural sense as stand establishment, the first stage of a useful crop, is critical to late season productivity and yield.

Many previous studies have focused on the primary root and determined that growth rate is less sensitive than the growth rate of the shoot under water-deficit conditions for multiple crops (Sharp et al., 1988; Spollen et al., 1993). The majority of subsequent studies on primary root growth in water-deficit conditions focused on maize and, to a lesser extent, soybean (Sharp et al., 2004; Yamaguchi et al., 2010; Voothuluru et al., 2016). These studies utilized a seedling model system that allowed for consistent and reliable assessment of primary root growth responses in a medium that could be manipulated to deliver precise water deficits (Sharp et al., 1988). It is with research utilizing this model system that much of what we understand about the physiology and underlying molecular mechanisms involved in the growth response of primary roots to a water-deficit stress has been amassed (Yamaguchi & Sharp, 2010).

However, little is known about growth response of primary roots to water-deficit stress for other species. Cotton is an important economic crop that produces fiber, seed oil, protein meal and hulls for the textile, food and livestock industries. Cotton production is facing a growing threat of increased yield losses as a result of drought as climate change has reduced available water resources (Chapagain et al., 2006; Fahad et al., 2017). Cotton, as a dicot plant, has a tap root system which is dependent upon a successful

establishment of the primary root. The cotton primary root also maintains growth relative to the growth of shoot, leaf and stem under water-deficit conditions (Spollen et al., 1993; Pace et al., 1999). These studies illustrate the importance of cotton primary root growth under water-deficit conditions and the need for further studies to identify the mechanisms underlying the growth responses.

As the organ for both water and nutrient uptake, the interaction between nutrient status and water stress has a major multifaceted impact on roots and the plant. Under water-deficit conditions, the addition of macronutrients, including nitrogen, phosphorus and potassium, promotes root growth and enhances the accumulation of carbohydrates and anti-oxidative enzymes in cotton (Price et al., 1989; Brouder & Cassman, 1990; Liu et al., 2008; Luo et al., 2015; Zahoor et al., 2017a; Zahoor et al., 2017b). Sulfur also promoted the growth of cotton reproductive organs (Mullins, 1998; Yin et al., 2011; Geng et al., 2016), perhaps through the maintenance of redox-homeostasis, relieving water deficit-induced oxidative stress via the regulation of sulfur containing amino acid metabolism, antioxidant production and other sulfur derivatives (Srivalli & Khanna-Chopra, 2008; Bürstenbinder et al., 2010; Noctor et al., 2011; Waduwara-Jayabahu et al., 2012). However, the effects of sulfur on cotton root growth are not well investigated.

The focus of this dissertation was to modify the well-developed seedling system (Sharp et al., 1988) to characterize the growth response of the cotton primary root to water deficits and to compare the underlying metabolic responses with maize in order to identify possible similarities or differences in the drought response between primary roots of a monocot and a dicot plant under water deficits. As the dissertation work progressed, as

will be described in later chapters, the focus shifted into an interest in the role that oxidative stress plays in the water-deficit response of the primary root of both cotton and maize, and within this context the role of sulfur-containing compounds in regulating oxidative metabolism. The role of sulfur in root growth, especially the primary root growth of cotton under water-deficit stress, has not been investigated to date. Thus, the research also led to an examination of the effects of sulfur on growth and metabolism of cotton primary roots under water-deficit conditions to gain a deeper understanding of the function of sulfur metabolism in water-deficit stress tolerance mechanisms.

Root responses to water deficit

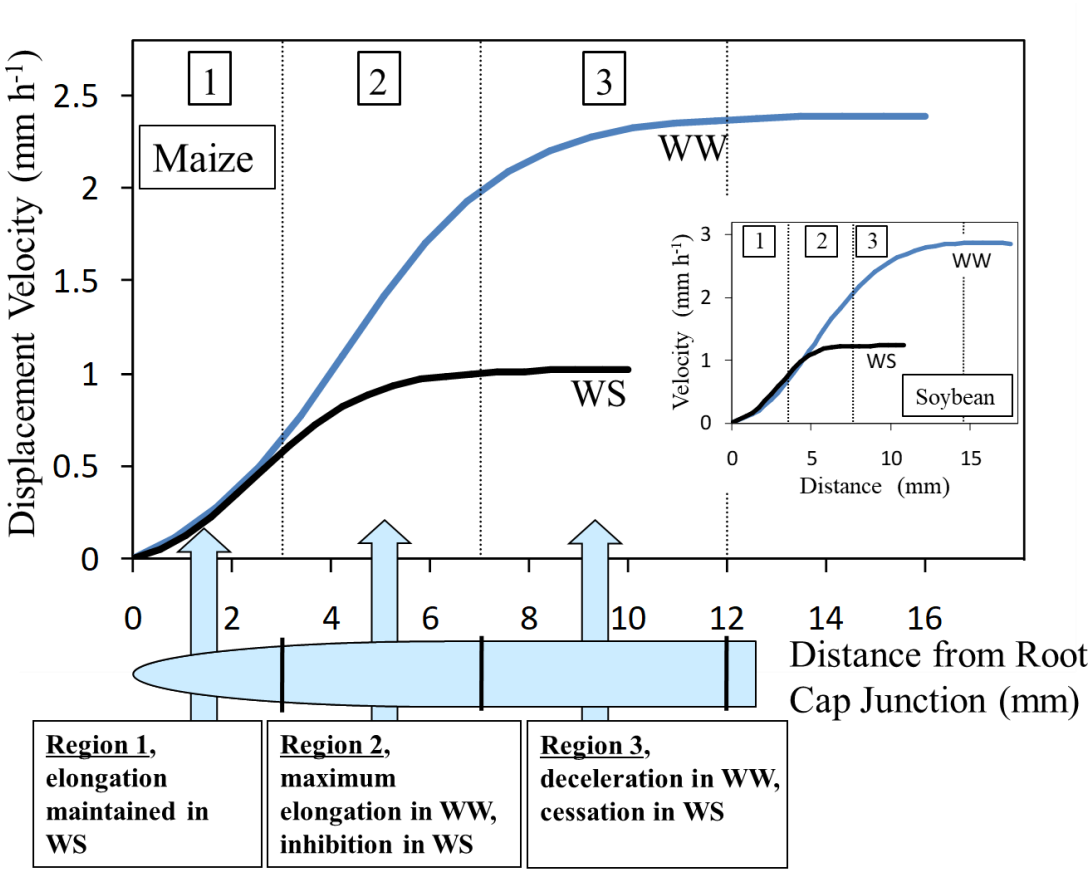
Under water-deficit conditions, plant roots are of prime importance as they make direct contact with the drying soil environment and are the first to sense the water deficit. Roots can be exposed to water-deficit stress from the beginning of seedling establishment. Under circumstances where soil water availability is limited, roots need to find ways to maintain growth. In the early stages of root system development, the primary root was reported to maintain growth, relative to shoot growth, under drought conditions in multiple crops including maize, cotton and soybean (Spollen et al., 1993). A seedling system based on vermiculite media (Sharp et al., 1988) was established for characterizing the growth responses of the primary root to water deficit and studying the mechanisms involved in primary root adaptation to water deficit with different physiological and metabolic approaches.

The most critical growth response found with this seedling system in both maize and soybean was the differential pattern of cell expansion in the growth zone of the primary

root under well-watered and water-stressed conditions (Sharp et al., 1988; Saab et al., 1992; Sharp et al., 2004; Yamaguchi et al., 2010). Using a kinematic approach (Silk et al., 1989), the growth zone can be divided into three distinct regions according to the response of cell expansion rate at different positions along the primary root axis (Figure 1-1). In region 1 (R1), encompassing the root meristem, growth rates are the same in both well-watered and water-stressed treatments. In region 2 (R2), the growth rate of the water-stressed treatment starts to decline while the well-watered treatment reaches the maximal growth rate. In region 3 (R3), growth ceases in the water-stressed treatment while starting to decline in the well-watered treatment. Beyond region 3, the well-watered treatment reaches final cell length and the beginning of the maturation zone (Yamaguchi & Sharp, 2010). The underlying mechanisms of how the maintenance and inhibition of growth in the different regions are regulated have been investigated in detail, mainly focused on the maize primary root.

Osmotic adjustment is an important mechanism regulating plant water relations and root growth under water-deficit conditions. Osmotic adjustment is manifested in water-stressed roots by a lowering of the osmotic potential of root tissues so as to maintain turgor (Sharp & Davies, 1979; Westgate & Boyer, 1985) at low water potentials (Ψ_w), and is achieved by accumulating compounds that have osmolyte functions (Sharp et al., 1990; Voetberg & Sharp, 1991). Carbohydrates are one of the most typical osmolytes that accumulate under water-deficit conditions (Kaplan & Guy, 2004; Iordachescu & Imai, 2008). Studies using the seedling system have characterized carbohydrates accumulated in the growth zone of water-stressed maize primary roots. Hexoses were found to

Figure 1-1. Displacement velocity as a function of distance from the root cap junction of primary roots of well-watered (WW) and water-stressed (WS) maize seedlings (cv. FR697). The inset shows the profile of displacement velocities for WW and WS soybean primary roots (cv. Magellan). Relative elongation rates (h^{-1}) are obtained from the derivative of velocity with respect to position (modified from Yamaguchi & Sharp, 2010).



accumulate primarily in the basal region of the growth zone and to contribute substantially to the regulation of osmotic potential (Sharp et al., 1990). A transcriptomics study indicated that this might be regulated by transcript abundance as transcripts of sucrose synthase increased in abundance in water-stressed maize primary roots (Spollen et al., 2008).

Proline is another osmolyte that accumulates in water-stressed conditions in plants (Stewart & Hanson, 1980). The accumulation of proline has been observed in roots of multiple plants (Voetberg & Sharp, 1991; Manivannan et al., 2007; Dobrá et al., 2010). For the maize primary root, the osmotic adjustment function of proline was located primarily in the apical region of the growth zone (Voetberg & Sharp, 1991). Transcriptional evidence indicated that genes involved in proline metabolism were induced by water-deficit stress in roots of different plants (Spollen et al., 2008; Dobrá et al., 2010; Dobrá et al., 2011). Other than osmotic adjustment, proline interacts with other water-deficit responses including anti-oxidants (Omidi, 2010; Lum et al., 2014) and plant hormones (Ober & Sharp, 1994), whose functions under water-deficit stress are reviewed below.

Plant hormone regulation is another important mechanism modulating plant growth under water-deficit conditions. Abscisic acid (ABA) is a well-known plant hormone that accumulates in water-stressed plant tissues (Zhang & Davies, 1989; Saab et al., 1992; Ober & Sharp, 2007). ABA is considered to be an important signaling molecule for root-shoot communication and regulation of stomatal closure (Wilkinson & Davies, 2010). Utilizing the seedling system, studies determined that ABA accumulation was related to

the maintenance of maize primary root growth under water-deficit conditions (Saab et al., 1990). Particularly, ABA is associated with growth maintenance in the apical region of the growth zone (Saab et al., 1992). ABA also regulates ROS generation and thus directs the protection of plant tissues from oxidative damage under stress conditions (Kwak et al., 2003; Yamaguchi & Sharp, 2010). High ROS content was found in the primary root of the ABA-deficient maize mutant *vp14* under water-deficit conditions and was associated with the reduction of growth, supporting the hypothesis that ABA regulates ROS production (Cho, 2006).

Ethylene is another important plant hormone induced by water-deficit stress in plants (Bergner & Teichmann, 1993, Huberman et al., 1993, Michelozzi et al., 1995; Morgan & Drew, 1997; Sobeih et al., 2004). Metabolically, the ethylene biosynthesis pathway is an important branch of sulfur metabolism. The precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC), is produced via the degradation of S-adenosylmethionine (SAM), which is derived from methionine, one of the sulfur containing amino acids (Morgan & Drew, 1997). SAM is an important intermediate of the sulfur pathway whose functions are reviewed below. Ethylene also interacts with ROS under water-stressed conditions. The ethylene biosynthesis enzyme 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) was upregulated under water-stressed conditions and triggered intracellular ROS generation, resulting in cellular damage in mungbean seedlings (Ke & Sun, 2004). Importantly, the interaction of these two important plant hormones, ABA and ethylene, has been recognized in the maize primary root under water-deficit conditions where the accumulation of ABA restricted excess ethylene production (Spollen et al., 2000; Sharp, 2002).

Reactive oxygen species and anti-oxidative mechanisms in plants exposed to water-deficit stress

Oxidative stress has a major impact on plant cells when coupled with water-deficit stress. Hydrogen peroxide (H_2O_2), superoxide, hydroxyl radicals and singlet oxygen are the major forms of ROS in plants, animals and other organisms. An increase in ROS production was observed in several studies of plant acclimation to various stresses including water deficit (Fujita et al., 2006; Yamaguchi & Sharp, 2010). ROS accumulation under stressed conditions can cause protein denaturation, lipid peroxidation in cellular membranes, DNA or RNA damage, negative effects on anti-oxidative enzyme activities, and ultimately cell death (Scandalios, 1993; Noctor & Foyer, 1998; Mittler et al., 2004; Torres & Dangl, 2005). Therefore, anti-oxidative mechanisms involving both ROS-scavenging enzymes and metabolites to protect cellular homeostasis by preventing ROS accumulation are considered to be critical protective mechanisms under water-deficit conditions (Noctor & Foyer, 1998; Bouché et al., 2003; Van Breusegem et al., 2008). Under water-deficit conditions, ROS scavenging enzymes and metabolites are activated in plant roots (Shalata et al., 2001; Mittova et al., 2004; Yoshimura et al., 2008) and have greater activities in drought-tolerant lines (Moumeni et al., 2011).

Catalases (CATs) and peroxidases (POXs) are two major classes of enzymes that function as ROS scavengers, particularly for H_2O_2 (Willekens et al., 1997; Smirnoff, 2000). Comparing these two enzyme classes, catalases have higher reaction rates but lower affinity for H_2O_2 (Willekens et al., 1997). Superoxide dismutases (SODs), another class of ROS-related enzymes, contain heavy metals (Cu, Zn, Mn), and can be divided

into different enzyme families according to the metal element. SODs do not have a scavenging function for most ROS radicals but can convert highly damaging superoxide radicals to H_2O_2, which is less harmful (Scandalios, 1997; Fry et al., 2002).

In addition to anti-oxidative enzymes, anti-oxidative metabolites also play important roles in plant resistance to oxidative stress. Ascorbate and glutathione (GSH) are important metabolites that have multiple functions in plant cells, and are involved in many metabolic reactions occurring in plants under stressed conditions. Ascorbate and glutathione protect plant cells against oxidative stress and damage by detoxifying ROS and ROS-generated toxic metabolic products (Arrigoni, 1994; Córdoba & GonzalesReyes, 1994; Noctor & Foyer 1998; Asada, 1999). They deal with ROS molecules either directly by scavenging them or indirectly through the activation of other defense mechanisms (Edwards et al., 1991; Gupta et al., 1991; Kocsy et al., 2000; Saito, 2004; Parisy et al., 2007). The major ROS scavenging function of ascorbate and glutathione is achieved via the ascorbate-glutathione cycle whose function is the detoxification of H_2O_2, which is generated as a waste product of several metabolic pathways. Firstly, H_2O_2 is reduced to H_2O by ascorbate peroxidase (APX) using ascorbate as the electron donor and conversion of ascorbate to its oxidized form, monodehydroascorbate (MDHA). MDHA is degraded into ascorbate and dehydroascorbate (DHA) by monodehydroascorbate reductase (MDAR). DHA is reduced to ascorbate by dehydroascorbate reductase (DHAR). This step expands a reduced glutathione (GSH) and yields an oxidized glutathione (GSSG). Finally, GSSG is reduced by glutathione reductase (GR) using NADPH as the electron donor. As a result, ascorbate and glutathione are not consumed at the end of the reaction (Noctor & Foyer, 1998). The

ascorbate-glutathione cycle occurs in mitochondria, chloroplasts, cytosol, peroxisomes and the apoplast (Smirnoff, 2000; Mittler, 2002; Pignocchi & Foyer, 2003; Mittler, 2004; Asada, 2006). Ascorbate and glutathione are also involved in maintaining cellular redox homeostasis and redox signaling (Foyer & Noctor, 2005; Joo et al. 2005; Scheibe et al., 2005; Noctor, 2006; Foyer & Noctor, 2009). They may also interact with other signaling pathways during stress resistance responses (Neill et al., 2002; Pignocchi & Foyer, 2003). The ratios between reduced and oxidized forms of ascorbate and glutathione function as biomarkers of oxidative stress levels, and also activate various defense mechanisms (Noctor & Foyer, 1998; Tausz et al., 2004). In plants, other metabolites like carotenoids (Baroli & Niyogi, 2000; Donget al., 2007; Qin et al., 2007; Avendaño-Vázquez et al., 2014) and α-tocopherol (Munné -Bosch & Falk, 2004; Munné -Bosch, 2005; Kanwischer et al., 2005; Miret & Munné -Bosch, 2015; Tung & Ng, 2016) also have anti-oxidative functions.

The anti-oxidative functions of these enzymes and metabolites have been demonstrated in roots under water-deficit conditions. The anti-oxidative enzymes are activated by water deficit stress in roots of different plants (Shvaleva et al., 2005; Nayyar & Gupta, 2006; Jaleel et al., 2008b; Bian & Jiang, 2009; Wang et al., 2009; Zhu et al., 2009; Silveira et al., 2017) and further enhanced in water deficit-tolerant genotypes (Fazeli et al., 2007; Jaleel et al., 2008a; Li et al., 2015). Antioxidative metabolites including ascorbate (Jaleel et al., 2008a; Jaleel et al., 2008b; Zhu et al., 2009), glutathione (Nayyar & Gupta, 2006; Jaleel et al., 2008a; Jaleel et al., 2008b) and α-tocopherol (Jaleel et al., 2008a; Jaleel et al., 2008b) were also observed to increase in abundance under water-deficit conditions in roots of different plants.

In the maize primary root under severe water deficit conditions, previous transcriptomics and proteomics studies identified increases in abundance of several anti-oxidative enzymes and their transcripts in the growth zone, including CATs and SODs (Zhu et al., 2007; Spollen et al., 2008). However, these studies did not provide direct evidence for alterations in the abundance of the anti-oxidative metabolites or how such alterations are regulated. Therefore, studies of how the levels of root anti-oxidative metabolites are altered by water deficit are important for gaining a deeper understanding of antioxidative mechanisms in water-stressed primary roots of maize and other species.

Transcriptomics and metabolomics approaches to study plant responses to water deficits

"Omics" technologies, including genomics, transcriptomics, proteomics and metabolomics, are designed for the characterization and quantification of pools of biological molecules that change under different perturbations. Omics studies have played an important role in plant research, efficiently generating useful and large datasets, "big data", without the need for large amounts of biological materials (Urano et al., 2010).

Transcriptomic studies make it possible to track the expression of multiple genes simultaneously in plant tissues by measuring transcript abundance. Transcriptomic studies in maize have identified many genes whose transcripts respond to water deficit, including those that are related to carbohydrate metabolism, plant hormone regulation, signaling regulation and anti-oxidative mechanisms (Kakumanu et al., 2012; Shan et al., 2013; Xu et al., 2014). Studies focused on the maize primary root also displayed such

responses to water-deficit stress (Spollen et al., 2008; Yamaguchi & Sharp, 2010; Opitz et al., 2014; Opitz et al., 2015), in addition to changes in transcript levels for many transcription factors (Seeve et al., 2017). For cotton, a genome-wide transcriptomic analysis of bolls, ovules, and leaves determined that drought stress induced an accumulation of transcripts from a large number of genes encoding transcription factors, osmoprotectants, ion transporters and heat shock proteins (Padmalatha et al., 2012). Pathways involved in hormone [ABA, jasmonic acid (JA) and ethylene] biosynthesis and signal transduction were also shown to be associated with transcript accumulation of the relevant genes in response to water deficits (Padmalatha et al., 2012). A comparative transcriptomics study identified a significant induction of transcripts encoding enzymes related to carbon/sugar metabolism in roots compared to leaf tissue under the same drought condition, suggesting an activated osmotic regulation process in cotton roots (Payton et al., 2011).

Beyond gene expression (transcript abundance), metabolites are considered to be the integration of gene expression, protein networks and other post-translational regulatory events. Metabolite levels are closer to the expression of the phenotype than mRNA transcript levels and protein abundance. It was reported that many major metabolites, including carbohydrates (Kaplan & Guy, 2004; Sharp et al., 2004; Iordachescu & Imai, 2008; Nishizawa et al., 2008; Sicher et al., 2012), amino acids (Arbona et al., 2008; Nishizawa et al., 2008), polyamines (Gill & Tuteja, 2010; Verslues & Juenger, 2011; Alet et al., 2012) and secondary metabolites (Munns & Tester, 2008; Karowe & Grubb, 2011) increased or decreased in abundance in response to exposure to water-deficit conditions. Metabolomics provides an approach to investigate and analyze the changes in the levels

of many different metabolites in tissues under water-deficit conditions simultaneously. Metabolomics has been utilized to investigate plant responses to water-deficit conditions in multiple species. The typical metabolites that accumulate under water-deficit conditions have been identified in different metabolomics studies. Carbohydrates, including glucose (Lugan et al., 2009; Warren et al., 2012), fructose (Lugan et al., 2009; Bowne et al., 2012; Warren et al., 2012) and raffinose (Lugan et al., 2009; Urano et al., 2009), are among the most commonly observed metabolites accumulating in water-stressed plants. Proline is another commonly increased metabolite found in plant tissues under water-deficit conditions (Charlton et al., 2008; Lugan et al., 2009; Urano et al., 2009; Bowne et al., 2012; Sanchez et al., 2012). Metabolites of the TCA cycle (Urano et al., 2009; Bowne et al., 2012; Silvente et al., 2012) and its derivative, γ-aminobutyric acid (GABA) (Charlton et al., 2008; Lugan et al., 2009; Urano et al., 2009; Silvente et al., 2012), were observed to increase under water-deficit conditions in several plants. A metabolomics study conducted with *Sporobolus stapfianus*, a desiccation-tolerant grass species, demonstrated that oxidized glutathione (GSSG) and multiple γ-glutamyl amino acids relating to glutathione biosynthesis increased in abundance in dehydrating leaves. Ophthalmate, a metabolite generated by the glutathione pathway and a biomarker of oxidative stress, also increased in abundance under dehydration conditions (Oliver et al., 2011). ABA accumulation is also commonly found in water-stressed plants (Alvarez et al., 2008; Hochberg et al., 2013).

Because transcriptomics and metabolomics methods can analyze plant responses to water deficit more directly and comprehensively than more traditional approaches, they offer a useful approach to compare responses of different species. However, it is notable that for

the primary root seedling system, even though a number of studies have utilized transcriptomics and proteomics approaches in maize and soybean, no metabolomics studies have been conducted with this system.

Cotton responses to water deficit

Cotton is naturally a perennial dicot plant but is managed as an annual for pest-control and harvestability reasons. Normally, cotton cultivation requires a long frost-free period, plenty of sunshine and a moderate rainfall. However, a large proportion of modern production occurs in areas with low rainfall and thus its cultivation requires not only new drought-tolerant cultivars but also irrigation, for example, in Xinjiang, China and some Central Asian countries. The South Plains area in the United States is known as the largest contiguous cotton producing region in the world and is classified as a semi-arid region. In most of the cotton production areas in the northern hemisphere, the planting time of cotton varies from the beginning of February to the beginning of June. The growing season is about six months until harvest. In traditional agriculture, cotton is considered as a salt and drought tolerant crop, which makes it an attractive crop for arid and semi-arid regions. However, cotton growth is still inhibited by drought stress and improper cropping and irrigation practices caused desertification in areas like Uzbekistan, where the Aral Sea almost dried up because of unregulated pumping of water for irrigating cotton (Mainguet & Létolle, 1998; Waltham & Sholji, 2001).

Although cotton is considered as a relatively drought tolerant plant and is often irrigated, US cotton productivity has suffered yield losses as a result of drought stress (Santa Ana, 2009; Kerr, 2012). Because of climate change that may further limit available water

resources, cotton is facing an increasing threat of even more yield loss by drought (Chapagain et al., 2006; Fahad et al., 2017). In drought conditions, the rate of plant height increase, leaf extension, net photosynthesis, bolling period and production of lint were all reduced in cotton under drought conditions (McMichael & Hesketh, 1982), ultimately leading to a reduction of lint yield. Further physiological research revealed that under drought conditions, ABA, proline, soluble sugars and potassium ions accumulated while stomatal conductance and the rates of photosynthesis and transpiration declined as leaf water potential was reduced (Shareef et al., 2018).

The variation of cotton responses to water stress was highlighted from early research reports. Screening of multiple cotton lines determined that different lines have variable responses under field drought conditions, differing in water use efficiency, days to permanent wilting and root/shoot biomass ratio (Quiseberry & McMichael, 1991; McMichael & Quiseberry, 1991). Tolerant lines were able to adjust osmotic potential by regulating the synthesis of osmolytes to maintain turgor under drought conditions, while susceptible lines could not maintain plant water content (Nepomuceno et al., 1998). Further genotypic investigations into cotton lines indicated that many root and shoot parameters varied among cotton genotypes including root length, shoot length, root and shoot fresh weight, root and shoot dry weight, lateral root numbers, excised leaf water loss, relative water content and total plant weight (Riaz et al., 2013; Hassan et al., 2015).

In the initialization of the cotton root system, the radicle emerges directly from the seed and becomes the primary/tap root. Lateral roots then grow from the primary/tap root. The lateral roots and primary/tap root form the fundamental root system of cotton. Other

lateral roots with higher orders develop from the fundamental root system and have a functional life of about three weeks. These roots form when environmental conditions are favorable, and then die when water and nutrient supplies are insufficient in the area of soil where they develop (Ritchie, 2007). Water and nutrients are thus limiting factors for cotton root development and growth.

Cotton roots develop certain growth phenotypes in response to water-limited conditions. The growth of the cotton root system was promoted in mild water-deficit conditions compared with fully irrigated plants (Hu et al., 2009). It was reported that in cotton seedlings, when height, leaf area and dry weights of leaf and stem were reduced as a result of drought, root biomass was maintained by having a longer primary/tap root (Pace et al., 1999). This study indicated that cotton roots reduced the thickness of the root in favor of maintaining root elongation, thereby reaching deeper soil layers that may contain more water. Other root traits also demonstrated variability under drought conditions. Tolerant cotton genotypes have a longer primary/tap root, more lateral roots and greater root dry and fresh weights than the susceptible lines (Basal et al., 2005). They also tend to have comparatively higher abilities to synthesize antioxidant enzymes [glutathione S-transferases (GST), SOD] and osmolytes like proline and soluble sugars in the root system under drought (Singh et al., 2016). Drought-tolerant cotton lines also have a better capability for maintaining plant water status. Early studies of cotton roots determined that the arrangement of the vascular bundle varied with genotype among cotton lines, which resulted in differences in the total cross-sectional vessel area in each root. Increased vascular bundle elements could increase the water transportation ability to maintain plant water potential under drought in some genotypes. The expanded vascular bundle

phenotype was found in both the primary and lateral roots (McMichael et al. 1985; McMichael et al. 1987).

Excluding the work on vascular bundle elements, the majority of cotton root research under drought has focused on the root system as a whole. Little is known about the responses of different root types (primary/tap root and lateral roots with different orders) under water-deficit conditions. Most of the studies discussed the basic traits (length and numbers) of the primary/tap root and lateral roots and there is a need for further analyses of physiological and metabolic processes associated with the drought response in different root types in order to manage or manipulate root growth in both vegetative and reproductive stages of cotton.

Cotton is also affected by the interaction of nutrient availability and water stress in many aspects. Nitrogen is one of the most important nutrients for plant growth. Nitrogen fertilization can affect cotton root development under drought stress by enhancing the root/shoot ratio, root density and increasing the activities of anti-oxidative enzymes (Liu et al., 2008; Luo et al., 2015). Analysis of the effect of potassium in cotton under drought stress determined that potassium fertilizer can promote the rate of photosynthesis (Zahoor et al., 2017a). Potassium also interacts with nitrogen to regulate osmotic adjustment in cotton leaves under drought (Zahoor et al., 2017b). An important micronutrient, zinc, was found to improve drought stress tolerance in cotton by enhancing photosynthesis and the biosynthesis of osmoregulatory compounds (proline, soluble sugars, soluble protein) and antioxidants (APX, CAT, SOD, carotenoid, glutathione, ascorbate) (Wu et al., 2015). Sulfur was reported to promote the growth of cotton reproductive organs (Mullins, 1998;

Yin et al., 2011; Geng et al., 2016). However, as an important macronutrient whose function is related to many drought resistance mechanisms that are reviewed below, the effects of sulfur on cotton root growth under water-deficit conditions are not well-studied.

With the development of modern breeding technologies, many cotton transgenic lines have been developed with enhanced stress tolerance abilities. It is notable that in recent years, the development and breeding of drought-tolerant cotton transgenic genotypes forms the major proportion of cotton drought research worldwide. In 2011, the International Service for the Acquisition of Agri-biotech Applications (ISAAA) reported that transgenic cotton was planted on an area of 25 million hectares, which was 69% of the worldwide total cotton planting area (ISAAA, 2012). Each transgenic line has investigated a specific promotion or repression of certain biological traits, and the fiber and seed yield were maintained or improved by the transgenic manipulations. The specifically enhanced functions in these transgenic lines that are directed at improving water-deficit stress resistance mechanisms include: increased enzyme activities for CAT (Liang et al., 2016; Yu et al., 2016), APX (Yue et al., 2012; Mishra et al., 2017), POX (Yue et al., 2012; Zhang et al., 2014) and SOD (Lv et al., 2007; Yue et al., 2012; Zhang et al., 2014; Liang et al., 2016; Yu et al., 2016; Mishra et al., 2017) as well as enhanced osmotic adjustment by increasing osmolyte (proline and soluble carbohydrates) production (Lv et al., 2007; Lv et al., 2009; Yue et al., 2012; Liu et al., 2014; Liang et al., 2016; Yu et al., 2016; Lisei-de-Sa et al., 2017), reduced lipid peroxidation (Lv et al., 2007) and reduced membrane damage (Lv et al., 2009; Yue et al., 2012). In some of the studies, root growth was promoted (Lv et al., 2009; Pasapula et al., 2011; Kuppa et al.,

2013; Zhang et al., 2014; Shen et al., 2015; Yu et al., 2016; Lisei-de-Sa et al., 2017; Mishra et al., 2017), and wilting (Maqbool et al., 2010) and senescence (Kuppa et al., 2013) times were delayed. ABA levels were also reported to increase in transgenic lines in response to water-deficit stress (Yue et al., 2012). Yield can also be increased under water-deficit conditions via transgenic manipulations (Pasapula et al., 2011, Shamim et al., 2013; Shen et al., 2015).

However, despite the increase in the knowledge of whole plant responses to water deficit, there are still many areas of research that need to be addressed to understand the tissue, cellular and subcellular level responses to water deficit in cotton and in particular with regards to the root.

The role of sulfur metabolism in plants under water-deficit stress

Sulfur is an essential macronutrient for plant growth and development. Multiple compounds in the sulfur metabolic pathways, especially sulfur-containing amino acids and their derivatives, are critical for plant health and growth (Hesse & Hoefgen, 2003; Saito, 2004). Sulfur metabolism not only plays a key role in the primary metabolism of plants providing the structural components of essential cellular molecules, but also some of the metabolites in the sulfur metabolism pathways are important for plant acclimation to stressful environments (Biswal et al., 2008). The major function of sulfur in responses to water deficit lie in the production of two sulfur containing amino acids cysteine (Cys) and methionine (Met) and their derivatives.

Cysteine is biosynthesized from serine by replacing the hydroxyl group attached to the β-carbon on the serine with a thiol group. Cysteine is a precursor of glutathione and the importance of cysteine to water-deficit stress resistance is achieved through glutathione. Glutathione is a tripeptide formed by glutamate, cysteine and glycine through a two-step reaction catalyzed by γ-glutamyl cysteine synthetase (γ-ECS) and glutathione synthetase (GSHS) (Srivalli & Khanna-Chopra, 2008). Cysteine availability can control the biosynthesis of glutathione as it is a limiting substrate for γ-ECS (Harms et al., 2000; Droux, 2004). Glutathione accumulation is found in tissues under water-deficit conditions in many different plants (Bartoli et al., 1999; Selote et al., 2004; Hicks et al., 2007; Choe et al., 2013). It is an essential metabolite that has multiple functions. Glutathione is involved in detoxification of heavy metals (Gullner et al., 2001; Koprivova et al., 2002; Mendoza-Cozatl et al., 2005; Sharma & Dietz, 2006), transfer and storage of sulfur (Fitzgerald et al., 2001), regulation of expression of defense-related genes (Després et al., 2003) and protein activity (Pastori et al., 2000; Rausch et al., 2007) under stressed conditions. Beyond these functions, the most important function of glutathione in plants is its anti-oxidative activity. As reviewed above, a large part of the antioxidative function of glutathione is achieved through the ascorbate-glutathione cycle. Additionally, a class of glutathione-dependent enzymes, GSTs, are involved in removing lipid peroxides, herbicides and methylglyoxal (Moons, 2005; Rausch et al., 2007; Yadav et al., 2008; Szalai et al., 2009). Some of the GSTs can catalyze the conjugation of glutathione to various metabolites and others have peroxidase or isomerase activities (Edwards et al., 2000; Noctor, 2006). Of particular importance to the focus of this dissertation, the peroxidase function of GST is achieved by conversion of the reduced form of glutathione

(GSH) to the oxidized form (GSSG) in the reduction of an organic hydroperoxide (ROOH) to the monohydroxy alcohol (ROH).

Methionine is biosynthesized from homoserine through multiple enzymatic reactions. Similar to cysteine, the importance of methionine in responses to water-deficit stress is achieved through a derivative, S-adenosylmethionine (SAM). SAM is a multi-functional amino acid derivative. It contains a reactive methylated sulfur moiety required for methylation of many essential molecules including DNA, RNA, proteins, lipids, lignin and flavonoids (Roeder et al., 2009, Gong et al., 2014). It also plays important roles in regulating plant development, stress responses and metabolite accumulation (Xu et al., 2006; Nagel et al., 2008; Köllner et al., 2010; Ghosh et al., 2012). The enhancement of SAM biosynthesis in a transgenic *Arabidopsis* line improved water stress tolerance (Kim et al., 2015). SAM is converted to ACC, the direct precursor of ethylene as mentioned earlier. SAM is also a precursor for polyamines which have an important role as osmoprotectants (Chan et al., 2013) due to their positive charge at physiological pH. This allows them to stabilize and moderate the activities of negatively charged molecules including DNA (Haworth et al., 1991), RNA (Bolton & Kearns, 1978) and proteins (Votyakova et al., 1999). Putrescine, spermidine, and spermine are major polyamines in plants. They are closely related because they are biosynthesized sequentially through the same metabolic pathway. Spermidine synthase (SPDS) combines putrescine and S-adenosylmethioninamine (decarboxylated SAM) to produce spermidine. Spermine synthetase (SPMS) in turn combines spermidine with another S-adenosylmethioninamine to produce spermine (Hanfrey et al., 2001). Both SPDS and SPMS require decarboxylated SAM as the aminopropyl group donor. The byproduct of these reactions

is 5-methylthioadenosine (MTA), which serves as an inhibitor of SPDS and SPMS. Plants use the methionine salvage pathway (Yang cycle) to degrade MTA and maintain the production of spermidine and spermine (Bürstenbinder et al., 2010; Waduwara-Jayabahu et al., 2012). Through the Yang cycle, the sulfur moiety of SAM is restored, however the function of the Yang cycle is regulated by the sulfur availability in the plant cell (Bürstenbinder et al., 2007). Additionally, polyamines can interact with ABA (Urano et al., 2003) and ROS (Cona et al., 2006) under water-deficit conditions.

As reviewed above, in plants, sulfur metabolism and some sulfur containing metabolites have important functions in responses to water-deficit stress. However, sulfur metabolism has not been well-studied in primary roots. Investigating how sulfur metabolism affects primary root growth under water-deficit condition is of great importance.

Justification

Root responses to water deficit are a vitally important component of plant adaptation to drought conditions. As the foundation of the root system, primary root responses to water deficit are critical but little is known about the responses of cotton primary roots to water deficits. To address this lack of knowledge a seedling system that allows detailed physiological studies of cotton primary roots under water-deficit stress was developed. It was based on the well-characterized system that has previously been used to investigate the physiology and molecular biology of maize and soybean roots in response to specific water deficits. This system was then employed in a detailed physiological and metabolic comparison of the response to water-deficit stress of the primary roots of cotton and maize.

Objectives

1. Establishment of a seedling system for characterization of cotton primary root growth responses to water deficits, and selection of a water stress-tolerant line.
2. Comparison of spatial growth distribution patterns and water stress levels of the maize and cotton primary root growth zones.
3. Comparisons of maize and cotton metabolic responses to water deficit in the primary root growth zone by metabolomic and transcriptomic analyses.
4. Characterization of the effect of sulfur deficiency on cotton root growth under simulated field water-deficit conditions.

Chapter 2

Establishment of a Seedling System for Cotton Primary Root Growth Studies

Introduction

As reviewed in Chapter 1, a key adaptation of plants to water-limited conditions is the maintenance of root growth as the soil dries, which enables the plant to access water from deeper in the soil profile. Previous research (Sharp et al., 1988; Spollen et al., 1993) discovered that under water-deficit conditions, the growth of the primary root is more tolerant of a water-deficit stress compared to the growth of the early shoot for several different plant species. Moreover, it has been shown that the cotton primary root may grow deeper under drought conditions, at the expense of shoot growth, which indicates that cotton plants survive drought by accessing water from deeper in the soil profile than the levels tapped during periods of adequate water supply (Pace et al., 1999). These features may be particularly important for cotton seedlings in dryland agriculture where soils are dry and stand establishment and early vigor are important characteristics for successful production (Hake et al., 1990; Guthrie et al., 1995).

One of the challenges to studying plant responses to soil drying is the difficulty of achieving reproducible water stress conditions and applying it to a specific developmental stage. Different growth and metabolic responses may be induced as the severity of water stress increases and if applied to the plant at different stages of development. Over several decades, a well-established seedling system has been used for the study of the responses of maize and soybean primary root growth and development to exposure to precise and repeatable soil water deficits (Sharp et al., 1988; Yamaguchi et al., 2010). This system utilizes vermiculite as the growth medium and a low concentration of calcium sulfate solution as the limited water supply. The vermiculite

allows the water potential of the medium to be controlled quantitatively and reproducibly. The water potential of the vermiculite is maintained at a constant level by growing the seedlings in the dark at near-saturation humidity and constant temperature to minimize evaporation from the media and transpirational water loss from the shoot. The system allows for stable primary root elongation rates with directly visible root growth patterns through the transparent face of the Plexiglas growth boxes for both maize and soybean seedlings at a range of soil water potentials from well-watered (-0.02 MPa) to severe water stress (-1.6 MPa) (Sharp et al., 1988; Yamaguchi et al., 2010).

Previous studies on maize primary roots revealed that different genotypes achieved various elongation rates under equivalent water-deficit conditions (Leach et al., 2011), and were categorized as either water stress sensitive or water stress tolerant dependent upon their root growth characteristics. The water stress tolerant line (cv. FR697) was selected as the model genotype for characterization of mechanisms of primary root growth maintenance at low water potentials (Sharp et al., 2004; Poroyko et al., 2005, 2007; Zhu et al., 2007; Spollen et al., 2008; Voothuluru & Sharp, 2013; Voothuluru et al., 2016). In cotton, many root and shoot parameters vary among genotypes in response to water-deficit conditions, including root length, shoot height, root and shoot fresh weight, root and shoot dry weight, lateral root number, excised leaf water loss, relative water content and total plant biomass (Riaz et al., 2013; Hassan et al., 2015). However, there are no studies that have focused on primary root growth under water-deficit stress as a means to select cotton lines for tolerance to low soil water contents. Investigating the variation in growth of seedling cotton primary roots between different genotypes in response to water deficit would be important to provide an easily measured trait for

classifying genotypes according to their potential tolerance of water deficits in the early stages of seedling development and stand establishment. Moreover, the cotton genotypes that perform better in the response of the root to water deficit could be used as a foundation for physiological research into mechanisms that impart the ability to maintain root growth in water-deficit conditions.

In order to better characterize primary root growth in cotton, whether in well-watered or water-deficit conditions, and reliably compare it to maize, it was necessary to adapt the vermiculite-culture seedling system for studies of cotton. The secondary purpose of the study was to select a cotton genotype that is potentially tolerant to water-deficit stress, as determined by primary root growth rate in the vermiculite system.

Materials and Methods

Growth media

Vermiculite (no. 2A, Therm-O-Rock East Inc., New Eagle, PA, USA) was prepared at a range of water potentials by thoroughly mixing with varying amounts of 1 mM $CaSO_4$ solution. Mixing was achieved by dripping the 1 mM $CaSO_4$ solution into the vermiculite in a sealed plastic bag followed by vigorous mixing to ensure homogeneity. The water potential of the media was measured by isopiestic thermocouple psychrometry (Boyer & Knipling, 1965). Four water potential treatments were used: well-watered (-0.02 MPa), mild stress (-0.4 to -0.5 MPa), moderate stress (-1.0 to -1.25 MPa) and severe stress (-1.4 to -1.9 MPa).

Cotton genotypes for selection

The candidate genotypes were selected in consultation with Cotton Incorporated based on their performance in field trials for yield. The genotypes tested were ARK0403-3, ARK0409-17, AU90810, AU90915, DP393, FM958, MD25-26ne, PD05064, and TAMCOT73.

Plant growth conditions

In the first round of genotype selection, cotton seeds were imbibed in 1 mM $CaSO_4$ solution for 24 h at 30ºC in a water bath. Seeds were aerated during imbibition by means of an air pump. Seeds were subsequently placed between two pieces of germination paper moistened with 1 mM $CaSO_4$ and germinated for 24 h. Seedlings with emerged radicals of 5-15 mm in length were transplanted against the face of transparent Plexiglas boxes containing the appropriate vermiculite media. Germination and cultivation of the seedlings were conducted in the dark at near-saturation humidity at 29 ºC, as described in Sharp et al. (1988) and Spollen et al. (2000). In the second round of genotype selection, the imbibition time and germination time of four selected lines were modified according to the characteristics of each genotype with the modified seedling system.

Measurement of primary root length and calculation of elongation rate

The lengths of the primary root at transplanting were marked on the side of the boxes and subsequent root tip positions were marked every 8-12 h until up to 84 h after transplanting, depending on genotype and treatment. For each treatment, lengths were plotted as a function of time and the slope of the response was recorded as the elongation

rate. Root elongation rates at low water potentials were calculated as a percentage of the well-watered elongation rate.

Modification of the seedling system

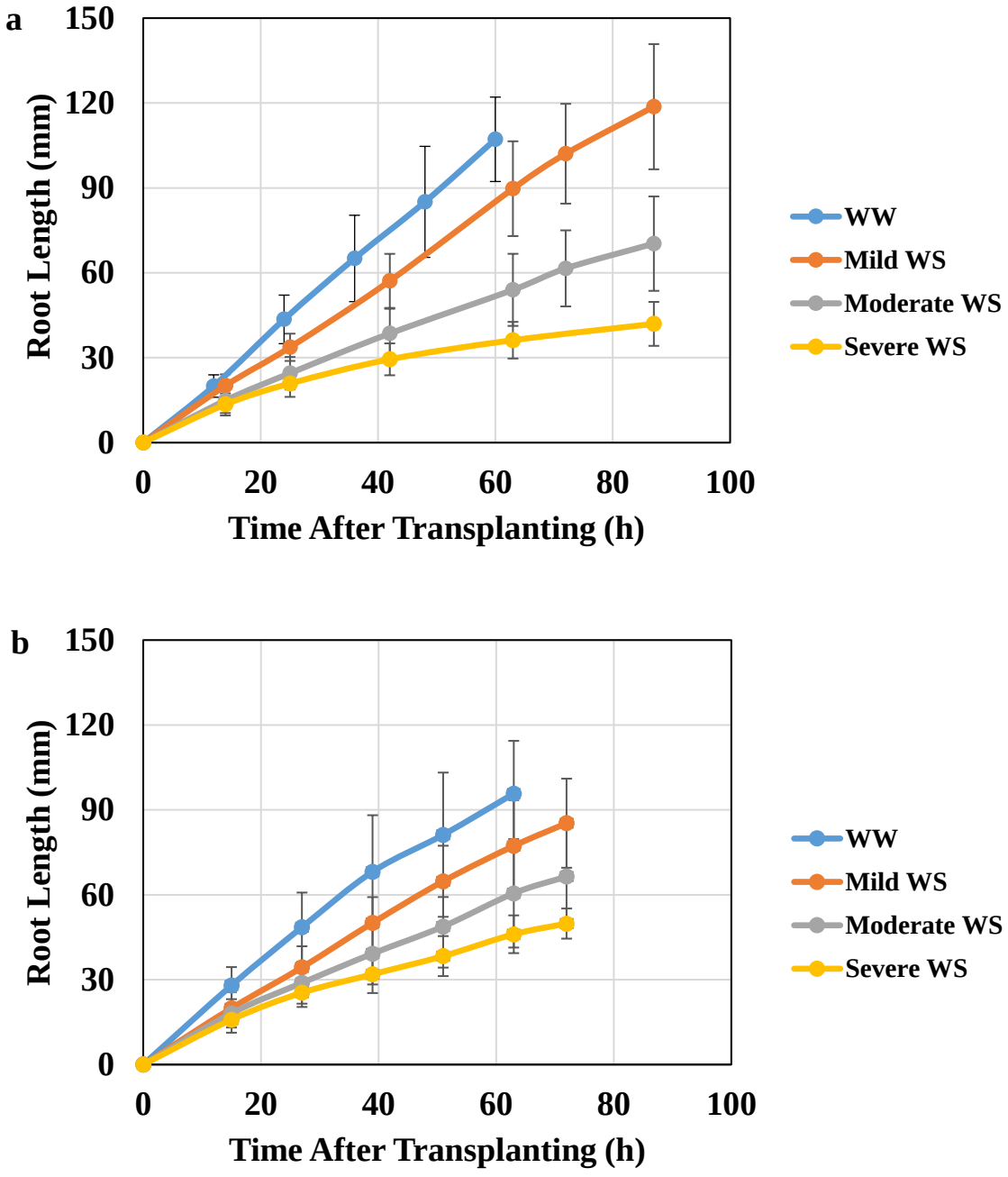
The vermiculite system was modified to obtain stable growth under water-stressed conditions, since in the first round of experiments it was observed that root elongation rates gradually slowed down, in particular for the severe water-stressed condition (water potential = -1.6 MPa). Therefore, the vermiculite fragment size was investigated to test the hypothesis that there was inadequate hydraulic contact between the root and vermiculite particles. The standard vermiculite was passed through sieves of 3 mm and 2 mm in size and the vermiculite that passed through each of the sieves was assessed using line AU90810 for suitability as a medium for the water-deficit experiments. Maize line FR697 was also tested with the 3 mm size vermiculite. Then the modified system was used with four genotypes selected from the first trial for the second trail of genotype selection.

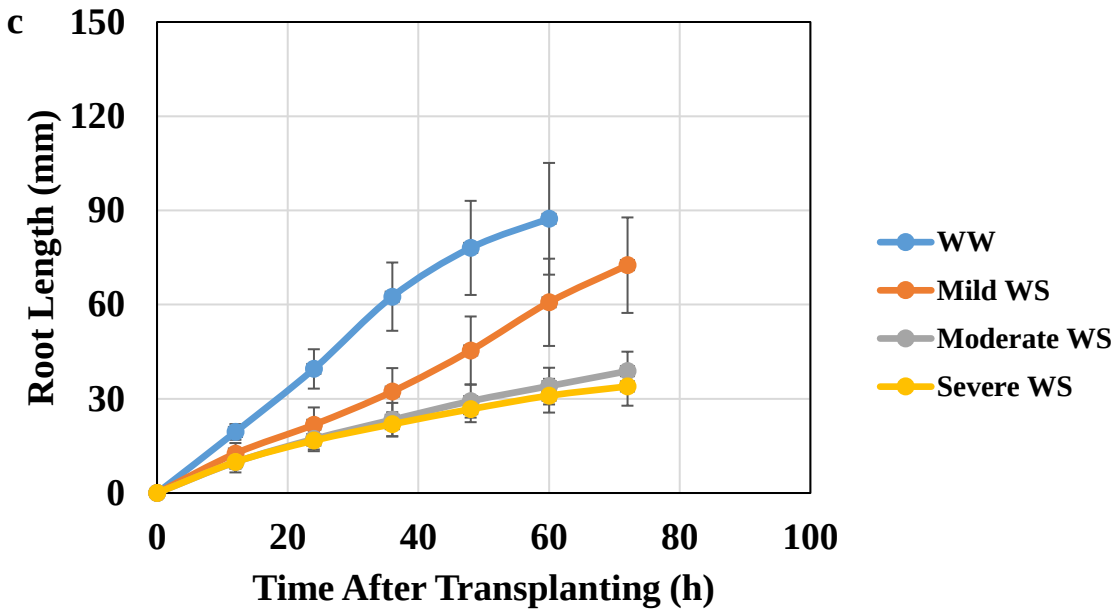
Results

First trial for genotype selection

In the first trial for genotype selection, the root elongation rates were not stable in many genotypes. Four genotypes are shown in Figure 2-1 as examples. This problem was particularly apparent in two situations. The first situation occurred in the well-watered treatment. In this case, root elongation slowed down substantially towards the end of the

Figure 2-1. Primary root length increase of four representative genotypes [cv. AU90810 (a), AU90915 (b), MD25-26ne (c), TAMCOT73 (d)] after transplanting to vermiculite under well-watered (WW) or different water stress (WS) levels in non-modified seedling system (n = 20-40 ± SD).




c
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Root Length (mm)
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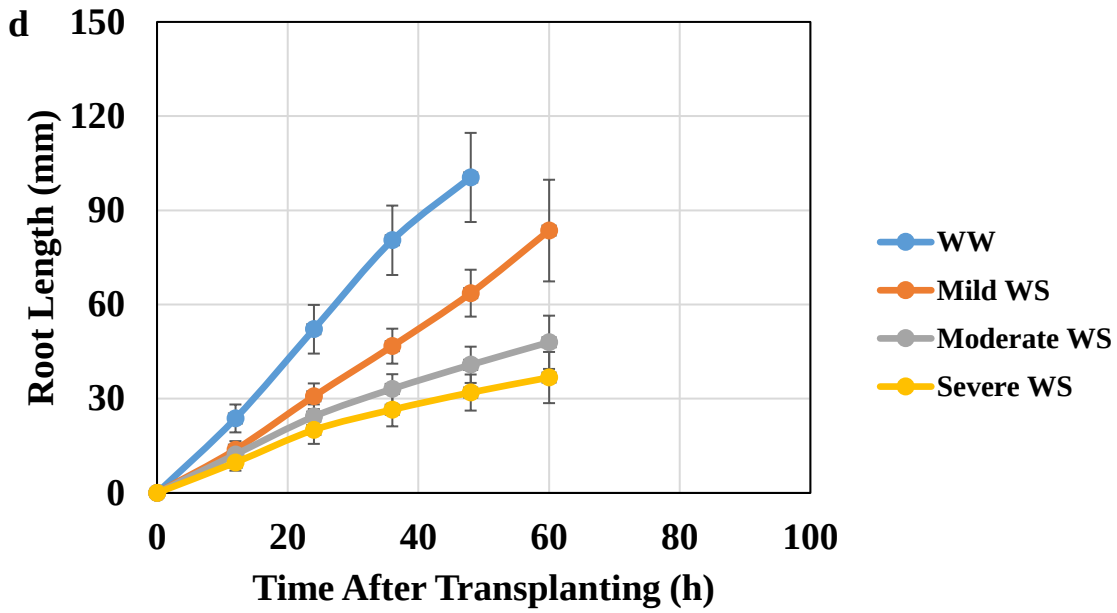
100
Time After Transplanting (h)
WW
Mild WS
Moderate WS
Severe WS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90
60
30
0
Root Length (mm)
0
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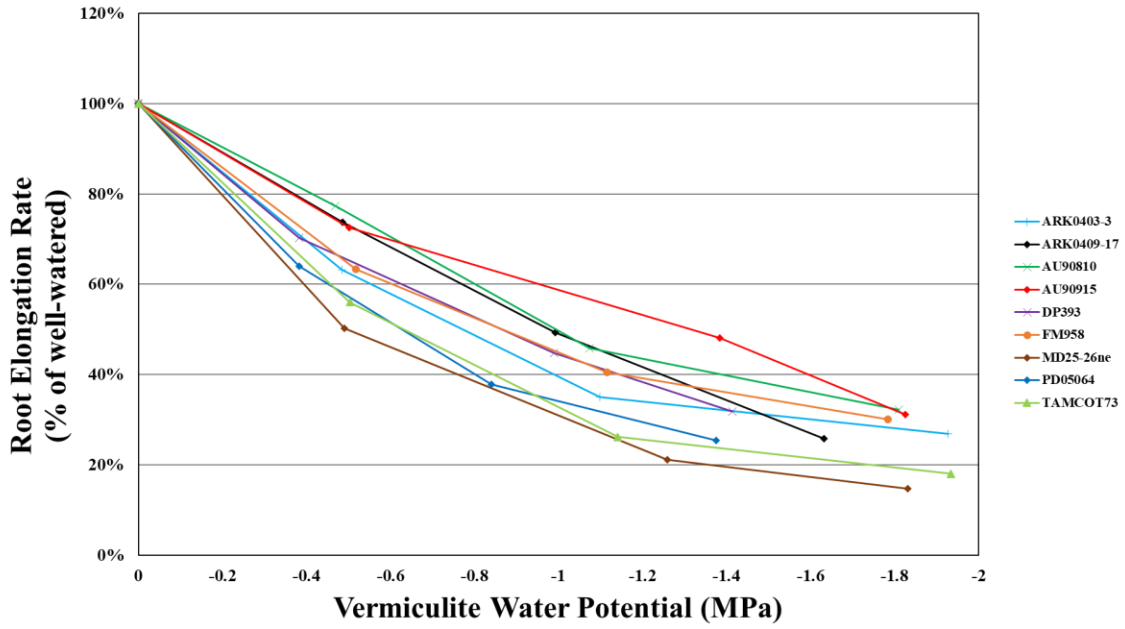
40
60
80
100
Time After Transplanting (h)
WW
Mild WS
Moderate WS
Severe WS

experiments (for example, MD25-26ne in Figure 2-1c). It is speculated that the non-steady elongation rates may be attributed to depletion of seed reserves due to the onset of etiolation and the initial shoot elongation in well-watered seedlings. Therefore, in subsequent experiments, well-watered treatments were grown only to 48 h after transplanting. The second situation occurred in moderate water stress (some genotypes) and severe water stress (all genotypes) treatments. In this case, root elongation progressively slowed down after transplanting to the water-stressed media (Figure 2-1). These problems presented a challenge for designating a specific root elongation rate to the particular genotype and treatment combination. Accordingly, root elongation rates of different genotypes under various water treatments were provisionally plotted from the slopes of root length increase over time during the first 40 h of the experiments.

The provisional root elongation rates of the nine different genotypes under various water treatments are displayed in Figure 2-2. The responses were readily distinguishable between genotypes when compared at different water deficit levels. The growth system was thus able to reveal variation in root elongation rates in response to water deficit among genotypes, but modification was required to obtain more reliable growth rates, as described below.

In addition, in the first trial, all genotypes were germinated under the same imbibition and germination times (24 h + 24 h). These conditions generated low germination rates for some of the genotypes and for some genotypes resulted in only radical emergence but further growth was arrested. The failure of germination of these seeds suggested that the previous germination and imbibition protocols for maize may not be suitable for cotton,

Figure 2-2. Primary root elongation rates of nine genotypes under a range of low water potential conditions normalized to the elongation rate under well-watered conditions using the standard vermiculite seedling system. The elongation rates were estimated from the slopes of root length increase over time during the first 40 h of the experiments (n = 20-40).



at least for the genotypes being tested. To address the issue with cotton germination and seedling growth and to have the maximum number of usable seedlings after germination, four genotypes were selected for optimization of imbibition and germination times. Two genotypes for which root elongation appeared to be particularly sensitive to water deficit (TAMCOT73 and MD25-26ne) and two genotypes that appeared to be less sensitive to water deficit (AU90810 and AU90915) were selected. Imbibition times did not affect seedling growth (Figure 2-3 shows AU90810 and TAMCOT73 as examples) but different combinations of imbibition and germination times did affect germination rates (Table 2-1). Considering the logistics of the experiment and length of root radicals in each treatment, the best combination of imbibition time and germination time for each genotype is shown in bold in Table 2-1. The optimized imbibition and germination times were used in the second trail of genotype selection.

System modification

According to the problem addressed in the first trail experiment, it was hypothesized that the contact of the cotton primary roots and the vermiculite media was limited by the size of the vermiculite fragments and thus some surfaces of the root were more exposed to dry air rather than in contact with fragments providing water. In this case, the inhibition of root growth would be influenced by the hydraulic properties of the root-media contact in addition to the water potential of the media. To investigate this hypothesis, the vermiculite media was sieved to obtain smaller fragment sizes which would increase the hydraulic contact with the root. The results indicated that a smaller vermiculite fragment size (0-3 mm) generated elevated and more stable root elongation rates (Figure 2-4). For

Figure 2-3. Length increase after transplanting of cotton [cv. AU90810 (a) and TAMCOT73 (b)] primary roots with different imbibition times under well-watered conditions (n = 20-40 ± SD).

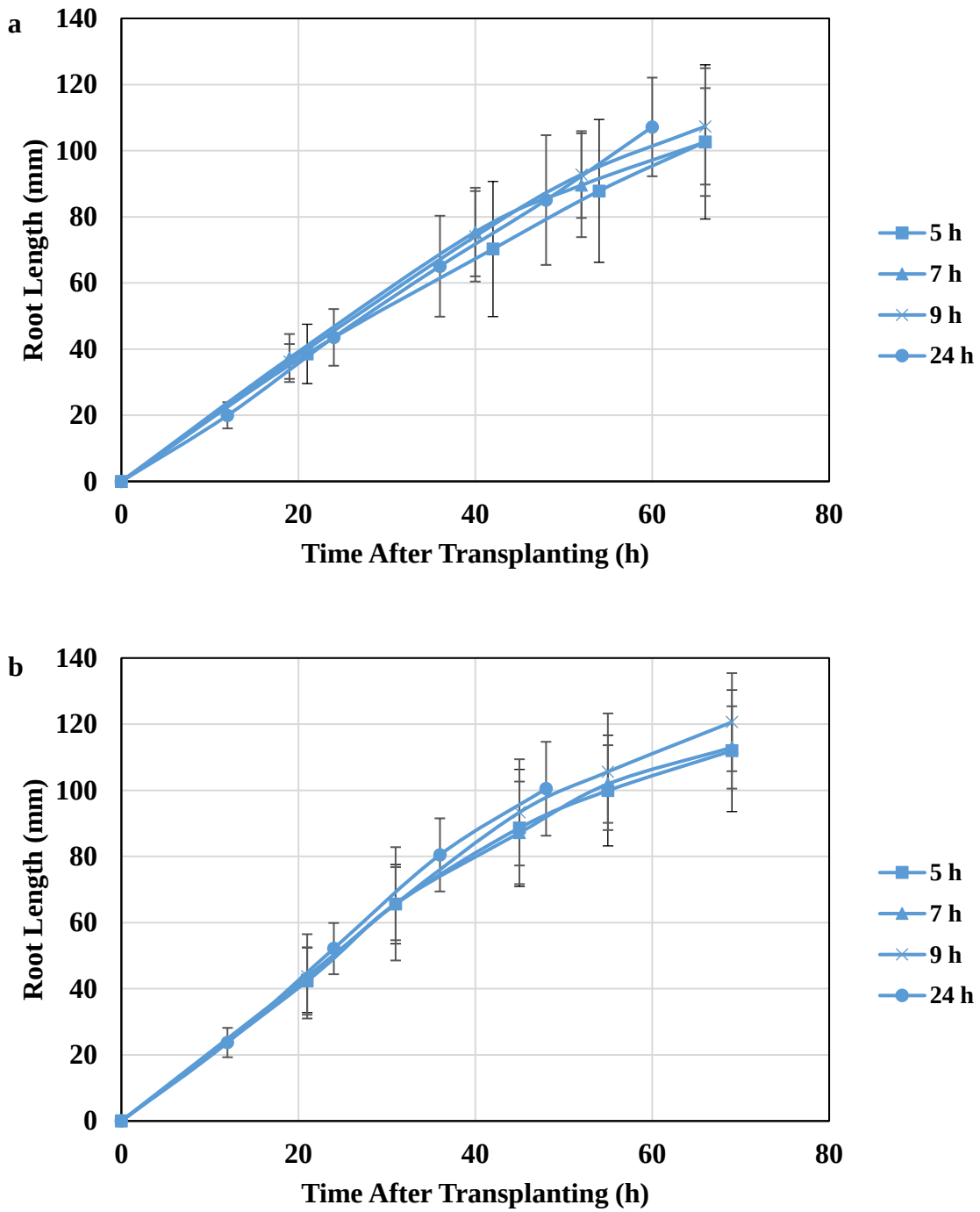
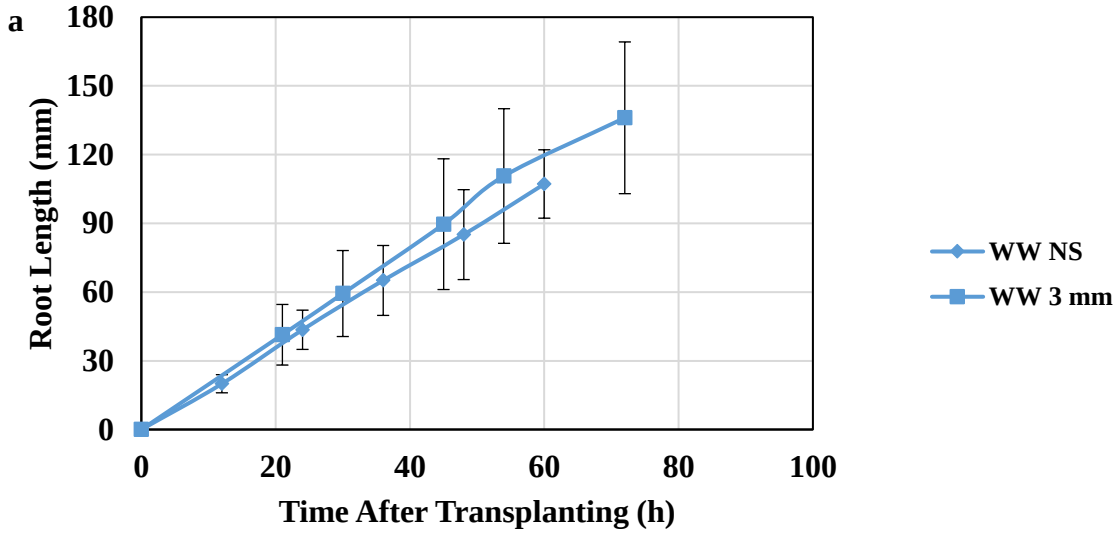
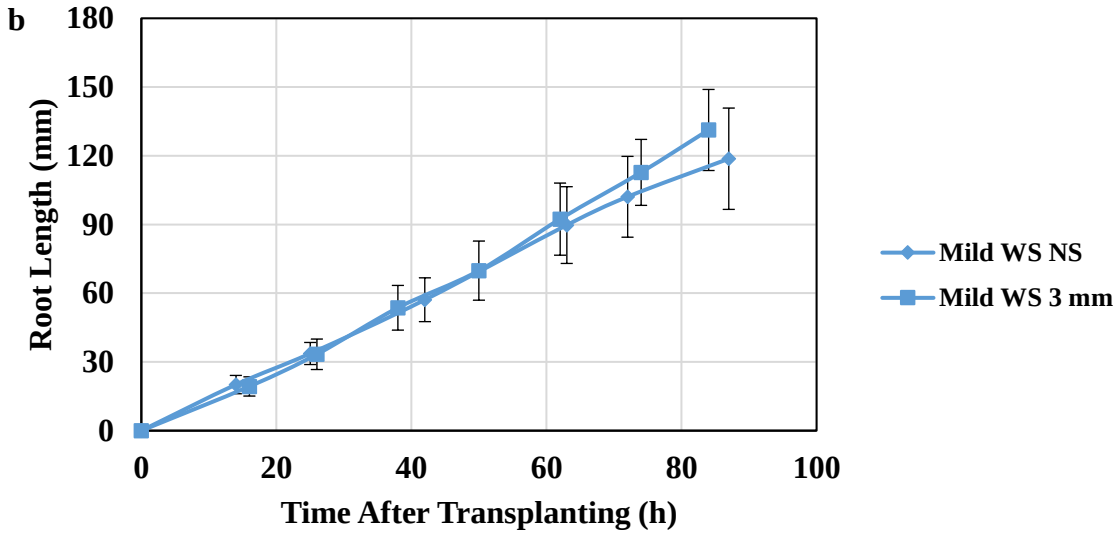


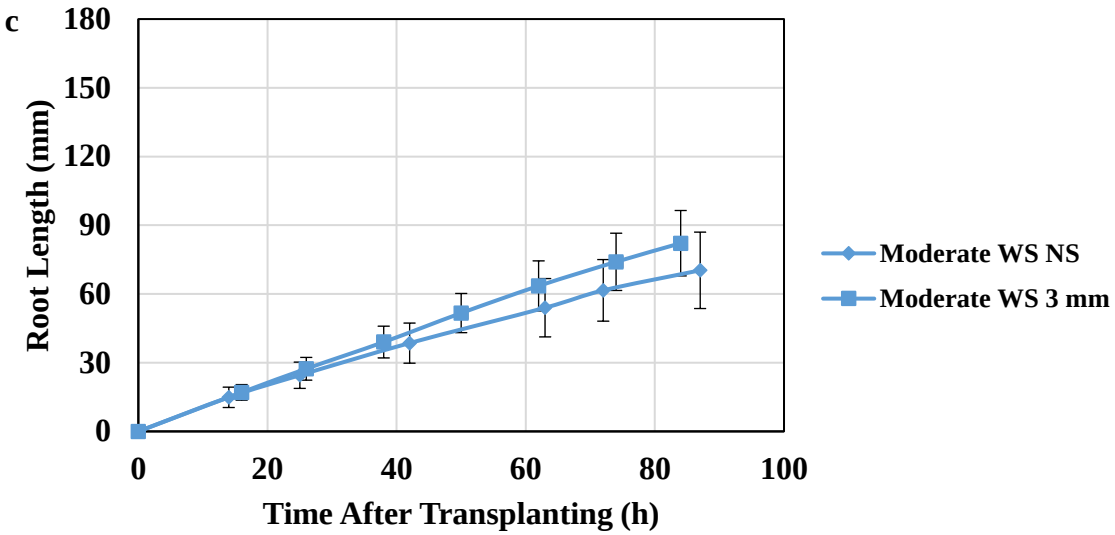
Table 2-1. Germination rates of different combinations of imbibition and germination time in four selected genotypes.

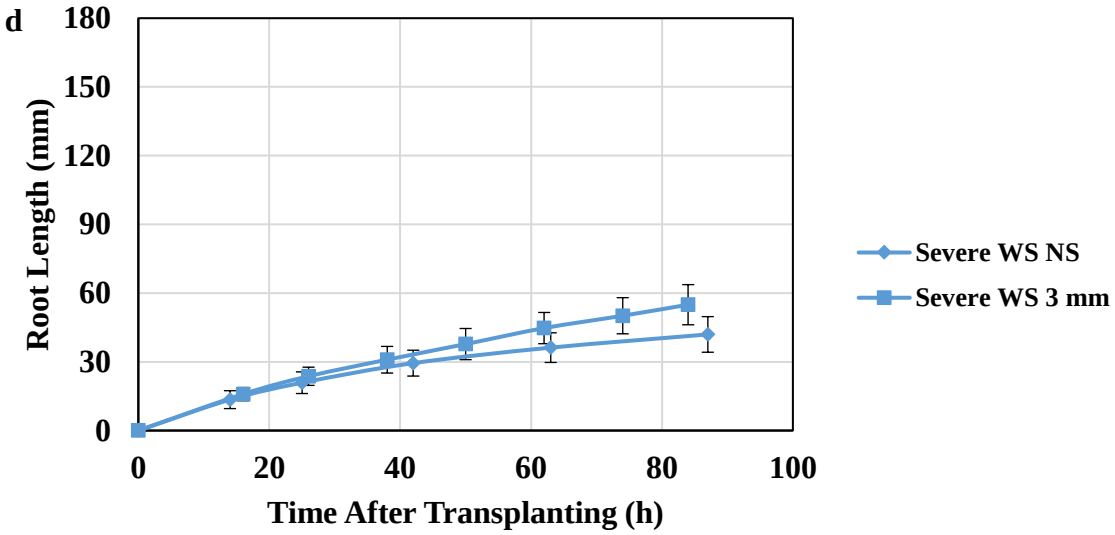
AU90915	**Germination Time**		
Imbibition Time	12 h	18 h	
3 h	46/101	**55/101**	
12 h	48/116	53/116	
24 h	43/110	47/110	
AU90810	**Germination Time**		
Imbibition Time	16 h	20 h	22 h
5 h	36/86	**46/86**	48/86
7 h	19/84	32/84	36/84
9 h	9/81	15/81	16/81
TAMCOT73	**Germination Time**		
Imbibition Time	16 h	20 h	24 h
5 h	**60/116**	62/116	64/116
7 h	50/116	52/116	55/116
9 h	43/117	43/117	46/117
MD25-26ne	**Germination Time**		
Imbibition Time	16 h	24 h	36 h
5 h	26/109	57/109	84/109
7 h	21/112	35/112	56/112
9 h	**58/114**	70/114	75/114

Figure 2-4. Effects of 3-mm sieved media on cotton (cv. AU90810) primary root length under (a) well-watered (WW), (b) mild water stress (Mild WS), (c) moderate water stress (Moderate WS) and (d) severe water stress (Severe WS) conditions (n = 20-40 ± SD). NS: non-sieved.






c
Root Length (mm)
180
150
120
90
60
30
0
0
20
40
60
80
100
Time After Transplanting (h)
Moderate WS NS
Moderate WS 3 mm


d
Root Length (mm)
180
150
120
90
60
30
0
0
20
40
60
80
100
Time After Transplanting (h)
Severe WS NS
Severe WS 3 mm

the well-watered treatment, the two sizes did not exhibit significant differences in length. For the mild water stress treatment, no significant differences in root length were observed in the early stages (0-63 h) but at later time points (63-87 h) root elongation rate was significantly increased in the sieved media. The promoting effects of sieved media on primary root growth were evident in both moderate and severe water stress treatments. Vermiculite media with a fragment size of 0-2 mm did not further improve the elongation rate compared with the 0-3 mm media (Figure 2-5). In consideration of the improvement of the stability and elevation of root elongation rates, vermiculite media of 0-3 mm fragment size was chosen as the standard media for all subsequent cotton experiments. In contrast, maize (cv. FR697) responded in an opposite fashion in that the average root elongation rate declined slightly in the sieved media (Figure 2-6). However, t-tests showed that the differences between the two media treatments was not significant ($p \geq 0.054$). Accordingly, non-sieved media was used for subsequent maize experiments, as used in previous studies (Sharp et al., 2004; Zhu et al., 2007; Spollen et al., 2008; Voothuluru et al., 2016).

Second trail of genotype selection

Using the improved and optimized seedling system, the four selected cotton genotypes were further evaluated for their root growth responses under water deficit stress. This experiment was conducted using three treatments: well-watered (water potential = -0.02 MPa), mild water stress (water potential = -0.4 MPa) and severe water stress (water potential = -1.6 MPa). The root elongation rates were plotted from the slopes of root length increase over time after 24 h from transplanting (Figure 2-7). The optimization of

Figure 2-5. Effects of 2-mm and 3-mm sieved media sizes on cotton primary root length under severe water stress (Severe WS) condition (n = 20-40 ± SD).

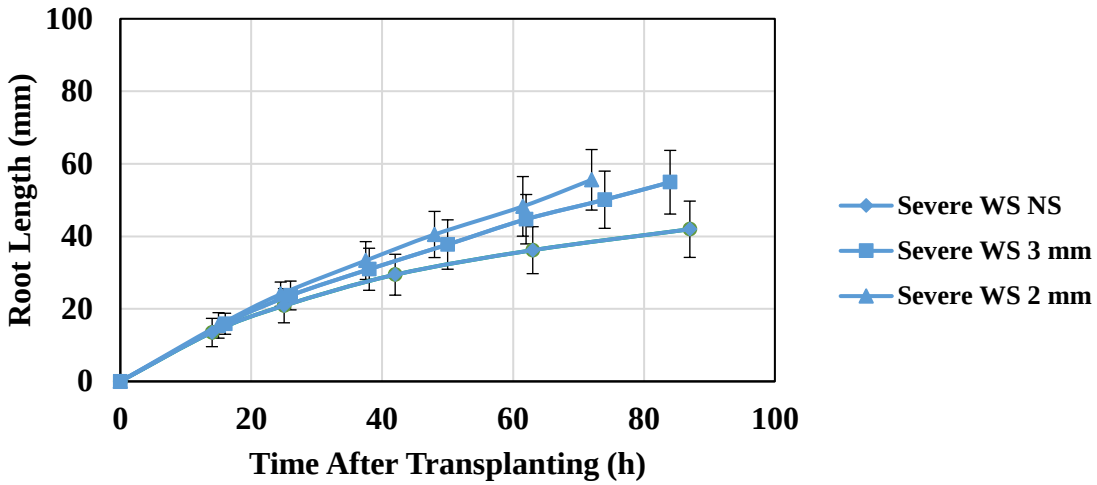


Figure 2-6. Effects of 3-mm sieved media on maize primary root length under severe water stress (Severe WS) condition (n = 20-40 ± SD).

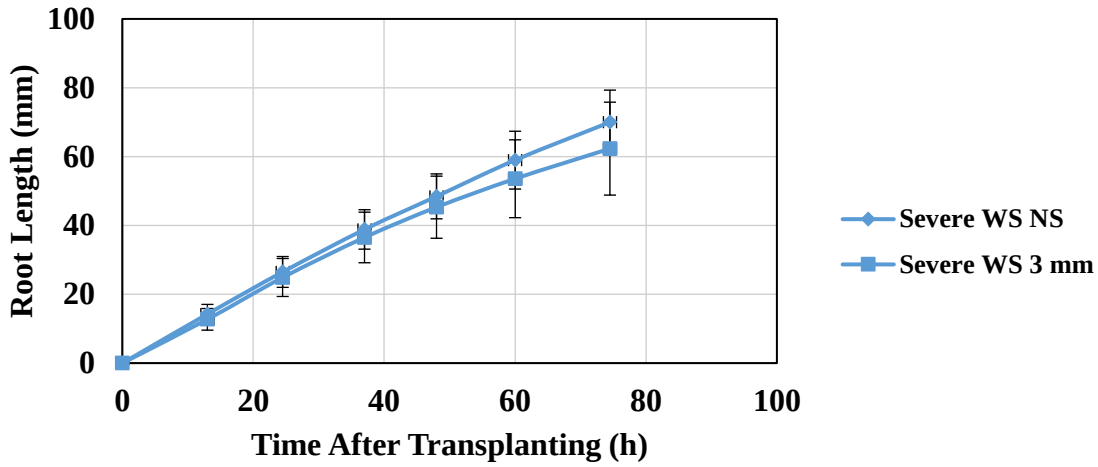
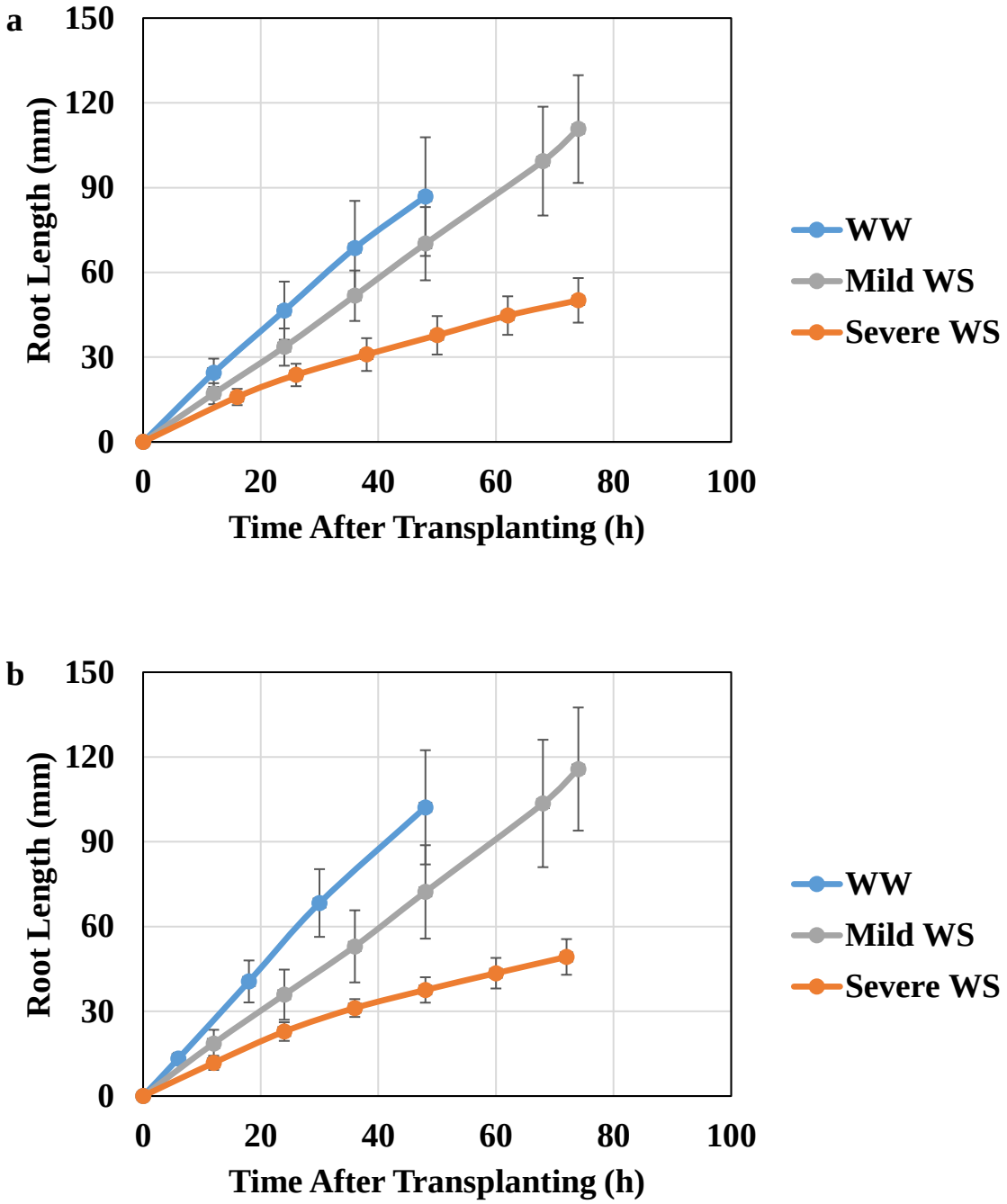
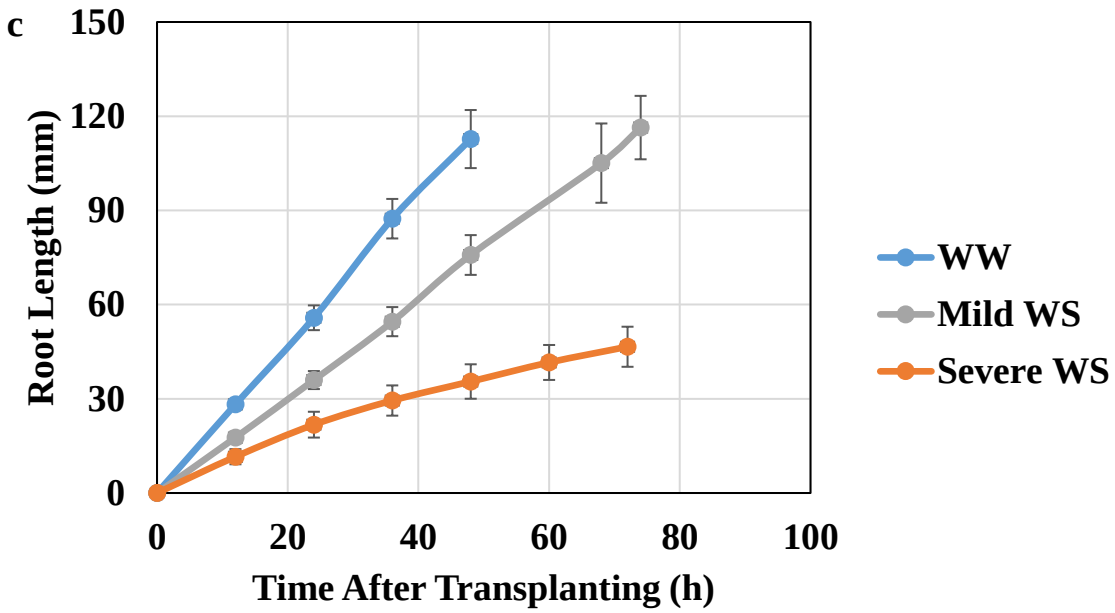
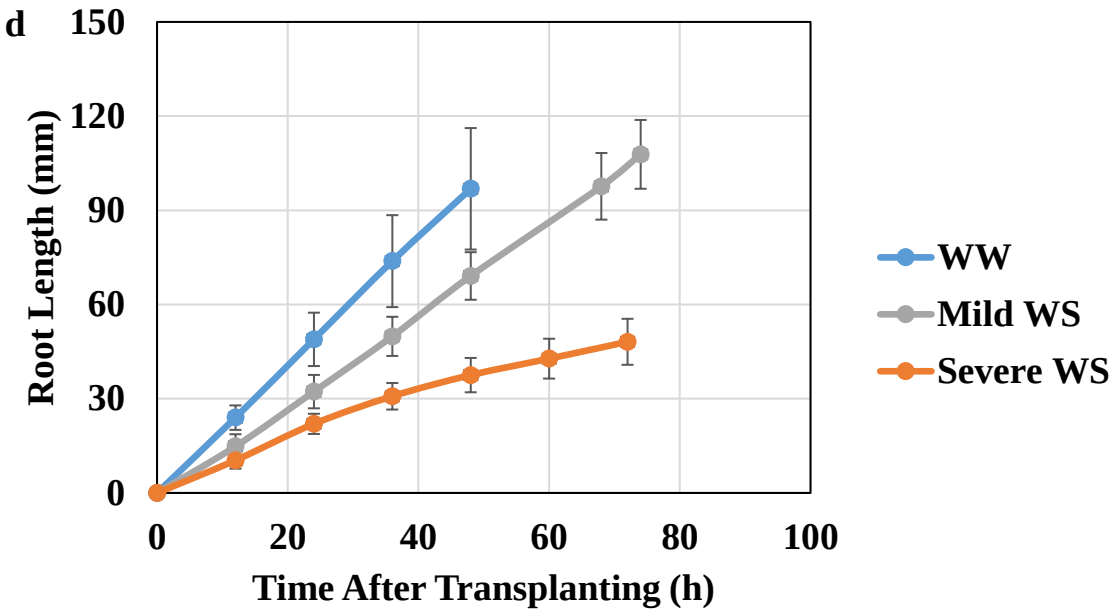


Figure 2-7. Primary root length increase of four selected genotypes [cv. AU90810 (a), AU90915 (b), MD25-26ne (c) and TAMCOT73 (d)] after transplanting to vermiculite under well-watered (WW) different water stress (WS) levels in the modified seedling system (n = 20-40 ± SD).




c
150
120
90
60
30
0
Root Length (mm)
0
20
40
60
80
100
Time After Transplanting (h)
WW
Mild WS
Severe WS

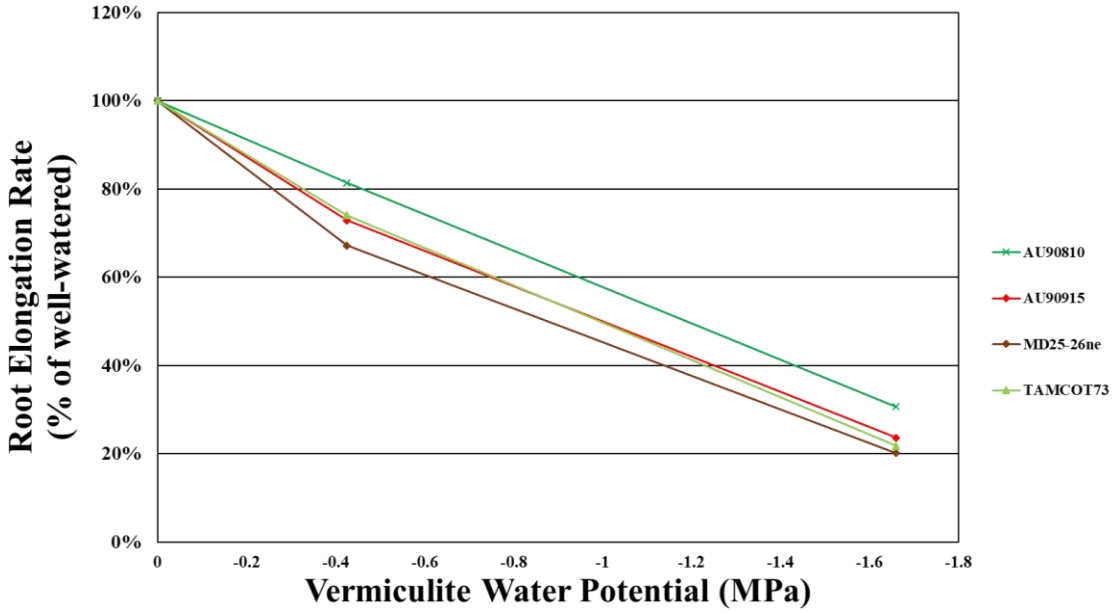

d
150
120
90
60
30
0
Root Length (mm)
0
20
40
60
80
100
Time After Transplanting (h)
WW
Mild WS
Severe WS

germination and imbibition conditions and the optimization of the hydraulic properties of the vermiculite medium reduced the variation in the root growth rate response to water stress among the genotypes, in particular when the roots were exposed to the severe stress treatment. As a result, the growth rates were more similar between genotypes under severe stress compared to those observed in Figure 2-2. Among the four genotypes, AU90810 maintained a superior growth rate under each stress level in both the first and the second trail experiments, and thus it was selected as the genotype for further studies.

Discussion

An optimized vermiculite culture system for the study of cotton primary root growth during exposure to water-deficit stress was established. The standard vermiculite system as reported in the literature (Sharp et al., 2004; Spollen et al., 1993; Leach et al., 2011) was determined to exhibit factors that prevented the accurate assessment of cotton root growth rates. The first trial experiment of cotton primary root elongation rates indicated that the different cotton genotypes have enough variation to permit classification into sensitive and tolerant genotypes, similar to the previous results for maize (Leach et al., 2011). However, steady-state root elongation rates were not obtained in many genotypes. The non-steady elongation rates may be attributed to inadequate hydraulic contact between the root and the dry vermiculite and relatively small water storage in water-stressed seedlings. It is notable that this problem has not been observed in previous studies using the seedling system for maize and soybean. By modification of media fragment size, stable root elongation rates under various water stress levels were obtained. The genotypic variation of root elongation rates was reduced in the modified

Figure 2-8. Primary root elongation rates of four selected cotton genotypes exposed to increasing water deficits normalized to the elongation rate under well-watered conditions with the optimized vermiculite seedling system. The elongation rates were calculated from the slopes of the growth rate curves (root lengths as a function of time) when stable growth was reached (normally after 24h) (n = 20-40).



media, indicating that the hydraulic properties of the media influence the root growth response to water deficits in cotton. A comparison experiment with maize genotype FR697 did not show such an effect. However, previous maize primary root studies indicated that media properties can also have an effect on root growth (Verslues et al., 1998). In that study, the response of root elongation rates to different water stress levels were investigated by comparing the effects of non-sieved vermiculite media and polyethylene glycol (PEG) media. Root elongation rate was less inhibited in PEG than in vermiculite under water-stressed conditions using media that had water potentials similar to those examined in this study (well-watered, mild (-0.3 MPa), moderate (-0.8 MPa) and severe (-1.6 MPa) stress). Therefore, media hydraulic properties clearly play an important role in determining variability in growth responses and have to be taken into consideration when applied to different species. Cotton roots appear to be more sensitive to the effect of root-soil hydraulic contact than maize, making it more challenging to cultivate them in the vermiculite system when compared to other plant species that have been studied previously. Among four genotypes selected from the first trial experiment, genotype AU90810 had a superior elongation rate under water-stressed conditions which indicated that this genotype has the greatest tolerance to water deficits among the genotypes tested. This genotype was therefore selected for further experimentation to investigate underlying mechanisms of root growth maintenance in cotton.

Chapter 3

Kinematic Analysis of the Cotton Primary Root Growth Zone under Water Deficit Conditions

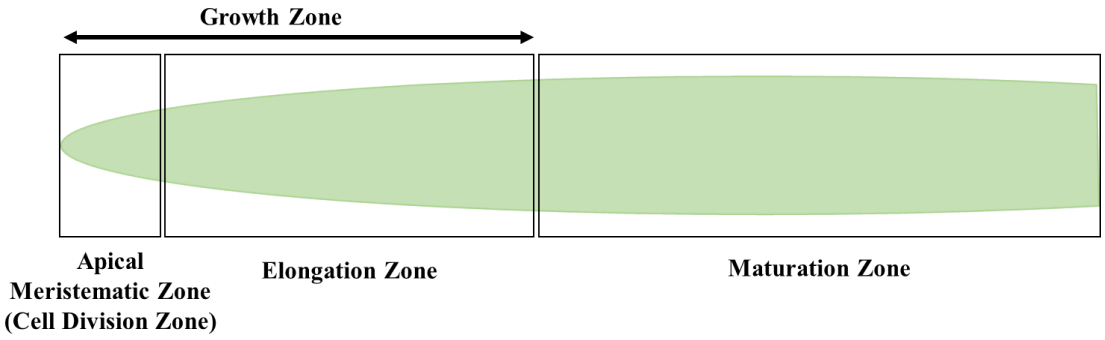
Introduction

The analysis of primary root growth responses to water-deficit stress presented in Chapter 2 indicated that the ability for root growth maintenance at low water potentials varies considerably among cotton genotypes. Cotton genotype AU90810 was selected as having a superior response to both mild and severe water stress conditions (Figures 2-2 and 2-8). In a similar study of primary root growth under water stress in maize, genotype FR697 was identified as exhibiting a superior response (Leach et al., 2011).

With a robust experimental system developed for studies of both cotton and maize primary roots, the objective was to utilize the selected water stress-tolerant genotypes for detailed comparative analyses of the physiological and metabolic processes involved in the growth responses of the primary root to water-deficit conditions.

The primary root growth zone encompasses the apical meristematic zone (cell division zone) and the zone of elongation (Figure 3-1). New cells are produced in the meristematic zone and then transition into the elongation zone where they expand in both length and diameter (Sharp et al., 1988; Liang et al., 1997). Previous studies showed that water deficit can alter the cell elongation pattern in the growth zone when compared to well-watered roots. In both maize and soybean primary roots, kinematic analysis showed that under water deficit cell elongation is maintained preferentially towards the root apex, whereas cell elongation is inhibited in the basal region of the growth zone, resulting in a shortening of the zone compared with well-watered roots, as described in Chapter 1 (Figure 1-1) (Sharp et al., 1988; Liang et al., 1997; Yamaguchi & Sharp, 2010). The growth pattern distribution of well-watered and water-stressed primary roots was

Figure 3-1. Regional organization of the apical region of the primary root.



characterized for the growth zone of maize cv. FR697 (Sharp et al., 2004), but such characterization has not been conducted for the cotton primary root.

Therefore, as an initial step to provide the foundation for the comparative studies between cotton and maize, it is important to characterize the spatial pattern of cell elongation within the growth zone in the primary root of cotton genotype AU90810 growing under well-watered and water-deficit conditions. By determining whether similar or different patterns of response occur in the cotton root, appropriate comparisons of tissue regions can be made in the subsequent metabolic analyses.

Materials and Methods

Plant growth conditions

Cotton seeds were imbibed in 1 mM $CaSO_4$ solution for 5 h and germinated on germination paper dampened with the same solution for 20 h. Seedlings with primary roots of 5-15 mm in length were transplanted against the face of transparent Plexiglas growth boxes containing the appropriate vermiculite media, and grown as described in Chapter 2. Three treatments were conducted: well-watered (water potential = -0.02 MPa), moderate water stress (water potential = -1.0 MPa) and severe water stress (water potential = -1.6 MPa).

Harvest of samples for kinematic analysis

Roots that were straight and had an elongation rate similar to the mean of the population were harvested for each treatment. The average root elongation rate was recorded for the

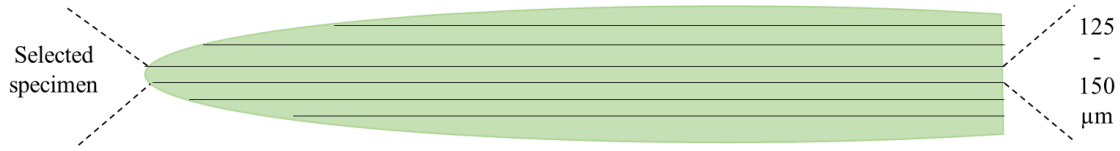
calculations described below. The first 20 mm of the primary root tips were collected at 45-48 h after transplanting when the elongation rates were stable. The root tips were placed on a Kimwipe® tissue dampened with melibiose solution that had the same water potential as each growth environment, to avoid rehydration or dehydration of the sample after harvest.

The root tip samples were sectioned longitudinally (4-5 sections) at a thickness of 125-150 µm using a Vibratome® 3000 Plus (Figure 3-2). The section that contained the most complete longitudinal root structure was stained with 1 mg/mL Calcofluor-white (Sigma-Aldrich) for 15 mins without exposing to light.

Cell length measurements

The sections were observed using a confocal microscope (Leica© TCS SP8) and images of the cells at intervals of 250 µm along the primary root were recorded photographically. Cell lengths were measured using the image processing software ImageJ (free access, NIH). Four to six cells were measured at each position and four roots were imaged in each treatment and the mean values of cell length were calculated for each position. The final cell length was determined as the average of all cell lengths in the last several millimeters region that constant cell lengths were reached in each treatment. The regions used for final cell length calculation were 12250-15000 µm in the well-watered treatment, 7250-10000 µm in the moderate water stress treatment, and 7250-10000 µm in the severe water stress treatment.

Figure 3-2. Sectioning protocol for kinematics analysis.



Kinematic analyses

The spatial distribution of elongation rate within the root growth zone was determined by a kinematic approach based on the analysis of cell length profiles along the longitudinal axis of the root tip (Silk et al., 1989; Saab et al., 1992). The cell lengths were converted to displacement velocities using the equation (1).

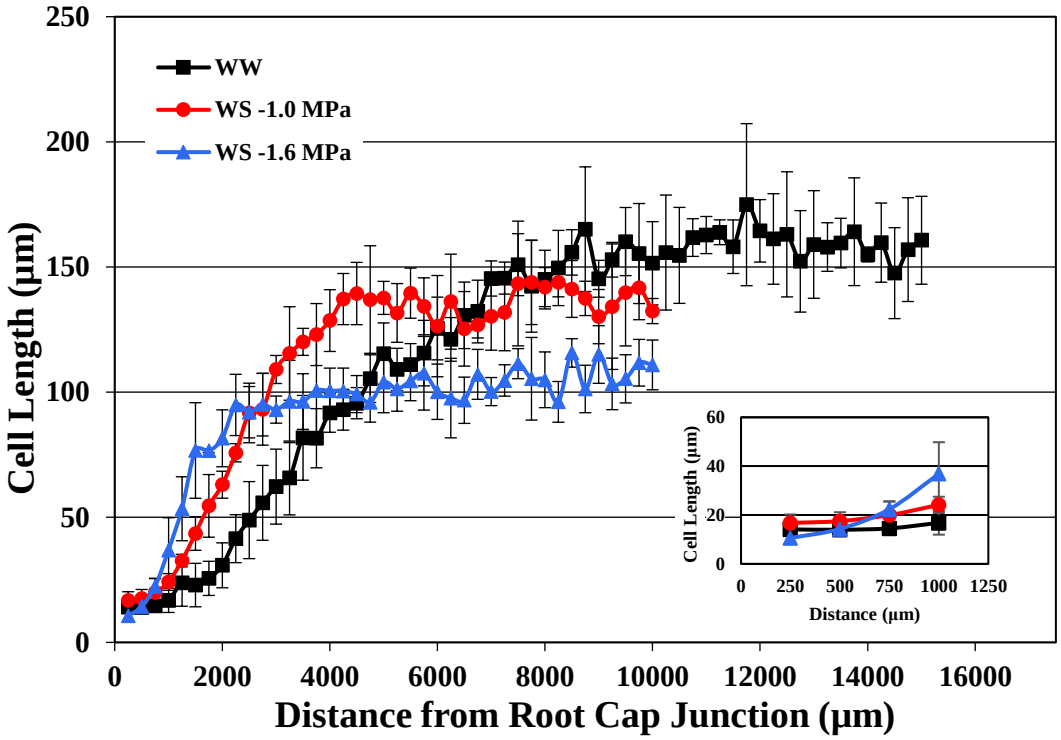
$$Displacement\ velocity = \frac{Cell\ length \times Root\ elongation\ rate}{Final\ cell\ length} \quad (1)$$

Displacement velocity is defined as the velocity of a cell as it passes a certain position within the growth zone. The calculation started from the distal end of the meristem, approximately at the position where cell lengths reached 2.5 times of the shortest cell length (Erickson, 1961). Displacement velocities for each position were plotted as a function of distance from the root cap junction and the scattered points were then fitted to logistic curves using Origin (OriginLab, Northampton, MA, U.S.A). Relative elongation rates can be obtained from the derivative of velocity with respect to position. Cell flux rates (the number of cells leaving the growth zone per hour) were calculated by dividing the root elongation rates by the final cell lengths of each treatment. Under steady conditions, cell flux rates approximate the rates of cell production in the meristem. Maize parameters were calculated using data from a previous study (Sharp et al., 2004).

Results

Cell length profile in different treatments

Figure 3-3. Cell length profiles as a function of distance from the root cap junction of the primary roots of cotton under three treatments with different water potentials: well-watered (WW), moderate water stress (WS -1.0 MPa), and severe water stress (WS -1.6 MPa) (n = 4 roots ± SD). The inset shows the profile of cell length from 250 μm to 1000 μm from the root cap junction.



The cotton primary root growth zone exhibited different patterns of cell expansion under well-watered and water-stressed conditions. Cell expansion patterns also differed with the level of water deficit to which the roots were exposed (Figure 3-3). Cell lengths were almost identical between water-stressed and well-watered roots in the region 250-750 µm from the root cap junction, which includes part of the meristematic zone. Beyond 750 µm, cell lengths started to differ between the treatments. Compared to the well-watered control, water-stressed cells reached constant lengths closer to the root apex. The cells in the well-watered control, the moderate water stress and the severe water stress treatments reached constant and steady lengths at 8750 µm, 4250 µm and 2750 µm from the root cap junction, respectively.

Spatial patterns of cell elongation rate

The displacement velocities at each position along the primary root growth zone were plotted as a function of distance from the root cap junction (Figure 3-4). Logistic curves were fitted to the points with the following equations.

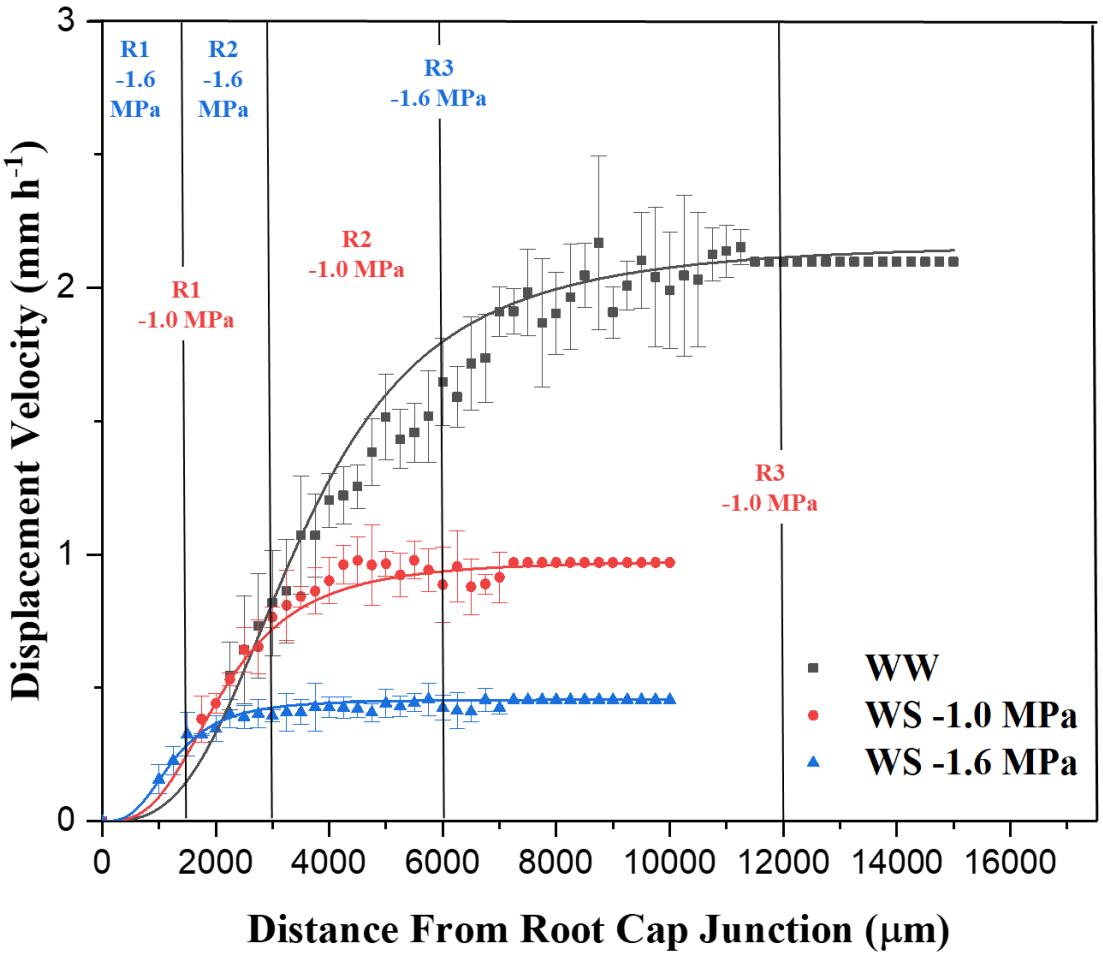
WW $$y = 2.16857 + (-2.16857)/(1 + \left(\frac{x}{3541.06295}\right)^3) \quad (2)$$

WS (-1.0 MPa) $$y = 0.97859 + (-0.97859)/(1 + \left(\frac{x}{2132.59214}\right)^3) \quad (3)$$

WS (-1.6 MPa) $$y = 0.45673 + (-0.45673)/(1 + \left(\frac{x}{1258.00712}\right)^3) \quad (4)$$

Similar to previous results for maize and soybean roots (Figure 1-1), the cotton primary root exhibits differential growth responses within the growth zone under water deficit conditions, with distinct regions of maintenance or inhibition of elongation. In the well-

Figure 3-4. Displacement velocity as a function of distance from the root cap junction of the primary roots of cotton under three treatments with different water potentials: well-watered (WW), moderate water stress (WS -1.0 MPa), and severe water stress (WS -1.6 MPa) (n = 4 roots ± SD).



watered to severe water stress (water potential = -1.6 MPa) comparison, region 1 is the region from 0 to 1.5 mm, region 2 is the region from 1.5 to 3 mm, and region 3 is the region from 3 to 12 mm. In the comparison of well-watered and moderate water stress (water potential = -1.0 MPa) treatments, region 1 is the region from 0 to 3 mm, region 2 is the region from 3 to 6 mm, and region 3 is the region from 6 to 12 mm (Figure 3-4). The delineation of the regions into numbered segments is as defined in Chapter 1 (Figure 1-1) where elongation is maintained at well-watered rates in region 1, elongation of the water-stressed treatment starts to decline in region 2 while the well-watered treatment reaches maximal growth rate, and growth ceases in the water-stressed treatment in region 3 while starting to decline in the well-watered treatment.

Root elongation rates, final cell lengths and cell flux rates of cotton roots under the different water stress levels are presented in Table 3-1, together with maize genotype FR697 (from Sharp et al., 2004). In both species, the root elongation rates, final cell lengths and cell flux rates were substantially inhibited under severe water stress condition (water potential = -1.6 MPa) compared with the well-watered control. However, the root elongation rate of cotton (0.455 mm h^{-1}) was much lower compared with maize (1.05 mm h^{-1}) under the severe water stress condition. This was also shown by the further shortening of the growth zone in cotton compared with maize (Figure 3-4). Final cell length and cell flux rate were also considerably smaller in cotton compared with maize under the severe stress condition.

However, cotton roots growing under moderate water stress (water potential = -1.0 MPa)

Table 3-1. Primary root elongation rate, final cell length, and cell production rate in well-watered (WW) and water-stressed (WS) primary roots of maize (cv. FR697) and of cotton (cv.AU90810).

Maize	**Root Elongation Rate (mm h^{-1})**		**Final Cell Length (mm)**		**Cell Flux Rate (h^{-1})**	
	WW	WS (-1.6 MPa)	WW	WS (-1.6 MPa)	WW	WS (-1.6 MPa)
	2.5	1.05	0.180	0.142	13.9	7.4

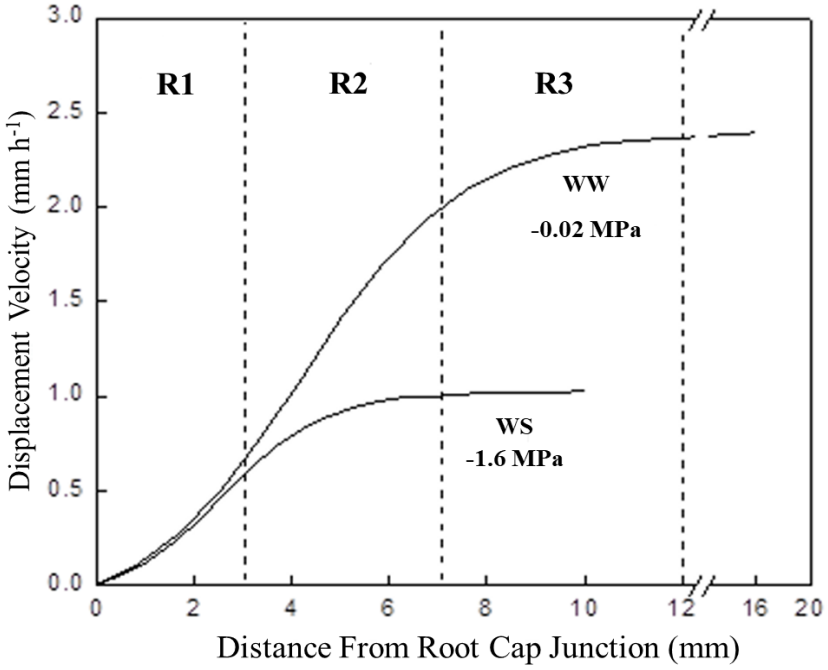
Cotton	**Root Elongation Rate (mm h^{-1})**			**Final Cell Length (mm)**			**Cell Flux Rate (h^{-1})**		
	WW	WS (-1.0 MPa)	WS (-1.6 MPa)	WW	WS (-1.0 MPa)	WS (-1.6 MPa)	WW	WS (-1.0 MPa)	WS (-1.6 MPa)
	2.098	0.970	0.455	0.160	0.138	0.107	13.1	7.0	4.25

Maize	**WS/WW Root Elongation Rate**		**WS/WW Final Cell Length**		**WS/WW Cell Flux**	
	-1.6 MPa		-1.6 MPa		-1.6 MPa	
	42.0%		78.9%		53.2%	

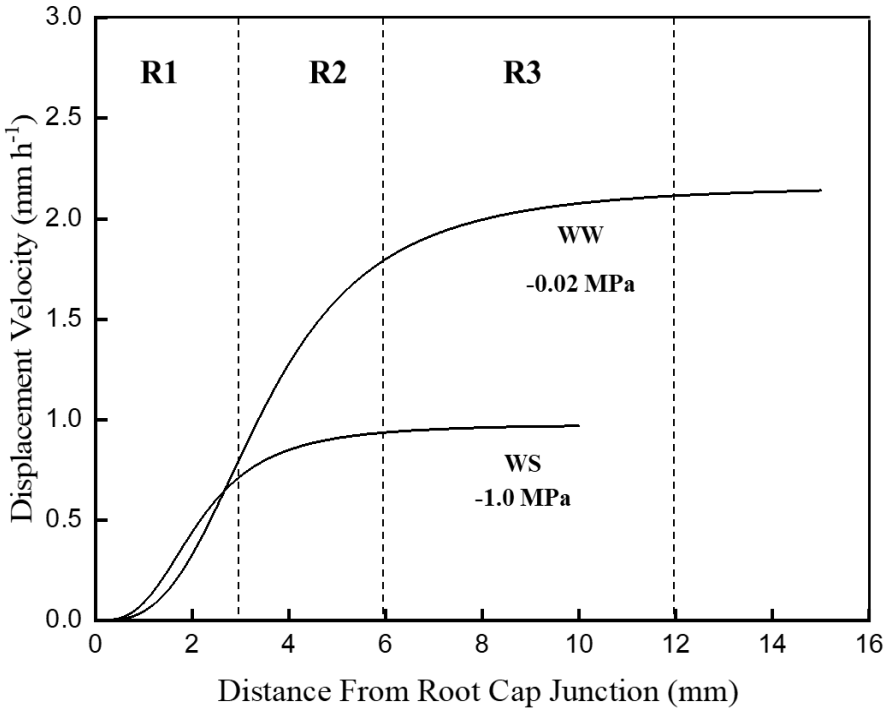
Cotton	**WS/WW Root Elongation Rate**		**WS/WW Final Cell Length**		**WS/WW Cell Flux**	
	-1.0 MPa	-1.6 MPa	-1.0 MPa	-1.6 MPa	-1.0 MPa	-1.6 MPa
	46.2%	21.7%	86.3%	66.9%	53.4%	32.4%

Figure 3-5. Comparison of the displacement velocity profiles in the primary roots of maize (a, reproduced from Zhu et al., 2007) and cotton (b) in well-watered (WW) and water-stressed (WS) conditions (n = 4 roots ± SD). The water potentials of the water-stressed treatment were -1.6 MPa and -1.0 MPa for maize and cotton, respectively.

a



b



exhibited very similar growth traits to those of maize under severe water stress (water potential = -1.6 MPa). Under these conditions, cotton and maize had similar root elongation rates, final cell lengths and cell production rates (Table 3-1). Moreover, the spatial growth patterns within their growth zones were also similar (Figure 3-5).

Discussion

Under water deficit conditions, one of the most important features of the root is the ability to continue elongation when soil water contents are low enough to inhibit shoot growth. For primary roots, which are critical for early seedling development, growth maintenance has been reported for multiple plant species including both maize and cotton (Sharp et al., 1988; Spollen et al., 1993). With a kinematics approach, the cell elongation patterns within the primary root growth zone can be monitored and regions exhibiting different responses can be discerned.

The comparison of the cell elongation patterns and primary root growth traits for cotton (cv. AU90810) and maize (cv. FR697) indicated that cotton primary root growth was more sensitive to water stress than maize under the same water stress level. The greater growth reduction in cotton may occur because of the apparently lower hydraulic contact with the vermiculite media, as discussed in Chapter 2. This possibility is investigated in the following chapter.

However, when cotton was placed in a moderate water stress condition (water potential = -1.0 MPa) the growth parameters and cell elongation profiles were comparable to maize when exposed to a severe water stress condition (water potential = -1.6 MPa). The relative

sensitivities of root elongation rate, final cell length, cell flux, and elongation spatial distribution patterns in response to water stress were also similar (Table 3-1; Figure 3-5). As a result, cotton and maize have similar and comparable regions of growth maintenance or inhibition under water stress conditions. The similar growth patterns within the growth zones provides the foundation for comparisons between the metabolic responses of maize and cotton to water-deficit stress in each region of the root growth zone.

Chapter 4

Comparison of Water Potentials in Maize and Cotton Primary Root Growth Zones under Water Deficit Conditions

Introduction

In Chapter 3, the cell elongation profiles for the primary root growth zones for cotton (cv. AU90810) exposed to different levels of water deficit were determined by a kinematics approach (Silk et al., 1989). It was determined that cotton (cv. AU90810) roots exposed to vermiculite media with a moderate water deficit (water potential = -1.0 MPa) had a similar cell elongation profile to that of maize (cv. FR697) roots when exposed to media with a severe water deficit (water potential = -1.6 MPa). When cotton primary roots were exposed to media at a water potential of -1.6 MPa, the growth zone was considerably further shortened compared with maize (Figure 3-4).

The major aim of this dissertation was to undertake a detailed comparative analysis of the physiological and metabolic processes involved in primary root growth adaptation to water deficits between cotton and maize using a metabolomics approach. The primary concern for such an undertaking is that comparisons should be made on root tissues that are at equivalent levels of water stress, i.e., the tissues should be at the same water potentials.

The similarity in growth patterns between the two species at different media water potentials could be explained by either a) greater sensitivity of root elongation to water stress in cotton compared with maize, and/or b) lower root water potentials at the same media water potential in cotton compared with maize. The latter possibility is suggested by the apparently lower root-media hydraulic contact in cotton compared with maize roots, as detailed in Chapter 2. Therefore, it was important that the root growth zone

tissue water potentials be assessed in order to establish experimental conditions that resulted in equivalent root water potentials in the two species.

Materials and Methods

Growth condition

Cotton seeds were imbibed in 1 mM $CaSO_4$ solution for 12 h and germinated on germination paper dampened with the same solution for 24 h. When the primary roots were of 5-15 mm in length, the seedlings were transplanted to vermiculite media in growth boxes as described in Chapter 2. The treatments were cultivated in water-stressed media with different water potentials (-1.6 MPa in maize; -1.0 MPa in cotton).

Primary root growth zone water potential measurements

Maize and cotton seedlings were cultivated for four different times after transplanting (12 h, 24 h, 36 h, 48 h). According to the previously determined growth zone lengths (Chapter 3), the whole growth zone for each treatment with an addition of 3-4 mm of attached mature tissue was collected for water potential measurement. The mature tissue provided a source of water for the growing region during the water potential measurements, without which the expanding cells could have exhibited continued wall relaxation and decrease in turgor after excision, resulting in erroneously low water potential measurements (Nonami & Boyer, 1989). Tissue water potentials for each time point were measured using isopiestic thermocouple psychrometry (Boyer & Knipling, 1965). This method measures the degree of cooling of a drop of sucrose solution with known water potential placed on a thermocouple as water evaporates from it and is

absorbed by the tissue. The thermocouple and the tissue are enclosed in a small chamber and kept at a constant temperature. The electrical output of the system indicates the rate of vapor transfer, which is proportional to the difference in water potential between the sucrose solution and the tissue. Therefore, when stable, the reading reflects the difference in water potential between the sucrose solution and the tissue water potential. Two measurements with sucrose solutions of different water potentials were conducted to determine the actual water potential of the tissue by interpolation. The isopiestic thermocouple psychrometry system was operated at two different temperatures: 5 ^{o}C in a cold room and 22 ^{o}C in the laboratory.

Statistical analysis

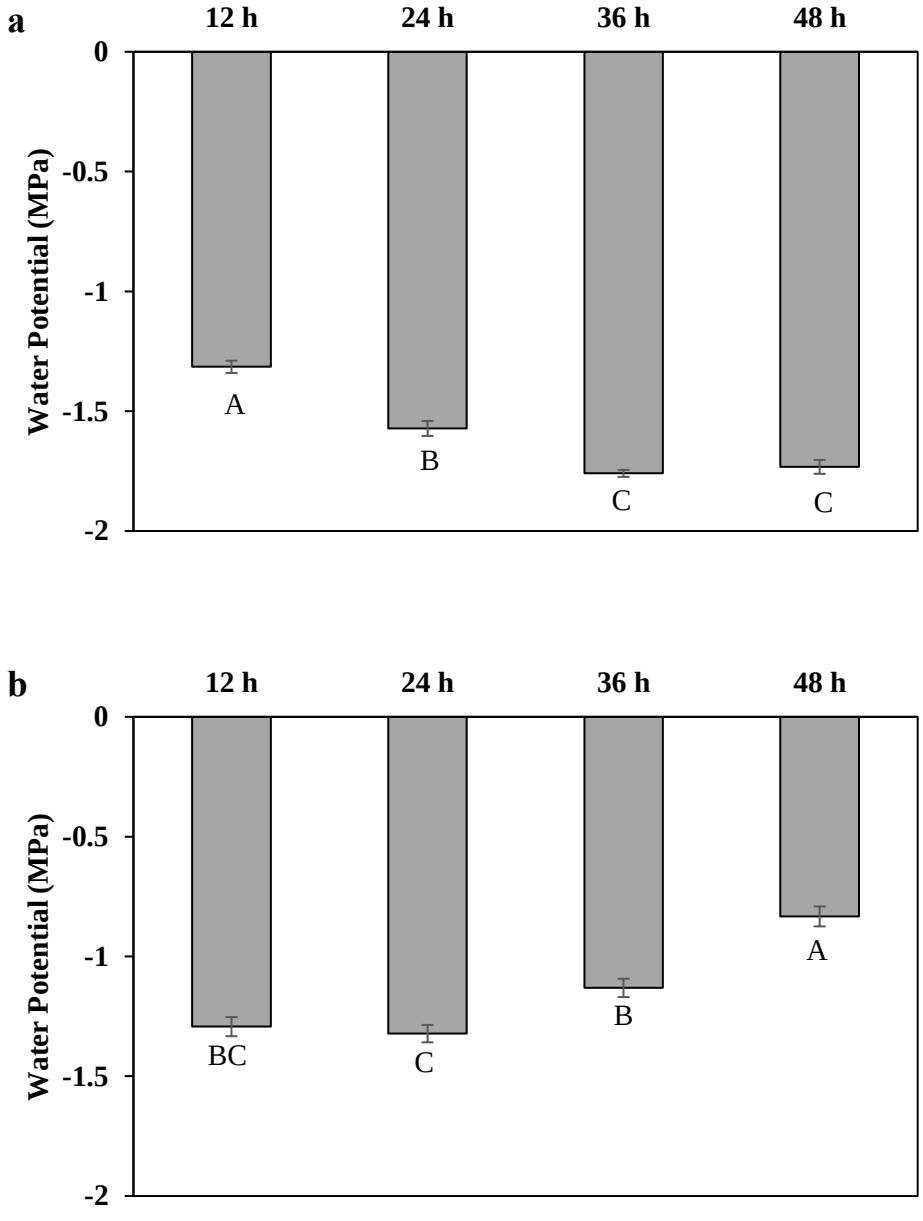
ANOVA analyses were carried out at the 0.05 significance level in Minitab (Minitab, LLC, PA, USA) to compare the water potential results at different time points in each species.

Results and Discussion

Comparison of maize and cotton growth zone water potential under water deficit conditions

Initially, the psychrometric measurements of tissue water potential were conducted under normal lab conditions of 22 ^{o}C. Under this environment, the water potentials of the maize primary root growth zone at different times of exposure to media at -1.6 MPa displayed a decreasing trend from -1.31 MPa at 12 h to -1.73 MPa at 48 h, which was slightly lower than the media water potential (Figure 4-1a). Root tip water potentials lower than the

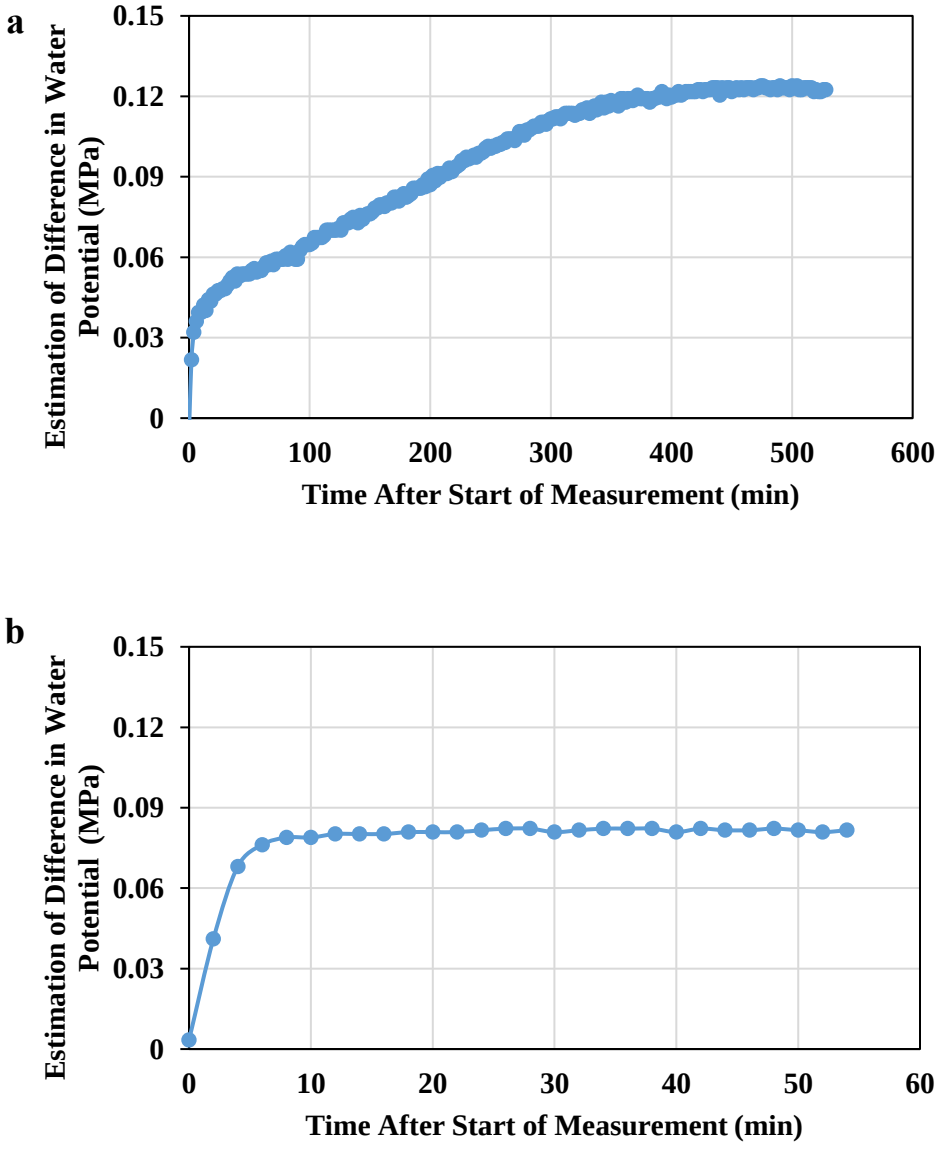
Figure 4-1. Time-course after transplanting of the growth zone water potential of maize (a) and cotton (b) primary roots grown in vermiculite with water potentials of -1.6 MPa and -1.0 MPa, respectively. Water potentials were measured using the thermocouple psychometry system at 22 ^{o}C (n = 4 ± SE). ANOVA analyses were conducted for each species. Different letters denote statistical difference at $p < 0.05$.



water potential of the surrounding media were expected because of the occurrence of "growth-induced" water potential gradients, resulting from the direction of water uptake from the media into the growing root (Nonami & Boyer, 1987). However, measurements of the water potential of the cotton primary root growth zone proved more challenging than anticipated because of an unexpected complexity in the measurements. With the increase of cultivation time under water stressed conditions, cotton primary root samples appeared to have erroneously high water potential measurements (up to -0.8 MPa, approximately) that were higher than the media water potential (-1.0 MPa) (Figure 4-1b). Root tissue water potentials higher than the media water potential are not expected and difficult to explain.

It was observed that during the measurements under room temperature, it took an extremely long time (up to 8 h) for the cotton samples to reach a stable reading in the psychrometry system (Figure 4-2a). Moreover, the trend exhibited an increasing water potential over time of equilibration, which should not be possible in the absence of a water supply inside the psychrometer chamber. In Figure 4-2a, zero on the y-axis means the sucrose solution water potential on the thermocouple. Positive readings mean that the water potential of the sample has a higher water potential than the sucrose solution water potential. The data indicated that the cotton root tissue water potential eventually equilibrated at a water potential approximately 0.12 MPa higher than the water potential of the sucrose solution, which in this measurement was -0.91 MPa. Accordingly, the tissue water potential calculated to be approximately -0.79 MPa, which was higher than the water potential of the growth media used (-1.0 MPa).

Figure 4-2. Examples of erroneous readings at 22 ºC (a) and corrected readings at 5 ºC (b) of the isopiestic thermocouple psychrometry system measuring cotton primary root growth zone water potential under water deficit conditions. Readings indicate the estimated difference between tissue water potential and the water potential of the sucrose solution on the thermocouple (see text for further details).

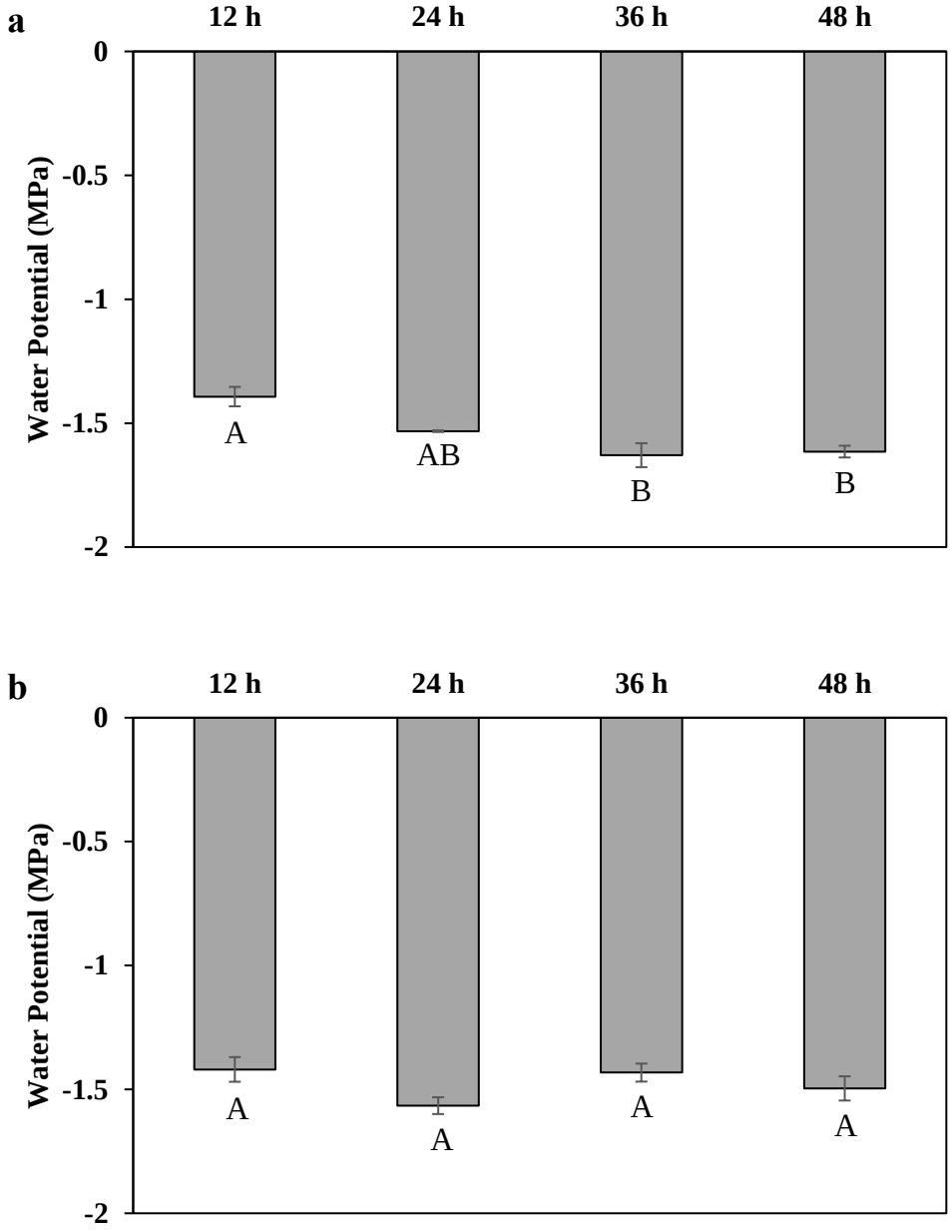


By wiping the surface of the root segments with Kimwipes®, we determined that the cotton primary root samples exuded liquid from the cut surface under room temperature. The liquid may be the result of some metabolic activity in the tissue that is active in the isopiestic thermocouple psychrometer chamber, and it was possible that this exuded liquid had a higher water potential such that the psychrometry system was measuring an average of the root water potential and the exudate water potential. However, because the amount of liquid was extremely small, it was not possible to measure the actual water potential of the liquid.

To resolve this problem, we tested whether conducting the psychrometric measurements inside a cold room at 5 °C would minimize metabolic activity and exudation from the tissues. At this temperature, the exudation of liquid was prevented and the psychrometer reading stabilized very quickly within 1 h (Figure 4-2b) at a water potential approximately 0.08 MPa higher than the sucrose solution. In this experiment, the sucrose solution water potential was -1.50 MPa, indicating that the tissue water potential was approximately -1.42 MPa, which was considerably lower than the media water potential (water potential = -1.0 MPa). As already mentioned, tissue water potentials lower than the growth media are expected, suggesting that the modified psychrometry system yielded accurate water potential measurements for the cotton samples. In maize root samples, in contrast, the water potential measurements were very similar when measured at 22^{o}C (Figure 4-1a) or at 5^{o}C (Figure 4-3a)

Utilizing the modified psychrometric procedure, we accomplished a time course comparison for water potentials of cotton and maize root tips. In cotton, the water

Figure 4-3. Time-course after transplanting of the growth zone water potential of maize (a) and cotton (b) primary roots grown in vermiculite with water potentials of -1.6 MPa and -1.0 MPa, respectively. Water potentials were measured with modified thermocouple psychometry system at 5 ^{o}C (n = 3 ± SE). ANOVA analyses were conducted for each species. Different letters denote statistical difference at $p < 0.05$.



potential of the primary root growth zone was about -1.4 MPa at 12 h after transplanting, which was already lower than the media water potential (-1.0 MPa) (Figure 4-3b).

The water potential then further declined to a stable value of approximately -1.50 MPa after 24 h when growth was also stabilized. In maize, the water potential of the primary root growth zone at 12 h after transplanting to vermiculite at -1.6 MPa was also about -1.4 MPa, and then exhibited a slightly decreasing trend as the time of stress exposure lengthened, stabilizing at approximately -1.61 MPa after 36 h (Figure 4-3a), which was very close to the water potential of the media. These results showed that maize and cotton primary root growth zones had similar and stable tissue water potential levels, of approximately -1.5 to -1.6 MPa, after 36 h of exposure to the different water deficit treatments. As suggested previously, the cotton primary root may have a lower root-media hydraulic contact than in maize, which would explain the larger media to root water potential gradient observed in cotton compared with maize in these experiments.

Conclusion

Cotton and maize primary root growth zones were shown to have similar tissue water potentials as well as similar spatial growth patterns when grown at vermiculite water potentials of -1.0 MPa and -1.6 MPa, respectively. These experimental conditions established a foundation for a direct comparison of the metabolic responses of maize and cotton primary roots to water deficit.

Chapter 5

Comparative Metabolite Studies of Primary Root Responses to Water Deficit in Maize and Cotton

Introduction

In Chapters 3 and 4, it was determined that under specific water-deficit conditions that resulted in equivalent root tip water potentials, maize and cotton primary roots displayed nearly identical spatial patterns of cell elongation within the growth zone. Based on these physiological observations, it was important to understand the extent to which the underlying metabolic mechanisms that maize and cotton roots employ to generate such comparable outcomes are similar or different.

Metabolite profiles are considered to be the integration of gene expression, protein networks and regulatory pathways. Metabolites are 'closer' to the phenotype than either mRNA transcript abundance or protein abundance alone. It was reported that many individual metabolites, such as carbohydrates (Kaplan & Guy, 2004; Sharp et al., 2004; Iordachescu & Imai, 2008; Nishizawa et al., 2008; Sicher et al., 2012), amino acids (Arbona et al., 2008; Nishizawa et al., 2008), polyamines (Gill & Tuteja, 2010; Verslues & Juenger, 2011; Alet et al., 2012) and secondary metabolites (Munns & Tester, 2008; Karowe & Grubb, 2011) increased or decreased in amounts during exposure to water deficit conditions in different plant tissues including roots. If all these metabolites were studied together rather than individually and with traditional biochemical methods, it would be time and labor consuming and inefficient. "Omics" technologies are recent high-throughput methods that can be used to examine, screen and compare plant responses under different conditions in a comprehensive manner, as summarized in Chapter 1. Among all of the "omics" technologies, metabolomics has been considered to

be the most easily utilized “omics” technology and can be used to analyze samples from many different organisms with little or no modification (Arbona et al., 2013).

Metabolomics has been utilized in studies of plant responses to water deficit conditions in multiple species. Some of typical metabolites that accumulate in plant tissues in response to water deficit conditions include intermediates or products of carbohydrate metabolism (Lugan et al., 2009; Bowne et al., 2012; Warren et al., 2012), amino acid metabolism (Charlton et al., 2008; Lugan et al., 2009; Urano et al., 2009; Bowne et al., 2012; Sanchez et al., 2012), the TCA cycle (Charlton et al., 2008; Lugan et al., 2009; Urano et al., 2009; Bowne et al., 2012; Silvente et al., 2012) and plant hormone metabolism (Alvarez et al., 2008; Hochberg et al., 2013). By delivering a global metabolic profile of a specific tissue, a metabolomics approach would serve to clarify the relationships and specific functions of individual and groups of metabolites that respond to water-deficit stress in plants, and in particular roots.

Although focused on leaves, previous research on C4 grasses in the *Sporobolus* genus provided an example of the successful use of untargeted global metabolomics analyses in elucidating plant responses to severe water deficits (Oliver et al., 2011). In this study, *Sporobolus stapfianus*, a desiccation-tolerant genotype, displayed an accumulation of common and unique metabolites from different metabolic pathways in response to dehydration. Osmolytes including proline (amino acid metabolism), sucrose and glucose (carbohydrate metabolism) increased in abundance in leaves during dehydration. The glutathione pathway, which is an essential component of the anti-oxidative mechanisms as reviewed in Chapter 1, also responded significantly to dehydration. In this pathway,

oxidized glutathione (GSSG) and multiple γ-glutamyl amino acids increased in abundance in *S. stapfianus* during dehydration. Ophthalmate, an analog of glutathione and a biomarker of oxidative stress, also increased in abundance during dehydration and was the first report of this particular metabolite in plants. Although *Sporobolus* is a grass and unlike cotton in fundamental ways, this study still provided a template for the use of untargeted global metabolite profiling to investigate responses to water deficit in a tolerant plant. The maize (cv. FR697) and cotton (cv. AU90810) lines studied in the previous chapters were also considered as relatively tolerant to water deficits, at least based on the primary root growth traits, and thus a comparative global metabolomics analysis was undertaken to understand how metabolic pathways were regulated in response to water stress conditions in the primary root of these two genotypes.

As discussed in the previous chapters, the specific study of the responses of primary root under water deficit provides valuable insight into the ability of a plant to successfully establish as a seedling and survive drought stress in the field. In light of the findings chronicled in Chapters 3 and 4 it was clear that a robust experimental system had been developed that ensured that comparable root growth zone regions could be accurately assessed for both cotton and maize, and equally importantly that the same degree of water-deficit stress could be imposed on the two primary root systems. Thus a comparative metabolomics study could be undertaken to determine if the two species respond metabolically in a similar or different fashion to achieve near identical root growth responses under water deficit conditions. The previous studies describing metabolite accumulation in maize primary roots in response to water deficits (as reviewed

in Yamaguchi & Sharp, 2010) led to a particular focus on osmolytes and antioxidants, as the *a priori* major targets, for both species.

Specifically, two untargeted global metabolomics studies were conducted on primary roots under well-watered and water-deficit conditions; one in maize (cv. FR697) and one in cotton (cv. AU90810). These analyses were designed to identify metabolites that may play important roles in distinguishing elongation maintenance or growth inhibition regions within the primary growth zones under water deficits and compare them in the two species. Detailed quantifications of oxidative stress levels and an essential antioxidant, glutathione, were also investigated so as to better understand the importance of anti-oxidative mechanisms in maize and cotton primary roots.

Materials and Methods

Collection of tissue samples

The optimized vermiculite culture system described in Chapter 2 was used for cotton in this experiment and the original vermiculite system (Sharp et al., 1988; Spollen et al., 2000) was used for maize. Three water-deficit treatments were employed for both cotton and maize. A well-watered developmental control (WW_D) with roots of the same length as in the water stress treatment was cultivated in fully moistened media (water potential = -0.02 MPa) for 24 h. A well-watered temporal control (WW_T) with roots of the same age as in the water-stressed treatment was cultivated in fully moistened media for 48 h. As described in Chapter 4, the water-stress treatment (WS) was cultivated for 48 h in media at the following water potentials: for maize, -1.6 MPa; and for cotton, -1.0 MPa. The

purpose of including both a developmental and a temporal control was to allow for the differentiation of metabolites that are truly responding to water stress from those whose changes result from differences in either the stage of seedling development or cellular maturation, as described in Voothuluru et al. (2016). The lengths of the different regions sampled were determined from the kinematic analyses of the spatial pattern of growth as described in Chapter 3. Each well-watered root tip was cut into three regions (WW_D 1, WW_D 2 and WW_D 3; WW_T 1, WW_T 2 and WW_T 3) and each water-stressed root tip was cut into two regions (WS1 and WS2) since region 3 of the water-stressed root tip had ceased elongation and reached the beginning of the maturation zone (Figure 3-4). For maize, the lengths of the different regions were: region 1 (0-3 mm), region 2 (3-7 mm) and region 3 (7-12 mm). For cotton, the lengths of the different regions were: region 1 (0-3 mm), region 2 (3-6 mm) and region 3 (6-12 mm). All distances are from the root cap junction. In order to have sufficient plant material for the metabolomics analyses, each sample consisted of root segments pooled to generate a minimum of 50 mg (based on dry weight) of each root segment for each species separately. For maize, 200-220 segments were collected for each region in each treatment. 320-440 cotton seedlings were collected for each region in each treatment. Four biological replicates were collected for both species.

Metabolomics profiling platform

After harvest, the samples were immediately frozen in liquid nitrogen, lyophilized and ground in liquid nitrogen into a powder and shipped to the metabolomics facility. The untargeted global metabolomics profiles were conducted by Metabolon Inc. (Durham,

NC, USA). Samples were extracted and prepared for analysis using Metabolon's standard methanol extraction, which has been described by Yobi et al. (2013). The extracted samples were split equally into two parts for analysis on GC-MS and UPLC-MS/MS platforms. A CMTRX technical replicate sample was created from a pool containing a small amount of extract from each sample used in the study to calculate an estimation of overall process and platform variability.

Ultrahigh performance liquid chromatography- tandem mass spectrometry (UPLC-MS/MS)

The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatograph (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried and reconstituted in acidic or basic LC-compatible solvents, each of which contained eight or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm). Extracts reconstituted in acidic conditions were gradient eluted from a C18 column using water and methanol containing 0.1% formic acid. The basic extracts were similarly eluted from C18 using methanol and water, however with 6.5 mM ammonium bicarbonate. A third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150

mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion.

Gas chromatography-mass spectrometry (GC-MS)

The samples destined for analysis by GC-MS were dried under vacuum for a minimum of 18 h prior to derivatization under dried nitrogen. Derivatization was achieved under standard conditions with bistrimethyl-silyltrifluoroacetamide as the derivatization agent. This agent replaces labile groups on metabolites, such as hydroxyls, with a stable trimethylsilyl group that protects compounds from the rigors of the gas chromatography separation conditions. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 μm film thickness) with helium as the carrier gas and a temperature ramp from 60° to 340°C in a 17.5 min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power.

Metabolite identification and quality control

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. The consistency of peak identification among the various samples was confirmed by proprietary visualization and interpretation

software. Library matches for each compound were checked for each sample and corrected if necessary.

Tissue sample collection for glutathione and hydrogen peroxide measurements

The whole growth zones of maize (well-watered: 0-12 mm; water-stressed: 0-7 mm) and cotton (well-watered: 0-12 mm; water-stressed 0-6 mm) primary roots were harvested 12 h, 24 h, 36 h and 48 h after transplanting into well-watered (water potential = -0.02 MPa) and low water potential (maize: -1.6 MPa; cotton: -1.0 MPa) vermiculite. The segments were excised and quickly frozen in liquid nitrogen. For the glutathione assays, 50-150 mg (based on fresh weight) of sample were collected for each treatment. Three biological replicates were conducted. For the hydrogen peroxide measurement, 50-100 mg (based on fresh weight) of sample were collected for each treatment. Six biological replicates were conducted.

Glutathione enzymatic assay

Glutathione was measured enzymatically using the dithio-bis-2-nitrobenzoic acid-glutathione reductase (DTNB-GR) recycling method, as described by Noctor and Foyer (2016) with some modification. Frozen samples were ground in liquid nitrogen, thoroughly mixed with 1.5 mL of 1 M $HClO_4$ and centrifuged for 15-20 mins at 14000 rpm in a desktop centrifuge (Eppendorf AG, 5418) at 4^oC. After centrifugation, the supernatant was collected and a solution of 120 mM KH_2PO_4 (pH = 5.6) was added in the ratio of supernatant: solution = 5:1. The pH of the mixture was adjusted to 5-6 using 5 M K_2CO_3. Samples were centrifuged to remove insoluble $KClO_4$ and decanted into fresh

tubes. Total and oxidized glutathione contents were measured by the interaction of reduced glutathione (GSH) with dithio-bis-2-nitrobenzoic acid forming 2-nitro-5-thiobenzoic acid, which has an absorbance peak at 412 nm. Glutathione reductase was used to reduce oxidized glutathione (GSSG) to GSH in the presence of NADPH. To measure GSSG, GSH in the sample was removed by adding 2-vinyl pyridine. The absorbance of each sample was referenced to a standard curve to calculate the total glutathione concentrations in each sample. The outliers were removed by Minitab using Grubbs' test. The final content was presented as ng of glutathione per mg fresh weight.

H_2O_2 measurement

H_2O_2 was measured using a modification of the method described by Le et al. (2016), using an Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (Invitrogen©). Frozen samples were ground in liquid nitrogen and thoroughly mixed with 50 mM $NaPO_4$ buffer (pH = 7.4). The buffer volume (μL) was calculated by multiplying the sample weight (mg) by 5/3. The sample was centrifuged for 10-15 mins at 14000 rpm in a desktop centrifuge (Eppendorf AG, 5418) at 4^oC. The supernatant was transferred to a new Eppendorf tube and a 25 μL aliquot was removed and added to the reaction buffer (0.25 μL Amplex Red + 0.25 μL hydrogen peroxidase + 24.5 μL 50 mM $NaPO_4$ buffer) and mixed thoroughly. The mixture was incubated for 30 min at room temperature before measuring absorbance. A Nanodrop® ND-1000 spectrophotometer was used for measuring absorbance at 560 nm. The absorbance was converted to concentration in each sample by reference to a standard curve. The final hydrogen peroxide content was presented as nmol per g fresh weight.

Statistical analyses

For the metabolomics study, the raw data were normalized for internal consistency by processing a constant weight per volume of extraction solvent for each sample. Data were scaled to the median value for each compound. The missing values were imputed with the minimum detected value for that compound. Statistical calculations were performed using natural log-transformed scaled imputed data. ANOVA contrasts and Welch's two-sample t-tests were used to identify metabolites that differed significantly between experimental groups at the 0.05 significance level. Analysis by two-way ANOVA identified metabolites exhibiting significant interaction and main effects for experimental parameters of treatment and region. The results were interpreted as fold changes between treatments.

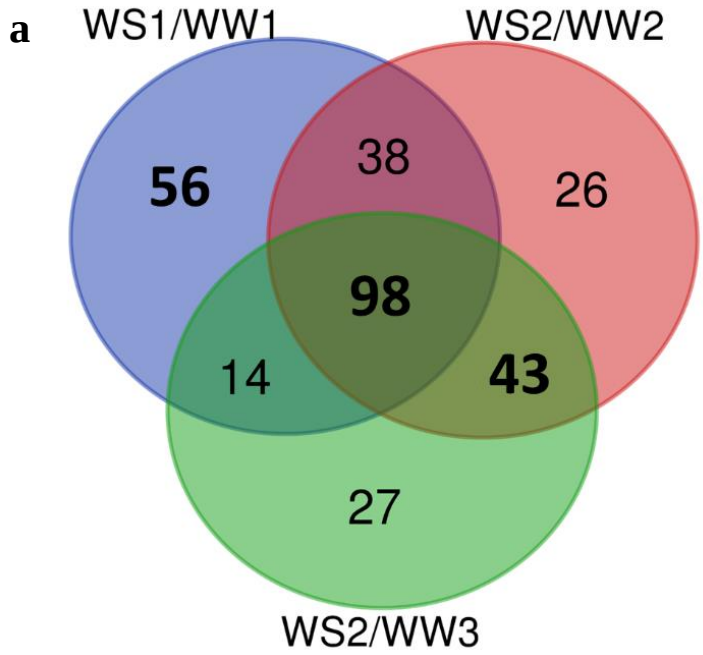
For glutathione and H_2O_2 measurements, ANOVA analyses were carried out at the 0.05 significance level in Minitab (Minitab, LLC, PA, USA) to compare the contents at different time points and different water stress treatments in each species.

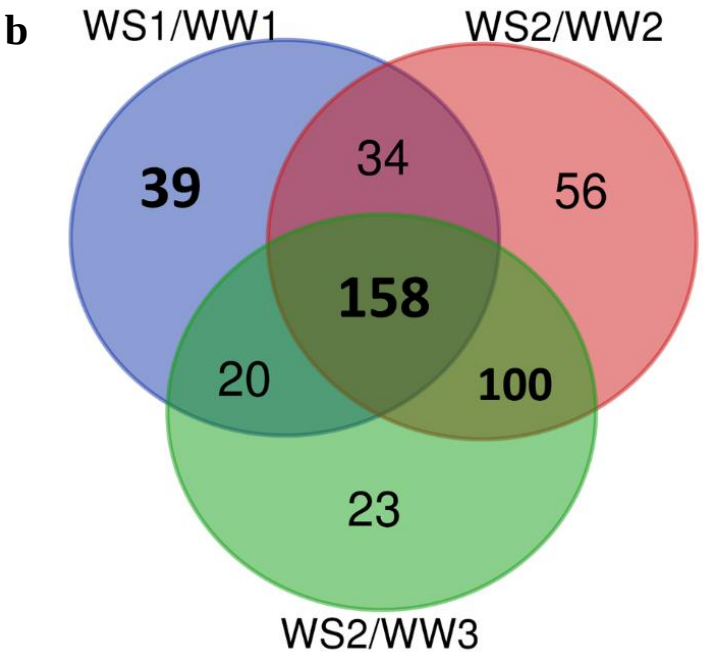
Results

Overview of maize and cotton metabolomics analysis

The metabolomics analyses for maize and cotton primary roots under water deficit stress indicated that the impact of water stress on the growth zone in both species was manifested as a major interruption of normal metabolic activity (Figure 5-1). The comparisons (WS/WW) have been filtered to include only those changes associated with

Figure 5-1. Changes in the abundance of metabolites in different regions within the growth zone of water-stressed (WS) compared with well-watered (WW) maize (a) and cotton (b) primary roots. Only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D) and temporal (WW_T) controls are included.





water stress, not with root development. A metabolite had to either increase or decrease in abundance significantly in both WS/WW_D and WS/WW_T comparisons to be considered as significantly changed in WS/WW and included in the results. The combination of the two comparisons is important to confirm that differences in metabolite profiles between WS and WW treatments are attributable to water stress rather than root age or development. Metabolites changing oppositely in WS/WW_D and WS/WW_T comparisons were not included even if the changes were significant. Abundance profiles of metabolites from region 2 of water-stressed roots (WS2) were compared with profiles from both region 2 (WW2) and region 3 (WW3) of well-watered controls. A metabolite had to significantly increase or decrease with the same trends in each of the comparisons $WS2/WW_D$ 2, $WS2/WW_T$ 2, $WS2/WW_D$ 3 and $WS2/WW_T$ 3 to be included as significantly changed in region 2. The purpose of this was to make a distinction between those metabolites that are associated with growth inhibition in region 2 specifically as a response to water stress, and those metabolites that are involved in root cell maturation whether under stress or control conditions when growth deceleration occurs in water-stressed region 2 or well-watered region 3, respectively, as detailed in previous studies (Zhu et al., 2007; Spollen et al., 2008; Yamaguchi & Sharp, 2010; Voothuluru et al., 2016).

A total of 302 and 430 metabolites changed in abundance in the maize and cotton primary root growth zones, respectively, in response to water-deficit stress (Figure 5-1). For maize, 56 metabolites changed in abundance specifically in region 1 (Figure 5-1, Table 5-1[1]), 43 metabolites changed in abundance specifically in region 2 (Figure 5-1,

[1] All Tables are shown at the end of this chapter.

Table 5-2), and 98 metabolites changed in abundance in both regions (Figure 5-1, Table 5-3). For cotton, 39 metabolites changed in abundance specifically in region 1 (Figure 5-1, Table 5-4), 100 metabolites changed in abundance specifically in region 2 (Figure 5-1, Table 5-5), and 158 metabolites changed in abundance in both regions (Figure 5-1, Table 5-6). Comparing the changes in maize and cotton, there were 27 metabolites that changed throughout the growth zone in both species, indicating some general commonalities in the responses of the root tissues to water deficit (Figure 5-2, Table 5-7). However, for the region-specific changes, only a small number of metabolites changed in the same way in both species. In region 2, maize and cotton had only three metabolites that changed in common (uridine-2',3'-cyclic monophosphate, cytidine 2',3'-cyclic monophosphate and N-acetylphenylalanine), while in region 1, only one metabolite (1-oleoyl-glycero-3-phosphoglycerol (18:1)) was altered in abundance in the same manner in both species. The metabolites that were significantly changed only in one region of root growth zone for the individual species are shown in Tables 5-8 through 5-11. In general, maize primary roots displayed a metabolic pattern that was predictable from data reported in previous studies, including the accumulation of osmolytes and antioxidants, whereas, cotton primary roots displayed some unique metabolic features in essential pathways that were significantly different from maize.

Carbohydrates and amino acids as osmolytes

Among the 27 metabolites changing in response to water stress throughout the growth zone that were common to both species, an increase in the osmolytes proline and sucrose, as well as glucose, fructose, raffinose and myo-inositol, were notable (Figures 5-3 and 5-

Figure 5-2. Metabolites that significantly changed in abundance in region 1 and region 2 of the growth zone in maize and cotton primary roots in response to water deficit compared to well-watered controls. Only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D) and temporal (WW_T) controls are included.

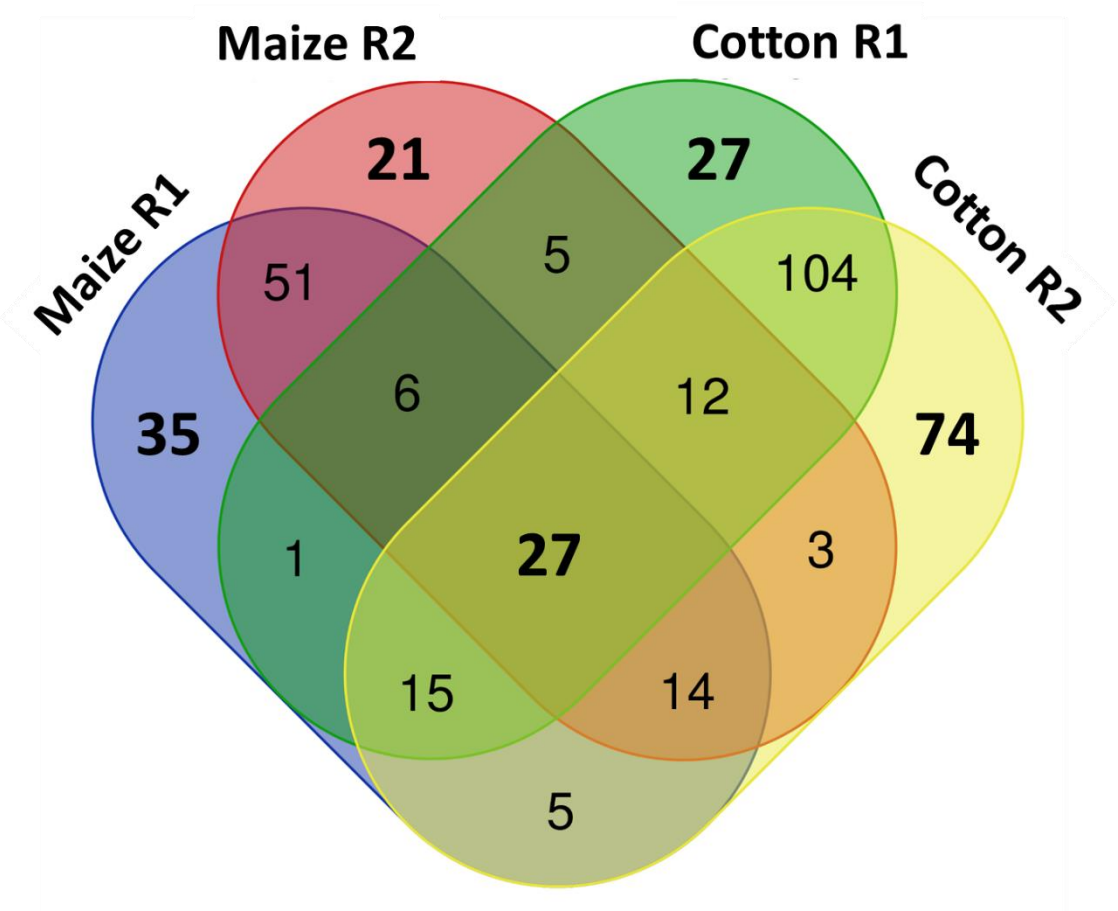


Figure 5-3. Changes in metabolites related to sucrose metabolism in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

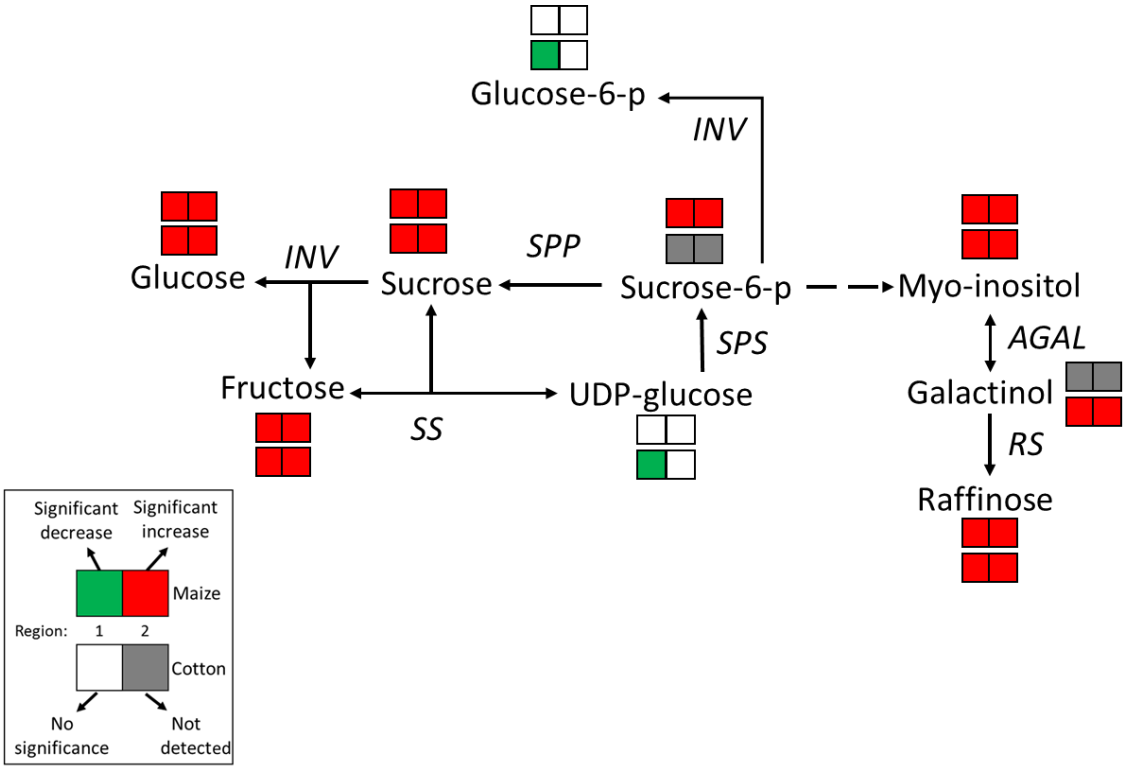


Figure 5-3-1. Fold changes in the abundance of raffinose in regions 1 and 2 (WS1 and WS2) of the growth zone of water-stressed maize and cotton primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red columns indicate significant increases in abundance (p-value ≤ 0.05). No minimum cutoff was applied to the fold changes.

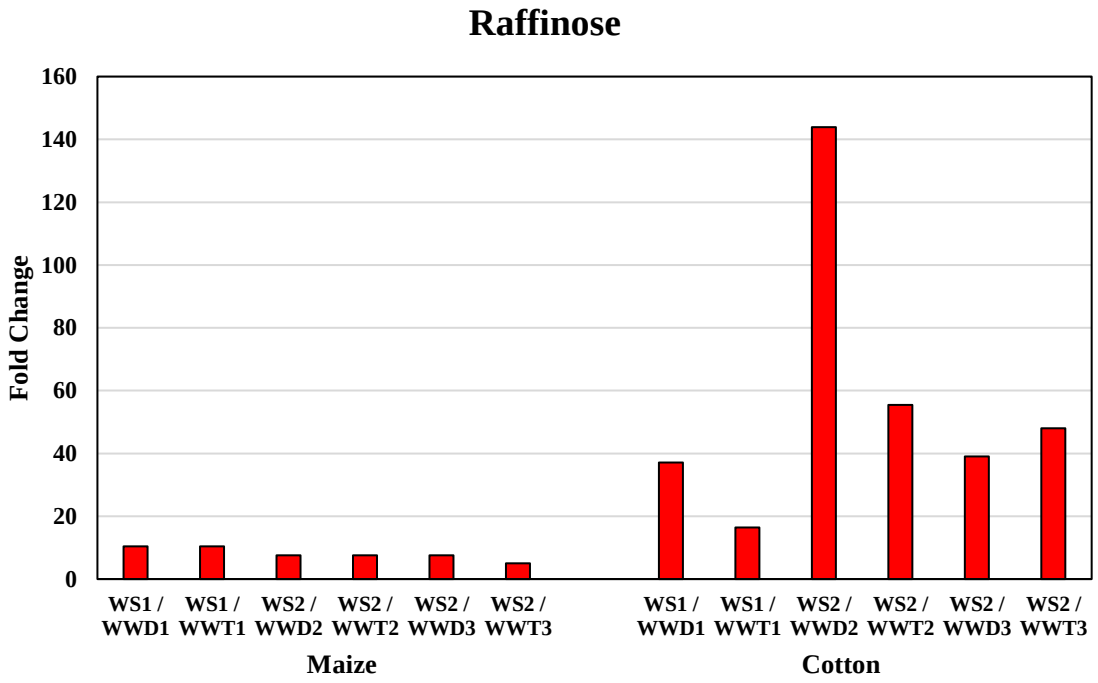


Figure 5-4. Changes in metabolites related to the TCA cycle, GABA shunt and glutamate metabolism in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

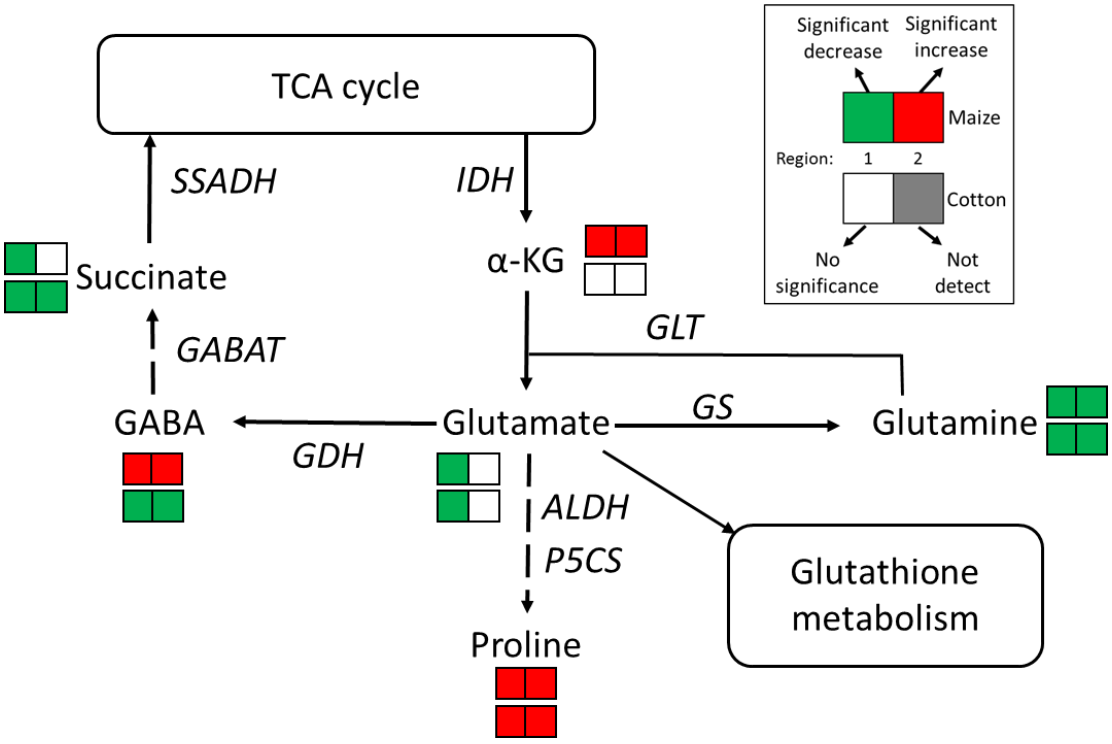
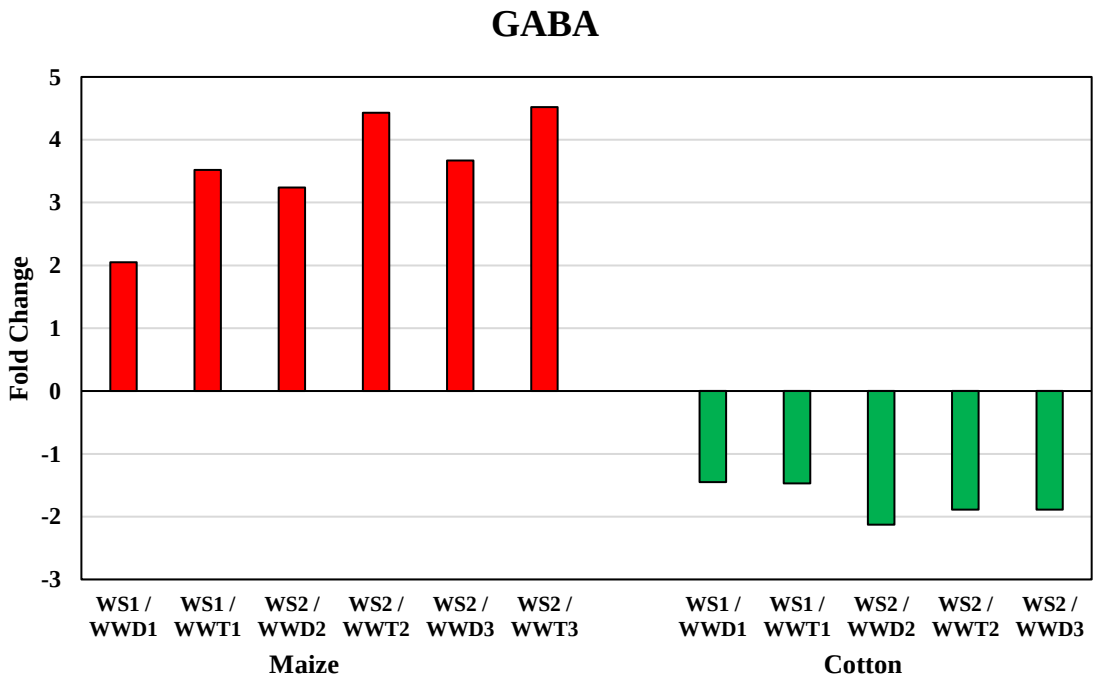


Figure 5-4-1. Fold changes in the abundance of γ-aminobutyrate (GABA) in regions 1 and 2 (WS1 and WS2) of the growth zone of water-stressed maize and cotton primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls Red columns indicate significant increases in abundance (p-value ≤ 0.05); green columns indicate significant decreases in abundance (p-value ≤ 0.05). No minimum cutoff was applied to the fold changes.



4). Raffinose, a trisaccharide that serves in both osmoregulation and anti-oxidative mechanisms, exhibited relatively large increases in abundance in water-stressed roots of both species compared to levels seen in the well-watered samples (Figure 5-3-1, Table 5-7).

TCA cycle, glutamate and GABA

Proline is biosynthesized from glutamate, which is a downstream product of α-ketoglutarate (Figure 5-4). α-ketoglutarate is an important intermediate in the TCA cycle, forming glutamate and other amino acids and their derivatives. From the metabolite profile comparisons, maize and cotton roots regulated the TCA cycle differently under water-stressed conditions. For example, α-ketoglutarate increased in maize but did not significantly change in cotton, while citrate decreased in both regions 1 and 2 in maize (Table 5-3) but only decreased in region 1 in cotton (Table 5-4). γ-aminobutyrate (GABA) decreased in abundance in both region 1 and region 2 in response to the stress treatment in the cotton primary root, but increased in abundance in both regions of the maize primary root (Figures 5-4 and 5-4-1).

Glutathione metabolism

As shown in Figure 5-5, glutathione abundance changed differently in maize and cotton. In maize, both the reduced (GSH) and oxidized forms (GSSG) of glutathione accumulated in region 1 but not significantly in region 2 of the primary root in response to water deficit. However, in the cotton primary root GSH and GSSG were reduced in both regions of the water-stressed roots (Figure 5-5-1). Among all of the compounds in

Figure 5-5. Changes in metabolites related to glutathione metabolism in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

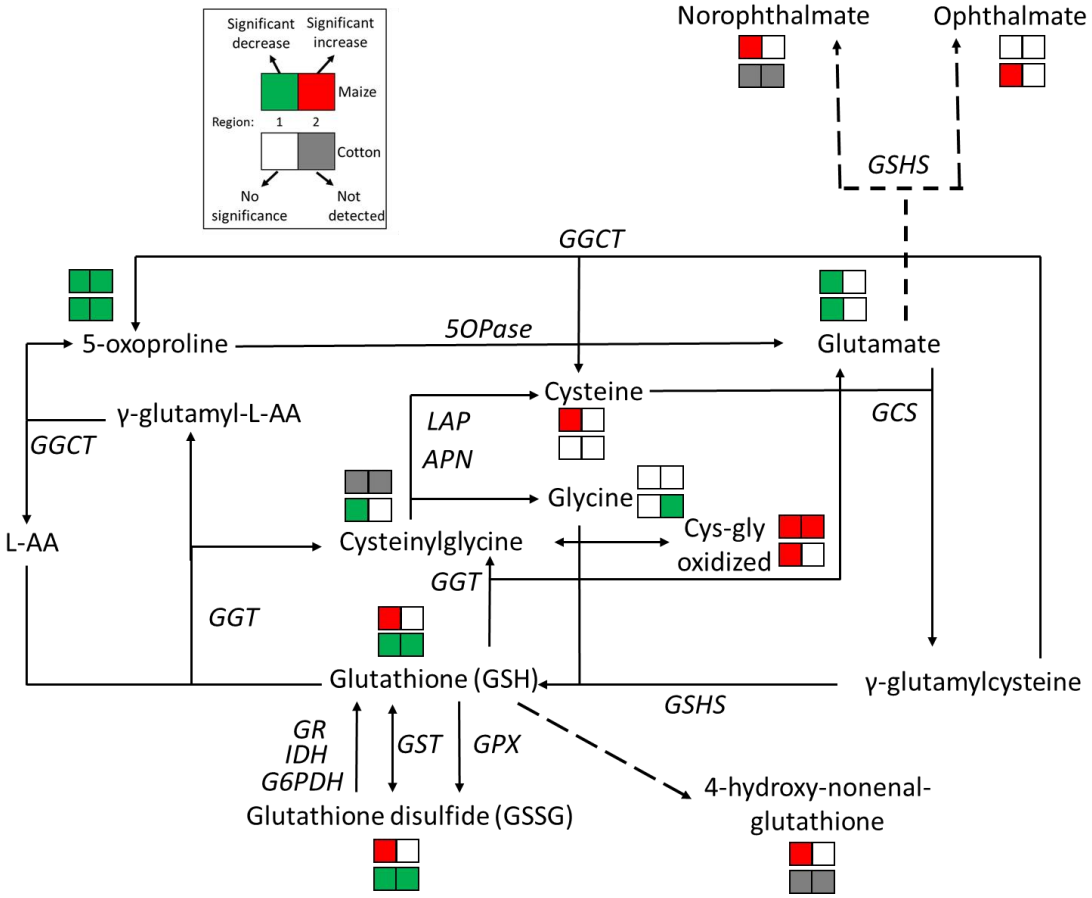
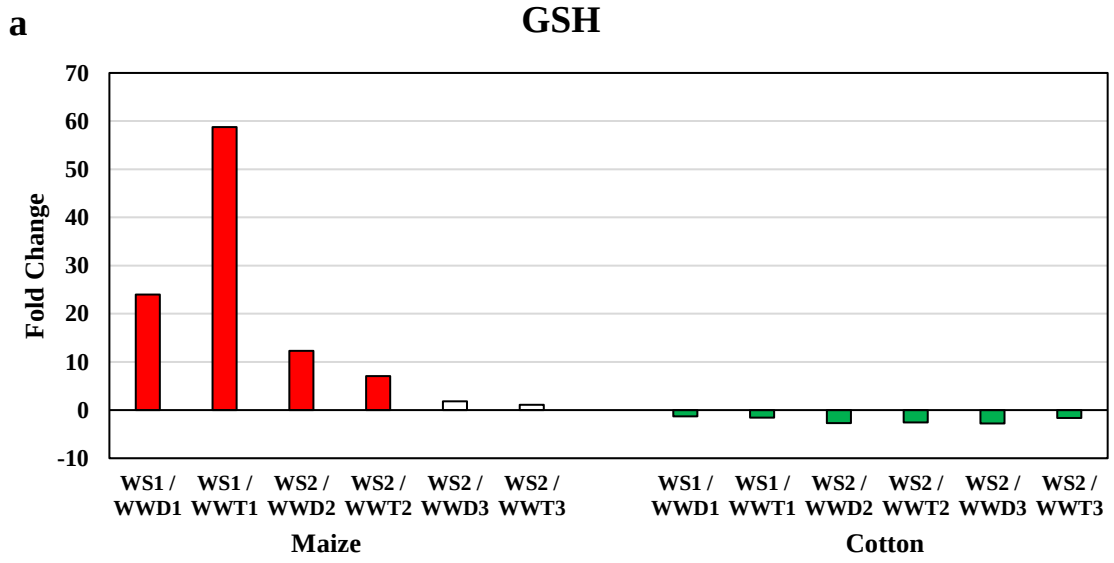
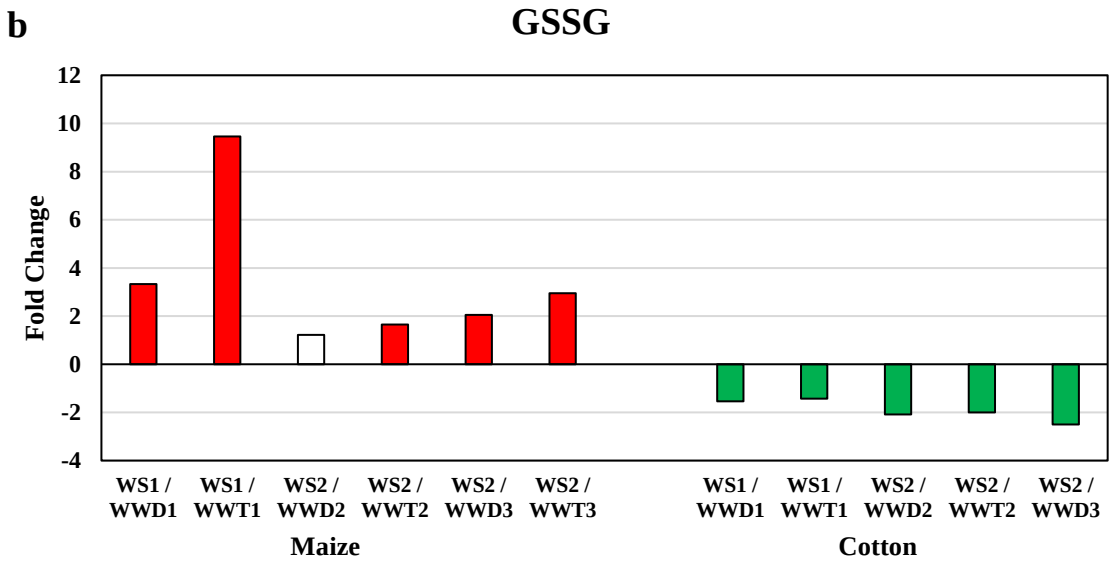


Figure 5-5-1. Fold changes in the abundance of reduced (GSH) (a) and oxidized (GSSG) (b) glutathione in regions 1 and 2 (WS1 and WS2) of the growth zone of water-stressed maize and cotton primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red columns indicate significant increases in abundance (p-value ≤ 0.05); green columns indicate significant decreases in abundance (p-value ≤ 0.05); white columns indicate non-significance. No minimum cutoff was applied to the fold changes.





the glutathione metabolic pathway, only the byproducts of glutathione synthesis, ophthalmate and oxidized cysteinylglycine, increased in abundance in region 1 of cotton roots in response to the stress treatment. Norophthalmate, a metabolite of similar structure to ophthalmate and another byproduct of this pathway, increased in region 1 of maize roots in response to the stress treatment. 5-oxoproline, an intermediate of glutathione degradation, was decreased in both regions of the root tip for both maize and cotton in response to stress. The glutathione derivative, 4-hydroxy-neoneal-glutathione (4HNE-GSH), increased in maize region 1 in response to stress but was undetectable in cotton.

Sulfur metabolism

Based on the differences in glutathione metabolism, it was logical to closely focus on sulfur metabolism and to identify if the two species differed in the response of sulfur metabolism to water-deficit stress. Sulfur metabolism in the primary root clearly responds to exposure to water deficit in a distinctly different manner in cotton compared to the response for maize. Sulfate levels in both root tip regions of cotton significantly declined in response to a water deficit, but in maize sulfate accumulated in both regions (Figures 5-6 and 5-6-1). Cysteine significantly increased in region 1 of the maize root but did not significantly change in region 2 of maize as well as all in both regions of the cotton root (Figure 5-6). Methionine increased in abundance in region 1 of the cotton root but did not change in region 2, whereas in maize methionine levels did not significantly change in region 1 but declined in region 2 (Figure 5-7). The methionine derivatives S-adenosylmethionine (SAM) (Figure 5-7-1a) and 5-methylthioadenosine (MTA) (Figure

Figure 5-6. Changes in metabolites related to sulfur-containing amino acid biosynthesis in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

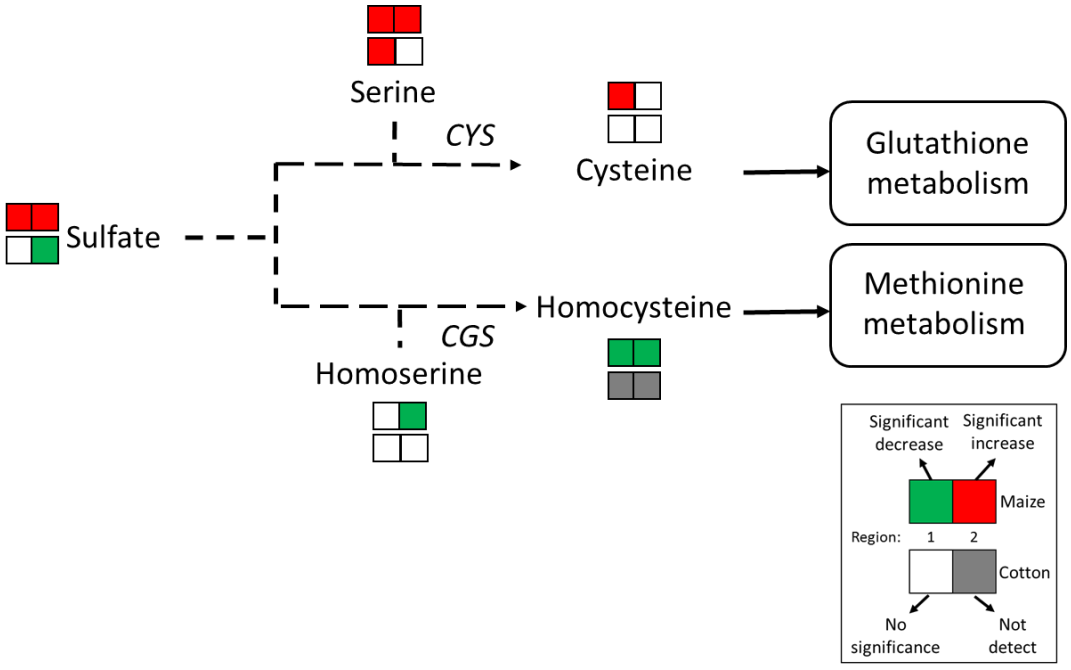


Figure 5-6-1. Fold changes in the abundance of sulfate in regions 1 and 2 (WS1 and WS2) of the growth zone of water-stressed maize and cotton primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red columns indicate significant increases in abundance (p-value ≤ 0.05); green columns indicate significant decreases in abundance (p-value ≤ 0.05); the pink column indicates increase in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); the white column indicates non-significant. No minimum cutoff was applied to the fold changes.

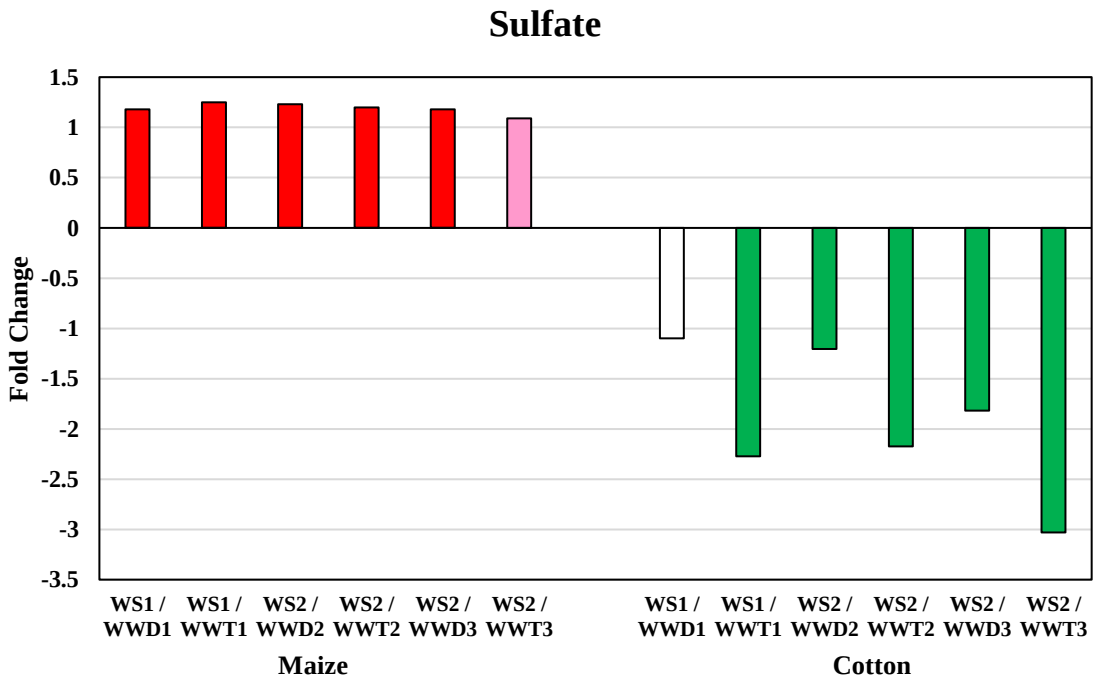


Figure 5-7. Changes in metabolites related to methionine metabolism in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

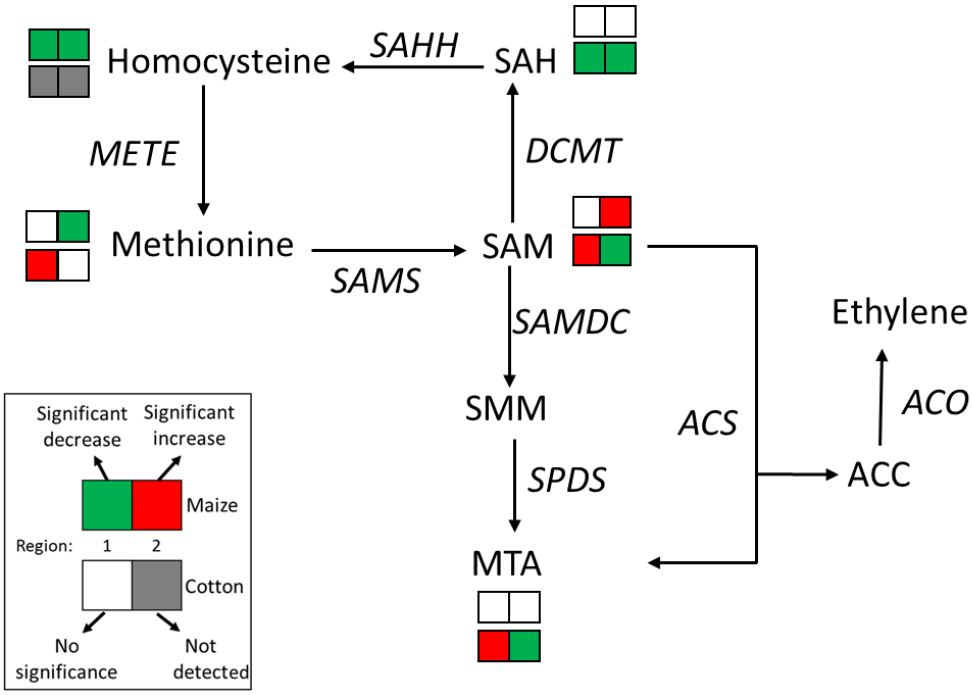
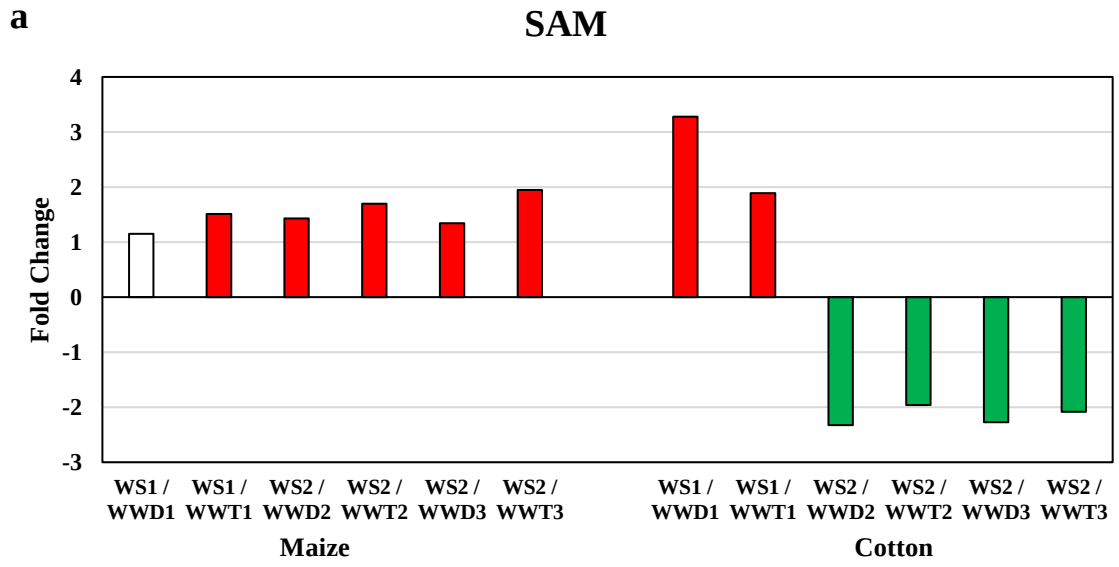
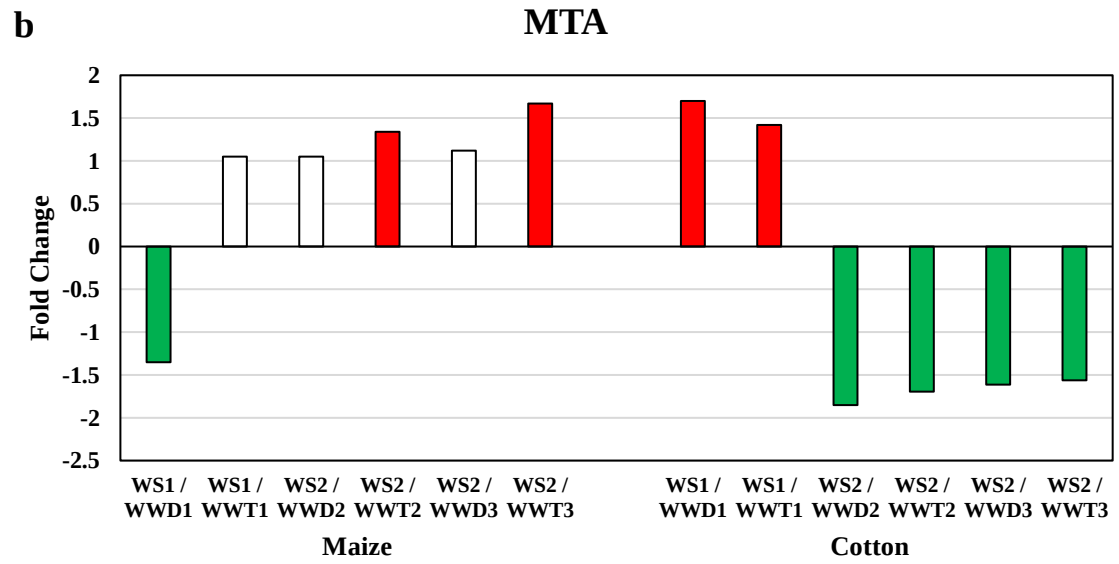


Figure 5-7-1. Fold changes in the abundance of S-adenosylmethionine (SAM) (a) and 5-methylthioadenosine (MTA) (b) in regions 1 and 2 (WS1 and WS2) of the growth zone of water-stressed maize and cotton primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red columns indicate significant increases in abundance (p-value ≤ 0.05); green columns indicate significant decreases in abundance (p-value ≤ 0.05); white columns indicate non-significant. No minimum cutoff was applied to the fold changes.





5-7-1b) accumulated in region 1 of the primary root of cotton but declined in region 2, a pattern that was rarely observed in the metabolite profiles (Figure 5-7, Table 5-6). In maize, SAM abundance was unchanged in region 1 and increased in region 2, but MTA did not significantly change in either region.

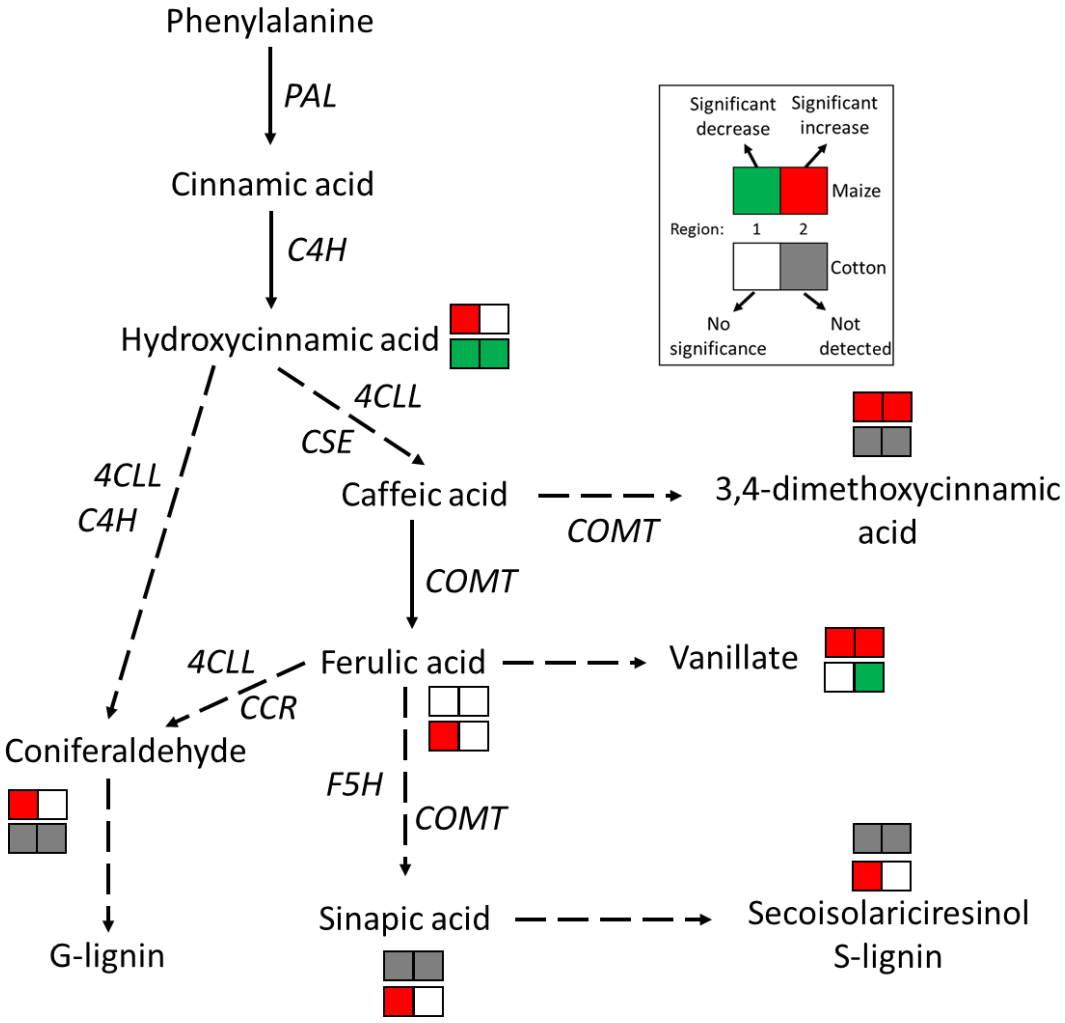
Phenylpropanoid metabolism

The phenylpropanoid pathway and associated downstream secondary metabolism also differed in the response to water-deficit stress in maize compared to cotton (Figure 5-8). Hydroxycinnamic acid (4-hydroxycinnamate), the precursor of some important phenylpropanoids including caffeic acid and 4-hydroxycinnamoyl-CoA, accumulated in region 1 of maize roots exposed to water deficit. Downstream metabolites including 3,4-dimethoxycinnamic acid and vanillate also accumulated throughout the maize root growth zone. In cotton roots, hydroxycinnamic acid decreased in abundance in the whole growth zone but the downstream metabolites ferulic acid, sinapic acid and secoisolariciresinol accumulated significantly in region 1.

Quantitative assessment of oxidative metabolism

It was clear from the metabolomics study that glutathione metabolism responded differently and in an opposite manner in maize compared to cotton roots when exposed to water deficit. The metabolomics analysis is primarily qualitative, examining differences compared to a well-watered control, and thus it was possible that the changes in relative abundance in either species were not physiologically relevant. To investigate glutathione

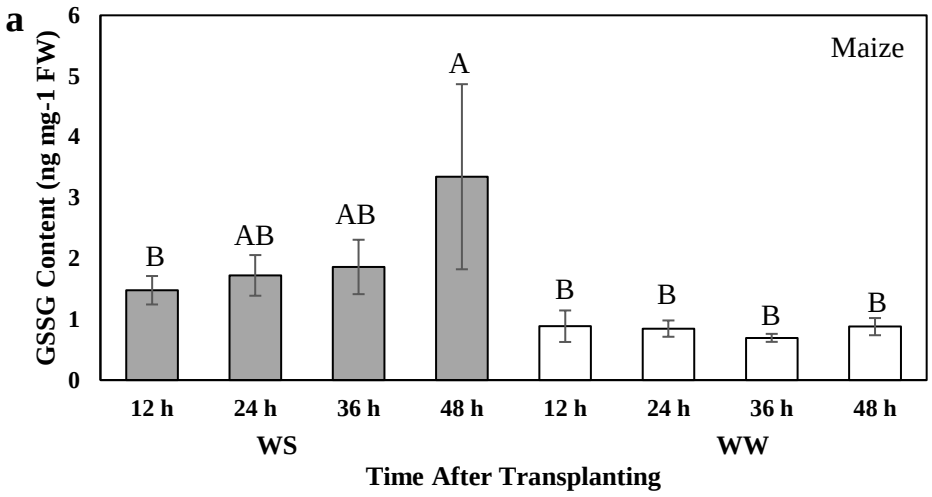
Figure 5-8. Changes in metabolites related to phenylpropanoid metabolism in the different regions of water-stressed compared with well-watered roots. Boxes next to the metabolites represent changes at p-value ≤ 0.05. In region 1, only metabolites that significantly changed in comparison with both the well-watered developmental (WW_D 1) and temporal (WW_T 1) controls are shown. In region 2, only metabolites that significantly changed in all of the comparisons WS2/WW_D 2, WS2/WW_T 2, WS2/WW_D 3 and WS2/WW_T 3 are shown.

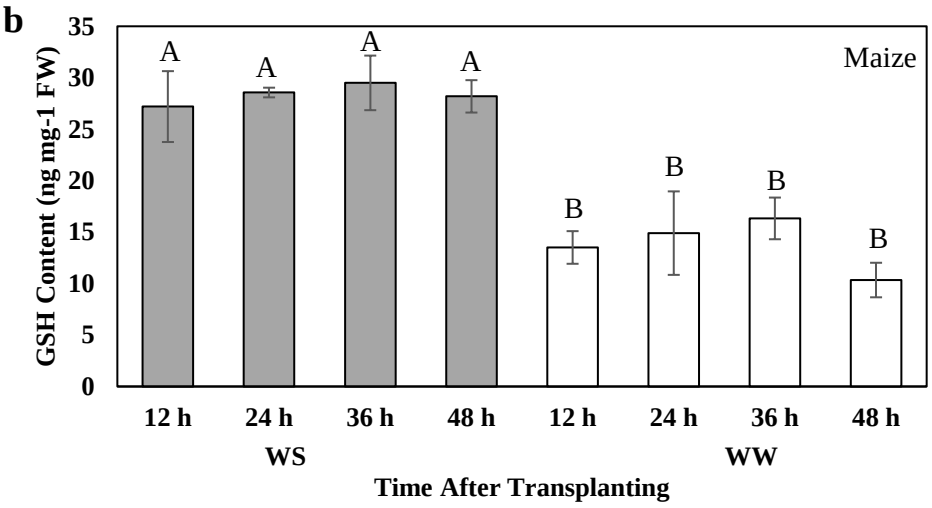


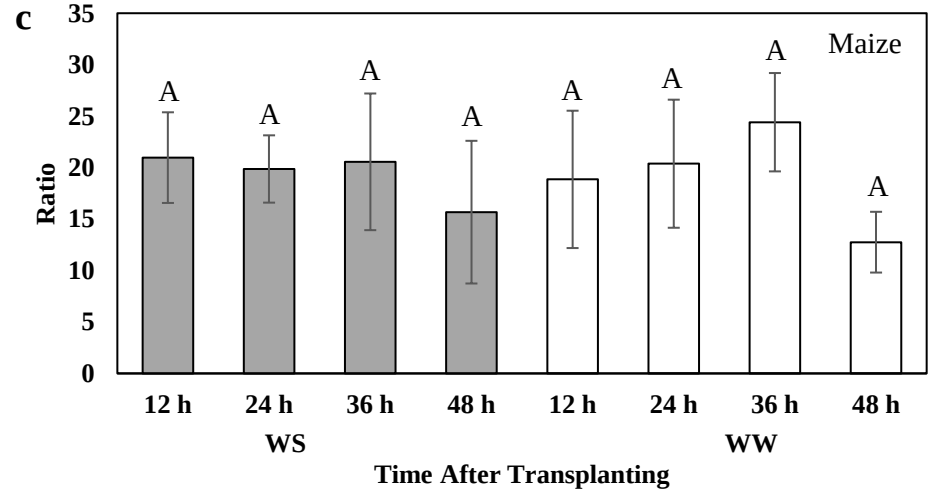
levels more precisely, a quantitative biochemical assay was undertaken to measure the amounts of glutathione during a 48 h period of exposure to either well-watered or water deficit conditions in the root growth zone of both species. For these measurements, the whole growth zone was sampled. The results of this analysis are presented in Figure 5-9.

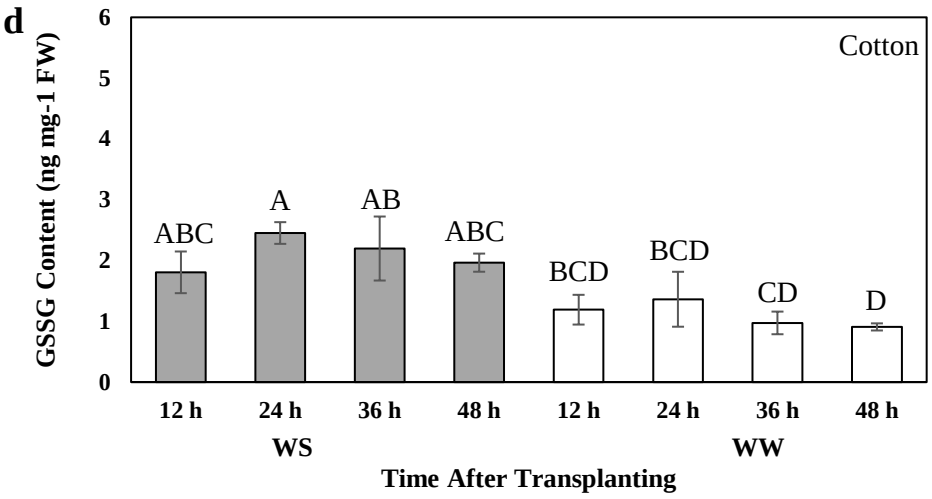
In maize, the oxidized (GSSG) form of glutathione only showed significant accumulation in the water-stressed 48 h treatment compared with the well-watered treatment (Figure 5-9a), while the reduced (GSH) form of glutathione significantly accumulated in the water-stressed treatment compared with the well-watered treatment at each time point (Figure 5-9b). In cotton, both GSSG and GSH accumulated in the water-stressed treatment compared with the well-watered treatment at time points after 12 h (Figures 5-9d and 5-9e). However, glutathione levels exhibited different trends over time in the maize and cotton primary roots. In maize, GSSG exhibited a significantly higher level in the water-stressed treatment at 48 h while GSH was maintained at high levels in the water-stressed treatment throughout the 48 h experiment with only minor fluctuations. In the well-watered treatment, levels of both GSH and GSSG remained constant. In contrast, in cotton roots exposed to water deficit, GSH increased in the first 24 h and then decreased at later time points. A similar trend was recorded for GSSG, although the decline was not significant as for GSH. In the well-watered treatment, GSH content reached maximum level at 12 h and subsequently declined. GSSG did not exhibit significant changes throughout the 48 h experiment. In both treatments, both cotton and maize maintained relatively constant GSH: GSSG ratios. Only the ratio at 12 h of well-watered cotton is significantly higher than the ratio at 48 h.

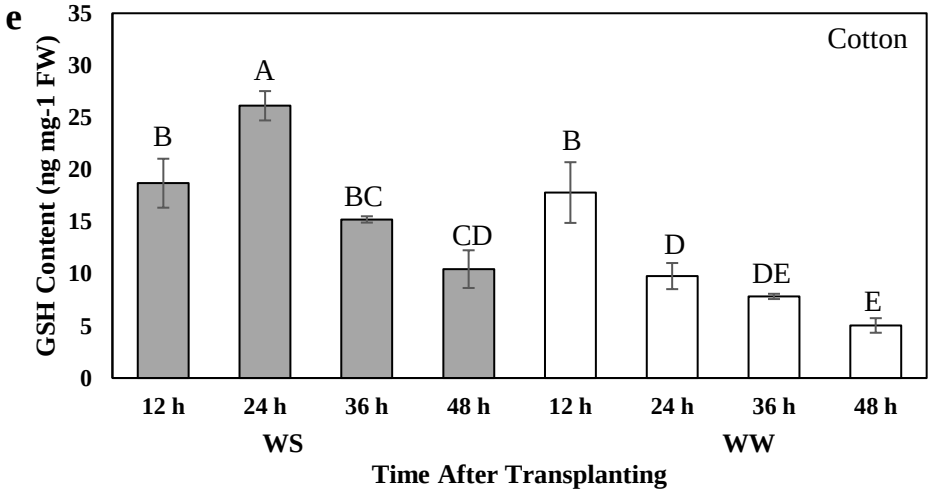
Figure 5-9. Quantitative measurements of oxidized (GSSG) (a, d) and reduced (GSH) (b, e) glutathione content, and their ratios (c, f), in the growth zone of maize (a-c) and cotton (d-f) primary roots during 48 h after transplanting to water-stressed (WS) and well-watered (WW) conditions. The whole growth zone (WS cotton: 0-6 mm; WS maize 0-7 mm; WW cotton: 0-12 mm; WW maize: 0-12 mm) was collected for the assay (n = 3-6 ± SE). ANOVA analyses were conducted at the 0.05 significance level to compare the contents and ratios at different time points and different water stress treatments in each species.

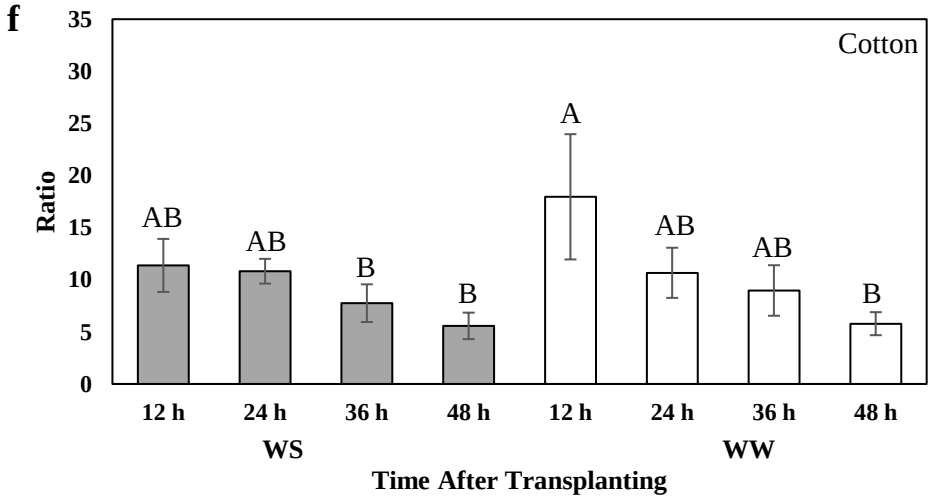

a
Maize
GSSG Content (ng mg-1 FW)
0
1
2
3
4
5
6
B
AB
AB
A
B
B
B
B
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting


b
Maize
GSH Content (ng mg-1 FW)
0
5
10
15
20
25
30
35
A
A
A
A
B
B
B
B
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting


c
Maize
Ratio
0
5
10
15
20
25
30
35
A
A
A
A
A
A
A
A
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting


d
Cotton
GSSG Content (ng mg-1 FW)
0
1
2
3
4
5
6
ABC
A
AB
ABC
BCD
BCD
CD
D
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting


e
Cotton
GSH Content (ng mg-1 FW)
0
5
10
15
20
25
30
35
B
A
BC
CD
B
D
DE
E
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting

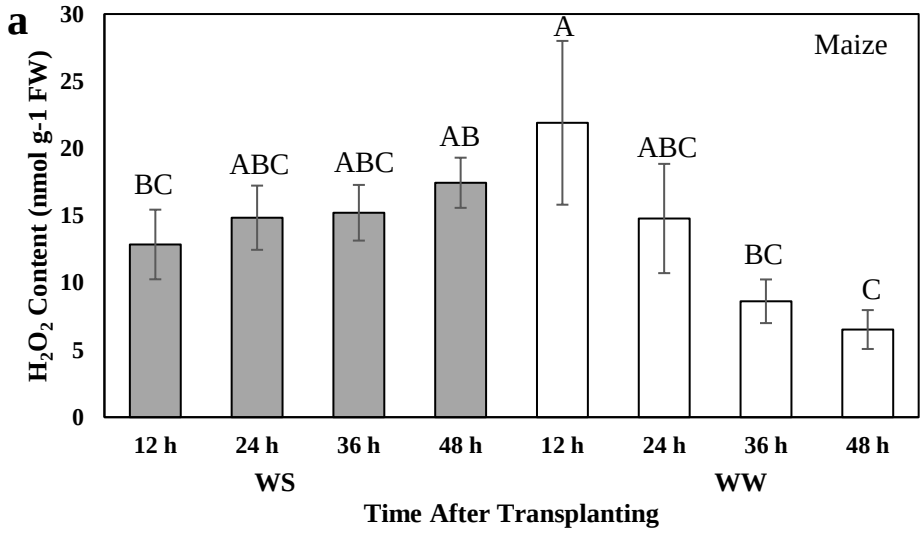

f
Cotton
Ratio
0
5
10
15
20
25
30
35
AB
AB
B
B
A
AB
AB
B
12 h
24 h
36 h
48 h
12 h
24 h
36 h
48 h
WS
WW
Time After Transplanting

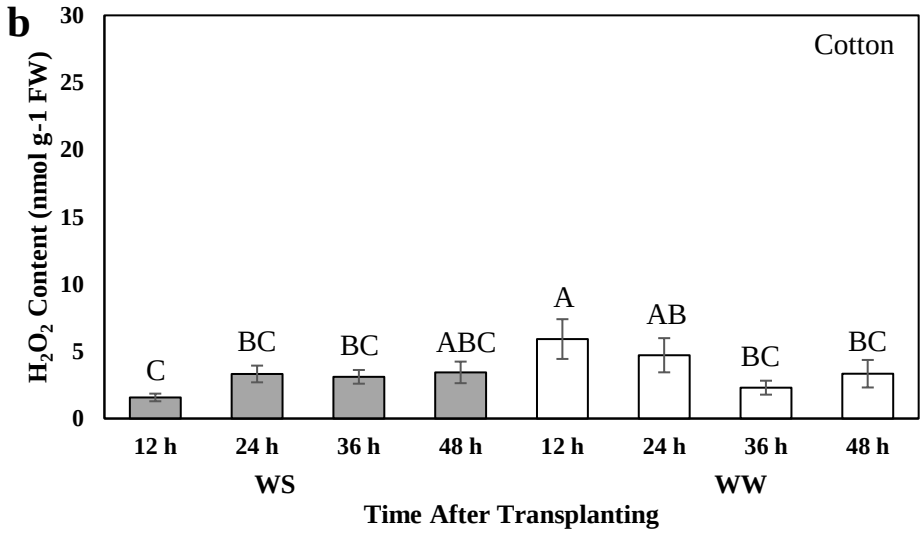
To explore the possibility that cotton roots were exposed to greater oxidative stress a direct measurement of the level of H_2O_2, a marker for oxidant levels, in the root growth zone of both maize and cotton was undertaken. H_2O_2 levels slightly increased in maize in response to water-deficit stress, with the opposite trend occurring in roots from the well-watered treatment (Figure 5-10), such that water-stressed roots had statistically higher levels of H_2O_2 than well-watered roots at 48 h. The high H_2O_2 content in the initial period after transplanting the seedlings presumably represents the sensitivity of response of the maize primary root to an environmental perturbation. In the maize roots, 36 h and 48 h exposure times to water-deficit stress exhibited a similar increasing trend in H_2O_2 content compared with the glutathione quantitative data. The H_2O_2 measurements for cotton roots were much lower than in maize roots in both treatments, and there were no clear trends of accumulation or depletion exhibited in either treatment. Only the well-watered treatment at 12 h had a significant increase compared with the water-stressed treatment.

Discussion

Osmotic adjustment plays an important role in maintaining primary root growth under drought stress. Osmolyte accumulation reduces the rate of intracellular water loss by lowering the osmotic potential of the cell (Delauney & Verma, 1993), thereby maintaining turgor for cell expansion as well as enabling the maintenance of cellular homeostasis. Metabolites that perform as osmolytes, including several sugars and other carbohydrates along with amino acids such as proline, were among the major compounds that increased in both cotton and maize root tips in response to water deficit. The maize

Figure 5-10. Quantitative measurements of hydrogen peroxide (H_2O_2) in the growth zone of maize (a) and cotton (b) primary roots during 48 h after transplanting to water-stressed (WS) and well-watered (WW) conditions. The whole growth zone of each treatment (WS cotton: 0-6 mm; WS maize 0-7 mm; WW cotton: 0-12 mm; WW maize: 0-12 mm) was collected for the assay (n = 6 ± SE). ANOVA analyses were conducted at the 0.05 significance level to compare the contents at different time points and different water stress treatments in each species.





genotype FR697 and cotton genotype AU90810 were selected as having better abilities for root growth maintenance under water-deficit stress among the genotypes studied.

Therefore, it is reasonable to assume that variation in the accumulation of osmolytes may be a critical factor in generating the variation in primary root growth rates between genotypes. Raffinose, apparently a significant osmolyte for both species, may also play a role as an anti-oxidant in the control of ROS accumulation during water-deficit stress (Nishizawa et al., 2008).

Glutathione is a tri-peptide synthesized from glutamate, glycine and cysteine. Glutathione has multiple biological functions as an important amino acid derivative, but perhaps its most important role is as an anti-oxidant within plant redox-metabolism as it is a key metabolite in the glutathione cycle (Noctor & Foyer, 2016). In previous water stress studies, compounds involved in this cycle were found to increase in plant tissues (Nayyar & Gupta, 2006; Jaleel et al., 2008a; Jaleel et al., 2008b) including maize roots (Ahmad et al., 2016), as confirmed in this study. In contrast, it was remarkable that the glutathione cycle, as a major drought induced and anti-oxidation pathway in plants, appeared to be repressed in cotton primary roots in response to water-deficit stress. A decrease in glutathione in response to stress in roots has been reported previously for canola but only in the presence of high levels of H_2O_2 (Lappartient & Touraine, 1997). It is possible that maintaining a relatively high growth rate during drought conditions results in the cotton primary root being more exposed to oxidative stress than in the maize root and that this consumes much of the reduced glutathione (GSH) leading to an overall loss of glutathione. It is also possible that glutathione is not needed in the cotton root as a

component of the anti-oxidative and drought resistance mechanisms. As reviewed in Chapter 1, glutathione has other functions in plants in addition to serving as an antioxidant, including involvement in cell signaling, redox balance, sulfur storage and as a regulator of stress defense genes (Khan et al., 2008).

These considerations, together with the fact that glutathione is an important intermediate in sulfur metabolism and, along with H_2O_2, serves as a marker for oxidative stress, led us to conduct quantitative and time-course based investigations. Although not entirely consistent with the metabolomics analysis, which was a single time point and did not include specific methodology to protect glutathione during extraction, we were able to establish different patterns of fluctuation in the glutathione content of the root tips of cotton and maize when exposed to water deficit. Maize responded rapidly upon exposure to water deficit by elevating glutathione levels, especially GSH, presumably because of the relatively high and increasing H_2O_2 content. The increase of GSH in maize was maintained throughout the 48 h exposure time indicating a large demand for the anti-oxidant as redox-homeostasis was challenged by the severe water-deficit stress. It is also notable that the H_2O_2 content decreased in well-watered maize primary roots. The elevated level of H_2O_2 in the WW12h and WW24h samples likely reflects the initiation of root growth and a significant change of environment during transplanting even though the media was well-watered throughout.

Unlike maize, cotton primary roots generated relatively low levels of H_2O_2 when transplanted to the media and during exposure to a water deficit. It appeared that the glutathione based anti-oxidative mechanism in cotton roots initiated an increase in the

generation of glutathione to prepare for scavenging activated H_2O_2, but by 24 h glutathione levels declined as an induction of H_2O_2 production did not occur. Thus, the cotton primary root may respond to a water deficit by increasing glutathione to counter a possible increase in oxidative metabolism but once that threat is not realized it reverts to utilizing the glutathione pathway towards accumulation of the amino acids glycine, cysteine and glutamate so as to preserve the amino acid resource for other water deficit-interrupted biological processes. A decreasing trend in glutathione levels in the well-watered treatment was also observed for cotton roots. The reduction of GSH was faster than GSSG which in turn resulted in a declining trend in the ratio of GSH: GSSG with time in the well-watered treatment but not in the water-stressed treatment. Under most conditions, a low GSH: GSSG ratio is considered to be an indication of exposure to a high degree of oxidative stress (Tausz et al., 2004). However, in cotton roots, where glutathione synthesis declined and H_2O_2 levels were low, the declining trend of the ratio does not appear to be indicative of a continuous and elevated oxidative stress level. Cotton primary roots may employ an alternative or combination of anti-oxidative mechanisms to render the observed low H_2O_2 levels. It is possible that cotton roots rely on a more effective enzymatic anti-oxidation defense than maize but this requires further research.

The abundance of some of the intermediates and byproducts of glutathione metabolism also displayed interesting differences in maize and cotton roots in response to water deficit. 5-oxoproline decreased in abundance significantly in the whole growth zone of both maize and cotton primary roots. In maize, the synthesis of glutathione appears relatively active (increase in glutathione), which would suggest that 5-oxoproline was

consumed in response to the water deficit. However, in cotton, since glutathione levels were reduced in the stress treatment, the downregulation of 5-oxoproline may indicate an overall inhibition of glutathione synthesis. Ophthalmate and norophthalmate are not well studied in plants but are used as biomarkers for oxidative stress in animal and bacterial systems, and are indicative of glutathione depletion (Soga et al., 2006; Narainsamy et al., 2016). The increase of ophthalmate accompanied by the decrease in glutathione in cotton roots under water stress may indicate a similar biomarker function for ophthalmate in plants. In contrast, the increase of norophthalmate in water-stressed maize may be indicative of a severe oxidative stress as the glutathione synthase activity was high enough to elevate levels of both glutathione and the oxidative stress biomarker, norophthatlmate. Cysteinyl-glycine is oxidized in the presence of oxidative stress and thus the increased level of oxidized cysteinylglycine (cys-gly, oxidized) indicates certain levels of oxidative stress in both maize and cotton roots under water-deficit conditions. Additionally, 4-hydroxy-neoneal (4HNE) is formed during ROS-induced lipid peroxidation (Zhong & Yin, 2015). When 4HNE is formed, GSH conjugates with 4HNE, forming 4HNE-GSH to remove it from the cytoplasm and representing an antioxidative function of glutathione that is not directly targeted to ROS molecules.

The dissimilarity of the metabolite abundance response between maize and cotton for TCA cycle intermediates may point to differences in the flux of compounds through the pathways, indicating different metabolic priorities for the TCA cycle in each species. This may be illustrated in the response of the γ-aminobutyric acid (GABA) shunt, as a branch pathway of TCA cycle. GABA is an important product of the TCA cycle and is known to accumulate to high levels in plants exposed to multiple abiotic stresses,

including oxygen deficiency, acidification, drought, salinity, heat shock, low temperature, osmotic stress and mechanical stimulation (Shelp et al., 1999; Kinnersley & Turano, 2000; Bouché & Fromm, 2004). The functions of the GABA shunt include pH homeostasis (Snedden et al., 1996; Shelp et al., 1999; Essah et al., 2003) and as an anti-oxidant (Liu et al., 2011). The decline in abundance of GABA in cotton primary roots (but not in maize) may be related to a repression of the glutathione cycle in the water-stress treatment and may indicate that the regulation of pH homeostasis under water deficit stress could differ in maize and cotton roots. When succinate semialdehyde is catalyzed to succinate by SSADH in the GABA shunt pathway, the reaction also converts NAD to NADH. NADH is an essential part of the glutathione cycle and is a cofactor in the reduction of GSSG to create GSH (Noctor & Foyer, 1998). Additionally, the GABA shunt is a branch of the TCA cycle, which can convert GABA to succinate and feed into the TCA cycle. These cycle pathways are closely linked in plant metabolism, and the metabolomics data indicated that the TCA cycle was repressed in cotton in response to a water deficit in contrast to maize where α-ketoglutarate accumulated, indicating an active TCA cycle. Therefore, the repression of both the glutathione cycle and TCA cycle may indicate an adaptation to water deficit in cotton that does not employ anti-oxidative metabolites.

The metabolomics comparison between maize and cotton primary root growth zones revealed a significant and contrasting response of sulfur metabolism in the response to water deficit. The contrast is highlighted in the behavior of sulfate, glutathione and methionine derivatives. In maize, sulfate increased in the two regions of the root tip along with glutathione. In cotton, there was a significant decrease of sulfate and glutathione in

the growth zone. In a review of plant sulfur metabolism (Chan et al., 2013), it was concluded that we have little knowledge of how plants regulate sulfur metabolism to balance the "competing interests" among pathways that require sulfur containing metabolites for growth, cell homeostasis and combating the rigor of stress. In maize primary roots, it appears the focus of sulfur metabolism was to generate glutathione to combat oxidative stress and perhaps to generate cysteine, which itself may be involved in redox homeostasis. In cotton roots, sulfur metabolism appears to primarily focus on the accumulation of S-adenosylmethionine (SAM) and 5-methylthioadenosine (MTA) in region 1. SAM is the precursor of MTA and the byproduct of this reaction is 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene. As reviewed in Chapter 1, SAM is also the precursor of some polyamines that have osmoprotective functions (Bolton & Kearns, 1978; Haworth et al., 1991; Votyakova et al., 1999; Bürstenbinder et al., 2010; Chan et al., 2013). Therefore, the accumulation of SAM and MTA may play critical roles in water deficit tolerance or response mechanisms in both cotton and maize primary roots. However, these metabolites accumulated in different regions of the growth zone between the two species (Table 5-7). ACC was not measured in the metabolomics study for either species so we cannot draw direct conclusions as to possible changes in ethylene, an important plant hormone involved in drought resistance mechanisms as reviewed in Chapter 1.

The shift of sulfur metabolism pathways may reflect differences in the mechanism of water stress resistance for maize and cotton under water deficit conditions. For cotton, the significant decline of sulfate levels in both regions of the root tip is the possible reason why sulfur metabolism is focused on SAM, as this is a central metabolite involved in

many aspects of growth and cellular homeostasis (Chan et al., 2013). The reason for the decline in sulfur in cotton is unclear. It could occur biochemically as it is utilized during the response or it is also possible that the uptake of sulfur is inhibited by water-deficit stress in cotton (but not in maize), but this requires further study.

The differential accumulation of phenylpropanoids in maize and cotton roots exposed to water deficit (Figure 5-8) revealed another possible difference in the mechanisms for water stress resistance that the two species employ. There are two pathway flows starting from hydroxycinnamic acid. Maize generated 3,4-dimethoxycinnamic acid, vanillate and coniferaldehyde in stressed roots whereas cotton accumulated ferulic acid, sinapic acid and secoisolariciresinol, especially in region 1. These hydroxycinnamic acid derivatives can function as antioxidants (Yobi et al., 2013; Roleira et al., 2015), and therefore for cotton, these metabolites may be an important part of an alternative anti-oxidative mechanism to glutathione. Moreover, the two directions lead to different lignin products, G-lignin and S-lignin, and this may indicate that maize and cotton primary roots have different mechanisms to alter cell walls in response to water deficit stress.

Conclusion

The similarities and differences in the metabolic responses of maize and cotton primary roots revealed that each species employs unique mechanisms to regulate metabolites under water stressed conditions even though the tissues exhibit similar growth phenotypes and root water relations. The similarities in the types of osmolytes that respond to water deficit indicate that there are common mechanisms for osmotic adjustment in the two species. However, maize and cotton exhibit very different

perturbations of sulfur metabolism including the key antioxidant glutathione. By measuring H_2O_2 content as an indicator of ROS levels, we determined that differences in the glutathione response to water deficit in the two species might reflect significant differences in ROS exposure experienced by the roots. The results also suggested that there are other compounds in cotton that may have an anti-oxidative function or that cotton has other ROS detoxification mechanisms, e.g., enzymatic antioxidative pathways, to avoid generating or exposure to ROS.

Table 5-1. Fold changes in the abundance of metabolites in region 1 (WS1) and 2 (WS2) of the growth zone of water-stressed **maize** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Metabolites specifically changing in **region 1** are included in this table. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Amines and polyamines	N-acetylputrescine	0.55	0.88	0.39	0.84	0.43	0.84
	Aromatic amino acid metabolism	tryptamine	2.17	1.77	2.86	2.86	0.85	2.86
	Aspartate family	aspartate	0.7	0.77	0.76	1.01	0.97	1.39
		N6-carboxymethyllysine	1.56	1.93	1.02	1.29	1.29	1.55
		threonine	1.37	1.68	1.03	1.2	0.82	1.03
	BCAA - isoleucine catabolism	2-methylglutarate	1.97	1.42	0.63	0.86	1.75	1.78
	Branched Chain Amino Acids	allo-isoleucine	0.29	0.83	0.28	1	1	1
		3-hydroxy-2-ethylpropionate	1.48	1.61	0.66	0.8	0.89	1.24
		α-hydroxyisovalerate	2.17	2.51	1.17	0.97	1.45	1.42
		levulinate (4-oxovalerate)	0.56	0.51	0.69	0.74	0.68	0.74
	Glutamate family	glutamate	0.7	0.71	0.79	1.04	0.97	1.29
		homocitrulline	0.47	0.87	0.36	1.12	0.61	1.56
		ornithine	0.7	0.63	1.36	0.74	1.42	0.51
		4-hydroxy-nonenal-glutathione	14.73	8.71	3.73	1.75	1.54	2.29
		γ-glutamylglutamine	0.34	0.6	0.34	0.63	0.64	1.29
		γ-glutamylglycine	1.78	1.55	1.16	1.96	1.47	3.19
		norophthalmate*	1.58	1.7	0.72	0.81	0.82	1.78
	Serine family)	cysteine	1.58	1.82	0.97	1.45	0.84	1.44
		erythrose	1.8	2.52	1.77	1.88	2.39	2.46

Carbohydrate	Sucrose, glucose, fructose metabolism	glucoheptose	0.36	0.22	0.73	0.33	1	1
		methyl glucopyranoside (α + β)	0.53	0.57	0.62	0.81	0.68	0.67
	TCA cycle	malate	0.7	0.64	0.89	0.71	1.01	0.87
Cofactors, Prosthetic Groups, Electron Carriers	CoA metabolism	phosphopantetheine	4.59	4.59	2.9	1.01	0.55	0.58
	Nicotinate and nicotinamide metabolism	nicotinamide	0.35	0.34	0.7	0.96	0.95	0.98
		nicotinamide adenine dinucleotide (NAD+)	0.74	0.77	0.72	1.06	1.11	3.12
		nicotinate ribonucleoside	0.4	0.45	0.6	0.86	0.83	1.27
		trigonelline (N'-methylnicotinate)	0.32	0.46	0.82	1.06	1.09	1.12
	Riboflavin and FAD metabolism	flavin adenine dinucleotide (FAD)	0.31	0.39	0.57	0.9	1.02	1.72
Lipids	Fatty acid, Dicarboxylate	ethylmalonate	0.39	0.53	0.65	0.79	0.74	0.82
		malonate	1.56	1.76	1.03	1.23	1.17	1.13
		pimelate (heptanedioate)	0.72	0.49	0.84	0.52	1.19	0.77
	Free fatty acid	2-hydroxypalmitate	0.5	0.64	0.68	1.03	1.14	1.62
		2-hydroxystearate	0.42	0.58	0.63	0.94	1.19	1.66
		arachidate (20:0)	0.49	0.57	0.58	0.94	0.93	1.52
		heptanoate (7:0)	0.44	0.22	0.69	0.5	0.59	0.77
		palmitate (16:0)	0.7	0.8	0.88	1.43	1.26	1.85
	Glycerolipids	1-oleoylglycerol (18:1)	0.47	0.43	0.6	0.76	1.38	1.75
		1-palmitoylglycerol (16:0)	0.48	0.38	0.91	1.08	1.46	1.12
		2-palmitoylglycerol (16:0)	0.44	0.38	0.88	0.94	1.09	1.04
		glycerol	1.19	1.3	0.84	1.13	1.1	1.58
	Phospholipids	1-linoleoyl-GPI (18:2)*	0.19	0.32	0.69	1.78	1.78	3.77
		1-oleoyl-GPA (18:1)	0.27	0.37	0.39	0.66	0.76	1.29
		1-oleoyl-GPE (18:1)	0.48	0.65	0.27	0.58	0.51	1.03
		1-oleoyl-GPG (18:1)*	0.34	0.34	1	1	1	1
		1-oleoyl-GPI (18:1)*	0.17	0.25	0.29	1.78	0.61	1.78
		1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4)*	2.81	4.8	1.16	2.06	2.06	2.06
		1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	0.37	0.35	0.43	0.97	1.04	1.39
	Sphingolipid	sphingosine	0.1	0.12	0.41	0.17	1.21	0.56
Nucleotide	Purine metabolism	2'-deoxyadenosine	0.64	0.64	0.76	0.89	1.38	1.12
		2'-deoxyguanosine 3'-monophosphate	0.5	0.39	0.39	0.89	0.92	1.54

	Pyrimidine metabolism	3-aminoisobutyrate	1.5	2.21	0.76	1.28	0.58	0.96
		orotate	0.19	0.24	0.44	0.77	0.96	1.72
		thymidine	0.58	0.73	0.64	0.82	1.24	0.57
		thymidine 3'-monophosphate	0.39	0.33	0.45	0.83	1.05	3.54
Peptide	Dipeptide	alanylleucine	0.65	0.75	0.61	0.88	0.76	1.26
		leucylglutamine*	0.61	0.61	0.59	0.83	0.83	1.43

Table 5-2. Fold changes in the abundance of metabolites in region 1 (WS1) and 2 (WS2) of the growth zone of water-stressed **maize** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Metabolites specifically changing in **region 2** are included in this table. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Aromatic amino acid metabolism	3-(4-hydroxyphenyl)lactate	0.72	0.51	0.68	0.33	0.57	0.31
		4-hydroxyphenylpyruvate	1	1	0.29	0.21	0.18	0.15
		N-acetylphenylalanine	0.36	0.25	0.32	0.27	0.4	0.36
		phenylalanine	1	0.84	0.86	0.69	0.51	0.55
		quinate	1.53	0.95	2.54	1.49	2.74	1.87
		tyrosine	0.93	0.9	0.69	0.55	0.37	0.39
	Aspartate family	alanine	1.17	1.16	1.5	1.25	0.83	0.81
		homoserine	0.76	1.48	0.57	0.88	0.56	0.69
		homoserine lactone	0.71	0.64	0.71	0.68	0.4	0.41
		methionine	0.62	0.92	0.35	0.38	0.22	0.29
		S-adenosylmethionine (SAM)	1.15	1.51	1.43	1.7	1.34	1.95
	Branched Chain Amino Acids	2,3-dihydroxyisovalerate	0.51	0.78	0.22	0.42	0.19	0.29
		leucine	0.88	0.52	0.69	0.44	0.43	0.35
		N-acetylleucine	0.38	0.2	0.41	0.3	0.41	0.48
	Glutamate family	2-pyrrolidinone	0.85	1.15	3.42	6.04	8.23	8.78
	Glutathione metabolism	γ-glutamylalanine	1.43	1.91	2.53	3.42	2.9	4.8
		γ-glutamylphenylalanine	1.25	1.33	1.44	1.47	1.66	2.05
Carbohydrate	Amino sugar and nucleotide sugar	ribulose/xylulose 5-phosphate	2.94	1.41	0.31	0.3	0.35	0.54

	Glycolysis	fructose-6-phosphate	1.15	0.94	0.25	0.19	0.18	0.21
		glucuronate	0.85	0.8	0.77	0.65	0.67	0.56
	Sucrose, glucose, fructose metabolism	mannose-6-phosphate	1.16	0.88	0.36	0.52	0.4	0.54
	TCA cycle	2-methylcitrate/homocitrate	0.83	1.35	5.02	5.02	5.02	3.83
		mesaconate (methylfumarate)	1.27	1.2	0.73	0.73	0.7	0.67
Cofactors, Prosthetic Groups, Electron Carriers	Nicotinate and nicotinamide metabolism	nicotinate	0.99	1.65	1.53	2.16	2.37	2.28
	Oxidative phosphorylation	acetylphosphate	1.99	1.04	0.46	0.34	0.37	0.36
	Thiamine metabolism	thiamin (Vitamin B1)	0.8	1.54	2.08	2.25	2.41	2.41
Hormone metabolism	Auxin metabolism	2-oxindole-3-acetate	0.83	1.05	0.49	0.66	0.41	0.68
Lipids	Fatty acid, Dicarboxylate	azelate (nonanedioate)	0.69	1.15	1.86	2.62	2.43	2.44
	Oxylipins	13-HODE + 9-HODE	0.69	0.39	0.67	0.77	1.18	1.3
	Phospholipids	1-palmitoyl-GPG (16:0)*	0.63	0.63	1.41	2.19	2.7	5.39
		1-palmitoyl-GPI (16:0)*	0.91	0.98	2.6	2.62	2.44	3.56
	Sterols	ergosterol	1.19	1.19	1.29	1.29	1.29	1.29
Nucleotide	Purine metabolism	adenosine 2'-monophosphate (2'-AMP)	1	0.68	0.53	0.57	0.55	0.47
		adenosine-2',3'-cyclic monophosphate	0.86	0.3	0.62	0.41	0.29	0.18
		guanosine 2'-monophosphate (2'-GMP)*	1.94	0.85	0.77	0.73	0.69	0.75
		guanosine 3'-monophosphate (3'-GMP)	0.66	0.14	0.15	0.14	0.07	0.07
		guanosine-2',3'-cyclic monophosphate	1.48	0.53	0.63	0.46	0.32	0.22
		xanthine	1.33	1.13	0.38	0.18	0.3	0.19
	Pyrimidine metabolism	cytidine 2',3'-cyclic monophosphate	0.87	0.29	0.76	0.58	0.47	0.41
		cytidine 3'-monophosphate (3'-CMP)	1.25	0.42	0.81	0.78	0.64	0.77
		uridine 2'-monophosphate (2'-UMP)*	0.76	0.36	0.62	0.59	0.63	0.75
		uridine 3'-monophosphate (3'-UMP)	1.01	0.38	0.69	0.71	0.71	0.81
		uridine-2',3'-cyclic monophosphate	0.84	0.3	0.66	0.52	0.44	0.33

Table 5-3. Fold changes in the abundance of metabolites that significantly changed in both **regions 1 and 2** (WS1 and WS2) of the growth zone of water-stressed **maize** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10). No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Amines and polyamines	nicotianamine	0.74	0.37	0.46	0.25	0.65	0.46
	Aromatic amino acid metabolism	N-formylphenylalanine	0.1	0.08	0.08	0.11	0.08	0.17
		N-methylphenylalanine	21.27	31.6	11.72	9.74	5.64	8.44
		O-methyl tyrosine	0.65	0.45	0.53	0.63	0.58	0.74
		phenylpyruvate	0.47	0.31	0.18	0.16	0.29	0.28
		shikimate	2.45	1.45	5.82	3.42	5.89	4.43
	Aspartate family	2-aminoadipate	0.09	0.12	0.17	0.28	0.22	0.43
		2-piperidinone	1.24	1.13	1.19	1.26	1.2	1.23
		6-oxopiperidine-2-carboxylic acid	2.03	2.19	2.41	3.03	2.09	3.1
		asparagine	3.43	4.3	2.32	2.49	1.66	1.88
		methionine sulfoxide	0.31	0.29	0.29	0.27	0.21	0.24
		N6-acetyllysine	0.33	0.35	0.39	0.48	0.47	0.74
		N-acetylasparagine	0.08	0.14	0.11	0.21	0.15	0.32
		N-acetylaspartate (NAA)	0.21	0.2	0.27	0.36	0.35	0.5
		N-formylmethionine	0.4	0.59	0.08	0.14	0.1	0.23
		pipecolate	1.72	5.53	1.96	6.72	1.48	5.92
		saccharopine	18.21	20.8	9.29	12.49	11.68	32.1
	Branched Chain Amino Acids	3-methyl-2-oxobutyrate	3.31	10.39	1.97	1.99	2.28	1.81
		β-hydroxyisovalerate	0.61	0.36	0.38	0.26	0.65	0.53
		N-methylleucine	7.94	5.95	5.2	4.03	3.27	3.52
		norvaline	0.45	0.54	0.41	0.52	0.54	0.59
	Glutamate family	1,3-diaminopropane	0.47	0.58	0.3	0.39	0.43	0.55
		4-hydroxy-2-oxoglutaric acid	0.16	0.18	0.25	0.28	0.76	0.45

		dimethylarginine (SDMA + ADMA)	0.55	0.49	0.54	0.51	0.5	0.66
		γ-aminobutyrate (GABA)	2.05	3.52	3.24	4.43	3.67	4.52
		glutamine	0.47	0.57	0.33	0.39	0.31	0.39
		histidine	0.49	0.73	0.64	0.81	0.51	0.65
		N-acetylglutamine	0.26	0.29	0.19	0.15	0.52	0.37
		N-methylglutamate	1.32	1.95	1.32	1.54	1.47	1.73
		N-methylproline	1.95	4.02	1.87	4.88	1.92	5.73
		proline	19.79	12.89	5.99	4.69	3.77	4.06
		trans-4-hydroxyproline	3.14	3.79	1.89	2.53	1.99	2.52
	Glutathione metabolism	5-oxoproline	0.47	0.58	0.37	0.45	0.27	0.38
		γ-glutamylthreonine*	1.52	2.21	1.92	2.51	2.51	4.05
		γ-glutamyltryptophan	2.09	2.94	3.63	5.22	4.07	6.98
		S-methylglutathione	2.49	2.41	1.39	2.04	1.8	2.72
	Serine family	homocysteine	0.36	0.42	0.15	0.16	0.12	0.14
		N-acetyltaurine	1.57	2.73	1.71	2.92	2.14	3.08
		O-acetylserine	0.47	0.58	0.09	0.18	0.1	0.21
		S-carboxymethyl-L-cysteine	0.26	0.26	0.34	0.35	0.43	0.46
		serine	2.58	2.58	2.04	2.2	1.38	1.66
		sulfate*	1.18	1.25	1.23	1.2	1.18	1.09
		taurine	3.15	2.85	2.1	1.74	2.06	1.88
Carbohydrate	Amino sugar and nucleotide sugar	maltol	0.54	0.46	0.26	0.23	0.45	0.73
		ribonate	0.44	0.38	0.45	0.43	0.57	0.4
		xylitol	0.73	0.75	0.58	0.67	0.61	0.68
		xylulose	0.65	0.59	0.47	0.54	0.61	0.69
	C5 branched dibasic acid metabolism	itaconate (methylenesuccinate)	0.35	0.57	0.32	0.38	0.4	0.44
	Calvin cycle and pentose phosphate	ribose 1-phosphate	0.53	0.5	0.24	0.3	0.35	0.47
		ribose 5-phosphate	0.61	0.6	0.3	0.37	0.45	0.62
	Glycolysis	glucose	2.03	1.5	1.56	1.26	1.37	1.19
		pyruvate	0.5	0.55	0.48	0.51	0.7	0.7
	Inositol metabolism	myo-inositol	1.45	1.45	1.64	1.78	1.63	1.96
	Sucrose, glucose, fructose metabolism	1-kestose	5	2.56	7.6	3.49	6.96	3.78
		fructose	2.96	2.26	2.8	2.02	2.28	2.1
		raffinose	10.42	10.42	7.61	7.61	7.61	5.03
		sucrose	8.63	15.26	5.36	5.16	4.66	4.33
		sucrose-6-phosphate	2.34	2.34	1.92	1.92	1.92	1.92
	TCA cycle	α-ketoglutarate	3.49	3.64	2.02	2.52	2.59	3.19

		cis-aconitate	0.55	0.71	0.7	0.78	0.85	0.82
		citrate	0.33	0.42	0.4	0.4	0.58	0.48
		trans-aconitate	0.56	0.71	0.69	0.78	0.86	0.82
Cofactors, Prosthetic Groups, Electron Carriers	Ascorbate metabolism	gulonic acid*	0.46	0.81	0.4	0.56	0.42	0.51
		threonate	0.52	0.62	0.61	0.75	0.65	0.76
	Nicotinate and nicotinamide metabolism	nicotinamide riboside	0.29	0.25	0.39	0.46	0.46	0.24
	Riboflavin and FAD metabolism	riboflavin (Vitamin B2)	0.46	0.54	0.57	0.71	0.64	0.76
Lipids	Fatty acid, Dicarboxylate	adipate	0.75	0.55	0.66	0.54	0.84	0.74
		dodecanedioate	0.4	0.26	0.19	0.2	0.29	0.37
	Free fatty acid	oleate (18:1n9)	0.59	0.55	0.38	0.51	0.6	0.81
	Glycerolipids	glycerophosphoglycerol	26.69	28.09	7.62	8.53	5.05	8.18
	Phospholipids	glycerol 3-phosphate	6.12	6.87	1.88	2.43	1.96	2.94
		glycerophosphoethanolamine	29.04	34.8	13.25	18.23	7.38	10.21
		glycerophosphoinositol*	6.08	7.15	3.55	3.99	3.09	4.74
		glycerophosphorylcholine (GPC)	237	439.83	301.06	698.94	94.85	274.26
		phosphoethanolamine	9.59	3.38	3.6	2.15	2.64	2.27
	Sphingolipid	sphinganine	0.17	0.19	0.11	0.08	0.09	0.17
	Sterols	3-hydroxy-3-methylglutarate	0.53	0.6	0.52	0.52	0.6	0.55
		campesterol	0.58	0.56	0.46	0.53	0.59	0.7
		fucosterol	0.57	0.42	0.32	0.27	0.39	0.36
		stigmasterol	0.41	0.47	0.39	0.5	0.46	0.57
Nucleotide	Purine metabolism	adenosine	0.59	0.62	0.6	0.66	0.73	0.72
		allantoic acid	0.61	0.38	0.16	0.17	0.17	0.22
		allantoin	3.38	2.7	2.11	1.64	1.2	1.14
		inosine	0.28	0.26	0.16	0.22	0.53	0.63
		N2,N2-dimethylguanosine	2.92	2.96	1.37	2.08	4.34	3.65
		urate	0.34	0.19	0.49	0.32	0.35	0.34
		xanthosine	0.37	0.32	0.25	0.22	0.31	0.33
	Pyrimidine metabolism	cytidine	0.51	0.5	0.65	0.76	0.75	0.78
		cytosine	0.11	0.11	0.12	0.16	0.18	0.29
		ectoine	0.08	0.05	0.13	0.12	0.25	0.14
Peptide	Dipeptide	cys-gly, oxidized	2.26	7.44	4.84	8.3	8.85	6.3
		phenylalanylalanine	0.42	0.49	0.4	0.6	0.47	0.77
Secondary metabolism	Fatty acid and sugar derivatives	galactarate (mucic acid)	0.55	0.65	0.63	0.56	0.91	0.71
	Phenylpropanoids	3,4-dimethoxycinnamic acid	2.89	6.58	4.93	18.82	5.43	16.25

		vanillate	1.89	1.81	1.79	2.73	2.26	2.28
	Terpenoids	mevalonate	0.52	0.53	0.38	0.38	0.63	0.75
		mevalonolactone	0.31	0.26	0.26	0.18	0.48	0.5
Xenobiotics	Chemicals	succinimide	0.2	0.19	0.28	0.33	0.44	0.42

Table 5-4. Fold changes in the abundance of metabolites in region 1 (WS1) and 2 (WS2) of the growth zone of water-stressed **cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Metabolites specifically changing in **region 1** are included in this table. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Aspartate family	methionine	4.28	1.87	1.91	1.19	1.13	1.04
		methionine sulfoxide	2.44	1.41	1.4	1.11	1.45	1
	Branched Chain Amino Acids	leucine	2.19	1.44	1.66	1.12	1.22	0.96
	Glutamate family	N-δ-acetylornithine	0.77	0.87	0.92	1.48	0.67	1.14
		trans-4-hydroxyproline	3.41	4.36	1.49	2.2	1.66	3.73
	Serine family	N-acetyltaurine	0.81	0.83	1.28	1.16	1.17	1.46
Carbohydrate	Glycolysis	glucose 6-phosphate	0.68	0.5	1.12	1.23	1.23	1.12
		lactate	2.21	1.62	1.98	1.12	1.61	1.24
		pyruvate	2.57	1.3	2.76	0.94	1.02	0.6
	Inositol metabolism	inositol 1-phosphate (I1P)	0.38	0.43	0.57	0.76	0.94	0.93
	Sucrose, glucose, fructose metabolism	galacturonate	4.04	3.23	0.81	0.42	1	0.73
		mannose	0.74	0.73	0.54	1	1	1
		mannose-6-phosphate	0.72	0.61	0.92	1.38	1.2	1.57
	TCA cycle	aconitate [cis or trans]	0.44	0.17	1.55	0.38	1.17	0.3
		citrate	0.61	0.24	8.69	0.59	1.9	0.32
		isocitrate	0.35	0.12	0.73	0.36	1.29	0.81
Cofactors, Prosthetic Groups, Electron Carriers	Vitamin B metabolism (B6 or B12)	pyridoxal phosphate	0.38	0.38	0.7	0.53	0.66	0.87
Hormone metabolism	Auxin metabolism	indoleacetylaspartate	0.45	0.41	1.08	0.94	1.12	1.09
Lipids		adipate	1.69	1.27	1.25	1.27	1.32	1.35

	Fatty acid, Dicarboxylate	suberate (octanedioate)	0.68	0.73	0.65	1.06	0.78	1.15
	Phospholipids	1-oleoyl-GPG (18:1)*	0.33	0.63	1	1	1	1
		1-palmitoleoyl-2-linolenoyl-GPC (16:1/18:3)*	1.64	1.67	0.58	0.61	0.68	2.23
		1-palmitoleoyl-GPA (16:1)*	0.3	0.45	0.28	0.54	0.39	0.53
		1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	1.76	1.38	0.63	0.53	0.87	1.36
		1-stearoyl-GPA (18:0)	0.12	0.28	0.18	0.39	0.32	0.26
		2-palmitoleoyl-GPC (16:1)*	2	2.04	0.83	0.55	0.81	1.67
Nucleotide	Purine metabolism	1-methylguanine	0.57	0.43	1.12	1.08	1.12	0.58
		2'-deoxyinosine	0.17	0.25	0.14	0.26	0.41	0.49
		guanine	0.71	0.63	1.27	1.29	1.37	1
		N1-methyladenosine	0.33	0.36	0.98	0.8	0.83	0.44
		N2-methylguanosine	0.4	0.48	0.89	1.02	0.94	0.8
		N6-carbamoylthreonyladenosine	0.75	0.76	0.92	0.97	0.98	0.89
		xanthosine 5'-monophosphate (xmp)	0.36	0.76	1	1	1	1
	Pyrimidine metabolism	cytidine 3'-monophosphate (3'-CMP)	0.6	0.58	0.82	0.99	0.95	0.77
		cytidine diphosphate	0.34	0.2	0.55	1	1	1
		pseudouridine	0.42	0.39	1.06	0.95	0.91	0.56
		uridine 2'-monophosphate (2'-UMP)*	0.61	0.61	0.91	1.04	0.83	1.11
Secondary metabolism	Phenylpropanoids	sinapate	3.82	3.82	0.42	0.8	0.6	1.16
Xenobiotics	Chemicals	thioproline	0.31	0.31	0.96	0.58	0.44	1

Table 5-5. Fold changes in the abundance of metabolites in region 1 (WS1) and 2 (WS2) of the growth zone of water-stressed **cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Metabolites specifically changing in **region 2** are included in this table. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Amines and polyamines	agmatine	0.5	0.97	0.15	0.19	0.08	0.14
		N-acetylputrescine	0.54	1.13	0.49	0.6	0.4	0.4
		spermidine	1.43	0.51	0.05	0.12	0.08	0.05
	Aromatic amino acid metabolism	N-acetylphenylalanine	0.65	0.84	0.41	0.6	0.45	0.64
		N-acetyltryptophan	1.21	1.42	1.63	2.28	1.72	2.48
		phenylpyruvate	1.38	0.66	0.26	0.32	0.25	0.31
		tryptophan	1.22	0.95	2.18	1.44	1.5	1.18
	Aspartate family	2-oxoadipate	7.95	1.21	3.25	1.18	1.6	1.53
		6-oxopiperidine-2-carboxylate	3.11	1.14	3.66	1.74	1.75	1.78
		N-acetylasparagine	0.88	1.35	0.48	0.59	0.55	0.7
		N-acetylmethionine sulfoxide	0.89	0.92	0.27	0.43	0.5	0.48
		N-formylmethionine	0.85	0.69	0.49	0.51	0.68	0.71
	Branched Chain Amino Acids	2-isopropylmalate	0.47	0.44	0.32	0.28	0.44	0.35
		3-methyl-2-oxobutyrate	1.48	0.71	0.52	0.51	0.76	0.84
		3-methyl-2-oxovalerate	3.72	0.67	0.57	0.39	0.81	0.75
		4-methyl-2-oxopentanoate	1.51	0.65	0.49	0.46	0.65	0.73
	Glutamate family	arginine	1.35	0.93	0.65	0.46	0.42	0.33
		argininosuccinate	0.78	0.53	0.12	0.12	0.43	0.49
		histamine	0.72	0.69	0.25	0.4	0.18	0.27
		imidazole lactate	2.73	0.92	3.1	1.45	2.29	1.47
		N-acetylglutamine	0.39	1.13	0.21	0.42	0.28	0.5

		N-α-acetylornithine	1.1	1.22	1.15	1.18	1.29	1.67
	Glutathione metabolism	γ-glutamylglutamine	0.42	0.77	0.09	0.31	0.17	0.64
		γ-glutamylhistidine	0.91	0.36	0.56	0.52	0.86	0.87
		γ-glutamyltryptophan	1.25	1	1.67	2.14	2.16	2.72
		γ-glutamyltyrosine	1.98	1.23	2.04	1.85	3.32	3.64
	Serine family	sulfate*	0.91	0.44	0.83	0.46	0.55	0.33
		glycine	1.03	0.93	0.52	0.48	0.29	0.3
Carbohydrate	Amino sugar and nucleotide sugar	UDP-galactose	0.87	0.63	0.53	0.41	0.22	0.24
		xylose	1.1	1.11	0.67	0.76	0.49	0.61
	Calvin cycle and pentose phosphate	3-phosphoglycerate	0.89	0.86	0.24	0.31	0.45	0.58
		sedoheptulose-7-phosphate	1.15	0.73	0.44	0.27	0.57	0.55
	Sucrose, glucose, fructose metabolism	6-phosphogluconate	1.02	0.49	0.08	0.06	0.13	0.08
		maltose	0.98	0.71	0.5	0.45	0.58	0.56
		methyl glucopyranoside (α + β)	1.19	1.41	2.4	2.87	2.87	1.94
	TCA cycle	fumarate	1.08	0.8	0.71	0.48	0.6	0.44
		isocitric lactone	0.86	0.42	0.29	0.29	0.2	0.23
		maleate	1.18	0.85	0.41	0.26	0.38	0.39
Cofactors, Prosthetic Groups, Electron Carriers	Folate Metabolism	biopterin	1	1	0.41	0.62	0.39	0.47
	Thiamine metabolism	thiamin monophosphate	0.62	0.67	0.14	0.17	0.18	0.17
	Tocopherol metabolism	α-tocopherol	1.23	1.22	0.46	0.54	0.54	0.5
	Vitamin B metabolism (B6 or B12)	pyridoxate	1.94	0.93	2.81	2.21	1.95	1.8
Hormone metabolism	Auxin metabolism	indoleacetate	1.1	1.23	0.6	0.57	0.4	0.42
Lipids	Fatty acid amide	oleoyl ethanolamide	0.64	1.04	0.13	0.31	0.25	0.48
		palmitoyl ethanolamide	0.67	1.66	0.2	0.55	0.31	0.63
	Fatty acid ester	oleoylcholine	0.81	1.15	0.1	0.26	0.15	0.4
	Free fatty acid	2-hydroxyglutarate	1.13	0.68	0.63	0.41	0.57	0.46
		linoleate (18:2n6)	0.21	0.54	0.06	0.14	0.15	0.12
	Galactolipids	1,2-dilinoleoyl-digalactosylglycerol (18:2/18:2)*	0.91	0.97	0.49	0.47	0.48	0.45
		1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3)*	0.95	1.04	0.39	0.4	0.42	0.45
		1-linoleoyl-2-linolenoyl-galactosylglycerol (18:2/18:3)*	0.66	0.91	0.29	0.4	0.34	0.39
		1-palmitoyl-2-linolenoyl-	0.62	0.99	0.52	0.76	0.51	0.63

		digalactosylglycerol (16:0/18:3)						
		1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3)*	0.58	0.89	0.5	0.57	0.41	0.41
		1-palmitoyl-2-linoleoyl-galactosylglycerol (16:0/18:2)*	1.01	0.92	0.51	0.43	0.5	0.4
	Glycerolipids	diacylglycerol (16:1/18:2 [2], 16:0/18:3 [1])*	1.11	1.04	0.21	0.27	0.37	0.53
		linolenoyl-linolenoyl-glycerol (18:3/18:3) [1]*	0.85	0.95	0.11	0.28	0.29	0.5
		linoleoyl-linolenoyl-glycerol (18:2/18:3) [1]*	0.99	0.86	0.13	0.2	0.34	0.46
		linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]*	1.45	1.17	0.33	0.48	0.57	0.66
		linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]*	0.81	0.58	0.22	0.32	0.52	0.45
		oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	1.15	0.93	0.21	0.21	0.36	0.41
		palmitoyl-linolenoyl-glycerol (16:0/18:3) [2]*	1.4	1.45	0.18	0.18	0.25	0.46
		palmitoyl-linoleoyl-glycerol (16:0/18:2) [1]*	1.02	0.9	0.35	0.39	0.59	0.52
		palmitoyl-linoleoyl-glycerol (16:0/18:2) [2]*	1.22	1.1	0.32	0.3	0.43	0.44
	Oxylipins	12,13-DiHOME	0.14	0.44	0.12	0.35	0.11	0.44
		9,10-DiHOME	1.07	1.54	0.32	0.79	0.27	0.75
	Phospholipids	1,2-dilinoleoyl-GPE (18:2/18:2)*	0.89	0.65	0.28	0.24	0.47	0.55
		1,2-dioleoyl-GPA (18:1/18:1)*	0.57	0.48	0.26	0.25	0.43	0.44
		1-linoleoyl-GPG (18:2)*	0.49	0.7	0.16	0.14	0.21	0.32
		1-oleoyl-2-linoleoyl-GPE (18:1/18:2)*	0.81	0.6	0.3	0.26	0.45	0.45
		1-oleoyl-2-linoleoyl-GPG (18:1/18:2)*	0.71	0.79	0.26	0.2	0.34	0.39
		1-oleoyl-GPC (18:1)	0.67	0.75	0.18	0.17	0.34	0.5
		1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	0.88	0.7	0.4	0.36	0.54	0.57
		1-palmitoyl-2-linoleoyl-GPG (16:0/18:2)	0.85	0.9	0.29	0.21	0.44	0.56
		1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	0.91	0.56	0.27	0.22	0.48	0.44

		1-palmitoyl-2-oleoyl-GPG (16:0/18:1)	0.81	0.78	0.25	0.2	0.46	0.53
		1-palmitoyl-2-oleoyl-GPI (16:0/18:1)*	0.94	0.77	0.26	0.23	0.53	0.64
		1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	0.84	0.68	0.29	0.21	0.56	0.46
		1-palmitoyl-GPI (16:0)	0.67	0.71	0.37	0.4	0.4	0.57
		1-stearoyl-2-linoleoyl-GPA (18:0/18:2)*	0.77	0.66	0.41	0.44	0.42	0.4
		1-stearoyl-2-oleoyl-GPC (18:0/18:1)	1.16	0.85	0.21	0.15	0.47	0.41
		1-stearoyl-GPC (18:0)	0.5	0.66	0.25	0.2	0.31	0.43
		glycerophosphoethanolamine	1.5	0.97	1.85	1.48	2.2	1.95
		glycerophosphoinositol*	1.06	0.62	1.36	1.34	2.21	2.51
	Sphingolipid	phytosphingosine	1.37	0.95	0.49	0.37	0.6	0.48
		sphinganine	1.11	0.66	0.28	0.16	0.47	0.28
Nucleotide	Purine metabolism	allantoic acid	1.3	0.69	0.42	0.42	0.27	0.37
		allantoin	0.79	0.71	0.7	0.61	0.49	0.57
		hypoxanthine	0.3	0.97	0.59	0.6	0.29	0.48
		inosine	0.75	1.74	0.4	0.47	0.32	0.47
		inosine 5'-monophosphate (IMP)	2.07	2.56	0.17	0.29	0.44	0.54
		N2,N2-dimethylguanosine	0.52	0.44	0.69	0.51	0.66	0.45
	Pyrimidine metabolism	cytidine 2',3'-cyclic monophosphate	0.98	0.77	2.65	3.14	2.21	1.91
		dihydroorotate	1.31	0.55	0.6	0.39	0.44	0.33
		uridine 5'-monophosphate (UMP)	0.98	0.71	0.46	0.47	0.53	0.62
		uridine-2',3'-cyclic monophosphate	1.11	0.77	1.88	1.94	2.43	1.61
Peptide	Dipeptide	alanylleucine	1.41	1.19	0.74	0.76	0.65	0.67
		glycylleucine	1.2	0.93	0.54	0.67	0.67	0.75
		valylglycine	1.28	1.43	0.68	0.8	0.67	0.71
Secondary metabolism	Phenylpropanoids	vanillate	0.76	0.71	0.32	0.36	0.38	0.62

Table 5-6. Fold changes in the abundance of metabolites that significantly changed in both **regions 1 and 2** (WS1 and WS2) of the growth zone of water-stressed **cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10). No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Amines and polyamines	5-methylthioadenosine (MTA)	1.7	1.42	0.54	0.59	0.62	0.64
		N(4)-acetylspermidine	0.54	0.34	0.12	0.11	0.12	0.09
		putrescine	0.33	0.4	0.03	0.03	0.02	0.02
	Aromatic amino acid metabolism	3-(4-hydroxyphenyl)lactate	4.12	3.99	8.78	4.05	5.01	3.31
		N-formylphenylalanine	0.41	0.36	0.33	0.38	0.34	0.53
		phenylacetate	0.42	0.44	0.26	0.35	0.31	0.34
		phenylalanine	0.72	0.72	0.44	0.5	0.32	0.42
		quinate	0.6	0.41	0.57	0.4	0.64	0.44
		shikimate	0.5	0.52	0.3	0.35	0.31	0.37
		tryptamine	2.35	7.58	2.91	4.87	1.63	2.27
		tyramine	1.48	5.33	2.45	4.26	1.49	2.21
	Aspartate family	2-aminoadipate	3.27	1.71	2	1.63	1.58	2.37
		2-hydroxyadipate	3.36	1.49	2.78	1.66	3.15	2.1
		alanine	1.35	2.33	0.71	0.82	0.4	0.49
		aspartate	0.4	0.55	0.54	0.53	0.71	0.69
		lysine	2.48	2.09	2.06	1.63	1.35	1.15
		N6-acetyllysine	5.15	2.34	3.89	2.11	1.72	1.41
		N6-formyllysine	0.4	0.33	0.35	0.35	0.25	0.3
		N-acetyl-β-alanine	3.97	2.21	2.66	1.68	3.01	2.28
		N-methylpipecolate	2	5.84	2	7.24	2.3	7.14
		pipecolate	7.07	10.44	3.89	6.28	3.64	6.37
		saccharopine	13.13	4.66	4.92	4.4	2.3	4.9
		S-adenosylhomocysteine (SAH)	0.4	0.46	0.19	0.38	0.28	0.61

		S-adenosylmethionine (SAM)	3.28	1.89	0.43	0.51	0.44	0.48
	Branched Chain Amino Acids	2,3-dihydroxyisovalerate	0.57	0.59	0.17	0.2	0.14	0.21
		2-isopropylmalate	0.47	0.44	0.32	0.28	0.44	0.35
		α-hydroxyisovalerate	2	1.55	1.75	1.36	2.32	2.31
		β-hydroxyisovalerate	8.04	2.06	4.47	1.44	5.42	2.38
		methylsuccinate	5.77	4.85	2.28	2.02	1.55	1.69
	Glutamate family	1-methylhistidine	2.65	6.43	2.74	6.45	2.74	4.78
		1-methylimidazoleacetate	2.21	2.98	1.65	2.25	1.35	2.07
		3-methylhistidine	1.57	2.3	1.41	2.02	1.5	2.13
		β-guanidinopropanoate	1.46	3.6	1.59	4.05	1.22	3.12
		carboxyethyl-GABA	2.11	1.72	1.65	1.36	1.85	1.69
		γ-aminobutyrate (GABA)	0.69	0.68	0.47	0.53	0.53	0.61
		glutamine	0.35	0.72	0.16	0.28	0.12	0.23
		guanidinoacetate	6.77	6.77	3.77	7.95	3.2	7.95
		histidine	0.74	0.32	0.61	0.42	0.49	0.38
		homocitrulline	1.43	1.85	1.76	3.11	1.71	3.27
		N-acetylarginine	0.63	0.52	0.39	0.4	0.53	0.48
		N-acetylproline	4.94	4.35	3.97	3.97	6.95	5.28
		N-methylproline	2.84	1.96	2.41	2.04	2.69	2.34
		N-monomethylarginine	0.68	0.52	0.62	0.53	0.44	0.41
		proline	4.93	6.3	3.96	6.37	3.63	6.18
		pyroglutamine*	5.81	18.51	3.98	11.61	2.99	10.3
		stachydrine	5.6	7.75	2.83	4.23	2.52	4.62
	Glutathione metabolism	5-oxoproline	0.52	0.75	0.32	0.53	0.28	0.43
		cysteine-glutathione disulfide	0.59	0.56	0.5	0.51	0.3	0.39
		γ-glutamyl-epsilon-lysine	3.23	2.91	1.52	1.34	1.66	1.94
		γ-glutamylleucine	3.29	1.7	1.26	1.38	1.36	1.7
		glutathione, oxidized (GSSG)	0.65	0.7	0.48	0.5	0.4	0.53
		glutathione, reduced (GSH)	0.77	0.64	0.37	0.39	0.36	0.61
		S-methylglutathione	0.59	0.39	0.73	0.54	0.68	0.58
	Serine family	betaine	4.43	3.96	2.81	2.89	2.5	2.67
		betaine aldehyde	6.55	3.09	18.92	9.28	17.71	7.92
		dimethylglycine	3.46	4.28	1.85	2.99	1.81	2.61

		N-acetylserine	0.57	0.52	0.39	0.3	0.49	0.41
		S-carboxymethyl-L-cysteine	0.76	0.51	0.61	0.46	0.76	0.65
		taurine	3.01	4.32	3.07	4.58	2.81	5.43
Carbohydrate	Amino sugar and nucleotide sugar	arabonate/xylonate	1.76	1.53	1.46	1.41	1.43	1.37
		ribose	0.58	0.65	0.41	0.65	0.47	0.55
		UDP-N-acetylgalactosamine	0.74	0.62	0.52	0.58	0.65	0.76
		UDP-N-acetylglucosamine	0.77	0.56	0.36	0.37	0.63	0.71
	C5 branched dibasic acid metabolism	citraconate/glutaconate	0.55	0.53	0.61	0.55	0.65	0.66
	Glycolysis	glucose	2.99	2.81	1.91	1.86	1.44	1.63
		glucuronate	0.49	0.71	0.44	0.62	0.39	0.6
	Inositol metabolism	myo-inositol	2.54	2.64	1.95	2.33	1.83	2.33
	Photorespiration	oxalate (ethanedioate)	0.84	0.83	0.75	0.81	0.79	0.74
	Sucrose, glucose, fructose metabolism	3-deoxyoctulosonate	0.74	0.71	0.49	0.55	0.48	0.51
		fructose	3.48	3.04	2.04	1.94	1.51	1.76
		galactinol	5.82	4.33	16.09	18.89	14.18	22.16
		melibiose	3.71	3.26	9.67	10.89	10.5	13.99
		raffinose	37.07	16.45	143.86	55.47	39.09	47.99
		sucrose	2.23	7	15.91	18.82	13.01	21.58
		trehalose	1.44	2.14	1.48	2.49	1.65	2.67
	TCA cycle	succinate	0.85	0.64	0.72	0.48	0.58	0.46
Cofactors, Prosthetic Groups, Electron Carriers	Ascorbate metabolism	threonate	0.83	0.71	0.83	0.72	0.74	0.61
	Carnitine metabolism	deoxycarnitine	3.01	19.08	2.75	11.99	2.44	8.31
	Nicotinate and nicotinamide metabolism	nicotinamide adenine dinucleotide (NAD+)	0.6	0.63	0.35	0.42	0.4	0.55
		nicotinate	0.41	0.43	0.53	0.56	0.45	0.43
		nicotinate ribonucleoside	0.78	0.72	0.71	0.59	0.69	0.6
		quinolinate	0.4	0.3	0.32	0.28	0.31	0.29
		trigonelline (N'-methylnicotinate)	1.73	2.91	2.26	3.71	2.09	3.2
	Riboflavin and FAD metabolism	flavin adenine dinucleotide (FAD)	0.5	0.52	0.34	0.51	0.57	0.53
		flavin mononucleotide (FMN)	0.4	0.4	0.38	0.6	0.63	0.51
		riboflavin (Vitamin B2)	0.65	0.73	0.46	0.41	0.46	0.47
	Thiamine metabolism	thiamin (Vitamin B1)	0.53	0.5	0.55	0.66	0.53	0.51
	Vitamin B metabolism	pyridoxal	0.56	0.54	0.33	0.4	0.4	0.51
		pyridoxamine	0.63	0.66	0.48	0.62	0.51	0.6

Hormone metabolism	Abscisic acid metabolism	abscisate	4.83	5.68	3.21	5.42	2.78	5.02
Lipids	Choline metabolism	cytidine 5'-diphosphocholine	0.53	0.49	0.13	0.18	0.34	0.47
	Fatty acid amide	palmitic amide	0.48	0.42	0.31	0.17	0.37	0.14
	Fatty acid, Amino	2-aminoheptanoate	5.09	1.8	5.11	1.52	4.19	1.5
	Fatty acid, Dicarboxylate	3-methylglutarate/2-methylglutarate	3.21	1.95	2.04	1.26	2.04	1.43
		azelate (nonanedioate)	0.75	0.86	0.5	0.55	0.76	0.76
		malonate	2.06	2	2.05	1.91	1.63	1.48
		pimelate (heptanedioate)	11	2.99	6.86	2.34	7.25	3.13
	Free fatty acid	3-hydroxybutyrate (BHBA)	0.8	0.77	0.37	0.36	0.47	0.51
		linolenate [α or γ; (18:3n3 or 6)]	0.24	0.57	0.06	0.15	0.15	0.17
		pelargonate (9:0)	0.58	0.59	0.46	0.5	0.58	0.84
	Galactolipids	1,2-dilinolenoyl-digalactosylglycerol (18:3/18:3)	0.67	0.77	0.25	0.27	0.33	0.41
		1,2-dilinolenoyl-galactosylglycerol (18:3/18:3)*	0.55	0.65	0.34	0.39	0.35	0.38
		1,2-dilinoleoyl-galactosylglycerol (18:2/18:2)*	0.75	0.76	0.29	0.29	0.35	0.39
	Phospholipids	1,2-dilinolenoyl-GPA (18:3/18:3)*	0.36	0.5	0.34	0.53	0.31	0.48
		1,2-dilinoleoyl-GPA (18:2/18:2)*	0.53	0.51	0.37	0.39	0.4	0.44
		1,2-dilinoleoyl-GPI (18:2/18:2)*	0.77	0.84	0.22	0.23	0.25	0.51
		1,2-dipalmitoyl-GPE (16:0/16:0)*	0.7	0.55	0.39	0.43	0.66	0.66
		1-linolenoyl-GPA (18:3)*	0.25	0.42	0.22	0.4	0.35	0.45
		1-linoleoyl-2-linolenoyl-GPA (18:2/18:3)*	0.45	0.49	0.38	0.49	0.36	0.44
		1-oleoyl-2-linoleoyl-GPA (18:1/18:2)*	0.56	0.69	0.4	0.36	0.54	0.46
		1-oleoyl-GPA (18:1)	0.43	0.5	0.22	0.27	0.3	0.36
		1-oleoyl-GPE (18:1)	0.6	0.59	0.22	0.19	0.34	0.41
		1-palmitoyl-2-linolenoyl-GPA (16:0/18:3)*	0.56	0.64	0.49	0.61	0.46	0.52
		1-palmitoyl-2-linolenoyl-GPG (16:0/18:3)*	0.71	0.82	0.24	0.24	0.37	0.53
		1-palmitoyl-2-linoleoyl-GPA (16:0/18:2)*	0.65	0.71	0.56	0.56	0.5	0.52
		1-palmitoyl-2-oleoyl-GPA (16:0/18:1)	0.54	0.54	0.35	0.38	0.4	0.42
		1-palmitoyl-GPA (16:0)	0.46	0.61	0.36	0.48	0.39	0.54

		1-palmitoyl-GPG (16:0)*	0.49	0.75	0.25	0.25	0.43	0.58
		1-stearoyl-GPE (18:0)	0.4	0.5	0.25	0.24	0.39	0.5
		choline	0.81	0.79	0.71	0.74	0.77	0.77
		ethanolamine	0.49	0.63	0.28	0.55	0.33	0.56
		glycerophosphorylcholine (GPC)	2.72	1.81	4.19	2.59	4.56	3.62
		phosphoethanolamine	5.81	1.5	2.02	1.18	2.16	1.58
	Sphingolipid	sphingosine	0.63	0.4	0.27	0.21	0.53	0.38
Nucleotide	Purine metabolism	1-methyladenine	2.47	1.68	3.69	1.95	3.31	2.06
		2'-deoxyadenosine	0.11	0.11	0.17	0.18	0.43	0.26
		2'-deoxyadenosine 5'-monophosphate	0.13	0.09	0.13	0.09	0.58	0.16
		2'-deoxyguanosine	0.12	0.11	0.18	0.21	0.62	0.33
		7-methylguanosine	0.61	0.54	0.64	0.48	0.48	0.24
		adenine	0.24	0.23	0.23	0.28	0.29	0.29
		adenosine	0.5	0.6	0.39	0.49	0.43	0.5
		adenosine 3'-monophosphate (3'-AMP)	9.33	9.45	8.01	10.15	5.87	7.85
		adenosine 5'-monophosphate (AMP)	0.63	0.58	0.42	0.44	0.54	0.6
		adenosine-2',3'-cyclic monophosphate	3.36	2.2	3.14	2.65	4.7	1.86
		adenylosuccinate	0.5	0.39	0.14	0.19	0.25	0.41
		guanosine	0.43	0.5	0.25	0.39	0.39	0.45
		guanosine 3'-monophosphate (3'-GMP)	3.05	4.82	4.16	8.98	2.8	2.16
		guanosine 5'-monophosphate (5'-GMP)	0.52	0.46	0.37	0.39	0.51	0.51
		inosine 5'-monophosphate (IMP)	2.07	2.56	0.17	0.29	0.44	0.54
		isopentenyl adenosine	0.17	0.21	0.13	0.25	0.2	0.28
		xanthosine	0.22	0.32	0.27	0.25	0.58	0.45
	Pyrimidine metabolism	2'-deoxycytidine 5'-monophosphate	0.16	0.11	0.22	0.16	0.63	0.29
		2'-deoxyuridine	0.15	0.16	0.11	0.1	0.19	0.14
		5-methylcytidine	0.24	0.25	0.39	0.34	0.33	0.31
		β-alanine	3.38	2.68	1.48	1.23	0.74	0.83
		cytidine 5'-monophosphate (5'-CMP)	0.33	0.3	0.21	0.25	0.3	0.36
		thymidine	0.17	0.16	0.26	0.27	0.39	0.31
		thymidine 5'-monophosphate	0.18	0.12	0.27	0.19	0.67	0.35

		uracil	0.4	0.54	0.1	0.3	0.17	0.53
		uridine	0.51	0.55	0.29	0.43	0.32	0.61
Secondary metabolism	Benzenoids	4-hydroxybenzoate	0.43	0.41	0.15	0.21	0.27	0.41
		benzoate	0.62	0.69	0.4	0.28	0.6	0.56
		hydroquinone β-D-glucopyranoside	0.59	0.65	0.36	0.48	0.3	0.42
		phenylacetylglutamate	0.43	0.3	0.47	0.42	0.54	0.47
	Flavonoids	catechin	0.53	0.44	0.23	0.38	0.24	0.44
		dihydrokaempferol	0.31	0.61	0.13	0.2	0.23	0.32
		procyanidin B1	0.22	0.21	0.18	0.25	0.17	0.28
	Phenylpropanoids	4-hydroxycinnamate	0.52	0.54	0.24	0.38	0.36	0.52

Table 5-7. Fold changes in the abundance of metabolites that significantly changed in both **regions 1 and 2** (WS1 and WS2) of the growth zone of both water-stressed **maize and cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Metabolites may increase or decrease to the same direction or change differently between the two species. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05). No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	Species	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Aromatic amino acid metabolism	N-formylphenylalanine	Maize	0.1	0.08	0.08	0.11	0.08	0.17
			Cotton	0.41	0.36	0.33	0.38	0.34	0.53
		shikimate	Maize	2.45	1.45	5.82	3.42	5.89	4.43
			Cotton	0.5	0.52	0.3	0.35	0.31	0.37
	Aspartate family	2-aminoadipate	Maize	0.09	0.12	0.17	0.28	0.22	0.43
			Cotton	3.27	1.71	2	1.63	1.58	2.37
		N6-acetyllysine	Maize	0.33	0.35	0.39	0.48	0.47	0.74
			Cotton	5.15	2.34	3.89	2.11	1.72	1.41
		pipecolate	Maize	1.72	5.53	1.96	6.72	1.48	5.92
			Cotton	7.07	10.44	3.89	6.28	3.64	6.37
		saccharopine	Maize	18.21	20.8	9.29	12.49	11.68	32.1
			Cotton	13.13	4.66	4.92	4.4	2.3	4.9
	Branched Chain Amino Acids	β-hydroxyisovalerate	Maize	0.61	0.36	0.38	0.26	0.65	0.53
			Cotton	8.04	2.06	4.47	1.44	5.42	2.38
	Glutamate family	γ-aminobutyrate (GABA)	Maize	2.05	3.52	3.24	4.43	3.67	4.52
			Cotton	0.69	0.68	0.47	0.53	0.53	0.61
		glutamine	Maize	0.47	0.57	0.33	0.39	0.31	0.39
			Cotton	0.35	0.72	0.16	0.28	0.12	0.23
		histidine	Maize	0.49	0.73	0.64	0.81	0.51	0.65
			Cotton	0.74	0.32	0.61	0.42	0.49	0.38
		N-methylproline	Maize	1.95	4.02	1.87	4.88	1.92	5.73
			Cotton	2.84	1.96	2.41	2.04	2.69	2.34
		proline	Maize	19.79	12.89	5.99	4.69	3.77	4.06
			Cotton	4.93	6.3	3.96	6.37	3.63	6.18
	Glutathione metabolism	5-oxoproline	Maize	0.47	0.58	0.37	0.45	0.27	0.38
			Cotton	0.52	0.75	0.32	0.53	0.28	0.43
		S-methylglutathione	Maize	2.49	2.41	1.39	2.04	1.8	2.72
			Cotton	0.59	0.39	0.73	0.54	0.68	0.58

	Serine family	S-carboxymethyl-L-cysteine	Maize	0.26	0.26	0.34	0.35	0.43	0.46
			Cotton	0.76	0.51	0.61	0.46	0.76	0.65
		taurine	Maize	3.15	2.85	2.1	1.74	2.06	1.88
			Cotton	3.01	4.32	3.07	4.58	2.81	5.43
Carbohydrate	Glycolysis	glucose	Maize	2.03	1.5	1.56	1.26	1.37	1.19
			Cotton	2.99	2.81	1.91	1.86	1.44	1.63
	Inositol metabolism	myo-inositol	Maize	1.45	1.45	1.64	1.78	1.63	1.96
			Cotton	2.54	2.64	1.95	2.33	1.83	2.33
	Sucrose, glucose, fructose metabolism	fructose	Maize	2.96	2.26	2.8	2.02	2.28	2.1
			Cotton	3.48	3.04	2.04	1.94	1.51	1.76
		raffinose	Maize	10.42	10.42	7.61	7.61	7.61	5.03
			Cotton	37.07	16.45	143.86	55.47	39.09	47.99
		sucrose	Maize	8.63	15.26	5.36	5.16	4.66	4.33
			Cotton	2.23	7	15.91	18.82	13.01	21.58
Cofactors, Prosthetic Groups, Electron Carriers	Ascorbate metabolism	threonate	Maize	0.52	0.62	0.61	0.75	0.65	0.76
			Cotton	0.83	0.71	0.83	0.72	0.74	0.61
	Riboflavin and FAD metabolism	riboflavin (Vitamin B2)	Maize	0.46	0.54	0.57	0.71	0.64	0.76
			Cotton	0.65	0.73	0.46	0.41	0.46	0.47
Lipids	Phospholipids	glycerophosphorylcholine (GPC)	Maize	237	439.83	301.06	698.94	94.85	274.26
			Cotton	2.72	1.81	4.19	2.59	4.56	3.62
		phosphoethanolamine	Maize	9.59	3.38	3.6	2.15	2.64	2.27
			Cotton	5.81	1.5	2.02	1.18	2.16	1.58
Nucleotide	Purine metabolism	adenosine	Maize	0.59	0.62	0.6	0.66	0.73	0.72
			Cotton	0.5	0.6	0.39	0.49	0.43	0.5
		xanthosine	Maize	0.37	0.32	0.25	0.22	0.31	0.33
			Cotton	0.22	0.32	0.27	0.25	0.58	0.45

Table 5-8. Fold changes in the abundance of metabolites exclusive to **region 1** (WS1) of the growth zone of water-stressed **maize** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significance; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Aspartate family	N6-carboxymethyllysine	1.56	1.93	1.02	1.29	1.29	1.55
		threonine	1.37	1.68	1.03	1.2	0.82	1.03
	BCAA - isoleucine catabolism	2-methylglutarate	1.97	1.42	0.63	0.86	1.75	1.78
	Branched Chain Amino Acids (OAA derived)	allo-isoleucine	0.29	0.83	0.28	1	1	1
	Branched Chain Amino Acids	3-hydroxy-2-ethylpropionate	1.48	1.61	0.66	0.8	0.89	1.24
		levulinate (4-oxovalerate)	0.56	0.51	0.69	0.74	0.68	0.74
	Glutamate family	glutamate	0.7	0.71	0.79	1.04	0.97	1.29
		ornithine	0.7	0.63	1.36	0.74	1.42	0.51
	Glutathione metabolism	4-hydroxy-nonenal-glutathione	14.73	8.71	3.73	1.75	1.54	2.29
		γ-glutamylglycine	1.78	1.55	1.16	1.96	1.47	3.19
		norophthalmate*	1.58	1.7	0.72	0.81	0.82	1.78
	Serine family	cysteine	1.58	1.82	0.97	1.45	0.84	1.44
Carbohydrate	Sucrose, glucose, fructose metabolism	erythrose	1.8	2.52	1.77	1.88	2.39	2.46
		glucoheptose	0.36	0.22	0.73	0.33	1	1
	TCA cycle	malate	0.7	0.64	0.89	0.71	1.01	0.87
Cofactors, Prosthetic Groups, Electron Carriers	CoA metabolism	phosphopantethеine	4.59	4.59	2.9	1.01	0.55	0.58
	Nicotinate and nicotinamide metabolism	nicotinamide	0.35	0.34	0.7	0.96	0.95	0.98
Lipids	Fatty acid, Dicarboxylate	ethylmalonate	0.39	0.53	0.65	0.79	0.74	0.82
	Free fatty acid	2-hydroxypalmitate	0.5	0.64	0.68	1.03	1.14	1.62

		2-hydroxystearate	0.42	0.58	0.63	0.94	1.19	1.66
		arachidate (20:0)	0.49	0.57	0.58	0.94	0.93	1.52
		heptanoate (7:0)	0.44	0.22	0.69	0.5	0.59	0.77
		palmitate (16:0)	0.7	0.8	0.88	1.43	1.26	1.85
	Glycerolipids	1-oleoylglycerol (18:1)	0.47	0.43	0.6	0.76	1.38	1.75
		1-palmitoylglycerol (16:0)	0.48	0.38	0.91	1.08	1.46	1.12
		2-palmitoylglycerol (16:0)	0.44	0.38	0.88	0.94	1.09	1.04
		glycerol	1.19	1.3	0.84	1.13	1.1	1.58
	Phospholipids	1-linoleoyl-GPI (18:2)*	0.19	0.32	0.69	1.78	1.78	3.77
		1-oleoyl-GPI (18:1)*	0.17	0.25	0.29	1.78	0.61	1.78
		1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4)*	2.81	4.8	1.16	2.06	2.06	2.06
Nucleotide	Purine metabolism	2'-deoxyguanosine 3'-monophosphate	0.5	0.39	0.39	0.89	0.92	1.54
	Pyrimidine metabolism	3-aminoisobutyrate	1.5	2.21	0.76	1.28	0.58	0.96
		orotate	0.19	0.24	0.44	0.77	0.96	1.72
		thymidine 3'-monophosphate	0.39	0.33	0.45	0.83	1.05	3.54
Peptide	Dipeptide	leucylglutamine*	0.61	0.61	0.59	0.83	0.83	1.43

Table 5-9. Fold changes in the abundance of metabolites exclusive to **region 2** (WS2) of the growth zone of water-stressed **maize** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Aromatic amino acid metabolism	4-hydroxyphenylpyruvate	1	1	0.29	0.21	0.18	0.15
		tyrosine	0.93	0.9	0.69	0.55	0.37	0.39
	Aspartate family	homoserine	0.76	1.48	0.57	0.88	0.56	0.69
		homoserine lactone	0.71	0.64	0.71	0.68	0.4	0.41
	Branched Chain Amino Acids	N-acetylleucine	0.38	0.2	0.41	0.3	0.41	0.48
	Glutamate family	2-pyrrolidinone	0.85	1.15	3.42	6.04	8.23	8.78
	Glutathione metabolism	γ-glutamylalanine	1.43	1.91	2.53	3.42	2.9	4.8
		γ-glutamylphenylalanine	1.25	1.33	1.44	1.47	1.66	2.05
Carbohydrate	Amino sugar and nucleotide sugar	ribulose/xylulose 5-phosphate	2.94	1.41	0.31	0.3	0.35	0.54
	Glycolysis	fructose-6-phosphate	1.15	0.94	0.25	0.19	0.18	0.21
	TCA cycle	2-methylcitrate/homocitrate	0.83	1.35	5.02	5.02	5.02	3.83
		mesaconate (methylfumarate)	1.27	1.2	0.73	0.73	0.7	0.67
Cofactors, Prosthetic Groups, Electron Carriers	Oxidative phosphorylation	acetylphosphate	1.99	1.04	0.46	0.34	0.37	0.36
Hormone metabolism	Auxin metabolism	2-oxindole-3-acetate	0.83	1.05	0.49	0.66	0.41	0.68
Lipids	Phospholipids	1-palmitoyl-GPG (16:0)*	0.63	0.63	1.41	2.19	2.7	5.39
	Sterols	ergosterol	1.19	1.19	1.29	1.29	1.29	1.29
Nucleotide	Purine metabolism	adenosine 2'-monophosphate (2'-AMP)	1	0.68	0.53	0.57	0.55	0.47

		guanosine 2'-monophosphate (2'-GMP)*	**1.94**	0.85	**0.77**	**0.73**	**0.69**	**0.75**
		guanosine-2',3'-cyclic monophosphate	1.48	**0.53**	**0.63**	**0.46**	**0.32**	**0.22**
		xanthine	1.33	1.13	**0.38**	**0.18**	**0.3**	**0.19**
	Pyrimidine metabolism	**uridine 3'-monophosphate (3'-UMP)**	1.01	**0.38**	**0.69**	**0.71**	**0.71**	**0.81**

Table 5-10. Fold changes in the abundance of metabolites exclusive to **region 1** (WS1) of the growth zone of water-stressed **cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Glutamate family	N-δ-acetylornithine	0.77	0.87	0.92	1.48	0.67	1.14
Carbohydrate	Glycolysis	glucose 6-phosphate	0.68	0.5	1.12	1.23	1.23	1.12
		lactate	2.21	1.62	1.98	1.12	1.61	1.24
	Inositol metabolism	inositol 1-phosphate (I1P)	0.38	0.43	0.57	0.76	0.94	0.93
	Sucrose, glucose,fructose metabolism	galacturonate	4.04	3.23	0.81	0.42	1	0.73
		mannose	0.74	0.73	0.54	1	1	1
	TCA cycle	aconitate [cis or trans]	0.44	0.17	1.55	0.38	1.17	0.3
		isocitrate	0.35	0.12	0.73	0.36	1.29	0.81
Cofactors, Prosthetic Groups, Electron Carriers	Vitamin B metabolism (B6 or B12)	pyridoxal phosphate	0.38	0.38	0.7	0.53	0.66	0.87
Hormone metabolism	Auxin metabolism	indoleacetylaspartate	0.45	0.41	1.08	0.94	1.12	1.09
Lipids	Fatty acid, Dicarboxylate	suberate (octanedioate)	0.68	0.73	0.65	1.06	0.78	1.15
	Phospholipids	1-palmitoleoyl-2-linolenoyl-GPC (16:1/18:3)*	1.64	1.67	0.58	0.61	0.68	2.23
		1-palmitoleoyl-GPA (16:1)*	0.3	0.45	0.28	0.54	0.39	0.53
		1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	1.76	1.38	0.63	0.53	0.87	1.36
		1-stearoyl-GPA (18:0)	0.12	0.28	0.18	0.39	0.32	0.26
		2-palmitoleoyl-GPC (16:1)*	2	2.04	0.83	0.55	0.81	1.67
Nucleotide	Purine metabolism	1-methylguanine	0.57	0.43	1.12	1.08	1.12	0.58
		2'-deoxyinosine	0.17	0.25	0.14	0.26	0.41	0.49
		guanine	0.71	0.63	1.27	1.29	1.37	1
		N1-methyladenosine	0.33	0.36	0.98	0.8	0.83	0.44

		N2-methylguanosine	0.4	0.48	0.89	1.02	0.94	0.8
		N6-carbamoylthreonyladenosine	0.75	0.76	0.92	0.97	0.98	0.89
		xanthosine 5'-monophosphate (xmp)	0.36	0.76	1	1	1	1
	Pyrimidine metabolism	cytidine diphosphate	0.34	0.2	0.55	1	1	1
		pseudouridine	0.42	0.39	1.06	0.95	0.91	0.56
Secondary metabolism	Phenylpropanoids	sinapate	3.82	3.82	0.42	0.8	0.6	1.16
Xenobiotics	Chemicals	thioproline	0.31	0.31	0.96	0.58	0.44	1

Table 5-11. Fold changes in the abundance of metabolites exclusive to **region 2** (WS2) of the growth zone of water-stressed **cotton** primary roots compared to well-watered temporal (WW_T 1, 2, and 3) or well-watered developmental (WW_D 1, 2, and 3) region-specific controls. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05); pink cells indicate increases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); light green cells indicate decreases in abundance that narrowly missed the statistical cutoff for significance (0.05<p-value<0.10); white cells indicate non-significant; grey cells indicate that the metabolite was not detected. No minimum cutoff was applied to the fold changes.

Super Pathway	Sub Pathway	Biochemical Name	WS1 / WWD1	WS1 / WWT1	WS2 / WWD2	WS2 / WWT2	WS2 / WWD3	WS2 / WWT3
Amino acid	Amines and polyamines	agmatine	0.5	0.97	0.15	0.19	0.08	0.14
		spermidine	1.43	0.51	0.05	0.12	0.08	0.05
	Aromatic amino acid metabolism	N-acetyltryptophan	1.21	1.42	1.63	2.28	1.72	2.48
		tryptophan	1.22	0.95	2.18	1.44	1.5	1.18
	Aspartate family	2-oxoadipate	7.95	1.21	3.25	1.18	1.6	1.53
		6-oxopiperidine-2-carboxylate	3.11	1.14	3.66	1.74	1.75	1.78
		N-acetylmethionine sulfoxide	0.89	0.92	0.27	0.43	0.5	0.48
	Branched Chain Amino Acids	3-methyl-2-oxobutyrate	1.48	0.71	0.52	0.51	0.76	0.84
		4-methyl-2-oxopentanoate	1.51	0.65	0.49	0.46	0.65	0.73
	Glutamate family	arginine	1.35	0.93	0.65	0.46	0.42	0.33
		argininosuccinate	0.78	0.53	0.12	0.12	0.43	0.49
		histamine	0.72	0.69	0.25	0.4	0.18	0.27
		imidazole lactate	2.73	0.92	3.1	1.45	2.29	1.47
		N-α-acetylornithine	1.1	1.22	1.15	1.18	1.29	1.67
	Glutathione metabolism	γ-glutamylhistidine	0.91	0.36	0.56	0.52	0.86	0.87
		γ-glutamyltyrosine	1.98	1.23	2.04	1.85	3.32	3.64
	Serine family	glycine	1.03	0.93	0.52	0.48	0.29	0.3
Carbohydrate	Amino sugar and nucleotide sugar	UDP-galactose	0.87	0.63	0.53	0.41	0.22	0.24
		xylose	1.1	1.11	0.67	0.76	0.49	0.61
	Calvin cycle and pentose phosphate	3-phosphoglycerate	0.89	0.86	0.24	0.31	0.45	0.58
		sedoheptulose-7-phosphate	1.15	0.73	0.44	0.27	0.57	0.55
	Sucrose, glucose, fructose metabolism	6-phosphogluconate	1.02	0.49	0.08	0.06	0.13	0.08
		maltose	0.98	0.71	0.5	0.45	0.58	0.56
	TCA cycle	fumarate	1.08	0.8	0.71	0.48	0.6	0.44

		isocitric lactone	0.86	0.42	0.29	0.29	0.2	0.23
		maleate	1.18	0.85	0.41	0.26	0.38	0.39
Cofactors, Prosthetic Groups, Electron Carriers	Folate Metabolism	biopterin	1	1	0.41	0.62	0.39	0.47
	Thiamine metabolism	thiamin monophosphate	0.62	0.67	0.14	0.17	0.18	0.17
	Tocopherol metabolism	α-tocopherol	1.23	1.22	0.46	0.54	0.54	0.5
	Vitamin B metabolism (B6 or B12)	pyridoxate	1.94	0.93	2.81	2.21	1.95	1.8
Hormone metabolism	Auxin metabolism	indoleacetate	1.1	1.23	0.6	0.57	0.4	0.42
Lipids	Fatty acid amide	oleoyl ethanolamide	0.64	1.04	0.13	0.31	0.25	0.48
		palmitoyl ethanolamide	0.67	1.66	0.2	0.55	0.31	0.63
	Fatty acid ester	oleoylcholine	0.81	1.15	0.1	0.26	0.15	0.4
	Free fatty acid	2-hydroxyglutarate	1.13	0.68	0.63	0.41	0.57	0.46
		linoleate (18:2n6)	0.21	0.54	0.06	0.14	0.15	0.12
	Galactolipids	1,2-dilinoleoyl-digalactosylglycerol (18:2/18:2)*	0.91	0.97	0.49	0.47	0.48	0.45
		1-linoleoyl-2-linolenoyl-digalactosylglycerol (18:2/18:3)*	0.95	1.04	0.39	0.4	0.42	0.45
		1-linoleoyl-2-linolenoyl-galactosylglycerol (18:2/18:3)*	0.66	0.91	0.29	0.4	0.34	0.39
		1-palmitoyl-2-linolenoyl-digalactosylglycerol (16:0/18:3)	0.62	0.99	0.52	0.76	0.51	0.63
		1-palmitoyl-2-linolenoyl-galactosylglycerol (16:0/18:3)*	0.58	0.89	0.5	0.57	0.41	0.41
		1-palmitoyl-2-linoleoyl-galactosylglycerol (16:0/18:2)*	1.01	0.92	0.51	0.43	0.5	0.4
	Glycerolipids	diacylglycerol (16:1/18:2 [2], 16:0/18:3 [1])*	1.11	1.04	0.21	0.27	0.37	0.53
		linolenoyl-linolenoyl-glycerol (18:3/18:3) [1]*	0.85	0.95	0.11	0.28	0.29	0.5
		linoleoyl-linolenoyl-glycerol (18:2/18:3) [1]*	0.99	0.86	0.13	0.2	0.34	0.46
		linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]*	1.45	1.17	0.33	0.48	0.57	0.66
		linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]*	0.81	0.58	0.22	0.32	0.52	0.45
		oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	1.15	0.93	0.21	0.21	0.36	0.41
		palmitoyl-linolenoyl-glycerol (16:0/18:3) [2]*	1.4	1.45	0.18	0.18	0.25	0.46

		palmitoyl-linoleoyl-glycerol (16:0/18:2) [1]*	1.02	0.9	0.35	0.39	0.59	0.52
		palmitoyl-linoleoyl-glycerol (16:0/18:2) [2]*	1.22	1.1	0.32	0.3	0.43	0.44
	Oxylipins	12,13-DiHOME	0.14	0.44	0.12	0.35	0.11	0.44
		9,10-DiHOME	1.07	1.54	0.32	0.79	0.27	0.75
	Phospholipids	1,2-dilinoleoyl-GPE (18:2/18:2)*	0.89	0.65	0.28	0.24	0.47	0.55
		1,2-dioleoyl-GPA (18:1/18:1)*	0.57	0.48	0.26	0.25	0.43	0.44
		1-linoleoyl-GPG (18:2)*	0.49	0.7	0.16	0.14	0.21	0.32
		1-oleoyl-2-linoleoyl-GPE (18:1/18:2)*	0.81	0.6	0.3	0.26	0.45	0.45
		1-oleoyl-2-linoleoyl-GPG (18:1/18:2)*	0.71	0.79	0.26	0.2	0.34	0.39
		1-oleoyl-GPC (18:1)	0.67	0.75	0.18	0.17	0.34	0.5
		1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	0.88	0.7	0.4	0.36	0.54	0.57
		1-palmitoyl-2-linoleoyl-GPG (16:0/18:2)	0.85	0.9	0.29	0.21	0.44	0.56
		1-palmitoyl-2-oleoyl-GPG (16:0/18:1)	0.81	0.78	0.25	0.2	0.46	0.53
		1-palmitoyl-2-oleoyl-GPI (16:0/18:1)*	0.94	0.77	0.26	0.23	0.53	0.64
		1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	0.84	0.68	0.29	0.21	0.56	0.46
		1-palmitoyl-GPI (16:0)	0.67	0.71	0.37	0.4	0.4	0.57
		1-stearoyl-2-linoleoyl-GPA (18:0/18:2)*	0.77	0.66	0.41	0.44	0.42	0.4
		1-stearoyl-2-oleoyl-GPC (18:0/18:1)	1.16	0.85	0.21	0.15	0.47	0.41
		1-stearoyl-GPC (18:0)	0.5	0.66	0.25	0.2	0.31	0.43
	Sphingolipid	phytosphingosine	1.37	0.95	0.49	0.37	0.6	0.48
Nucleotide	Purine metabolism	hypoxanthine	0.3	0.97	0.59	0.6	0.29	0.48
	Pyrimidine metabolism	dihydroorotate	1.31	0.55	0.6	0.39	0.44	0.33
		uridine 5'-monophosphate (UMP)	0.98	0.71	0.46	0.47	0.53	0.62
Peptide	Dipeptide	glycylleucine	1.2	0.93	0.54	0.67	0.67	0.75
		valylglycine	1.28	1.43	0.68	0.8	0.67	0.71

Chapter 6

Transcriptomics Analysis of Sulfur Metabolism and Anti-oxidative Mechanisms in the Cotton Primary Root under Water Deficit Conditions

Introduction

The metabolomics study described in Chapter 5 revealed that the growth zone of the cotton primary root displayed changes in the abundance of several metabolites in response to water deficit stress that differed from those seen in the maize primary root at almost identical tissue water potentials. The major differences were focused on metabolites involved in anti-oxidative stress mechanisms and sulfur metabolism and included glutathione (Figure 5-5), GABA (Figure 5-4), sulfate (Figure 5-6), SAM and MTA (Figure 5-7). The critical roles of these metabolites in the water-deficit response were discussed in Chapter 5.

Oxidative stress is a critical stress factor that contributes to water deficit stress (see Chapter 1). Oxidative stress has a major negative impact on metabolism and homeostasis of plant cells. Glutathione and GABA function as antioxidants (Bouché & Fromm, 2004; Noctor et al., 2016), and in the comparative metabolomics analysis, these two compounds decreased in abundance in the growth zone of the cotton primary root under water deficit compared to the opposite response in the maize primary root. However, quantitative measurements of H_2O_2, as a surrogate measure of oxidative stress levels, indicated that cotton primary roots accumulated much less H_2O_2 when exposed to water deficit, unlike maize that had high levels of H_2O_2 (Figures 5-9 and 5-10). As discussed in Chapter 5, cotton may have alternative mechanisms to quickly scavenge ROS or avoid generating ROS (or both). To further investigate these questions, a transcriptomic study was conducted to provide an additional 'global' analysis of sulfur and anti-oxidative pathways in the water deficit response of the cotton primary root growth zone with the aim of

further clarifying the role of sulfur metabolism in the response and to gain an understanding of possible alternate anti-oxidative mechanisms that may play a role in ROS scavenging.

Chan et al. (2013) suggested that there are "competing interests" for precursors among sulfur metabolic pathways involved in the production of key metabolites important for plant survival during exposure to water deficit stress. The metabolomics and quantitative metabolite data indicated that in cotton primary roots exposed to water deficit, the impact on sulfur metabolism was manifested by a decreased abundance of both sulfate and glutathione in the growth zone. At 48 h after exposure to water deficit, sulfur metabolism had apparently shifted from glutathione synthesis to methionine metabolism, as evidenced by increased levels of S-adenosylmethionine (SAM). The fact that cotton either generates less H_2O_2 or has a very efficient scavenging mechanism that does not require glutathione in response to water deficit may allow the cotton root to distribute its limited sulfur resources to a more demanding pathway, in this case, methionine metabolism. As a key derivative of methionine, SAM is a central metabolite of sulfur metabolism and is known as a universal methyl donor (Sauter et al., 2013). It promotes resistance to water deficit and salinity stresses in plants (Kim et al., 2015; Ma et al., 2017) and also serves as a precursor of ethylene and polyamines, as discussed in Chapter 5. Ethylene and polyamines are involved in stress tolerance mechanisms and it is possible that for the cotton primary root, the "competing interests" for sulfur metabolism may also involve these pathways to activate drought resistance mechanisms.

Transcriptomics is an “omics” technology that enables the tracking of expression of multiple genes, as measured by transcript abundance, simultaneously for multiple plant tissues at the same time. Multiple transcriptomic studies in maize have characterized transcripts (and by inference genes) that respond to water deficit by a change in abundance (up and/or down). These studies revealed the involvement of transcripts in carbohydrate metabolism, plant hormone regulation, signal transduction and anti-oxidative mechanisms (Kakumanu et al., 2012; Shan et al., 2013; Xu et al., 2014). There are also studies that focused on the maize primary root response to water deficit stress that report similar results (Spollen et al., 2008; Yamaguchi & Sharp, 2010; Opitz et al., 2014; Opitz et al., 2015). The transcriptomes of cotton reproductive tissues and leaves have been investigated to reveal alterations induced by drought stress. Alterations in the abundance of transcripts for a large number of transcription factors, osmoprotectant pathway enzymes, ion transporter proteins and heat shock proteins were reported (Padmalatha et al., 2012). Transcripts that represent genes that control hormone biosynthesis pathways and signal transduction (for ABA, jasmonic acid, and ethylene) increased in abundance in response to a drought stress (Padmalatha et al., 2012). Notably, cotton roots exhibited a significant accumulation of transcripts related to carbon/sugar metabolism when compared with leaf tissue under the same drought condition, suggesting an activated osmotic regulation process in cotton roots (Payton et al., 2011).

It is reasonable to assume that alterations in sulfur metabolism and anti-oxidative mechanisms can be tracked by measuring transcript abundance as there are several reported examples of changes in transcript levels for key sulfur metabolism genes (Koprivova et al., 2008; Queval et al., 2009; Chan et al., 2013; Gallardo et al., 2014) and

anti-oxidative related genes (Baek & Skinner, 2003; Smeets et al., 2008) in response to stress. Therefore, a transcriptomics analysis was conducted to test the hypothesis that sulfur metabolism and anti-oxidative mechanisms are impacted at the transcript level in cotton roots upon exposure to water deficit.

Materials and Methods

Tissue sample collection

The optimized vermiculite seedling system described in Chapter 3 was used for growing the cotton line AU90810 in this experiment. The whole growth zones of cotton (WW: 0-12 mm; WS: 0-6 mm) primary roots were harvested at 24 h, 48 h and 72 h after transplanting into well-watered (water potential = -0.02 MPa) and water-stressed (water potential = -1.0 MPa) vermiculite, as described in Chapter 2. The root segments were immediately frozen in liquid nitrogen. Approximately 50-100 mg (fresh weight) per sample were collected for each treatment in one experiment, with three biological replicates.

RNA extraction

The RNeasy® Plant Mini Kit (QIAGEN) was used for RNA extraction. The tissue samples were finely ground in liquid nitrogen in a 2 mL Eppendorf microcentrifuge tube (RNase-free). The liquid nitrogen was allowed to evaporate and before the tissue thawed, 450 µL RLT Buffer was added to the tube and mixed by vigorous vortexing. The total lysate was transferred to a QIAshredder™ spin column and placed in a 2 mL collection tube. The spin column and tube were centrifuged in a desktop centrifuge (Eppendorf AG,

5418) for 2 mins at 14,000 rpm. The supernatant was transferred to a new Eppendorf microcentrifuge tube without disturbing the pelleted cell-debris, and 98% ethanol (50% of the total supernatant volume) was added to the cleared lysate and drawn up and down in the pipet to mix thoroughly. The sample was transferred to a RNeasy Mini spin column in a 2 mL collection tube. The spin column and tube were centrifuged for 15 s at 14,000 rpm and the flow-through was discarded. 700 µL RW1 Buffer (QIAGEN) was added to the RNeasy spin column and the spin column and tube were centrifuged for 15 s at 14,000 rpm and the flow-through was discarded. A 500 µL aliquot of RPE Buffer (QIAGEN) was added to the RNeasy spin column and the spin column and tube were centrifuged for 15 s at 14,000 rpm. The flow-through was discarded and repeated with a further 500 µL aliquot of RPE Buffer. When the membrane was dry, an 80 µL aliquot of DNase I (0.34 unit/µL) from the RNase-Free DNase Set (QIAGEN) was placed on the center of the membrane to remove all contaminating DNA. Finally, the RNeasy spin column was placed in a new 1.5 mL collection tube and 30–50 µL RNase-free water was added directly to the membrane. Then the spin column and tube were centrifuged for 1 min at 14,000 rpm to elute the RNA. The quality and quantity of the RNA in each sample were assessed by use of a Bioanalyzer® (Agilent) and Qubit® Fluorometer (Invitrogen).

Preparation for cDNA library

The Illumina RNASeq libraries were constructed using the NEBNext® Ultra™ II RNA Library Prep Kit from Illumina®. Each sample was diluted with nuclease-free water to a final volume of 50 µL. The samples were extracted with 50 µL Oligo dT Beads $d(T)_{25}$ at 65 °C for 5 min and cooled to 4 °C with the heated lid set at ≥ 75 °C. Then 10 µL of

RNA sample was fragmented in 11.5 µL NEBNext First Strand Synthesis Reaction Buffer and Random Primer Mix at 94 °C for 15 min with the heated lid set at 105 °C. The first strand was assembled by adding 20 µL NEBNext First Strand cDNA Synthesis Reaction Mix at 25 °C for 10 min, 42 °C for 15 min and 70 °C for 15 min with the heated lid set at ≥ 80 °C. 80 µL NEBNext Second Strand Synthesis Reaction Mix was added to 20 µL of the first-strand synthesis product and incubated for 1 h at 16 °C with the heated lid set at ≤ 40 °C. The produced double-strand cDNA was purified, end-repaired and then ligated with NEBNext Adaptor. Next, the cDNA sample was enriched by PCR in the following conditions: with the heated lid set at 105 °C, 98 °C for 30 s; 98 °C for 10 s then 65 °C for 75 s, 7-15 cycles; 65 °C for 5 min; and then held at 4 °C. The PCR-enriched cDNA libraries were purified by adding 45 µL NEBNext Sample Purification Beads and eluted in 23 µL 0.1× TE Buffer and stored at -20 °C. 1 µL of the end product was run on a DNA High Sensitivity Chip by Bioanalyzer® (Agilent) to check whether the libraries were within the expected size range of approximately 300 bp.

Sequencing and data processing

The samples were sent to the University of Missouri DNA Core Facility for sequencing. The raw sequencing data quality was checked with FASTQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to filter the quality of raw sequence data coming from high throughput sequencing pipelines and remove low quality sequences. The data were processed with Hisat2-Cuffcompare software (Pertea et al., 2016; Nelson et al., 2017), which uses Hisat2 for alignment of sequence reads to the upland cotton (*Gossypium hirsutum*) genome (UTX-JGI v1.1), and Cuffcompare to

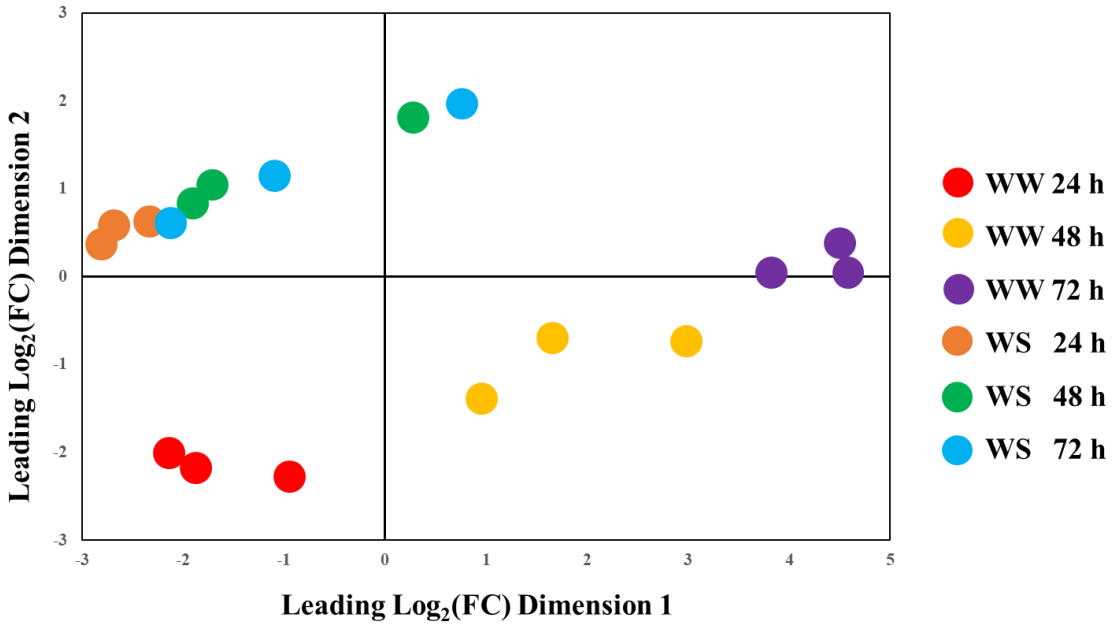
generate output files for Htseq-count, which counts how many aligned reads overlap the exon of each gene (Anders et al., 2015). Using normalized counts, egdeR was used to identify transcripts that were differentially regulated via an associated statistical algorithm (Robinson et al., 2010). All the software packages were provided by Cyverse, an online public bioinformatics analysis platform (www.cyverse.org)[2]. The final output was a database of the normalized read counts for the transcripts of each gene, which was sorted to provide a list of differentially expressed genes (DEGs) by comparing the well-watered samples with those exposed to water deficit. The final results were presented as fold changes in transcript abundance between the two treatments at the same time point at a significance level of 0.05 without cutoff.

Results

Overall, the data analysis revealed that water-deficit stress dramatically alters the transcriptome of the cotton root growth zone, as evidenced by the separation of the individual sample transcriptomes in the Multidimensional scaling plot (Figure 6-1). All water-stressed samples separate into the upper left quadrant of the plot and the well-watered samples to the lower left and right quadrants. The transcriptomes of the water-stress treatment were highly reproducible over the time course of the experiment as evidenced by the clustering of the data point positions. The well-watered samples were more separated by time (development) with the 24, 48, and 72 h samples clustering with each other, in contrast to the water-stressed samples.

[2] *This material is based upon work supported by the National Science Foundation under Award Numbers DBI-0735191, DBI-1265383, and DBI-1743442. URL: www.cyverse.org*

Figure 6-1. Two-dimensional Multidimensional scaling plot of all biological replicate samples in each treatment showing transcriptional similarities. Each sample is represented as a single point. Different colors represent the different treatments, well-watered (WW) and water-stressed (WS), at different times. Proximity indicates transcriptional similarity between two samples. Euclidean distance of log2-transformed fold-change (FC) was used to measure the pairwise differences.



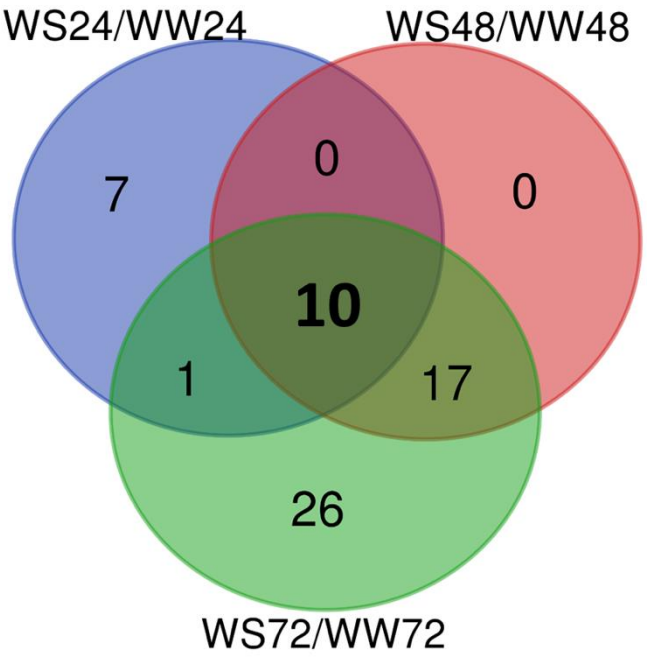
The current upland cotton (*Gossypium hirsutum*) genome (UTX-JGI v1.1) was used to annotate the assembled reads and to identify those transcripts that relate to either sulfur or oxidative metabolism. The enzymes related to the sulfur metabolism pathways and those related to the major anti-oxidative stress mechanisms were selected (Table 6-1[3]). Among the enzymes involved in the glutathione pathway, glutathione dehydrogenase/transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR), isocitrate dehydrogenase (IDH) and glucose-6-phosphate dehydrogenase (G6PDH) were considered as anti-oxidative enzymes because their major function is to scavenge ROS through the glutathione cycle; glutathione hydrolase (GGT) and glutathione synthase (GSHS) were considered as sulfur metabolism-related enzymes because their functions are glutathione biosynthesis and degradation. For sulfur metabolism, 238 transcripts encoding 12 enzymes (some of which were represented by multiple distinct transcripts) were identified. For the anti-oxidative enzymes, 498 transcripts encoding 10 enzymes were identified.

Transcript abundance for each gene and for each time point was determined and the values for samples collected for the water stress treatment were compared to the values for the well-watered treatment. The significances of the differential transcript abundance results were filtered by p-value (≤ 0.05). It was determined that 61 of the 238 identified transcripts for sulfur metabolism increased in abundance and 43 decreased in abundance in response to water stress in at least one comparison between water-stressed and well-watered treatments at a single time point (WS/WW) (Figure 6-2, Table 6-2). For the

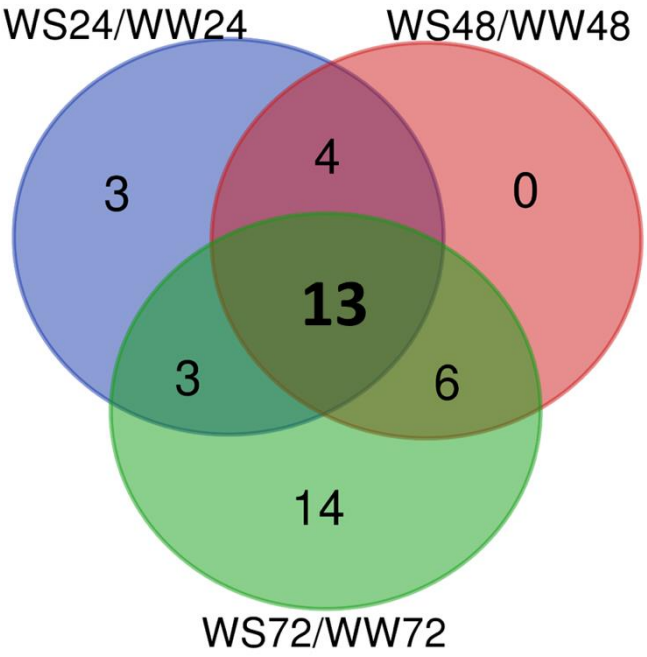
[3] All Tables are shown at the end of this chapter.

Figure 6-2. The number of significantly increased (a) and decreased (b) transcripts related to sulfur metabolism in comparisons of water-stressed (WS) and well-watered (WW) cotton primary root growth zones at 24 h, 48 h, and 72 h after transplanting.

a



b

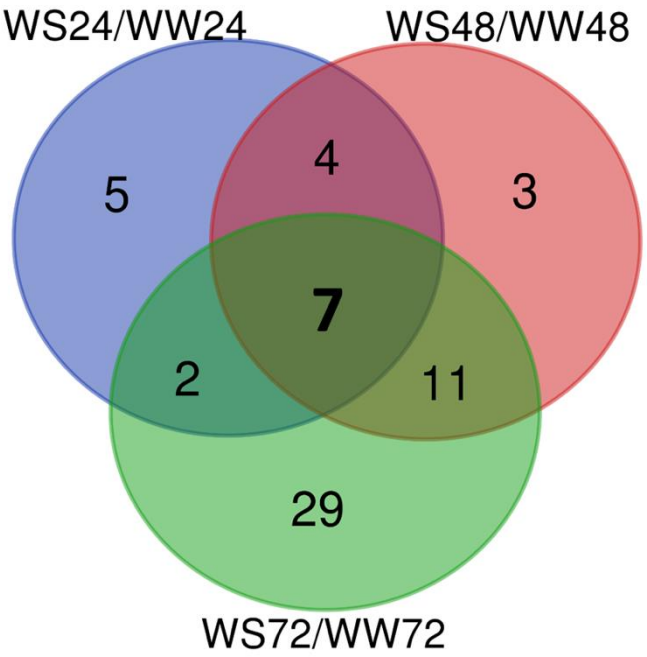


transcripts encoding anti-oxidative related enzymes, it was determined that 61 of the 498 identified transcripts increased in abundance and 139 decreased in abundance in response to water stress in at least one WS/WW comparison (Figure 6-3, Table 6-3).

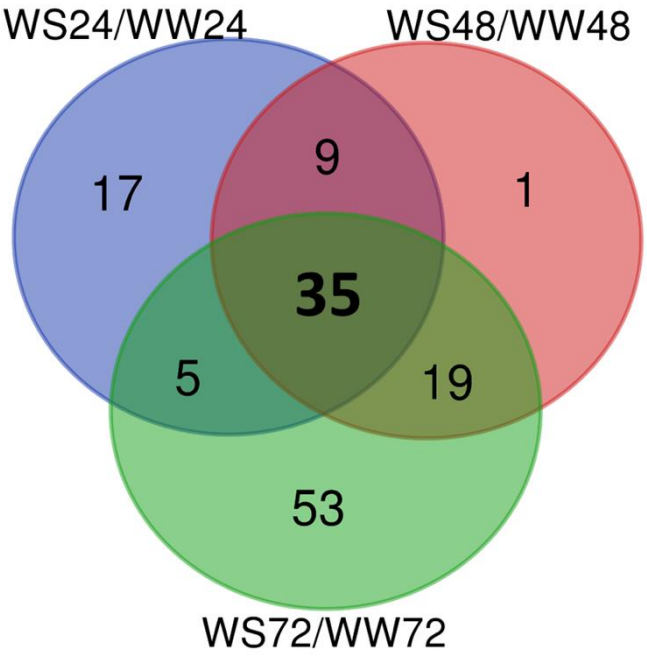
The transcripts that were significantly increased or decreased in abundance in every WS/WW comparison are located in the center section of the Venn diagrams and are highlighted in Tables 6-2 and 6-3. These transcripts are important since the uniform trends in change of abundance indicated that these genes were consistently responding to the water deficit stress throughout the period of cultivation and were specific to the water-deficit treatment and not the result of developmental change. Transcripts encoding proteins involved in sulfur metabolism that increased in abundance in response to water deficit were associated with the SAM biosynthesis pathway (Figures 5-6 and 5-7). However, the analysis is complicated by the fact that each enzyme appears to be encoded by either a multigene family or, because the cotton genome is an alloploid (two different sub-genomes), different alleles, either of which results in multiple transcripts that were annotated as functionally identical. The majority of transcripts encoding cystathionine γ-synthase (CGS), 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (METE) and S-adenosylmethionine synthetase (SAMS) increased in abundance under water stress in each comparison (Table 6-2). However, the transcripts for the downstream enzymes in the pathway, S-adenosylmethionine decarboxylase (SAMDC) and 1-aminocyclopropane-1-carboxylate synthase (ACS), which are involved in 5-methylthloadenosine (MTA) and 1-aminocyclopropane-1-carboxylic acid (ACC) biosynthesis (SAM catabolism), exhibited a mixed pattern, with some transcripts

Figure 6-3. The number of significantly increased (a) and decreased (b) transcripts related to anti-oxidative mechanisms in comparisons of water-stressed (WS) and well-watered (WW) cotton primary root growth zones at 24 h, 48 h, and 72 h after transplanting.

a



b



increasing in abundance while others decreased in abundance in each comparison. The mixed transcript abundance response was also observed for transcripts encoding aminocyclopropanecarboxylate oxidase (ACO), which is the ethylene-forming enzyme, and γ-glutamyltranspeptidase (GGT) that degrades glutathione.

For the major anti-oxidative enzymes (Table 6-3), ascorbate peroxidase (APX), superoxide dismutase (SOD) and peroxidase (POX), only five out of 271 transcripts were observed to increase in abundance in all comparisons: one SOD and four POX transcripts. In contrast, 27 transcripts encoding these enzymes significantly decreased in abundance. For the glutathione-related anti-oxidative enzymes, one transcript of GST and one transcript of dehydroascorbate reductase (DHAR) increased but 6 transcripts of GST decreased in abundance while one transcript of G6PDH decreased in abundance in all WS/WW comparisons. However, GR and GPX, two major enzymes in the glutathione cycle that scavenge hydrogen peroxide, only showed significant changes in WS/WW comparisons in some time points. Only one transcript of GPX exhibited a relatively high (5.85 fold) increase in abundance in the WS24/WW24 comparison. Additionally, one transcript encoding 4-aminobutyrate---pyruvate transaminase (GABAT) decreased in abundance in all WS/WW comparisons.

Discussion

The transcriptomics data provided some insight into alternative modifications of sulfur metabolism and additional anti-oxidative stress mechanisms in the cotton primary root under water-deficit conditions, as discussed in Chapter 5. However, the complexity of the

cotton genome and the possibility that each enzyme may be represented by multi-gene families makes it difficult to derive any robust conclusions from the transcriptome data.

Overall, more transcripts encoding anti-oxidative enzymes decreased in abundance than increased in water-stressed roots. Transcripts encoding sulfur metabolism enzymes, however, were more balanced in their response and generally increased in abundance. The decline in the large number of anti-oxidative enzyme transcripts may be related to the lack of an oxidative response for the cotton primary root, as described in Chapter 5. The lack of water deficit-induced increase in H_2O_2 for the cotton primary root appears to not only be manifested by decreases in glutathione and GABA but also by a decline in transcripts encoding anti-oxidative enzymes. It is unclear, at this juncture, why cotton primary roots do not exhibit an increase in ROS production when exposed to water deficit as seen in maize. Either there are mechanisms to prevent ROS production or there is a highly efficient ROS-scavenging system that does not entail glutathione or require an increase in transcript abundance for antioxidant enzymes. There is a caveat to these observations, however. It is possible that the few transcripts for anti-oxidative enzymes that do increase in abundance may represent stress-inducible gene family members that do provide efficient ROS protection. One transcript encoding SOD and four transcripts encoding POX increased in abundance in water-stressed roots. SOD-encoding transcripts were previously reported to accumulate under drought conditions and enhanced in drought-tolerant cotton lines (Lv et al., 2007; Chen et al., 2015; Singh et al., 2016; Yu et al., 2016). Similarly, POX-encoding transcripts were also reported to accumulate under drought conditions or enhanced in drought tolerant cotton lines (Ranjan et al., 2012; Yue et al., 2012; Zhang et al., 2014). Although the actual levels of these enzymes in either

report were not measured, it is possible that these enzymes may play critical roles in ROS abatement under water stress conditions in cotton primary roots. Thus, it is possible that SOD and POX may combine to form an alternative ROS protection pathway for cotton primary roots, as discussed in Chapter 5.

Transcripts for the glutathione pathway enzymes DHAR, GPX, GR and GST exhibited a mixed response in the three transcriptome comparisons, although, some of the transcripts encoding these four enzymes showed increases in abundance in response to water deficit in particular comparisons. Notably, two transcripts encoding GST and one transcript encoding GPX displayed high and increased fold-changes early (24 h) after exposure of the root to water stress. Two transcripts encoding DHAR also exhibited increases in abundance at the same time point. Considering the increasing trend of glutathione content from 12 h to 24 h under water deficit conditions (Figure 5-9), the increased transcript abundance may indicate that the glutathione cycle was activated early after transplanting but became less active as time progressed, resulting in the declining trend of glutathione content from 24 h to 48 h. Moreover, a decrease in abundance of a G6PDH transcript was found in all comparisons. Although there was only one transcript, this change might be critical since GR needs NADPH to convert oxidized (GSSG) to reduced (GSH) glutathione. Considering the numerous roles of NADPH/ NADP+ in plants, the data suggested that the conversion of NADP+ to NADPH may be an important factor in the cotton root response to water deficit, impacting both redox balance and biosynthetic capabilities associated with growth. This might be the reason for the non-significant change of GR transcripts in the initial stages and subsequent decrease in abundance in latter time points. Thus, it is possible that GST and DHAR may be the only enzymes of

the glutathione cycle that have promoted activities in cotton primary roots in response to water deficit. A previous proteomics study on soybean using the vermiculite-culture seedling system (Yamaguchi et al., 2010) also identified increased protein abundances of GST, DHAR and GPX in the primary root growth zone under water-stressed conditions. It is likely that GST and DHAR have more uniform functions across dicot plants but that GPX is functioning differentially according to species in different conditions. The non-robust transcript abundance levels of GPX and GR might be related to low levels of oxidative stress in the cotton primary root, which would reduce the importance of the glutathione cycle in the stress response. The reduction of glutathione content was not coupled with a reduction in transcripts encoding enzymes for degradation and biosynthesis of glutathione, as the abundance of the related transcripts was not altered by exposure to water deficit.

Transcripts encoding enzymes for GABA biosynthesis (GDH) and degradation (GABAT) displayed a general decline in abundance in response to water deficit. The decreased abundance of the metabolite GABA may be related to the decreased abundance of transcripts related to its biosynthesis.

A uniform increase in transcripts of enzymes involved in the SAM biosynthesis pathway in the response to water deficit was consistent with the metabolomics data described earlier, and suggests that SAM levels are controlled directly by transcript abundance. The enhancement of SAMS transcripts, the enzyme that directly directs the synthesis of SAM, has been reported in multiple plants under drought conditions (Kasim et al., 2013; Wang et al., 2016). A reduction in the catabolism of SAM, indicated by the decrease in

transcript abundance of enzymes involved in the metabolism of ACC from SAM, may also play a role in reducing ethylene production in the root in response to water stress.

Even though this study displayed changes of mRNA transcripts in different comparisons, only actual measurements of protein abundance and enzyme activities can validate if the changes in transcript abundance deliver meaningful changes in the tissue or plant. Therefore, further investigations of protein abundance and enzyme activities need to be conducted to draw a conclusion on whether the sulfur metabolism and anti-oxidative mechanisms in the cotton primary root under water-deficit stress are truly operative.

Conclusion

The transcriptomics study of the cotton primary root growth zone supported the indications from the metabolomics analysis that alterations in sulfur metabolism and anti-oxidative mechanisms occur in water-stressed cotton primary roots and perhaps involve the redistribution of sulfur resources to specific metabolic pathways. The results suggest that selective anti-oxidative enzymes other than those related to the glutathione pathway may have important roles dealing with ROS in the cotton root. Further investigation of these enzymes should be conducted to verify their actual activities under water-stressed conditions.

Table 6-1. Enzymes targeted in transcriptomics analysis.

Sulfur Metabolism Related Enzymes	
CYS	cysteine synthase
CGS	cystathionine γ-synthase
METE	5-methyltetrahydropteroyltriglutamate---homocysteine S-methyltransferase
SAMS	S-adenosylmethionine synthetase
DCMT	DNA (cytosine-5)-methyltransferase
SAHH	adenosylhomocysteinase / S-adenosylhomocysteine hydrolase
SAMDC	S-adenosylmethionine decarboxylase
ACS	1-aminocyclopropane-1-carboxylate synthase
ACO	1-aminocyclopropane-1-carboxylate oxidase
SPDS	spermidine synthase
GSHS	glutathione synthase
GGT	γ-glutamyltranspeptidase/glutathione hydrolase
Anti-oxidative Related Enzymes	
APX	ascorbate peroxidase
SOD	superoxide dismutase
POX	peroxidase
GST	glutathione dehydrogenase/transferase
DHAR	dehydroascorbate reductase
GPX	glutathione peroxidase
GR	glutathione reductase

IDH	isocitrate dehydrogenase
G6PDH	glucose-6-phosphate dehydrogenase
GDH	glutamate decarboxylase
GABAT	4-aminobutyrate---pyruvate transaminase

Table 6-2. Transcripts related to sulfur metabolism that were changed in abundance in the growth zone of water-stressed compared with well-watered cotton primary roots at 24 h, 48 h and 72 h after transplanting. No minimum cutoff was applied to the fold changes. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05). Key transcripts discussed in the text are highlighted.

Enzyme	ID	Ref-seq	WS24/ WW24	WS48/ WW48	WS72/ WW72
CYS	Gohir.A13G171200	cysteine synthase C1			0.34
	Gohir.A13G171100	cysteine synthase D1	0.26		0.39
	Gohir.D13G176700	cysteine synthase D1			0.30
	Gohir.A05G368400	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	1.70		
CGS	Gohir.A07G206200	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein			1.67
	Gohir.D04G046900	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	2.06	1.58	1.88
	Gohir.D07G212300	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein		0.15	0.04
METE	Gohir.A13G097500	Cobalamin-independent synthase family protein	1.67	2.13	5.88
	Gohir.D13G102200	Cobalamin-independent synthase family protein	1.60	2.11	4.81
	Gohir.A07G114900	Cobalamin-independent synthase family protein			2.46
	Gohir.A08G219500	Cobalamin-independent synthase family protein		1.96	5.24
	Gohir.A11G231900	Cobalamin-independent synthase family protein			1.50
	Gohir.A11G300000	Cobalamin-independent synthase family protein			1.94
	Gohir.A13G164600	Cobalamin-independent synthase family protein		1.66	4.44
	Gohir.D07G119200	Cobalamin-independent synthase family protein			2.92
	Gohir.D08G236300	Cobalamin-independent synthase family protein		1.94	4.81
	Gohir.D11G243100	Cobalamin-independent synthase family protein			2.42

	Gohir.D11G314900	Cobalamin-independent synthase family protein			1.98
	Gohir.D13G167400	Cobalamin-independent synthase family protein		1.82	4.41
SAMS	Gohir.D08G142400	S-adenosylmethionine synthetase 1	0.70		
	Gohir.D12G124900	S-adenosylmethionine synthetase 1		1.42	2.62
	Gohir.A12G121100	S-adenosylmethionine synthetase 2	1.46	1.54	2.44
	Gohir.A11G106400	S-adenosylmethionine synthetase 2		1.46	2.46
	Gohir.D11G111300	S-adenosylmethionine synthetase 2		1.41	2.26
	Gohir.A02G057600	S-adenosylmethionine synthetase family protein			2.42
	Gohir.A08G121100	S-adenosylmethionine synthetase family protein			1.82
	Gohir.D02G063400	S-adenosylmethionine synthetase family protein		1.54	2.70
	Gohir.D08G220000	NAD(P)-binding Rossmann-fold superfamily protein	1.54		1.59
	Gohir.D07G077800	NAD(P)-binding Rossmann-fold superfamily protein	0.54	0.46	
	Gohir.A07G073200	NAD(P)-binding Rossmann-fold superfamily protein	0.49	0.47	
	Gohir.D03G053400	NAD(P)-binding Rossmann-fold superfamily protein			1.90
	Gohir.D08G176300	NAD(P)-binding Rossmann-fold superfamily protein			2.19
	Gohir.D11G091000	NAD(P)-binding Rossmann-fold superfamily protein			1.47
	Gohir.A04G071900	methionine adenosyltransferase 3			1.86
	Gohir.A09G145900	methionine adenosyltransferase 3			3.16
	Gohir.A11G323900	methionine adenosyltransferase 3			2.11
	Gohir.D04G110400	methionine adenosyltransferase 3			1.72
DCMT	Gohir.A02G086500	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein			0.73
	Gohir.D02G062500	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein			0.70
	Gohir.A08G236800	DNA methyltransferase-2			1.88
	Gohir.A05G373000	methyltransferase 1		1.39	2.31
	Gohir.D04G042500	methyltransferase 1			1.55
SAHH	Gohir.A03G063300	S-adenosyl-L-homocysteine hydrolase			1.87
	Gohir.A09G206600	S-adenosyl-L-homocysteine hydrolase		1.57	3.31

	Gohir.A10G127200	S-adenosyl-L-homocysteine hydrolase			1.95
	Gohir.D03G103000	S-adenosyl-L-homocysteine hydrolase			1.74
	Gohir.D09G200200	S-adenosyl-L-homocysteine hydrolase		1.75	3.62
	Gohir.D10G138700	S-adenosyl-L-homocysteine hydrolase			2.02
SAMDC	Gohir.D08G046500	S-adenosylmethionine decarboxylase	0.48	0.41	0.14
	Gohir.A08G035800	S-adenosylmethionine decarboxylase	0.42	0.27	0.10
	Gohir.A05G007500	S-adenosylmethionine decarboxylase		2.00	3.45
	Gohir.D05G008400	S-adenosylmethionine decarboxylase			2.30
	Gohir.D13G089400	Adenosylmethionine decarboxylase family protein	3.28	3.86	4.07
	Gohir.A13G095200	Adenosylmethionine decarboxylase family protein	1.99	2.22	1.68
	Gohir.A09G231800	Adenosylmethionine decarboxylase family protein			0.44
ACS	Gohir.D08G134700	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.40	0.40	
	Gohir.D03G078200	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.27	0.34	0.30
	Gohir.A02G105600	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.24	0.26	0.19
	Gohir.A08G114500	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.16	0.35	0.27
	Gohir.D11G022600	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.12	0.08	0.11
	Gohir.A11G023000	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	0.08	0.06	0.06
	Gohir.A12G233600	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6			0.25
	Gohir.D12G233800	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6			0.12
	Gohir.A07G010000	1-amino-cyclopropane-1-carboxylate synthase 7		5.15	13.16
	Gohir.D07G010300	1-amino-cyclopropane-1-carboxylate synthase 7		5.08	3.64
	Gohir.A11G211400	1-amino-cyclopropane-1-carboxylate synthase 8	4.17	2.51	22.22
	Gohir.A05G263600	1-amino-cyclopropane-1-carboxylate synthase 8	0.06	0.01	0.001
	Gohir.D05G265900	1-amino-cyclopropane-1-carboxylate synthase 8		0.09	0.01
	Gohir.D11G211700	1-amino-cyclopropane-1-carboxylate synthase 8		2.43	5.52

ACO	Gohir.A05G173900	ethylene-forming enzyme	3.37		
	Gohir.A08G050300	ethylene-forming enzyme	2.51	3.32	3.42
	Gohir.D05G176900	ethylene-forming enzyme	2.03		
	Gohir.A07G084100	ethylene-forming enzyme	0.26		0.41
	Gohir.A07G083900	ethylene-forming enzyme	0.05	0.09	0.03
	Gohir.A11G152600	ethylene-forming enzyme			0.001
	Gohir.D07G090100	ethylene-forming enzyme			0.14
	Gohir.D07G090200	ethylene-forming enzyme			0.30
	Gohir.D11G159500	ethylene-forming enzyme		0.06	0.001
	Gohir.D10G067400	downstream target of AGL15-4	0.65		
	Gohir.A10G066100	downstream target of AGL15-4	0.60		
	Gohir.D07G010700	ACC oxidase 1		1.70	2.96
	Gohir.D08G186600	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.47		0.51
	Gohir.A10G091100	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.47	0.36	0.22
	Gohir.A08G167900	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	0.38	0.39	0.28
	Gohir.A02G155800	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein		0.34	0.04
	Gohir.A08G167800	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein			0.39
	Gohir.A08G168100	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein			0.60
	Gohir.D08G186700	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein		0.66	0.31
	Gohir.D10G097000	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein			0.70
SPDS	Gohir.A03G140700	spermidine synthase 1			1.99
	Gohir.D02G163500	spermidine synthase 1			1.70
	Gohir.D12G251800	spermidine synthase 3	1.64		
	Gohir.D08G103400	spermidine synthase 3	1.61		
	Gohir.A12G251000	spermidine synthase 3	1.55		

	Gohir.1Z011900	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein			2.23
	Gohir.A09G221300	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein		1.52	1.48
GSHS	Gohir.D11G232600	glutathione synthetase 2	0.69	0.74	
	Gohir.1Z044900	glutathione synthetase 2		0.65	0.70
	Gohir.A05G061100	DnaJ/Hsp40 cysteine-rich domain superfamily protein			0.38
GGT	Gohir.D03G045100	γ-glutamyl transpeptidase 1	2.28	1.79	3.34
	Gohir.D06G067900	γ-glutamyl transpeptidase 4	0.55	0.61	0.54
	Gohir.A06G069200	γ-glutamyl transpeptidase 4	0.31	0.47	0.35
	Gohir.D02G159500	alanine-2-oxoglutarate aminotransferase 2	1.64	1.45	1.32
	Gohir.A03G136200	alanine-2-oxoglutarate aminotransferase 2	1.45		

Table 6-3. Transcripts related to anti-oxidative mechanisms that were changed in abundance in the growth zone of water-stressed compared with well-watered cotton primary roots at 24 h, 48 h and 72 h after transplanting. No minimum cutoff was applied to the fold changes. Red cells indicate significant increases in abundance (p-value ≤ 0.05); green cells indicate significant decreases in abundance (p-value ≤ 0.05). Key transcripts discussed in the text are highlighted.

Enzyme	ID	Ref-seq	WS24/ WW24	WS48/ WW48	WS72/ WW72
APX	Gohir.A05G100600	ascorbate peroxidase 1			2.17
	Gohir.A08G195700	ascorbate peroxidase 1			2.65
	Gohir.A08G196100	ascorbate peroxidase 1			1.39
	Gohir.D05G100400	ascorbate peroxidase 1			2.20
	Gohir.D08G213200	ascorbate peroxidase 2			1.61
	Gohir.A02G172100	ascorbate peroxidase 3	0.42	0.50	
	Gohir.A03G204900	ascorbate peroxidase 3	1.36		
	Gohir.D03G008000	ascorbate peroxidase 3	0.35		0.37
	Gohir.A05G236200	stromal ascorbate peroxidase			1.95
	Gohir.D05G238400	stromal ascorbate peroxidase			1.60
	Gohir.D06G046100	thylakoidal ascorbate peroxidase			1.71
SOD	Gohir.A13G100600	copper/zinc superoxide dismutase 1	0.65	0.59	0.69
	Gohir.A13G166100	copper/zinc superoxide dismutase 1	0.36	0.41	
	Gohir.D13G104200	copper/zinc superoxide dismutase 1	0.57	0.56	0.68
	Gohir.D13G169000	copper/zinc superoxide dismutase 1	0.60	0.57	0.65
	Gohir.A09G088700	copper/zinc superoxide dismutase 2			1.61
	Gohir.D09G088400	copper/zinc superoxide dismutase 2			2.08
	Gohir.D07G049600	Fe superoxide dismutase 2	9.26	8.77	3.66
	Gohir.A05G278100	manganese superoxide dismutase 1			0.47
	Gohir.D10G185400	manganese superoxide dismutase 1			1.39
POX	Gohir.A08G229000	peroxidase 2	0.36	0.02	0.01
	Gohir.A09G128500	peroxidase 2	0.04	0.09	0.19
	Gohir.A11G201500	peroxidase 2	0.28	0.29	
	Gohir.D08G249300	peroxidase 2	0.18	0.03	0.03
	Gohir.D11G221700	peroxidase 2	0.16	0.09	0.26
	Gohir.A07G001600	Peroxidase family protein		0.49	

	Gohir.D04G076500	Peroxidase family protein			0.16
	Gohir.D07G002000	Peroxidase family protein	0.31	0.28	
	Gohir.1Z055700	Peroxidase superfamily protein	0.57	0.64	
	Gohir.A01G015100	Peroxidase superfamily protein	0.44	0.23	0.46
	Gohir.A02G063000	Peroxidase superfamily protein			0.14
	Gohir.A02G093200	Peroxidase superfamily protein		1.68	3.61
	Gohir.A02G173600	Peroxidase superfamily protein			0.37
	Gohir.A03G024000	Peroxidase superfamily protein	0.25		
	Gohir.A03G108000	Peroxidase superfamily protein			0.07
	Gohir.A03G109800	Peroxidase superfamily protein	0.005	0.09	0.01
	Gohir.A03G110100	Peroxidase superfamily protein	0.42	0.44	0.30
	Gohir.A03G172900	Peroxidase superfamily protein	0.09	0.07	0.09
	Gohir.A03G173100	Peroxidase superfamily protein		0.08	0.04
	Gohir.A04G120900	Peroxidase superfamily protein	0.003	0.01	0.02
	Gohir.A05G170100	Peroxidase superfamily protein	1.80	1.68	
	Gohir.A05G184200	Peroxidase superfamily protein	0.23		
	Gohir.A05G190300	Peroxidase superfamily protein	0.09		
	Gohir.A05G338100	Peroxidase superfamily protein		0.19	0.07
	Gohir.A05G361500	Peroxidase superfamily protein		2.17	5.78
	Gohir.A05G374600	Peroxidase superfamily protein	0.10	0.14	0.22
	Gohir.A05G401800	Peroxidase superfamily protein		0.19	0.11
	Gohir.A07G217900	Peroxidase superfamily protein		2.20	4.42
	Gohir.A07G228900	Peroxidase superfamily protein			0.33
	Gohir.A07G229000	Peroxidase superfamily protein	0.45		
	Gohir.A08G078800	Peroxidase superfamily protein		0.06	0.04
	Gohir.A08G079100	Peroxidase superfamily protein	0.33	0.15	0.07
	Gohir.A08G083500	Peroxidase superfamily protein	1.49	2.18	3.45
	Gohir.A08G203900	Peroxidase superfamily protein			0.08
	Gohir.A09G151200	Peroxidase superfamily protein		0.23	0.11
	Gohir.A10G064200	Peroxidase superfamily protein	0.51		
	Gohir.A10G148700	Peroxidase superfamily protein		0.11	0.14
	Gohir.A10G181500	Peroxidase superfamily protein	4.02	16.39	5.26
	Gohir.A11G042900	Peroxidase superfamily protein	0.18		
	Gohir.A11G181300	Peroxidase superfamily protein	0.14	0.41	0.37
	Gohir.A11G205200	Peroxidase superfamily protein	0.37	0.34	0.34
	Gohir.A12G008200	Peroxidase superfamily protein	0.08	0.18	0.19
	Gohir.A12G086800	Peroxidase superfamily protein			0.36
	Gohir.A12G156000	Peroxidase superfamily protein			2.78
	Gohir.A12G262000	Peroxidase superfamily protein		10.42	10.20
	Gohir.A13G091800	Peroxidase superfamily protein			2.02
	Gohir.D01G010900	Peroxidase superfamily protein	0.47	0.46	

	Gohir.D02G068700	Peroxidase superfamily protein		0.28	0.26
	Gohir.D02G111300	Peroxidase superfamily protein		1.61	2.88
	Gohir.D02G134600	Peroxidase superfamily protein	0.02	0.04	0.01
	Gohir.D02G134700	Peroxidase superfamily protein	0.54	0.49	0.36
	Gohir.D02G196200	Peroxidase superfamily protein	0.22	0.54	
	Gohir.D03G144500	Peroxidase superfamily protein			0.35
	Gohir.D04G041100	Peroxidase superfamily protein	0.12	0.12	0.15
	Gohir.D04G154100	Peroxidase superfamily protein	0.02	0.01	0.03
	Gohir.D04G162400	Peroxidase superfamily protein	0.03	0.01	0.02
	Gohir.D05G017400	Peroxidase superfamily protein	0.09	0.16	0.09
	Gohir.D05G173200	Peroxidase superfamily protein	1.58	1.54	
	Gohir.D05G187000	Peroxidase superfamily protein	0.16		
	Gohir.D05G193700	Peroxidase superfamily protein	0.08	0.36	0.37
	Gohir.D06G115300	Peroxidase superfamily protein			0.67
	Gohir.D07G225000	Peroxidase superfamily protein		2.52	5.32
	Gohir.D07G236000	Peroxidase superfamily protein	0.13		0.28
	Gohir.D08G088500	Peroxidase superfamily protein	0.39	0.14	0.06
	Gohir.D08G088600	Peroxidase superfamily protein			0.08
	Gohir.D08G093200	Peroxidase superfamily protein		3.44	3.41
	Gohir.D08G221200	Peroxidase superfamily protein			0.01
	Gohir.D08G237300	Peroxidase superfamily protein	0.52		
	Gohir.D09G060200	Peroxidase superfamily protein	6.90	7.04	15.87
	Gohir.D09G146800	Peroxidase superfamily protein		0.19	0.05
	Gohir.D09G209300	Peroxidase superfamily protein			0.02
	Gohir.D09G209400	Peroxidase superfamily protein			0.05
	Gohir.D09G209500	Peroxidase superfamily protein			0.05
	Gohir.D09G213600	Peroxidase superfamily protein			0.03
	Gohir.D09G213700	Peroxidase superfamily protein			0.01
	Gohir.D09G213900	Peroxidase superfamily protein			0.02
	Gohir.D10G065800	Peroxidase superfamily protein	0.47		
	Gohir.D10G098100	Peroxidase superfamily protein			0.31
	Gohir.D10G165300	Peroxidase superfamily protein		0.54	0.39
	Gohir.D10G165400	Peroxidase superfamily protein	0.35	0.31	
	Gohir.D10G188500	Peroxidase superfamily protein	2.89	4.88	4.88
	Gohir.D11G046400	Peroxidase superfamily protein	0.44		
	Gohir.D11G187800	Peroxidase superfamily protein	0.14		
	Gohir.D11G219400	Peroxidase superfamily protein	0.32	0.37	0.21
	Gohir.D11G239800	Peroxidase superfamily protein	0.56		
	Gohir.D12G007700	Peroxidase superfamily protein	0.48		
	Gohir.D12G089200	Peroxidase superfamily protein	0.02	0.04	0.07
	Gohir.D12G159100	Peroxidase superfamily protein			2.91

	Gohir.D12G262700	Peroxidase superfamily protein		7.94	8.00
	Gohir.D13G087000	Peroxidase superfamily protein			2.87
DHAR	Gohir.A07G078600	dehydroascorbate reductase 1	1.87		2.16
	Gohir.D07G083700	dehydroascorbate reductase 1			2.00
	Gohir.D04G061500	dehydroascorbate reductase 2	1.46	1.83	2.40
	Gohir.A05G351200	dehydroascorbate reductase 2			0.63
GST	Gohir.D08G138300	glutathione S-transferase 6	8.85	2.02	2.38
	Gohir.A12G010100	glutathione S-transferase 6		1.59	2.20
	Gohir.D12G010200	glutathione S-transferase 6			1.63
	Gohir.A01G218300	Glutathione S-transferase family protein		2.91	
	Gohir.A02G110700	Glutathione S-transferase family protein			0.56
	Gohir.A04G097000	Glutathione S-transferase family protein			0.16
	Gohir.A09G160500	Glutathione S-transferase family protein			0.39
	Gohir.A11G115700	Glutathione S-transferase family protein			2.43
	Gohir.D04G136900	Glutathione S-transferase family protein			0.32
	Gohir.D09G156100	Glutathione S-transferase family protein			0.29
	Gohir.D11G238600	Glutathione S-transferase family protein			0.60
	Gohir.D12G095900	glutathione S-transferase phi 8	0.36	0.40	0.27
	Gohir.D11G222600	glutathione S-transferase phi 8		0.42	0.43
	Gohir.A11G157300	glutathione S-transferase PHI 9	1.50		
	Gohir.D13G137000	glutathione S-transferase PHI 9	1.50		1.89
	Gohir.D02G110900	glutathione S-transferase PHI 9		1.65	
	Gohir.A04G097100	glutathione S-transferase TAU 16			3.06
	Gohir.A04G097300	glutathione S-transferase TAU 18	0.55		
	Gohir.D04G136800	glutathione S-transferase TAU 18	0.32	0.34	0.17
	Gohir.A13G152500	glutathione S-transferase TAU 19			1.55
	Gohir.D13G156800	glutathione S-transferase TAU 19		3.15	3.02
	Gohir.D13G156900	glutathione S-transferase TAU 19			1.36
	Gohir.A08G164600	glutathione S-transferase TAU 23			0.60
	Gohir.A06G077600	glutathione S-transferase TAU 24	1.63		
	Gohir.A04G047800	glutathione S-transferase TAU 25	0.23	0.35	0.37
	Gohir.A04G047500	glutathione S-transferase TAU 25	0.22		0.33
	Gohir.D11G236100	glutathione S-transferase tau 7	0.65		
	Gohir.D04G089000	glutathione S-transferase tau 7	0.33		0.47

	Gohir.A13G088500	glutathione S-transferase tau 7	0.31	0.13	0.06
	Gohir.A02G025700	glutathione S-transferase tau 7			0.13
	Gohir.A02G026600	glutathione S-transferase tau 7			0.18
	Gohir.A02G026700	glutathione S-transferase tau 7			0.22
	Gohir.A02G027200	glutathione S-transferase tau 7			0.22
	Gohir.D02G034200	glutathione S-transferase tau 7			0.16
	Gohir.D02G034300	glutathione S-transferase tau 7			0.22
	Gohir.D02G034500	glutathione S-transferase tau 7			0.17
	Gohir.D02G034800	glutathione S-transferase tau 7			0.66
	Gohir.D13G101500	glutathione S-transferase tau 7			0.22
	Gohir.A09G161200	glutathione S-transferase TAU 8	0.33	0.11	0.06
	Gohir.A09G161400	glutathione S-transferase TAU 8	0.22	0.22	0.07
	Gohir.A02G026900	glutathione S-transferase TAU 8			0.09
	Gohir.A07G202800	glutathione S-transferase TAU 8			0.11
	Gohir.D07G209000	glutathione S-transferase TAU 8			0.10
	Gohir.D09G053100	glutathione S-transferase TAU 8			0.06
	Gohir.D09G157000	glutathione S-transferase TAU 8		0.23	0.11
	Gohir.D11G021800	glutathione S-transferase THETA 1	3.28	2.02	
	Gohir.D08G200300	glutathione S-transferase THETA 1	1.88		
	Gohir.A08G181800	glutathione S-transferase THETA 1			2.10
	Gohir.A11G022200	glutathione S-transferase THETA 1		0.50	0.18
	Gohir.A12G227600	glutathione S-transferase THETA 1			0.72
	Gohir.D11G021900	glutathione S-transferase THETA 1			0.24
	Gohir.D12G230100	glutathione S-transferase THETA 1		0.68	0.52
	Gohir.A10G068200	glutathione S-transferase zeta 1			0.34
	Gohir.A12G101900	glutathione S-transferase zeta 1			0.36
	Gohir.D10G083000	glutathione S-transferase zeta 1			0.37
	Gohir.D12G104900	glutathione S-transferase zeta 1		0.64	0.44
	Gohir.D07G184000	microsomal glutathione s-transferase, putative	0.64		
	Gohir.D08G048100	microsomal glutathione s-transferase, putative			0.61
GPX	Gohir.D08G078300	glutathione peroxidase 4		0.35	0.22
	Gohir.D12G230200	glutathione peroxidase 6	5.85	1.63	
	Gohir.A08G182000	glutathione peroxidase 6	1.39		
	Gohir.D08G200600	glutathione peroxidase 6			0.63
GR	Gohir.A03G093500	glutathione-disulfide reductase			1.56
	Gohir.A10G122600	glutathione-disulfide reductase			0.35
	Gohir.D10G143400	glutathione-disulfide reductase		0.69	0.41
IDH	Gohir.A10G103000	Isocitrate/isopropylmalate dehydrogenase family protein			2.11

	Gohir.D10G087200	Isocitrate/isopropylmalate dehydrogenase family protein			2.93
	Gohir.A13G127400	isocitrate dehydrogenase			0.69
	Gohir.D13G131100	isocitrate dehydrogenase		0.62	0.57
	Gohir.A11G169500	cytosolic NADP+-dependent isocitrate dehydrogenase			1.56
G6PDH	Gohir.D11G156300	glucose-6-phosphate dehydrogenase 1	0.47	0.47	0.39
	Gohir.A11G149300	glucose-6-phosphate dehydrogenase 1		0.50	0.38
	Gohir.A03G149400	glucose-6-phosphate dehydrogenase 2			2.41
	Gohir.D01G020700	glucose-6-phosphate dehydrogenase 2			1.52
	Gohir.D02G172300	glucose-6-phosphate dehydrogenase 2			2.84
	Gohir.A09G033500	glucose-6-phosphate dehydrogenase 6			0.44
	Gohir.D07G233000	glucose-6-phosphate dehydrogenase 6			0.76
	Gohir.D09G033700	glucose-6-phosphate dehydrogenase 6		0.49	0.33
GDH	Gohir.A12G153200	glutamate decarboxylase 4	0.64		
	Gohir.D12G156600	glutamate decarboxylase 4	0.40	0.72	
	Gohir.D01G136200	glutamate decarboxylase 4	0.14		0.20
	Gohir.A01G144400	glutamate decarboxylase 4			0.22
	Gohir.A12G272000	glutamate decarboxylase 4		4.05	
	Gohir.D09G168600	glutamate decarboxylase 5		3.98	8.20
GABAT	Gohir.A11G156000	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	0.59	0.39	0.28
	Gohir.D11G163100	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein			0.62

Appendix I

Effect of Soil Sulfur Supplementation on the Growth Response in Water-stressed Cotton Roots

Introduction

Sulfur metabolism appears to play critical roles in the primary root of both maize and cotton when exposed to water deficit, but the control of the sulfur metabolic pathways differs between the two species (see Chapters 5 and 6). In cotton, as highlighted by the metabolomics data, sulfur metabolism during exposure to water deficit was characterized by a general decrease in the metabolites involved in the glutathione cycle whereas in maize they were elevated. More importantly, sulfate levels were significantly reduced in water-deficit stressed cotton primary roots but in maize sulfate levels increased. These observations suggested that the uptake and/or assimilation of sulfur may be limiting glutathione production in cotton roots. Alternatively, although the sulfate resource was limited in the cotton primary root, the increase in other sulfur-related metabolites indicated that there was competition for this resource and that this may have resulted in the decline in glutathione. The accumulation of methionine, SAM and MTA in region 1 of the cotton primary root could thus be a critical process that enables growth maintenance in water deficit conditions.

As reviewed in Chapter 1, sulfur has important functions in plants exposed to drought conditions. As the precursor of multiple important amino acids, antioxidants and their derivatives, sulfur is critical to cellular homeostasis and for the relief of water deficit and oxidative stresses (Srivalli & Khanna-Chopra, 2008; Bürstenbinder et al., 2010; Noctor et al., 2011; Waduwara-Jayabahu et al., 2012). Sulfur is an essential nutrient that needs to be supplied to field-grown cotton to obtain a commercially viable yield when the levels in the soil are relatively low (Stevens & Dunn, 2008). Increases in the sulfur supply were

reported to promote growth of reproductive organs including lint length, boll number, boll weight and seed yield (Mullins, 1998; Yin et al., 2011; Geng et al., 2016). However, there are no reports describing cotton root responses in the field to sulfur supplementation. The studies on the effects of water deficit on cotton primary root growth and metabolism presented in this dissertation have focused on a lab-based seedling system, so it was important to conduct a field test to determine if a low sulfur supply limits cotton root growth under water deficit conditions, as the data might suggest. It would be an important line of evidence that could support the lab-based findings if sulfur supply does affect cotton root growth in the field. However, it is not possible to conduct a full field evaluation of soil sulfur effects on cotton root growth in Columbia, MO, as the soils are relatively rich in sulfur. In light of this, we designed an initial growth tube experiment with low sulfur soils treated with sulfate supplementation and conducted the experiment under field conditions as a reasonable alternative. Our hypothesis was that supplemental sulfate would improve the response of root growth under water-deficit conditions.

Materials and Methods

The field experiment was conducted under a rain-out shelter located in Rollins Bottom Farm, Columbia, MO. A 180 cm × 180 cm wooden frame was built to hold 5-L Plexiglas tubes with a width of 10.2 cm and a height of 76.2 cm (TP430, Stuewe & Sons, Inc., Tangent, OR, USA) that were filled with 80:20 (V : V) dry sand and low-sulfur soil (Ri-Mor Topsoil, Columbia, MO, USA). The plants were grown in tubes to facilitate root harvest (Figure AI-1).

Figure AI-1. Setting of the field experiment

Four treatments were applied: well-watered with supplemental sulfate (WW S+), well-watered without supplemental sulfate (WW S-), water-stressed with supplemental sulfate (WS S+), and water-stressed without supplemental sulfate (WS S-). The sulfur supplements were applied with whole nutrient solutions modified from the standard Hoagland's solution with K_2SO_4 as the sulfur resource (Table AI-1). The sulfur-deficient treatments were applied with the same solution minus K_2SO_4. The solutions were mixed thoroughly with the soil before filling the tubes. The contents of major elements in the two types of soil were measured and are shown in Table AI-2. Soil sulfate levels were 21.7 ppm (S+) and 2.7 ppm (S-) before transplanting.

Each treatment included 64 plants, and additional rows of plants, treated as well-watered and sulfur sufficient, were placed between each treatment block to prevent an edge effect. Cotton (cv. AU90810) seeds were imbibed and germinated as described in Chapter 2 and taken to the field and transplanted into the tubes, one seedling per tube. Well-watered treatments were fully watered every three days. For the water-stressed treatments, 500 mL of water was supplied to the soil surface in each tube at the beginning of the experiment to promote seedling establishment, and no subsequent irrigation was supplied. Thus, the tube system was designed to simulate a dryland condition with limited irrigation such that the primary root grew into dryer soil layers towards the end of the experiment.

To assess if an appropriate root water potential was obtained in each of the treatments, nine plants from each treatment were harvested on day 24 for water potential measurements of the primary root growth zone and surrounding soil. The nine seedlings

Table AI-1. Ingredient concentrations (mol/L) in solution supplied to sulfur supplementation (S+) and sulfur deficiency (S-) soil treatments.

Soil Treatment	S+	S-
KNO_3	0	0.09
NH_4NO_3	0.48	0.44
KH_2PO_4	0.23	0.23
Fe-EDTA	4.83×10^{-03}	4.83×10^{-03}
K_2SO_4	0.10	0
$MnCl_2$	8.19×10^{-04}	8.19×10^{-04}
$ZnSO_4$	6.88×10^{-05}	6.88×10^{-05}
$CuSO_4$	2.83×10^{-05}	2.83×10^{-05}
H_3BO_3	4.16×10^{-03}	4.16×10^{-03}
Na_2MoO_4	9.38×10^{-06}	9.38×10^{-06}

Table AI-2. Content of major nutrients and pH in sulfur supplementation (S+) and sulfur deficiency (S-) soil treatments before transplanting.

Soil Treatment	S+	S-
pH	6.98	7.08
Bray I P (lb/Ac)	102.4	100.4
Ca (lb/Ac)	1237	1177.4
Mg (lb/Ac)	138	127
K (lb/Ac)	276.8	265.2
S in SO_4^{2-} form (ppm)	21.7	2.7
N in NO_3^- form (ppm)	23.8	29.2
N in NH_4^+ form (ppm)	31.2	25

were also assessed for root length and dry weight. Based on the data collected from the nine plants harvested on day 24, all remaining plants were harvested on day 29. Primary root tip water potentials, water potentials of soil in different layers (root tip area: soil surrounding the primary root tip; middle: soil from the region approximately half way between the soil surface and primary root tip area), total root length and total root dry weight were measured for selected seedlings from the harvested plants. The root water potentials were measured using the modified low-temperature isopiestic thermocouple psychrometry system described in Chapter 4. Soil water potentials were measured using a WP4C Dewpoint PotentiaMeter (METER Group, Inc., Pullman, WA, USA).

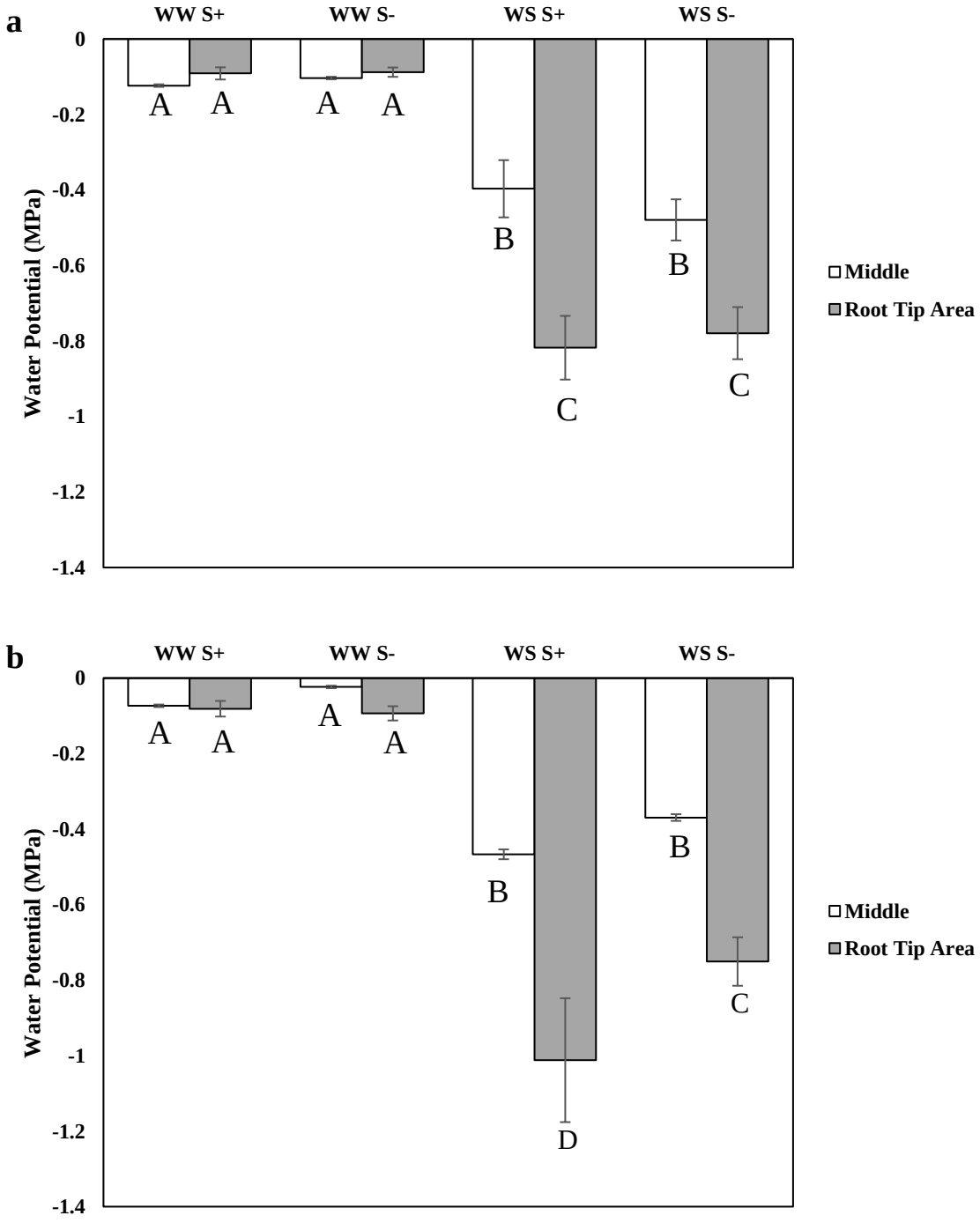
The whole root system was imaged using a flat-bed scanner and the total root length was measured using the WinRhizo® root analysis system. Unfortunately, independent measurements of primary root length were not possible because the intact primary root could not be retrieved from the harvested root system. After scanning, the whole root systems were oven dried and the dry weights were recorded once the samples reached a constant weight.

Results

Water potential measurements of the primary root tip and surrounding soil

Well-watered controls of both S+ and S- treatments had relatively high soil water potentials in the middle and root area soil samples, as expected (Figure AI-2). In the water-stress treatments, as anticipated, the soil layer in the middle region was wetter than the soil surrounding the primary root tip in both the S+ and S- treatments, with water

Figure AI-2. Water potentials of the soil surrounding the cotton primary root tip and the middle part of the root system on day 24 (a) and day 29 (b) after transplanting in the simulated field drought experiment (n = 3-5 ± SE). ANOVA analyses were conducted for all treatments in each harvest. Different letters denoted statistical difference at $p < 0.05$.



potential of approximately -0.4 MPa compared to -0.8 MPa, respectively (Figure AI-2). The water potential of the soil surrounding the primary root tip was comparable to that used in the cotton seedling studies with the vermiculite-based system in the lab-based experiments (-1.0 MPa), as described in the previous chapters. The different sulfur treatments did not have significant differences in soil water potentials for well-watered and most of the water-stressed treatments. The only significant difference was seen between WS S+ and WS S- in the comparison of root area soil water potentials at the second harvest, in that the WS S+ treatment showed a significantly lower water potential.

In the water-stressed treatment, primary root tip water potentials were approximately -1.1 MPa in both the S+ and S- treatments at day 24 and declined further to approximately -1.25 MPa at day 29, almost reaching the same degree of tissue water stress as seen in the lab-based seedling studies (Figure AI-3). The root tip water potentials in the S+ and S- treatments were not significantly different. In the well-watered treatments, root tip water potentials were approximately -0.6 MPa in both S treatments at both harvests, indicating either a mild water deficit stress or perhaps a normal water potential for cotton roots grown in a sandy soil under simulated field conditions.

Effect of sulfur supplementation on root system length and dry weight under well-watered and water-stressed conditions

In the well-watered treatment, the total length of the root system was not significantly different between the S+ and S- treatments at either 24 d or 29 d after transplanting (Figure AI-4). In contrast, in the water-stressed treatment, root system length was significantly higher in the S- treatment than in the S+ treatment at 24 d. This trend was

Figure AI-3. Water potentials of well-watered (WW) and water-stressed (WS) cotton primary root tips with (S+) and without (S-) supplemental sulfur on day 24 (a) and day 29 (b) after transplanting in the simulated field drought experiment (n = 3 ± SE). ANOVA analyses were conducted for all treatments in each harvest. Different letters denoted statistical difference at $p < 0.05$.

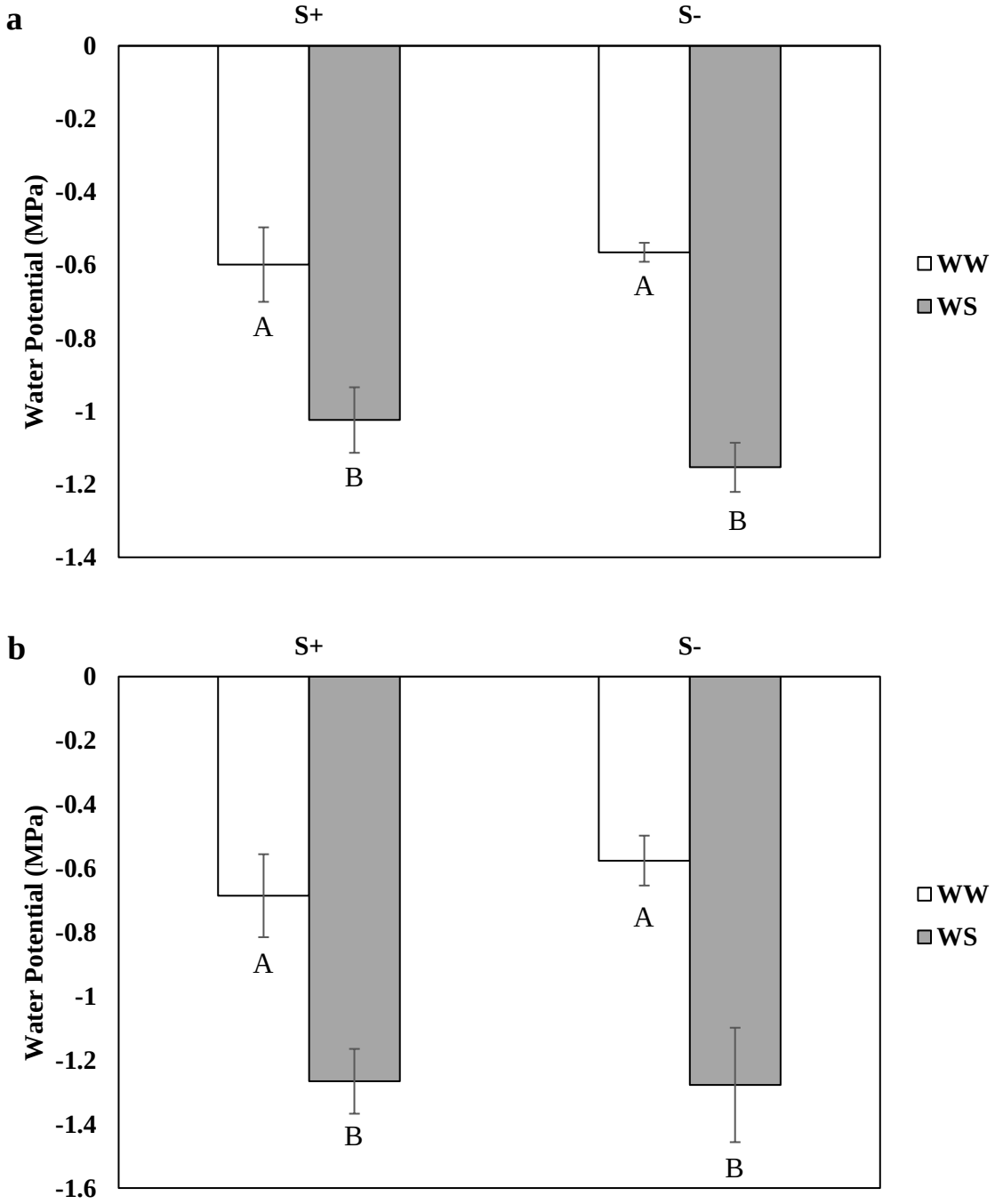
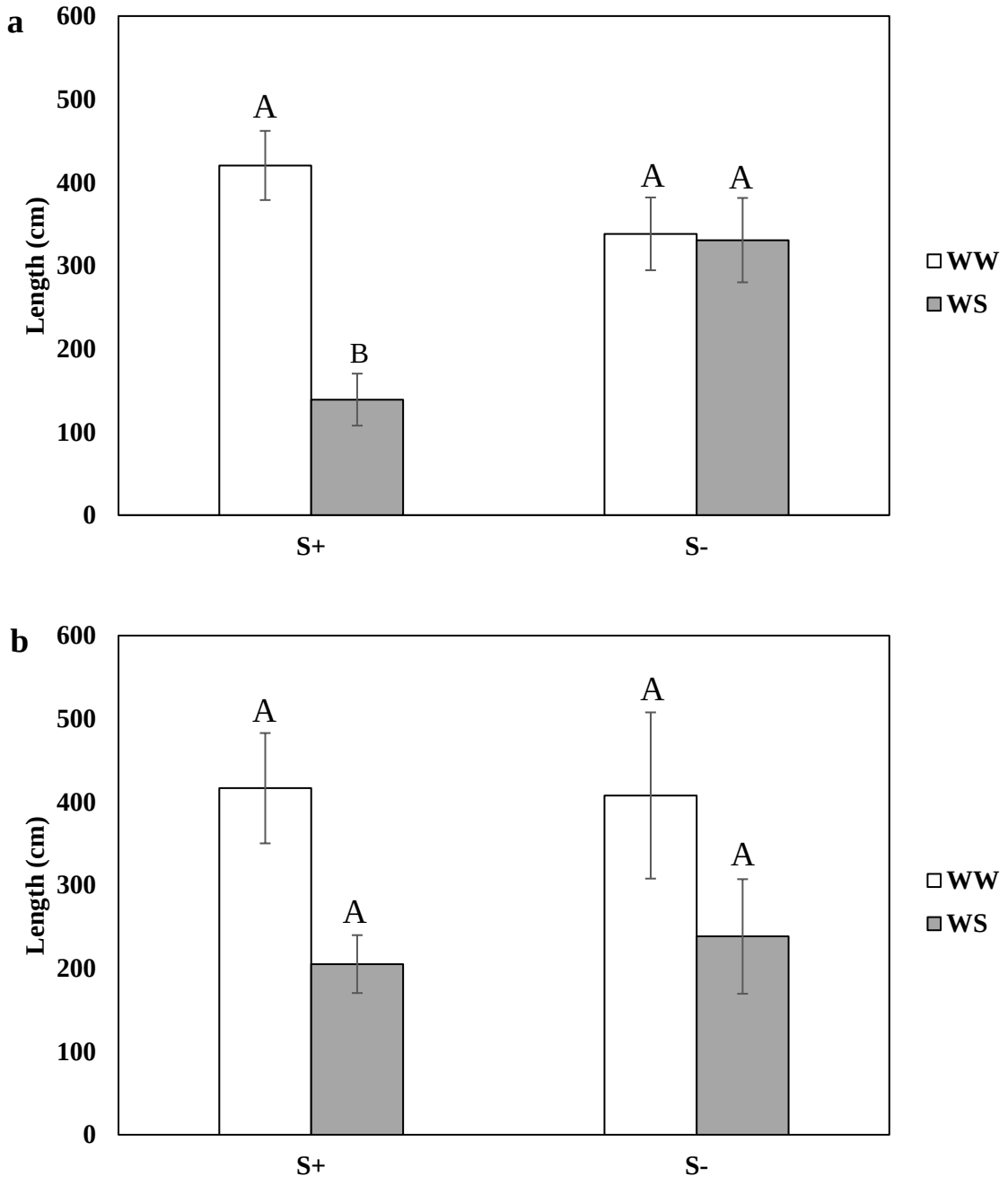


Figure AI-4. Total root length of well-watered (WW) and water-stressed (WS) cotton with (S+) and without (S-) supplemental sulfur on day 24 (a) and day 29 (b) after transplanting in the simulated field drought experiment (n = 5-9 ± SE). ANOVA analyses were conducted for all treatments in each harvest. Different letters denoted statistical difference at $p < 0.05$.



also apparent at 29 d although the treatments were not significantly different at this time. As a result of the differences in response to sulfur supply between the well-watered and water-stressed treatments, the water-stressed treatment exhibited a total root length that was much less than the well-watered controls in the S+ treatment at the first harvest, with a similar trend at the second harvest although the treatments were not significantly different (Figure AI-4b). In the S- treatment, in contrast, the water-stressed and well-watered treatments did not exhibit significant differences in root length at either harvest.

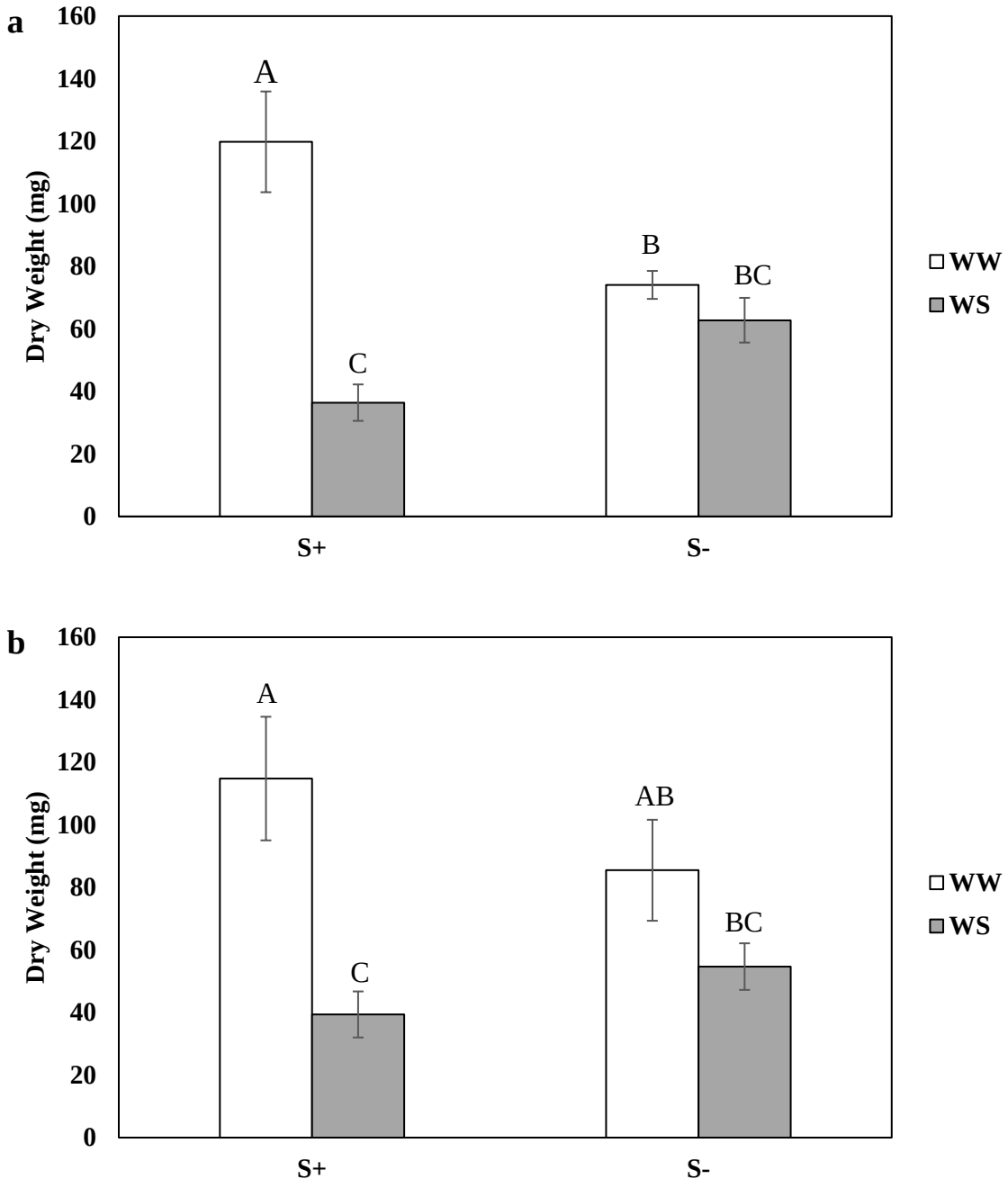
The trends in total root system dry weight changes were similar to the total root length measurements (Figure AI-5), with the exceptions that in the well-watered treatment the dry weight of the S- treatment was lower than the S+ treatment at the first harvest time (Figure AI-5a), and in the water-stressed treatment the dry weight data did not exhibit a significant difference between the S- treatment and the S+ treatment at the first harvest.

Thus, opposite to our hypothesis, root system development under water-deficit conditions exhibited a trend of enhancement in the S- treatment compared with the S+ treatment, especially for the total length of the root system.

Discussion

The experimental field system created a simulated dryland condition with limited irrigation such that the primary root grew into dryer soil layers towards the end of the experiment. The bottom layer of soil where the primary root tip was located had relatively low moisture content. The system also delivered closely similar soil water potentials to those achieved in the lab-based vermiculite system, which enables more

Figure AI-5. Total root dry weight of well-watered (WW) and water-stressed (WS) cotton with (S+) and without (S-) supplemental sulfur on day 24 (a) and day 29 (b) after transplanting in the simulated field drought experiment (n = 5-9 ± SE). ANOVA analyses were conducted for all treatments in each harvest. Different letters denoted statistical difference at $p < 0.05$.



reliable comparisons to be made. The root water potentials of the field-grown seedlings were substantially lower than those of the surrounding soil, as also observed in the lab-based seedling experiments, indicating a low hydraulic conductance for water movement into the cotton root growth zone, as discussed in Chapter 4. However, cotton root tips from the well-watered treatments in the field also displayed relatively low root tip water potentials compared to those seen in the well-watered lab environment that has a near-saturated water supply. The lower root tip water potentials for the well-watered field-grown seedlings were not expected. One possible explanation is that in the well-watered field treatments some of the primary roots were observed to have reached the bottom of the tubes. It is possible that these roots were partially exposed to the air (which is much drier than the soil) and thus experienced a certain level of water-deficit stress. Thus, the field system we developed may not be a good simulation of a true field condition when the soil is wet. Nevertheless, the sulfur supply did not affect the primary root tip water potentials in either the well-watered or water-stressed conditions.

The sulfur supplement appeared to have an additive effect on the water stress-induced reduction in root growth, more so at the early harvest date. This was unexpected since sulfur supplementation in field plots was reported to promote growth in cotton (Mullins, 1998; Yin et al., 2011; Geng et al., 2016), and in field-grown alfalfa, sulfur was observed to promote root length (Razmjoo & Henderlong, 1997). It is possible to speculate that cotton seedlings under drought conditions responded to the lack of sulfur (the soil used in the experiment was almost devoid of sulfur) by promoting root growth to search for this essential nutrient. For those seedlings with a substantial sulfur supply, a favorable environment decreased the demand for deeper penetration of the soil. However, since the

sulfur uptake under this field condition was not measured, we cannot reach a conclusion on whether sulfur uptake was inhibited, promoted or maintained in the water-stressed treatment plus or minus sulfur. A previous study (Schwab et al., 2000) showed that cotton total root length was promoted by increased sulfur uptake in a non-irrigated natural field environment. However, the sulfur uptake (measured as nutrient influx) was only assessed starting at 37 d after transplanting and the increase of sulfur uptake was observed after 87 d, and the results are therefore not directly comparable to the seedling system in this study.

Given that sulfur supplementation did not promote root growth, then, if sulfur uptake was not inhibited under the water-deficit condition, the channeling of sulfur in the cotton primary root towards methionine and SAM was not preventing root growth maintenance under the water-deficit condition. Thus, glutathione becomes a minor part of the water deficit responses in cotton. If sulfur uptake was inhibited under the water-deficit condition, the major problem would be that cotton could not get enough sulfur resources even when the surrounding environment is rich for sulfur. Therefore, either the plus or minus sulfur treatment could result in similar sulfur availability in water-stressed conditions. The channeling of sulfur in cotton metabolism in response to water deficit may reflect the selection of required metabolism when nutrient resources are limited. However, this needs further confirmatory studies before any conclusions can be drawn.

Conclusion

Root growth was affected by sulfur under drought conditions, but the effects were not as expected in that root growth was promoted when sulfur was absent. Further investigation

on the possible mechanisms underlying this result are needed.

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