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The morphological, physiological, and genetic underpinnings of intraspecific salinity tolerance in *Sorghum bicolor*

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**THE MORPHOLOGICAL, PHYSIOLOGICAL, AND GENETIC UNDERPINNINGS OF
INTRASPECIFIC SALINITY TOLERANCE IN SORGHUM BICOLOR**

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A dissertation submitted
to the Eberly College of Arts and Sciences
at West Virginia University

in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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ABSTRACT

The morphological, physiological, and genetic underpinnings of intraspecific salinity tolerance in *Sorghum bicolor*

Ashley N. Henderson

Decreases in land quality and quantity threaten the efficient production of agriculturally and economically pivotal crops. Such reductions in arable lands are a consequence of population growth and urbanization, and often result in the introduction of various abiotic stresses. The most common abiotic stressors include water limitation (drought), water logging (over watering), poor water quality (salinity), and extreme temperatures (cold, frost, heat). Each of these stressors negatively impact plant growth, development, and yield. Soil salinity, specifically, is a considerable constraint affecting lands used in agriculture. Salts in the soil rise both naturally and through anthropogenic factors making the abundance a threat. Salt toxicity affects plants in two phases, the first of which is osmotic stress. Similar to drought stress, salinity imposed osmotic stress is when soil water potential is lower than the osmotic potential of the plant, therefore limiting water and nutrient extraction. Following osmotic stress, plants accumulate salt ions (e.g. Na^+ , Cl^- , SO_4^{2-} , NO_3^-) that can increase to toxic concentrations and disrupt normal metabolic processes. Such toxicity results in reduction of important traits such as root biomass, live aboveground biomass, height, and grain yield. The goals of my dissertation work involved dissecting the various morphological, physiological, and genetic underpinnings of salinity tolerance in *Sorghum bicolor*. Since research to date demonstrates a significant amount of underlying genetic variation, I designed various projects to investigate the genetic controls associated with phenotypic responses to salinity stress among a diverse group of *Sorghum* genotypes. In my first research chapter, I screened 21 sorghum accessions and interpreted tolerance as the ability to maintain biomass, similar to plants in control conditions, in response to a long-term treatment of 75 mM NaCl. Findings from this research chapter, when combined with published phylogenetic relationships, suggest that the greater salinity tolerance observed in some accessions of *Sorghum bicolor*, specifically in the landrace durra, are a byproduct of drought tolerance acquired during domestication. To further expand on these findings, I selected two accessions that showed significant variation in tolerance and used qPCR to investigate expression variation of genes associated with salt detoxification. During the secondary phase of salinity stress (referred to as ion toxicity) Na^+ and Cl^- ions enter the plant and disrupt normal metabolic processes. If the ions are not effectively managed, the primary evident effect is increased leaf senescence. Tolerant genotypes, however, are able to overcome ion toxicity if they can 1) continue production of new growth and 2) efficiently and effectively exclude, extrude, sequester, and transport ions. Results from this chapter indicate that the tolerant accession experienced an earlier onset of osmotic adjustment, promoting the efficient uptake and transport of water. Additionally, the sensitive accession experienced an earlier onset of ionic stress, suggesting poor exclusion at the root level. These findings suggest that the tolerant genotype has better control over osmotic adjustment and ion transport systems, therefore requiring fewer resources to be diverted for the stress response, providing more available energy that can be utilized for new growth and development. Finally, to further investigate the variation in genetic

response to salt exposure, I evaluated the salinity tolerance that existed within a recombinant inbred line (RIL) population produced from a cross between *Sorghum bicolor* and *Sorghum propinquum*, two species that differ in response to salt exposure. In this study, I used a high-density genetic map to identify genetic markers correlated with salinity tolerance. I identified 146 candidate genes within the 18 QTL intervals. QTL containing candidate genes that aid in the alleviation of osmotic stress (i.e. water acquisition, osmotic adjustment) were significantly associated with live aboveground biomass, and QTL containing candidate genes that aid in ionic detoxification (i.e. sensing, signaling, transporting) were significantly associated with an increase in dead aboveground biomass. Given the QTL and their associated phenotypes observed in the study, I suspect that the increased tolerance observed in *S. bicolor* is a result of early osmotic adjustment followed by effective sensing and signaling during the ionic phase of salinity response. In summary my dissertation work suggests that salinity stress in sorghum triggers a complex network of tightly regulated response elements, where the accumulation of ions, if properly transported and sequestered, aid in osmotic adjustment and ionic alleviation. Further, given *Sorghum bicolor*'s domestication history, it appears that increased salinity tolerance arose as a byproduct of the drought tolerance acquired during domestication, therefore aiding in an early osmotic adjustment and subsequent water acquisition.

*For my fiancé, Justin,
who has been supportive and patient while I figured out how to
'finish this thing' called a Ph.D.
Thank you for taking my late-night-post-data-analysis (grumpy) comments, with a
grain of salt.
Your love and encouragement have never gone unnoticed,
I love you.*

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CHAPTER 1

OVERVIEW AND OBJECTIVES

Due to myriad factors that include rapid population growth and global climate change, agriculture is being forced onto marginal lands. Currently, it is estimated that the Earth's population is 7.7 billion, but this number is continuing to rise, and by 2100 it is expected to reach 11.18 billion (Frejka; Population Reference Bureau). It is critically and immediately necessary to efficiently use both arable and marginal lands to increase the food, fuel, water, and shelter available in order to meet the demands of the increasing population (Food and Agriculture Organization (FAO), 2008, 2009; Food and Agriculture Organization of the United Nations, 2017). Marginal lands are areas where crop production is currently limited in the absence of human inputs due to naturally occurring abiotic and biotic stressors (Elbersen *et al.*, 2018; Von Cossel *et al.*, 2019). Common abiotic stressors associated with marginal lands include low pH, high concentrations of salts, and high concentrations of heavy metals (Shahid & Al-Shankiti, 2013). Therefore, a deep understanding of the overall plant responses to abiotic stress will be crucial in order to meet global production needs.

To fully characterize abiotic stress tolerance from a whole plant perspective, we need to understand not only the gross plant response, but also the cellular events that contribute to variation in sensing and signaling among different genotypes and species. The overall objectives of my dissertation work include the identification of salt tolerant and salt sensitive *Sorghum* genotypes and their associated physiological, morphological, and genetic responses. I chose *Sorghum bicolor* as my primary study system to answer several questions in the field of salinity stress in crops:

1. What is the extent of intraspecific variation in salinity tolerance within *S. bicolor*?
2. Are there key morphological and physiological indicators associated with tolerance within *S. bicolor*?
3. What are the spatial and temporal sensing and signaling responses at the onset of salt exposure?
4. Do sensitive and tolerant genotypes differ in the spatial and temporal genetic response at the onset of salt exposure?
5. What regions of the genome correlate with higher degrees of salt tolerance?
6. What are the candidate genes for increased tolerance observed in domesticated sorghum?

Dissertation Organization

The body of my dissertation is organized into six chapters, with this chapter (Chapter 1: Overview and Objectives) being the first. Chapter 2, entitled “An Introduction to Salinity Stress”, provides a brief overview of the current literature addressing the intensifying constraints on agriculture. In this chapter, I discuss soil salinity and its overall impact on agriculture. I then elaborate on the osmotic and ionic effects of salinity stress on plants, including the genetic regulators, physiological responses, and morphological consequences. Finally, I introduce

Sorghum as a model system for my dissertation work. Subsequently, I present three original research chapters. Chapter 3, entitled “Phenotypic and physiological responses to salt exposure in *Sorghum* reveal diversity among domesticated landraces” and accepted in the *American Journal of Botany*, describes a study in which I screened a diverse group of *Sorghum* species and *S. bicolor* landraces and rank-ordered the accessions for salinity tolerance based on biomass traits. I then analyzed foliar proline and ion accumulation of individuals spanning a wide range of responses and compared the observed variation to known phylogenetic relationships (Mace *et al.*, 2013; Henderson *et al.*, 2019). Chapter 4, entitled “Variation in the expression of detoxification genes suggests that early water acquisition combined with Na⁺ exclusion leads to increased salt tolerance in *Sorghum bicolor*” describes a study in which I addressed early spatial and temporal variation of salt detoxification genes in both a tolerant and a sensitive accession of *Sorghum bicolor*. Chapter 5, entitled “Domestication & Detoxification: Early sensing, signaling, and osmotic adjustment aid in the increased salinity tolerance of *Sorghum bicolor*” extends the findings of Chapter 3 and Chapter 4. In this chapter, I use a recombinant inbred line population that was derived from a cross between a wild sorghum species, *S. propinquum*, and a domesticated genotype, *S. bicolor*. I characterized QTL related to several stress-associated phenotypes and discovered candidate genes that may play a role in increased tolerance in *Sorghum bicolor*. A final conclusion chapter (Chapter 6, entitled “General Conclusions”) summarizes the key findings, results, and importance of this dissertation work.

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CHAPTER 2

AN INTRODUCTION TO SALINITY STRESS

Environmental Stress, Marginal Soils, and the Impacts on Agriculture

Rapid increases in population size will have a significant impact on our ability to ensure the provision of basic human needs such as food, water, and shelter (Food and Agriculture Organization (FAO), 2008, 2009). As such, population growth is forcing housing and industrial developments onto lands that have been traditionally used for crop production. Such shifts limit the use of these lands for agriculture, and forces production on to marginal lands. It is estimated that the population will reach 9.3 billion by 2050, which is 34% higher than today's population (Food and Agriculture Organization (FAO), 2009), and with this, a 70-110% increase in food production will be required (Tilman *et al.*, 2011; Munns *et al.*, 2012). To achieve these challenging levels of production, increased yield will be required on both developed arable land as well as marginal soils where crops will need to be effectively cultivated.

Marginal soils are lands that have limitations in their use, especially without inputs (Elbersen *et al.*, 2018). Common characteristics of marginal soils include high pH, high concentrations of salts, and high concentrations of heavy metals that cause stress on plants, impacting growth and yield (Shahid & Al-Shankiti, 2013; Von Cossel *et al.*, 2019). Saline soils are a result of an overabundance in the following ions: sodium, chloride, calcium, sulphate, and magnesium, with sodium chloride (NaCl) being the most prominent and destructive to plants (Tari *et al.*, 2013; Munns *et al.*, 2020). Salt-affected soils have long been a concern in agriculture, but the magnitude and intensity is becoming progressively imperative, given that 6% of total land (Food and Agriculture Organization (FAO), 2008) and 33% of irrigated land is affected by salinity, and these numbers are increasing at a rate of 10% per year (Shrivastava & Kumar, 2015).

Some salts within the soil are expected, but large increases in abundance are often interrelated, via both human interference and natural processes. For example, global climate change has triggered more frequent and prolonged periods of drought, which has ultimately affected the rate of soil water evaporation (increasing soil salt content) and this has led to an increased need for irrigation (Asif *et al.*, 2018). Irrigation leads to increases in soil salinity in both new areas where irrigation was not previously needed and in areas where soil salinity was already a problem (Abrol *et al.*, 1988; Flowers & Yeo, 1995; Munns *et al.*, 2020). Additional examples include land clearing, ingress of sea water, changes in the rate of soil water evaporation, the use of animal manures as fertilizers, and soil run off (Abrol *et al.*, 1988; Munns, 2005, 2010; Mahajan & Tuteja, 2005; Jenks *et al.*, 2007; Shabala, 2012; Asif *et al.*, 2018; Vaishnav *et al.*, 2019). For these reasons, soil salt concentrations will continue to rise. Therefore, understanding the effects of soil salinity on crop growth and production is critical to meet demands.

Osmotic and Ionic Effects of Salinity Stress

Salinity stress, induced by an over-abundance of ions in the soil, leads to both osmotic and ionic stress (Rengasamy, 2006; Munns & Tester, 2008; Morton *et al.*, 2019). Osmotic stress results from the inhibition of water absorption, rather than overall toxicity (Bernstein & Hayward, 1958;

Munns & Tester, 2008). Highly saline environments are soils where ions are present in the area surrounding the rhizosphere and therefore generate a low water potential in the soil (Mahajan & Tuteja, 2005; Iqbal *et al.*, 2014). Even when soil conditions are wet, because soil water potential is lower than the plants osmotic potential, water and nutrients will not be extracted from the soil by the roots, therefore simulating drought-like conditions. The overall major effect of osmotic stress observed in both tolerant and sensitive species and genotypes is decreased above- and belowground growth due to lack of water (**Figure 1**) (Munns, 2005; Iqbal *et al.*, 2014). The initial shock of salinity stress causes cells to lose water, resulting in decreased volume and turgor; however, this decrease is not permanent. The initial loss of cell size is regained within hours, but the cell elongation rate is reduced, resulting in decreased leaf size (Yeo *et al.*, 1991; Passioura & Munns, 2000; Munns & Tester, 2008). Generally, leaf surface area is affected to a greater extent than depth, therefore resulting in smaller but thicker leaves. In order to mitigate the primary osmotic effect, plants must maintain cell turgor, which can be accomplished by osmotic adjustment (Flowers & Colmer, 2008; Turner, 2018). Plants produce and accumulate compatible solutes, which lowers the plants osmotic potential to assist in maintenance of turgor and enable the movement of water through the plant, therefore aiding in osmotic adjustment (Bernstein, 1961, 1963; Hsiao *et al.*, 1976; Turner, 2018). Examples of compatible solutes, or osmolytes, include various amino acids, carbohydrates, methylamines, sugars, and polyols (Negrão *et al.*, 2017; Turner, 2018).

The secondary phase of salinity stress results from the accumulation of ions as they infiltrate the plant during water uptake. Once the plant has overcome the primary phase of salinity stress by altering osmotic potential, ions enter the root symplast by passive influx through non-selective cation channels (NSCC) from the soil (Tyerman & Skerrett, 1998; Davenport & Tester, 2000; Demidchik & Tester, 2002; Munns *et al.*, 2020). Once Na^+ enters the root symplast, it is actively transported to the xylem where ions enter the shoot symplast from the xylem apoplast and move into mesophyll cells via NSCCs (Munns *et al.*, 2020). This overabundance of ions in the cytoplasm of cells becomes toxic and ultimately leads to ionic disequilibrium that results in premature leaf senescence, inactivation of enzymes, oxidative stress, and overall toxicity (Iqbal *et al.*, 2014). Because salts can be compartmentalized into older leaves or sequestered into vacuoles, plants can survive the ionic component of salt stress if the rate of new leaf emergence exceeds the rate of leaf death (Koyama *et al.*, 2001). This enables the plant to continue photosynthesizing and therefore producing enough carbon for growth and development (Munns, 2005, 2010).

Because cells have a negative electrical potential, Cl^- is less of a concern because uptake is less energy-demanding, and Cl^- cannot passively enter a cell unless cytosolic concentrations are extremely low (Feng *et al.*, 2015). The accumulation of Na^+ , on the other hand, disrupts the uptake and distribution of K^+ , an essential ion for basic biological functions (Tari *et al.*, 2013). The ability to maintain a high K^+/Na^+ ratio is a strong indication of a tolerant genotype (**Figure 1**). Much of the research aimed at dissecting the underpinnings of salinity tolerance indicates that survival on saline soils depends on the ability to exclude toxic sodium ions. However, recent studies have suggested that targeting additional mechanisms of tolerance is critical to fully understand osmotic and ionic tolerance.

Morphological Consequences of Salinity Stress

A myriad of morphological changes are associated with increased salinity (Munns & Tester, 2008; Tari *et al.*, 2013). For example, it has been observed that decreases in total germination and increases in the time taken to germinate result from salt exposure (Gill *et al.*, 2003; Almodares *et al.*, 2007; Tari *et al.*, 2013). Following germination, biomass accumulation is negatively affected, predominantly as a result of the initial osmotic stress (**Figure 1**) (Munns & Tester, 2008; Negrão *et al.*, 2017). Osmotic stress reduces cell elongation and division, and directly affects leaf area, plant height, and stem diameter (Yeo *et al.*, 1991; Passioura & Munns, 2000; Cramer, 2002; Fricke & Peters, 2002; Munns & Tester, 2008; Silva *et al.*, 2019). Further, with long-term osmotic stress, lateral shoot development becomes inhibited and overall aboveground biomass is reduced (Silva *et al.*, 2019). While reductions in root growth have been observed (Rahnama *et al.*, 2011; Jung & McCouch, 2013), root growth is not as severely affected as leaf growth. This is likely an adaptive response because with decreased leaf biomass, less water is extracted from the soil, ultimately affecting soil salt concentrations and ion uptake (Munns & Tester, 2008; Munns & Gilliham, 2015; Hanin *et al.*, 2016). Additionally, the ability to maintain lateral root growth is important for the exploration of non-saline water (Galvan-Ampudia *et al.*, 2013; Munns & Gilliham, 2015). In cereal crops, tillering is correlated with increased biomass, but with increased salinity, there is a decrease in tillering, resulting in reduced growth and early maturation (Munns, 2002; Jenks *et al.*, 2007; Shabala, 2012). Given what we know about osmotic and ionic stress and the morphological consequences associated with salinity, it is important to note that tolerant genotypes can be identified by their ability to maintain live aboveground biomass and limit dead aboveground biomass.

Physiological Response to Salinity Stress

Physiological responses associated with salinity stress include changes in potassium (K^+) uptake, photosynthetic efficiency (Nazar *et al.*, 2011), chlorophyll content, antioxidant metabolism, hormone signaling (Wang & Huang, 2019), and the accumulation of compatible solutes (Misra & Gupta, 2005; Khan *et al.*, 2012; Tari *et al.*, 2013). Potassium uptake (K^+) is critical for normal metabolic function (Wu *et al.*, 2018). Specifically, K^+ is an essential nutrient for protein synthesis (Jones *et al.*, 1979; Blaha *et al.*, 2000; Wu *et al.*, 2018), phloem transport (Gajdanowicz *et al.*, 2011; De Schepper *et al.*, 2013), photosynthesis (Fischer, 1968; Wu *et al.*, 2018), enzyme activity (Bhandal & Malik, 1988), and signaling (De Schepper *et al.*, 2013; Shabala, 2017). Because of the similarity in size and weight of Na^+ and K^+ , Na^+ competes with K^+ , and therefore disrupts normal ion ratios (Pardo & Quintero, 2002; Munns & Tester, 2008; Wu *et al.*, 2018). Photosynthesis is affected most in expanded leaves, not due to the accumulation of toxic salt ions, but due to stomatal closure, a plant response to avoid excess loss of water (Munns & Tester, 2008; Munns, 2010). When stomata close, the light-independent reactions of photosynthesis are inhibited and photosynthesis halts (Liu *et al.*, 2017; Nounjan *et al.*, 2018). Impaired photosynthesis leads to leaf chlorosis and results in leaf senescence (Munns, 1993, 2002; Tari *et al.*, 2013).

Molecular Responses to Salinity Stress

Osmotic stress is the result of limited water absorption, regardless of the abiotic stress causing it; however, research indicates that the underlying genetic responses differ depending on the specific type of stress to which the plant is exposed. For example, in *Arabidopsis*, gene expression profiles during exposure to a suite of abiotic stresses (drought, salt, temperature, and UV-B light) provided evidence that there is a common set of genes that are initially induced. Over time, however, gene expression patterns diverge in a stress-dependent manner (Kilian *et al.*, 2007), indicating that it is critical to delineate the expression patterns for the initial response (generalized) from the specialized response (specific).

The specific genetic responses to salinity stress can be grouped into three broad functional categories: 1) genes that control uptake and transport of toxic ions, 2) genes that aid in osmotic adjustment, and 3) genes that aid in increased growth and tissue tolerance, regardless of salinity (Munns, 2005; Munns & Tester, 2008). Most genes are not exclusive to one functional category, but rather play a role in all three components of salinity tolerance. For example, Munns and Tester (2008) organized six groups of genes that are involved in different tolerance processes and described the role of each in aiding in salinity tolerance. Processes described included: (1) sensing and signaling in roots (2) shoot growth (3) photosynthesis (4) accumulation of Na⁺ in shoots (5) accumulation of Na⁺ in vacuoles and (6) accumulation of organic solutes. The genes in each of these processes play a role in both osmotic and ionic stress tolerance (Na⁺ exclusion and tissue tolerance).

Genes known to play a role in sensing and signaling in roots include salt-overly sensitive3 (SOS3) and sucrose nonfermenting-1-related kinases (SnRK) (Munns & Tester, 2008). During osmotic stress and adjustment (category 2), these genes aid in the modification of long-distance signaling, and during ionic stress they aid in net ion transport to the shoot (category 1) and vacuoles (category 3). Genes that are involved in the photosynthetic response include ERA1 (enhanced response to ABA1), PP2C (protein phosphatase 2C), AAPK (abscisic acid-activated protein kinase), and PKS3 (phytochrome kinase substrate-like protein). These genes are involved in stomatal closure during the osmotic phase of salinity stress (category 2) and aid in decreased toxicity in chloroplasts (category 1 and 3) during the ionic phase. Genes involved in the accumulation of Na⁺ in shoots include high-affinity K transporter (HKT) and SOS1. These genes aid in tolerance via increased osmotic adjustment (category 2) by altering Na⁺ transport and extrusion during ionic stress (category 1 and 3). HKT, specifically, unloads Na⁺ ions from the xylem sap to prevent overaccumulation in the aboveground portion of the plant (Sunarpi *et al.*, 2005; Ren *et al.*, 2005; Huang *et al.*, 2006). With decreased sodium in the leaf sheaths, plants are able to maintain photosynthetic efficiency and capacity. The SOS1 antiporter removes Na⁺ from the cytoplasm in a process known as extrusion (Batelli *et al.*, 2007; Yue *et al.*, 2012; Lekshmy Sathee *et al.*, 2015; Miranda *et al.*, 2017; Mahi *et al.*, 2019; Baghour *et al.*, 2019). Genes involved in the accumulation of Na⁺ in vacuoles include NHX (sodium-hydrogen exchanger), a plasma membrane-localized transporter that is important in sequestration of Na⁺ into vacuoles, and AVP (inorganic H pyrophatase) (Shi & Zhu, 2002; Leidi *et al.*, 2010; Bassil *et al.*, 2011; Li *et al.*, 2011; Barragán *et al.*, 2012; Baghour *et al.*, 2019, p. 2011). During the osmotic phase, these genes aid in increased osmotic adjustment (category 2), and during the ionic phase they control the sequestration of Na⁺ into root and leaf vacuoles (category 1 and 3). Lastly, candidate genes that have been shown to play a role in osmotic regulation as a compatible solute include

P5CS (putative delta-1-pyrroline-5-carboxylate synthase), OTS (overly tolerant to salt), MT1 (metallothionein-like protein 1), M6PR (mannose-6-phosphate receptor), S6PDH (nadp-dependent d-sorbitol-6-phosphate dehydrogenase), and IMT1 (initiator methionine tRNA). During the osmotic phase, these genes aid in increased osmotic adjustment (category 2), and during the ionic phase they control the transport of Na⁺ (category 1 and 3) (Munns & Tester, 2008).

Sorghum as a Model for Salinity Stress

Crops that are most affected by global climate change, and therefore increased salinity, include the major grain crops maize, sorghum, wheat, rye, barley, oat, millets, and rice. Among these is *Sorghum bicolor* (L.) Moench, which is a C4 crop used for a variety of purposes (Wiersema & Dahlberg, 2007; Paterson, 2008; Ali & Idris, 2015; McCormick *et al.*, 2018). *Sorghum* is important in grain (ranks fifth), biofuel (ranks second), animal feed, fiber, fermentation, and fertilizer production (Paterson, 2008; Tari *et al.*, 2013). Sorghum is especially well adapted to hot, dry, saline, and waterlogged environments (Steduto *et al.*, 1997; Almodares *et al.*, 2008b,a; Promkhambhu *et al.*, 2010; Morris *et al.*, 2013; Mace *et al.*, 2013; Zhang *et al.*, 2016; Kadam *et al.*, 2017). It is, therefore, tolerant to most abiotic stresses, even with limited input (Doggett, 1970; Steduto *et al.*, 1997; Almodares *et al.*, 2008b,a; Paterson, 2008). With millet being an exception, sorghum is ranked the highest within agricultural crops for drought tolerance, which is consistent with a historical domestication background in Africa (Steduto *et al.*, 1997; Almodares *et al.*, 2008a). *Sorghum* was first domesticated >8,000 years ago in Ethiopia and Sudan (Wendorf *et al.*, 1992). Following domestication, *S. bicolor* diffused to various regions in Africa, and adaptation to various environments resulted in four landraces (Morris *et al.*, 2013; Mace *et al.*, 2013; Smith *et al.*, 2019). Kafir, which tends to predominate in South Africa, shows the largest genetic variation compared to other landraces, likely due to migration into a contrasting agroclimate (Morris *et al.*, 2013). Guinea tends to be widely distributed in western Africa in tropical savannas. A subgroup of guinea, referred to as guinea-margaritifera, is present in the same geographical area as guinea but appears to have undergone a separate, more recent, domestication event (Morris *et al.*, 2013; Mace *et al.*, 2013; Mullet *et al.*, 2014). Caudatum, primarily found in central-west Africa in tropical savanna climates, displays the least amount of population structure due to exposure of adjacent and varying climates. Lastly, durra is distributed in the warm semiarid deserts of northern Africa and India (Morris *et al.*, 2013; Mace *et al.*, 2013; Mullet *et al.*, 2014; Smith *et al.*, 2019). Given that each landrace adapted in various geographical regions, and each landrace possesses a diverse gene pool, *Sorghum* is a valuable resource for dissecting genetic adaptation to various environments.

Further, *Sorghum* shares a common ancestor with *Zea mays* (~12 mya); however *Sorghum* has a simple genome compared to maize (Gaut *et al.*, 1997; Swigoňová *et al.*, 2004). Since their divergence, maize has undergone a whole-genome duplication event followed by rediploidization resulting in massive genomic reorganization and a ~2.5 Gb genome (2n=20) with 85% repetitive DNA content. In contrast, sorghum has a much smaller (~730 Mb) diploid genome with less repetitive DNA (~61%) making it an ideal model system for genomic studies (Paterson *et al.*, 2009). The genome of *S. bicolor* has been well annotated and the transcriptome has been fully interrogated across the juvenile, vegetative, and reproductive stages of development (McCormick *et al.*, 2018). Further, more than 49 additional diverse genotypes have

been sequenced (Mace *et al.*, 2013; McCormick *et al.*, 2018; Boyles *et al.*, 2019). This makes thorough investigations in more specific studies of the genome and transcriptome manageable because we can utilize the information already available in conjunction with our salt-specific data. These key features of sorghum make it easier to dissect the genetic underpinnings associated with morphological and physiological responses, and, due to the close evolutionary relationship, it is an excellent model for crop improvement in maize and other related cereal grasses.

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Figures

Figure 1. Effects on Biomass During Osmotic and Ionic Phases of Salinity Stress.

Figure 1. Effects on Biomass During Osmotic and Ionic Phases of Salinity Stress. The initial decline in total plant biomass is a result of osmotic stress, but after a prolonged period of time, a salt specific response occurs. Plants that are better able to handle the ionic component via extrusion and compartmentalization display increased tolerance. Modified from “Mechanisms of salinity tolerance” (Munns & Tester, 2008).

CHAPTER 3

PHENOTYPIC AND PHYSIOLOGICAL RESPONSES TO SALT EXPOSURE IN SORGHUM REVEAL DIVERSITY AMONG DOMESTICATED LANDRACES

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Abstract

Soil salinity negatively impacts plant function, development, and yield. To overcome this impediment to agricultural productivity, variation in morphological and physiological response to salinity among genotypes should be explored in agriculturally and economically important crops. *Sorghum bicolor* is a staple crop that has adapted to a variety of environmental conditions and contains a significant amount of standing genetic diversity, making it an exemplary species to study phenotypic and physiological variation in salinity tolerance. In our study, a diverse group of sorghum landraces and accessions were first rank-ordered for salinity tolerance and then individuals spanning a wide range of response were analyzed for foliar proline and ion accumulation. We found that, while proline is often a good indicator of osmotic adjustment and is historically associated with increased salt tolerance in many species, proline accumulation in sorghum reflects a stress response injury rather than acclimation. When combining ion profiles with growth responses and stress tolerance indices, the variation observed in tolerance was not a sole result of Na⁺ accumulation, but rather reflected accession-specific mechanisms that may integrate these and other metabolic responses. We then compared our results with known phylogenetic relationships within sorghum. The most parsimonious explanation for our findings is that salinity tolerance was acquired early during domestication and subsequently lost in areas that vary in soil salinity.

Key words: comparative analysis; environmental adaptation; osmotic adjustment; potassium sodium ratio; proline; relative decreases in plant biomass; salinity stress; stress tolerance; stress tolerance index

Abbreviations:

RDPB: relative decrease in plant biomass
ST: stress tolerance
STI: stress tolerance index

Introduction

Soil salinity is a major constraint to agricultural crop productivity, limiting the provision of food, fuel, and fiber to large portions of the world's population (Munns & Tester, 2008; Qadir *et al.*, 2014; Morton *et al.*, 2019). Soil salinity, defined as concentrations of soluble salts above 40 mM sodium chloride (NaCl) or greater than 4 dSm⁻¹ electrical conductivity (Jamil *et al.*, 2011; Shrivastava & Kumar, 2015), is a global problem affecting more than 20% of the irrigated land used for agriculture (Qadir *et al.*, 2014). Salts increase in soils naturally through the rise and ingress of sea water (Abrol *et al.*, 1988; Singh, 2015; Liu *et al.*, 2017), weathering of soil parent material (Abrol *et al.*, 1988), and high surface evaporation associated with low precipitation (Chhabra, 1996; Shrivastava & Kumar, 2015; Singh, 2015). Anthropogenic factors, such as irrigation with saline water, inadequate field drainage, and over application of animal waste, also result in increased soluble salts in agricultural soils (Munns & Tester, 2008; Thomson *et al.*, 2010; Singh, 2015; Lemanowicz & Bartkowiak, 2017).

Increased salinity negatively impacts plant function and development through both osmotic and ionic effects (Munns & Tester, 2008; Negrão *et al.*, 2017). In the osmotic phase, salinity impedes plant water acquisition. Water uptake is disrupted even when soils contain adequate moisture due to lower soil water potentials compared to plant osmotic potentials. This imbalance inhibits water extraction by plant roots, simulating drought-like conditions (Munns & Tester, 2008; Negrão *et al.*, 2017). In response to osmotic stress, leaf emergence and growth rate is reduced, stomata close resulting in decreased/inhibited photosynthesis, and leaf temperature increases (Munns & Tester, 2008; Alqahtani *et al.*, 2019). In the ion-dependent phase, ions such as Na⁺ and Cl⁻ enter the plant, accumulate to toxic levels in the cytoplasm, and disrupt normal metabolic function (Munns & Tester, 2008).

Various plant responses result from both ion-independent and dependent phases. Key growth responses to osmotic stress include decreased leaf and root growth due to lack of turgor (Munns, 2005). Leaf growth is affected to a greater extent than root growth, resulting in a decreased shoot to root ratio (Negrão *et al.*, 2017). Toxic ions accumulate in leaves and affect ion homeostasis and photosynthesis, resulting in premature leaf senescence (Munns, 1993, 2002). As ions accumulate, Na⁺ specifically disrupts the uptake and distribution of K⁺, an essential ion for basic biological functions such as stomatal opening, enzyme activity (Tari *et al.*, 2013), or cellular metabolism (Zhu, 2003).

Sorghum bicolor (L.) Moench is an African grass that is cultivated for food, fuel, and fiber. There are five landraces of sorghum (bicolor, kafir, guinea, caudatum, and durra) that are classified based on morphology (Shehzad *et al.*, 2009) and reflect genetic diversity associated with different geographical regions of adaptation (Price *et al.*, 2005; Morris *et al.*, 2013; Mace *et al.*, 2013; Mullet *et al.*, 2014; Smith *et al.*, 2019). There are also 10 intermediate landraces that are a combination of the five landraces (Oliveira *et al.*, 1996; Price *et al.*, 2005). Worldwide, sorghum ranks fifth as a contributor to grain production and second as a biofuels feedstock (Wiersema & Dahlberg, 2007). Sorghum thrives in areas that are often not suitable for other crops and requires minimal human input while delivering high yields (Mullet *et al.*, 2014). Given these traits, sorghum provides a model system for studying the complex basis of salt tolerance because it is relatively drought tolerant (Mullet *et al.*, 2014; Fracasso *et al.*, 2016; McCormick *et al.*

al., 2018) and, as with drought stress, salinity stress results in osmotic imbalance (Munns & Tester, 2008).

Here, we evaluated the variation in whole-plant response to salt exposure in a diverse panel of sorghum accessions that are representative of a diversity of sorghum landraces and species. Specifically, we included a hybrid species, three wild progenitors, and a variety of cultivated landraces in order to evaluate the association between standing genotypic and phenotypic diversity and salinity tolerance. Because *Sorghum bicolor* was originally domesticated >8,000 years ago in eastern Africa and has since adapted to a variety of geographical regions (Wendorf *et al.*, 1992; Mace *et al.*, 2013; Winchell *et al.*, 2017; Smith *et al.*, 2019), we hypothesized that variation in salt tolerance may be a result of post-domestication adaptation to areas varying in soil salinity. Therefore, we expected to observe landraces that adapted in regions with high soil salt content to have increased salinity tolerance compared to landraces that adapted in low soil salt content.

Materials and Methods

Plant Material

Seeds for 21 diverse *Sorghum* accessions representative of the different landraces were obtained from the *Germplasm Resources Information Network (GRIN)*. Landrace information was provided by GRIN and arbitrary codes were assigned and used to reference specific accessions throughout this study (**Table 1**).

NaCl Exposure

A pilot study, in which five randomly selected accessions were exposed to increasing salt concentrations, was used to determine an appropriate experimental treatment level. Replicates were treated with 0 mM, 25 mM, 75 mM, 125 mM, 150 mM, or 200 mM NaCl beginning at the third leaf stage of development and for a period of four weeks. There was a clear reduction in growth and biomass as NaCl increased (**Appendix S1**, see the Supplementary Data with this article). Because soil is considered to be saline at concentrations greater than 40 mM (Shrivastava & Kumar, 2015) and we observed growth reduction without mortality at 75 mM NaCl, we used 75 mM NaCl as our treatment.

Twenty seeds of each accession (10 replicates per treatment and a total of two treatments) were germinated in metromix soil in 5 cm × 5 cm × 5 cm planting plugs in a controlled greenhouse. Target germination conditions were: 29/24°C (day/night temperatures), 60% humidity, and with ambient daylight at 14 hours a day. During germination, all seedlings were misted regularly with non-saline tap water and watered with a 20-10-20 N-P-K fertilizer (J.R. Peters, Inc., Allentown, PA, USA) fertilizer diluted to 200 mg N L⁻¹ once a week. When 90% of the seedlings were at the third leaf stage of development, seedlings were transplanted into 5 cm × 5 cm × 25 cm tree pots (Stuewe and Sons, Tangent, OR, USA) filled with a 1:1 mix of #2 and #4 silica sand. Seedlings were watered with tap water for one-week post-transplant to provide a period of establishment.

At the seedling stage and well before flowering (the 4th–6th leaf stage of development), plants were watered to saturation daily with tap water (control) or tap water containing 75 mM NaCl

solution (treatment). Twice each week, all plants were additionally watered to saturation with a 20-10-20 N-P-K fertilizer at a rate of 200 mg N L⁻¹ (J.R. Peters, Inc., Allentown, PA, USA). Treatment was carried out for a total of 12 weeks.

Biomass Measurements

At 12 weeks post-treatment, five of the ten replicates were collected for biomass measurements. At harvest, plants were separated into roots and shoots. Roots were brushed free of adhering sand and the biomass of each plant was dried in five categories (root biomass, main stem, live leaves [$>50\%$ green], dead leaves [$<50\%$ green], and tillers [shoots from the base of the stem]). All biomass samples were dried at 65°C for a minimum of 72 hours.

Throughout this study, the following terms were used to describe the following tissues: live aboveground biomass was the sum of the live stem, live leaves, and live tillers. Dead aboveground biomass was the sum of the dead stem, dead leaves, and dead tillers. Total aboveground biomass was the sum of live and dead aboveground biomass. Percent of alive aboveground biomass was the ratio of live aboveground biomass by the total aboveground biomass as a fraction of 100.

Phenotype Measurements

The remaining five replicates were used for phenotypic measurements. The following measurements were recorded after 12 weeks of treatment: total number of leaves, total number of live leaves, percent live leaves (calculated from live leaves and total leaves), mortality (defined as 1 for alive and 0 for dead), and height (cm). Height was measured from the base of the main stem to the tip of the newest emerged leaf.

Physiology Measurements

Physiology measurements were taken at 12 weeks post treatment on the third leaf from the top because it was the oldest living leaf across all plants. The same five replicates used for phenotypic measurements were used for quantification of chlorophyll content (SPAD 502 Plus Chlorophyll Meter, Konica Minolta, Osaka, Japan) and quantification of proline concentration. Leaf ion profiles were measured on the same five replicates used for biomass measurements. Proline and ion concentrations were quantified on a subset of accessions that showed variation in phenotypic responses.

Foliar sodium and potassium concentrations were determined on microwave-assisted acid digests (MARSPress, CEM Corporation, Matthews, NC, USA). Leaf tissue was dried for 72 h at 70°C, ground in a CyclotecTM 1093 sample mill (FOSS, Hillerød, Denmark), and digested in 4 mL of 70% HNO₃ and 1 mL of 30% H₂O₂ (Carrilho *et al.*, 2002). Digests were analyzed for elemental concentrations by inductively coupled plasma optical emission spectrometry (ICP-OES) by the Pennsylvania State University Analytical Laboratory (State College, PA, USA). Elemental yields were obtained using ground apple leaves from the National Institute of Standards and Technology and were used to calculate elemental content from the ICP-OES data.

Quantification of proline was determined colorimetrically by comparisons with standards. Following harvest, samples were flash frozen and immediately stored at -80°C. Tissue was

ground to a fine powder and 2 mL of 70% ethanol was added to each sample. Samples were incubated at room temperature with continuous agitation for 24 h, after which they were centrifuged, and the supernatant was transferred to a new tube. The ground tissue was then re-suspended in fresh 2 mL of 70% ethanol for an additional 24 h at room temperature with agitation. After the second extraction, both 2 mL extracts were combined. Samples were then incubated at 95°C for 20 min with a 1% ninhydrin and 60% acetic acid reaction mix and quantified on a Tecan Infinite® 200 PRO plate reader (Tecan, Grödig, Austria) at 520 nm.

Tolerance Indices

Salinity tolerance in plants is often defined as the ability of a plant to sustain growth in the presence of salts (Munns, 2010). In our study, several traits were evaluated and tolerance was defined by the ability to maintain biomass (live and total) when comparing salt exposure to control conditions (Negrão *et al.*, 2017).

Stress Tolerance (ST) – The stress tolerance value was calculated for SPAD of the oldest living leaf across all plants, percent of live leaves, height (cm), mortality, live aboveground biomass (dry weight in g), dead aboveground biomass (dry weight in g), and root biomass (dry weight in g) as (Negrão *et al.*, 2017):

$$ST = \frac{Y_{Salt\ at\ T_2}}{Y_{Control\ at\ T_2}}$$

Where Y is a growth-related trait measured at the end of the experiment (T₂) under control and salt treatments as indicated. The ST value normalizes performance by accession.

Relative Decrease in Plant Biomass (RDPB) – The sum of biomass for all tissues separated during a destructive harvest was used to determine the relative decrease in plant biomass (RDPB, Negrão *et al.*, 2017) for each accession and landrace. The RDPB describes the reduction of growth in stressed conditions compared to control conditions. The RDPB is calculated as:

$$RDPB = \frac{M_{f\ control} - M_{f\ salt}}{M_{f\ control}}$$

Where M_f is plant mass under control and salt treatments as indicated. Lower RDPB values indicate less reduction in biomass under stress conditions and are representative of higher degrees of tolerance. RDPB was converted to percent of plant biomass retained (1-RDPB). Tolerant genotypes were individuals with high amounts of biomass retained, while sensitive individuals retained less biomass in response to treatment.

Stress Tolerance Index (STI) – The stress tolerance index (STI, Negrão *et al.*, 2017) was calculated for biomass traits (live aboveground biomass, dead aboveground biomass, root biomass). The STI was calculated as:

$$Stress\ Tolerance\ Index = \frac{Y_{control}}{Y_{control\ average}} \times \frac{Y_{salt}}{Y_{control\ average}}$$

Where Y_{control} and Y_{salt} are measured traits for control and salt treatments for each accession, and $Y_{\text{control average}}$ is the trait response under control conditions for the entire population evaluated. A greater STI for an accession indicates higher degrees of salt tolerance. The STI accounts for genotypic response to salinity stress and compares it to a population response to reveal accessions that are performing superior to others. Raw STI values for live aboveground biomass, dead aboveground biomass, and root biomass (**Appendix S2**) were converted to a rank order. STI was rank ordered with 0 indicating missing data, 1 indicating the lowest STI, and 21 indicating the highest possible STI.

Statistical Analyses

Treatment Effects – Non-metric multidimensional scaling (NMDS) (Julkowska *et al.*, 2019), performed in R v. 3.6.0 (R Core Team, 2013), was used to evaluate plant response to salt exposure and to determine groupings among accessions across treatments. The `dimcheckMDS` function in the `geoveg` package generated the associated stress value with each reduction in dimension. A lower stress value indicates higher conformity between the true multivariate distance between samples and the distance between samples in reduced dimensions. Two dimensions were deemed appropriate. NMDS was paired with analysis of similarity (ANOSIM), which statistically tests clusters and ordination results from the NMDS. The ANOSIM determines whether the dissimilarity matrix used in the NMDS ordination is significantly different. Using an ANOSIM, we tested treatment effects. Dissimilarities were determined using a Bray-Curtis similarity to test whether accessions were more similar within a treatment compared to among treatments.

Landrace and Accession Effects – To determine if plant response to increased salt was a result of genetic mechanisms (accession response or landrace structure), an NMDS was coupled with ANOSIM. The Bray-Curtis dissimilarity coefficients for ST values were used in the NMDS to visualize patterns in the data. Two dimensions were specified. NMDS was paired with ANOSIM to statistically test clusters and ordination results. We tested whether individuals were more similar with an accession compared to among accessions; we tested whether individuals were more similar within a landrace compared to among landraces.

Treatment Effects on Growth – One-way analysis of variance (ANOVA) was used to deduce whether there was a statistical difference among accessions for live aboveground biomass STI values, dead aboveground biomass STI values, and root biomass STI values in response to salt exposure. An ANOVA was used to evaluate differences between landraces in response to salt exposure. If significant differences were found, Tukey's HSD was used to separate accession/landrace means.

In R v. 3.6.0 (R Core Team, 2013), Shapiro-Wilk tests were used to test population response normality, and Levene's test, in the `car` package (Fox & Weisberg, 2019), was used to test homogeneity of variance. Response variables that did not pass a threshold of $\alpha = 0.05$ in the Shapiro-Wilk tests were transformed prior to ANOVA. For the accession ANOVA, STI values for live aboveground biomass, dead aboveground biomass, and root biomass were square-root transformed. For the landrace ANOVA, STI values for live aboveground biomass and dead aboveground biomass were log transformed. STI values for root biomass were square-root transformed.

Treatment Effects on Sodium and Potassium Accumulation – To determine whether there was significant variation among treatments and accessions with respect to Na⁺ content, K⁺ content, and the potassium to sodium ratio (K⁺/Na⁺), a two-way ANOVA was performed in R v. 3.6.0 (R Core Team, 2013). If a significant difference was found ($P < 0.05$), Tukey's HSD was performed to determine which treatments and accessions were significantly different from one another.

Treatment Effects on Proline Accumulation – To determine whether there was significant variation among treatments and accessions for proline accumulation, a two-way ANOVA was performed on proline values that were log transformed using R v. 3.6.0 (R Core Team, 2013). If a significant difference was found ($P < 0.05$), Tukey's HSD was performed to determine which treatments and accessions significantly differed from one another.

Results

Treatment Effects

Salt exposure reduced live aboveground biomass, root biomass, the shoot-to-root ratio, height, the percent of live leaves, and foliar SPAD across all accessions and landraces, while dead aboveground biomass and mortality increased (**Appendix S3**). We observed a differential response to NaCl exposure among accessions indicating that variation in salt tolerance exists within our tested population.

Landrace and Accession Effects

Based on accession and landrace ST values calculated for the measured growth parameters, plants were more similar within an accession rather than across accessions and within a landrace rather than across landraces (**Fig. 1**) when exposed to salt.

Relative Decrease in Plant Biomass (RDPB)

The percent of biomass retained in response to NaCl ranged from 98% to 3% across accessions (**Fig. 2**). Accessions showing sustained growth included V-1 (subs. *verticilliflorum*), Sb-18 (durra), Sb-7 (caudatum), Sb-9 (guinea-margaritiflorum), Sb-10 (durra), and Sb-3 (guinea-margaritiflorum). These six accessions retained >90% of live aboveground biomass when exposed to NaCl. RDPB values within the NaCl treatment also varied among landraces ($F_{8,83} = 5.22$, $P < 0.001$; **Appendix S4**).

Stress Tolerance Index (STI)

STI values for live aboveground biomass, dead aboveground biomass, and root biomass differed among accessions ($F_{20,66} = 3.82$, $F_{17,56} = 61.65$, $F_{20,60} = 30.36$, respectively, $P < 0.001$ for each). STI values ranged from 0.01 to 1.51 for live aboveground biomass, 0.10 to 3.35 for dead aboveground biomass, and 0.05 to 1.97 for root biomass. Some accessions ranked high for all three traits while others ranked high for only one or two of the traits. For example, P-1 ranked low for live aboveground biomass (1st out of 21st) but ranked 17th out of 21st for root biomass (**Fig. 3**). The largest overall scores (additive rank score for live aboveground biomass, dead aboveground biomass, and root biomass) were observed for the accessions Sb-10, V-1, Sb-9, Sb-3, Sb-2, and Sb-12, indicating overall better performance compared to other accessions (**Fig. 3**).

When comparing the STI values among landraces, differences were observed for live aboveground biomass, dead aboveground biomass, and root biomass ($F_{8,83} = 38.93$, $F_{7,72} = 4.68$, $F_{8,71} = 32.87$, respectively, $P < 0.001$ for each; **Appendix S5**). STI values ranged from 0.01 to 1.28 for live aboveground biomass. *S. propinquum* had the lowest STI for live aboveground biomass with a mean of 0.01 and landrace durra had the highest STI for live aboveground biomass with a mean of 1.28. STI values ranged from 0.32 to 2.08 for dead aboveground biomass with the intermediate landraces displaying the least STI values and the landrace guinea-margaritifera displaying the highest. STI values ranged from 0.11 to 1.69 for root biomass. The landrace guinea-margaritifera had the highest STI for root biomass (1.69), while most other landraces averaged about 0.2 to 0.5 (**Appendix S2**).

Sodium and Potassium Accumulation

A subset of accessions that showed variation in growth under salt treatment were evaluated for ion accumulation. Variation in Na^+ content was found among treatment and accessions ($F_{11,87} = 3.04$, $P < 0.01$). Foliar Na^+ under control conditions was low but varied 35-fold across accessions (**Table 2**). When exposed to NaCl, Sb-3 and Sb-4 accumulated the least amount of Na^+ while P-1 and V-2 accumulated the most (**Table 2**).

As with Na^+ , foliar K^+ concentrations also varied among accessions and these differed in response to treatments ($F_{11,87} = 13.83$, $P < 0.001$). For example, P-1 exhibited similar K^+ content to the tested population mean under control conditions and then declined more than other accessions under NaCl exposure (79% decline), whereas K^+ increased significantly in Sb-3 and Sb-9 under NaCl exposure (**Table 2**).

In sorghum, we found variation among treatments and accessions for the K^+/Na^+ ratio ($F_{11,87} = 4.44$, $P < 0.001$). Under control conditions, V-2 had the lowest K^+/Na^+ ratio and Sb-16 the greatest (**Table 2**). The ratio declined (by 49% to 99%) in many accessions under NaCl exposure, most notably in P-1 (99%) and Sb-15 (97%), while the ratio did not change in Sb-3.

Proline Accumulation

In response to salt exposure, proline accumulation in sorghum foliage increased, with the magnitude of increase depending on the accession ($F_{11,87} = 4.44$, $P < 0.001$; **Fig. 4**). Proline accumulation ranged from 0.07 to 0.26 gfw^{-1} in the control treatment and 0.07 to 2.63 gfw^{-1} in the salt treatment (**Appendix S6**).

Discussion

Phenotypic Responses to Salinity Stress

Salinity tolerance is a product of maintenance mechanisms that occur during both the osmotic and ionic phases of salinity stress (Munns & Tester, 2008). During the osmotic phase, continued growth of aboveground biomass indicates the ability to overcome osmotic stress, since sensitivity to water deprivation typically results in decreased growth (Munns & Tester, 2008).

During the ionic phase, mechanisms of tolerance include compartmentalization of toxic ions into vacuoles and/or extrusion of Na^+ from cells and the removal of Na^+ from the xylem stream, which reduces potential exposure in the leaf. Therefore, the accumulation of dead aboveground biomass can be used as proxy for evaluating compartmentalization and extrusion efficiency (Munns & Tester, 2008; Deinlein *et al.*, 2014). We found that accessions with high STI values for dead aboveground biomass included both tolerant (Sb-10, Sb-9, and V-1) and sensitive (Sb-16 and V-2) accessions. This, combined with the results for live aboveground biomass, suggests that tolerance in sorghum is correlated to a greater extent with the plant's ability to overcome the osmotic phase via continued growth rather than exclusion and/or compartmentalization of ions during the ionic phase. This is most evident for accessions such as Sb-10, Sb-9, and V-1. These tolerant accessions accumulated large amounts of both live and dead aboveground biomass (**Fig. 3**), reflecting the ability to maintain continued growth under salt exposure. The ability to continue new growth aids in the dilution of Na^+ in leaves, and if new growth exceeds the rate of leaf senescence, plants can continue photosynthesizing and producing enough carbon to support overall plant growth (Munns & Tester, 2008; Carillo *et al.*, 2011).

Plants may exhibit limited root growth as a result of low soil water potential, or conversely, increased growth as a search response for non-saline water (Rahnama *et al.*, 2011; Jung & McCouch, 2013; Munns & Gilliham, 2015; Hanin *et al.*, 2016). In our study, we found that three of the overall most tolerant accessions (Sb-10, Sb-9, Sb-3) ranked in the top five highest STIs for root biomass (**Fig. 3**). This suggests that maintenance of root biomass in response to treatment is associated with salinity tolerance (Galvan-Ampudia *et al.*, 2013; Munns & Gilliham, 2015). *S. propinquum*, one of the most sensitive accessions, had the largest RDPB and the lowest live aboveground biomass STI, yet had the fifth highest overall STI for root biomass (**Fig. 2** and **Fig. 3**, respectively). The larger STI for root biomass in *S. propinquum* is most likely a product of its naturally more extensive root system compared to *S. bicolor*, rather than tolerance, *per se*.

In our study, we assessed tolerance by relative decrease in plant biomass (RDPB) and the stress tolerance index (STI) (Negrão *et al.*, 2017). We observed that some accessions displayed less than 10% decrease in plant biomass (RDPB) but ranked low in the STI analysis. For example, V-1 and Sb-10 displayed 2% and 10% decreases in plant biomass respectively (or 98% and 90% retained biomass, respectively), in response to treatment and ranked in the top 5 most tolerant accessions in the STI analysis for live aboveground biomass, dead aboveground biomass and root biomass. However, Sb-18, which lost only 4% of its biomass (retained 96% of its biomass) in response to treatment, ranked 16th, 1st, and 14th for live aboveground biomass, dead aboveground biomass, and root biomass respectively. The discordance between a high rank in the RDPB analysis versus STI analysis suggests that different modes of tolerance may exist in sorghum. Different modes of tolerance may reflect reductions in Na^+ accumulation achieved by multiple mechanisms, such as reduction in root uptake, reduction in xylem loading, increased extrusion, and increased retrieval from aboveground tissue (Munns & Tester, 2008; Deinlein *et al.*, 2014; Wu *et al.*, 2019). Each of these mechanisms results in reduced Na^+ in the cytoplasm. Regardless of the mechanism, reduced Na^+ typically results in increased tolerance. Therefore, we propose that the RDPB analysis is the better indicator of tolerance because it depicts the outcome of NaCl exposure regardless of the mechanism operating in tolerant genotypes.

Physiological responses to salinity stress

Historically, proline accumulation under salt and/or osmotic stress has been used as an indicator of tolerance (Iqbal *et al.*, 2014). When comparing proline accumulation across accessions, we found that leaf proline increased between the control and NaCl treatment, although this increase was accession dependent (**Fig. 4**). V-1 and Sb-10, two of our most tolerant accessions according to RDPB and STI analysis, displayed low amounts of proline in both control and treatment conditions. In contrast, Sb-7 and Sb-17 exhibited large NaCl-induced increases in proline content but were only moderately salt tolerant. The lack in correlation of proline accumulation with the rank score of tolerant accessions (**Appendix S7**) suggests that, in sorghum, proline accumulation may reflect stress injury rather than osmotic adjustment/osmoprotection for increased tissue tolerance (Munns & Tester, 2008; Roy *et al.*, 2014). The QTLs for proline accumulation under salinity stress and for stress tolerance are not linked in barley (Fan *et al.*, 2015) and, in rice, salt-sensitive accessions accumulated higher levels of Na⁺ and proline compared to salt-tolerant accessions (Lutts *et al.*, 1999; Vaidyanathan *et al.*, 2003; Theerakulpisut *et al.*, 2005). Therefore, although proline accumulation does occur in sorghum in response to NaCl (Weimberg *et al.*, 1982; Surender Reddy *et al.*, 2015), our results suggest that it is not an accurate predictor of protective capacity against stress injury.

Significant variation in sodium and potassium concentrations among accessions suggests that differences in the mechanisms responsible for sodium uptake and distribution and/or regulation of potassium concentration exist in sorghum (**Table 2**). When comparing the variation in Na⁺ accumulation with tolerance categories, we do not observe patterns suggestive of a unifying mechanism of sorghum response to excess Na⁺ (**Appendix S8** and **Appendix S7**). For example, Sb-1 and Sb-10, a sensitive and a tolerant accession, respectively, did not significantly differ in foliar Na⁺ accumulation. In control conditions, both accessions averaged approximately 0.02 mg Na⁺ g⁻¹, and in treatment conditions both averaged about 0.59 mg Na⁺ g⁻¹; however, in terms of relative decreases in plant biomass, Sb-10 displayed less than 10% loss in live aboveground biomass while Sb-1 had greater than 50% loss. Although our analysis of foliar Na⁺ by ICP is unable to assess subcellular localization, Sb-10 may have elevated tissue tolerance as a result of better compartmentalization of Na⁺ ions into vacuoles, resulting in less cell death due to ionic imbalance.

Salt sensitivity is often associated with changes in K⁺ uptake resulting from competition between Na⁺ and K⁺ (Deinlein *et al.*, 2014). In sorghum, we observed variation in K⁺ among accessions and NaCl treatments. Most variation in K⁺ was observed between accessions and not between treatments. The only accession exhibiting a decline in K⁺ between the control and NaCl treatments was P-1, whereas exposure led to an increase in K⁺ in Sb-3 and Sb-9. Sb-3 and Sb-9 are both from the landrace guinea-margaritifera and both exhibit low RDPB. In contrast, P-1 had a high RDPB. These patterns suggest that, at least in the sorghum accessions included in this study, the loss of K⁺ homeostasis may not underlie NaCl toxicity, but rather may represent the basis of salt sensitivity in the wild relative, *S. propinquum*.

Evolution, domestication, and adaptation of salt tolerant sorghum accessions

Where population structure and geographic distribution of sorghum has been studied, landraces show genetic diversity and racial structure with strong geographical patterning (Morris *et al.*,

2013; Mace *et al.*, 2013). We found, however, that salinity tolerance was not solely associated with landrace, suggesting that accessions exposed to high local and regional soil salt contents may have adapted mechanisms to overcome the stresses associated with NaCl exposure. We initially hypothesized that the driving force of variation in salt tolerance may be a result of post-domestication adaptation to saline environments; however, when we evaluate our findings within the phylogenetic framework presented in Mace *et al.* (2013), we observe that the most tolerant *S. bicolor* accessions are those that originated shortly after the domestication event, particularly those accessions within the durra clade (**Fig. 5**). Further, the two *S. verticilliflorum* accessions included in both this study and the Mace *et al.* (2013) study displayed significantly different responses to salinity. V-1 (PI226096), which had the lowest RDPB and ranked 5th largest for live aboveground biomass STI, dead aboveground biomass STI, and root biomass STI, is positioned in the first post-domestication clade; however, V-2 (PI300119), which lost approximately 70% of its biomass in response to treatment and ranked in the 3rd to last position for live aboveground biomass and the last position for root biomass, is placed in the clade prior to the domestication event (**Appendix S9**). This, combined with the observations for the durra accessions, suggests that salinity tolerance was gained during or shortly after sorghum domestication. In contrast, accessions from the landrace caudatum, which displayed a diversity of stress tolerance rankings, are not monophyletic, and are found in diverse positions throughout the tree (**Fig. 5**). Interpretation of these results within this phylogenetic context suggests that, during further selection and improvement, salinity tolerance was lost in lineages that were no longer subjected to continued environmental salt exposure. Lastly, given that *S. bicolor* and especially the landrace durra (Smith *et al.*, 2019) is known to be relatively drought tolerant (Mullet *et al.*, 2014; Fracasso *et al.*, 2016; McCormick *et al.*, 2018; Guo *et al.*, 2018) and, as with drought stress, salt stress has an initial osmotic component, we propose that salinity tolerance in sorghum originated in combination with, or as a by-product of, drought tolerance during domestication.

Conclusions

With more than 500 million people relying on food, fuel, and fiber production from sorghum (Mace *et al.*, 2013), the standing genetic diversity of this staple crop should be utilized to maximize production needs, especially in adverse soils. Because of its ability to thrive in environments associated with high degrees of abiotic stressors, it is imperative that the genetic, physiological, and morphological responses to salt exposure in sorghum are understood and utilized to enhance production on saline soils. We identified significant variation in response to salinity exposure among a diverse group of sorghum accessions, and we conclude that the variation seen in tolerance is not due to landrace alone, but rather a byproduct of domestication and improvement. Given our results, and in combination with results of Mace *et al.* (2013), we propose that accessions from the landrace durra would serve as valuable resources for genetic improvement of sorghum salinity tolerance in agriculture.

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Tables

Table 1. Summary of sorghum accessions. Sorghum accessions and associated information (identification code used to reference accessions throughout the study and landrace). Accession information and landrace information was supplied by GRIN.

Accession	ID	Landrace	Sorghum Association Panel
PI33027204SD	D-1	drummondii	
subs. propinquum	P-1	subs. <i>propinquum</i>	
PI57112801SD	Sb-1	caudatum	
PI53412801SD	Sb-2	durra	SAP-208
PI52569503SD	Sb-3	guinea-margaritifera	
PI57613001SD	Sb-4	kafir	SAP-65
PI53391004SD	Sb-5	caudatum	SAP-268
PI53383401SD	Sb-6	caudatum	
PI53379202SD	Sb-7	caudatum	SAP-140
PI65606902SD	Sb-8	intermediate (unknown)	
PI58643001SD	Sb-9	guinea-margaritifera	
PI58574902SD	Sb-10	durra	
PI56512103SD	Sb-11	caudatum	SAP-80
PI53413301SD	Sb-12	durra	SAP-233
PI53375201SD	Sb-13	caudatum	SAP-127
PI65361702SD	Sb-14	intermediate (unknown)	SAP-73
PI61353602SD	Sb-15	durra-caudatum	SAP-74
PI56351602SD	Sb-16	durra-caudatum	
PI60933601SD	Sb-17	intermediate (unknown)	
PI65602902SD	Sb-18	durra	SAP-37
Tx7000	Tx-1	durra	
PI22609603SD	V-1	subs. <i>verticilliflorum</i>	
PI30011903SD	V-2	subs. <i>verticilliflorum</i>	

Note: Tx-1 and Sb-6 were excluded from the study

Table 2. Summary of Sorghum ion profiles. Sodium (Na⁺), potassium (K⁺), and potassium sodium (K⁺/Na⁺) molar ratios for NaCl treatments for a subset of accessions that showed variability in phenotypic responses. Data shown are means ± (the standard error) of Na⁺ content, K⁺ content, and K⁺/Na⁺ ratio for each accession in the third leaf from the top. Different letters represent significant differences when comparing accessions (P<0.05).

Accession	Mean Na ⁺ mg/g				Mean K ⁺ mg/g				Mean K ⁺ /Na ⁺			
	Control		75 mM NaCl		Control		75 mM NaCl		Control		75 mM NaCl	
P-1	0.13	(0.03) ^{bcdefg}	2.58	(0.48) ^{gh}	14.32	(1.54) ^{bcde}	3.06	(1.08) ^a	81.98	(25.47) ^{cdefg}	0.67	(0.12) ^a
Sb-1	0.02	(0.01) ^{abc}	0.59	(0.16) ^{efgh}	17.89	(1.27) ^{ef}	18.18	(3.03) ^{ef}	564.71	(122.52) ^{gh}	23.72	(6.36) ^{bcde}
Sb-3	0.19	(0.14) ^{abcde}	0.15	(0.07) ^{abcde}	9.45	(0.29) ^b	14.52	(0.82) ^{cde}	124.51	(46.12) ^{cdefg}	142.67	(45.08) ^{defg}
Sb-4	0.17	(0.10) ^{abcdef}	0.22	(0.11) ^{bcdefg}	13.05	(0.69) ^{bcde}	17.11	(0.96) ^{def}	147.21	(85.36) ^{cdefg}	74.87	(20.78) ^{cdefg}
Sb-7	0.03	(0.01) ^{abc}	0.22	(0.08) ^{cdefg}	22.54	(1.34) ^f	22.84	(0.62) ^f	806.73	(240.01) ^{gh}	108.48	(41.20) ^{cdefg}
Sb-8	0.04	(0.01) ^{abcd}	0.92	(0.27) ^{efgh}	17.06	(0.80) ^{def}	18.85	(0.90) ^{ef}	246.46	(41.32) ^{fgh}	14.55	(4.68) ^{abcde}
Sb-9	0.03	(0.01) ^{abc}	0.51	(0.12) ^{efgh}	10.93	(0.85) ^{bc}	16.70	(0.81) ^{def}	235.69	(30.71) ^{fgh}	24.12	(4.75) ^{bcde}
Sb-10	0.02	(0.01) ^{ab}	0.60	(0.23) ^{defgh}	10.28	(0.68) ^{bc}	11.43	(0.92) ^{bcd}	703.49	(466.73) ^{fgh}	18.92	(5.44) ^{bcd}
Sb-15	0.09	(0.07) ^{abc}	1.95	(0.64) ^{gh}	18.68	(0.81) ^{ef}	19.34	(2.23) ^{ef}	936.91	(475.01) ^{fgh}	25.15	(20.61) ^{abc}
Sb-16	0.01	(0.01) ^a	0.13	(0.06) ^{abcde}	17.86	(1.22) ^{ef}	23.22	(1.07) ^f	1569.94	(674.41) ^h	351.98	(179.91) ^{efgh}
Sb-17	0.35	(0.24) ^{bcdefgh}	1.79	(0.74) ^{fgh}	14.88	(0.91) ^{bcdef}	18.28	(0.64) ^{def}	94.21	(65.45) ^{cdefg}	9.46	(4.46) ^{abc}
V-2	0.32	(0.20) ^{bcdefg}	3.47	(1.19) ^h	11.28	(1.52) ^{bcd}	12.28	(1.72) ^{bcde}	56.12	(22.40) ^{cdef}	2.80	(0.74) ^{ab}
SEM	0.45				0.08				0.45			
P _{Accession}	P<0.001				P<0.001				P<0.001			
P _{Treatment}	P<0.001				P<0.050				P<0.001			
P _{Interaction}	P<0.001				P<0.001				P<0.001			

Figures

Fig. 1. Non-metric multidimensional scaling using Bray-Curtis dissimilarity coefficient to two-dimensionally visualize plant response to treatment. For the NaCl treatment, accessions were ordinated in two-dimensional space. The following measurements were analyzed for dissimilarity among individuals: SPAD, percent of live leaves (live leaf count/total leaf count), height (cm), mortality, live aboveground biomass (dry weight in g), dead aboveground biomass (dry weight in g), and root biomass (dry weight in g). Shapes indicate the landrace grouping for each accession. The analysis of similarity revealed plants were more similar within a landrace than among landraces ($R=0.31$, $P<0.001$).

Fig. 2. Relative percent of plant biomass retained in response to 75 mM NaCl for each accession. Relative percent of plant biomass retained was calculated by 1-RDPB. Shapes indicate the landrace grouping for each accession. Larger percentages indicate higher amounts of biomass retained in response to NaCl. Lower percentages indicate higher amounts of biomass lost in response to NaCl. RDPB was calculated on mean live aboveground biomass in control and treatment conditions.

Fig. 3. Rank ordered stress tolerance index (STI) scores for live aboveground biomass, dead aboveground biomass, and root biomass, for each accession in response to NaCl. Accessions were arranged with the lowest overall STI rank on the left and the largest overall STI rank on the right. Overall rank was calculated by the sum of live aboveground biomass, dead aboveground biomass, and root biomass rank. Colors indicate portion of overall rank contributed by live aboveground biomass, dead aboveground biomass, and root biomass. Higher values indicate better performers compared to other individuals within the population. Lower values indicate poor performers compared to other individuals within the population. Note: Sb-14, Sb-1, and P-1 are missing STI values for dead aboveground biomass.

Fig. 4. Proline accumulation in a subset of accessions. Some accessions showed no increase in proline accumulation in response to 75 mM NaCl; however, trends for Sb-17 and Sb-7 show that, with increased salt exposure, proline accumulated. Statistical significance was found among accessions and proline accumulation in response to treatment ($P_{\text{Accession}} < 0.001$, $P_{\text{Treatment}} < 0.001$, $P_{\text{Treatment} \times \text{Accession}} < 0.01$). Values are the mean of five biological replicates with $\pm$ standard error. Different letters represent significant differences. Note: Break in axis to account for scale differences.

Supporting Information

Appendix S1. A pilot study showing the effect of increasing concentrations of NaCl on biomass accumulation.

Appendix S2. (A) Accession STI (B) Growth variation in response to NaCl. Data shown are means $\pm$ the standard error. Different letters represent significant differences when comparing accessions ($P < 0.05$).

A.		Stress Tolerance Index											
Landrace	Accession	STI _{LAGB}				STI _{DAGB}				STI _{RB}			
drummondii	D-1	0.18	±	0.01	i	0.99	±	0.13	d	0.11	±	0.03	gh
subs. <i>propinquum</i>	P-1	0.01	±	0.00	j					0.57	±	0.00	cde
caudatum	Sb-1	0.56	±	0.00	efgh					0.22	±	0.00	efgh
durra	Sb-2	1.51	±	0.16	a	1.79	±	0.16	bc	0.39	±	0.04	def
guinea-margaritifерum	Sb-3	0.80	±	0.08	cdef	0.95	±	0.01	d	1.97	±	0.34	a
kafir	Sb-4	0.88	±	0.08	bcde	0.75	±	0.09	def	0.31	±	0.01	defg
caudatum	Sb-5	0.41	±	0.03	ghi	0.22	±	0.01	hi	0.20	±	0.03	efgh
caudatum	Sb-7	0.87	±	0.06	cde	0.40	±	0.07	fgh	0.58	±	0.08	cde
Intermediate (unknown)	Sb-8	0.55	±	0.12	efgh	0.31	±	0.05	ghi	0.21	±	0.06	efgh
guinea-margaritifерum	Sb-9	0.69	±	0.04	defg	3.23	±	0.26	a	1.32		0.09	ab
durra	Sb-10	1.32	±	0.05	ab	3.35	±	0.33	a	0.85	±	0.10	bc
caudatum	Sb-11	1.13	±	0.13	abc	0.50	±	0.06	defgh	0.27	±	0.03	efg
durra	Sb-12	1.12	±	0.07	abc	0.43	±	0.04	efgh	0.63	±	0.04	cd
caudatum	Sb-13	0.43	±	0.07	ghi	0.55	±	0.09	defgh	0.20	±	0.05	fgh
Unknown	Sb-14	0.47	±	0.05	fgh					0.13	±	0.04	gh
durra-caudatum	Sb-15	0.65	±	0.04	efg	0.68	±	0.08	defg	0.18	±	0.01	fgh
durra-caudatum	Sb-16	0.64	±	0.02	efg	1.11	±	0.26	cd	0.28	±	0.09	efgh
Intermediate (unknown)	Sb-17	0.69	±	0.03	defg	0.33	±	0.08	ghi	0.26	±	0.05	efgh
durra	Sb-18	1.06	±	0.06	abcd	0.10	±	0.02	i	0.45	±	0.03	cdef
subs. <i>verticilliflorum</i>	V-1	1.37	±	0.11	a	2.17	±	0.16	b	0.52	±	0.07	cde
subs. <i>verticilliflorum</i>	V-2	0.28	±	0.03	hi	0.88	±	0.05	de	0.05	±	0.01	h
	SEM			0.01				0.01				0.01	
	P _{Landrace}			p<0.001				p<0.001				p<0.001	

LAGB = live aboveground biomass; DAGB = dead aboveground biomass; RB = root biomass; STI = stress tolerance index

B.		Control			75 mM NaCl					
Landrace	Accession	Total LAGB	Total DAGB	RB	Total LAGB	Total DAGB	RB			
drummondii	D-1	2.49	0.41	1.18	0.83 ± 0.03	0.25	0.29 ± 0.09			
subs. <i>propinquum</i>	P-1	2.01 ± 0.15	0.09 ± 0.02	1.04 ± 0.11	0.07 ± 0.01		1.41 ± 0.00			
caudatum	Sb-1	3.91 ± 0.36	0.59 ± 0.07	1.29 ± 0.18	1.62 ± 0.01		0.45 ± 0.01			
durra	Sb-2	5.11 ± 0.60	0.63 ± 0.08	1.64 ± 0.33	3.64 ± 0.32	0.34 ± 0.03	0.65 ± 0.07			
guinea-margaritifera	Sb-3	3.04 ± 0.47	0.51 ± 0.01	4.76 ± 0.74	3.29 ± 0.30	0.21 ± 0.00	1.24 ± 0.03			
kafir	Sb-4	3.68 ± 0.48	0.27 ± 0.05	1.28 ± 0.21	2.48 ± 0.19	0.28 ± 0.03	0.65 ± 0.09			
caudatum	Sb-5	2.60 ± 0.13	0.11 ± 0.02	1.18 ± 0.09	1.95 ± 0.23	0.20 ± 0.01	0.54 ± 0.12			
caudatum	Sb-7	3.57 ± 0.00	0.18 ± 0.02	2.06 ± 0.03	2.49 ± 0.17	0.22 ± 0.04	0.67 ± 0.14			
Intermediate (unknown)	Sb-8	2.85 ± 0.16	0.06 ± 0.02	1.00 ± 0.10	2.70 ± 0.08	0.52 ± 0.04	0.69 ± 0.09			
guinea-margaritifera	Sb-9	2.92 ± 0.15	0.92 ± 0.19	4.02	3.07	0.48				
durra	Sb-10	4.31 ± 0.20	0.94 ± 0.11	1.92 ± 0.04	3.46 ± 0.18	0.52 ± 0.14	1.11 ± 0.11			
caudatum	Sb-11	4.52 ± 0.23	0.24 ± 0.06	1.30 ± 0.10	2.96 ± 0.54	0.25 ± 0.04	0.55 ± 0.10			
durra	Sb-12	3.83 ± 0.41	0.25 ± 0.03	3.18 ± 0.74	3.36 ± 0.19	0.19 ± 0.02	0.51 ± 0.03			
caudatum	Sb-13	2.89 ± 0.24	0.21 ± 0.03	0.99 ± 0.10	1.96 ± 0.19	0.32 ± 0.05	0.62 ± 0.09			
Unknown	Sb-14	4.51 ± 0.47	0.44 ± 0.03	0.96 ± 0.05	1.10 ± 0.11		0.26 ± 0.06			
durra-caudatum	Sb-15	2.87 ± 0.11	0.26 ± 0.02	0.91 ± 0.05	2.70 ± 0.18	0.30 ± 0.03	0.50			
durra-caudatum	Sb-16	3.13 ± 0.27	0.21 ± 0.04	1.29 ± 0.08	2.34 ± 0.06	0.59 ± 0.14	0.55 ± 0.10			
Intermediate (unknown)	Sb-17	2.96 ± 0.32	0.15 ± 0.04	0.88 ± 0.19	2.62	0.33	1.18			
durra	Sb-18	3.61	0.05	1.62	2.98 ± 0.26	0.19 ± 0.03	0.71 ± 0.04			
subs. <i>verticilliflorum</i>	V-1	3.74 ± 0.00	0.56 ± 0.03	1.46 ± 0.11	3.78 ± 0.69	0.38 ± 0.07	0.81 ± 0.19			
subs. <i>verticilliflorum</i>	V-2	3.22 ± 0.49	0.40 ± 0.05	0.96 ± 0.12	1.06 ± 0.18	0.25 ± 0.03	0.16 ± 0.06			
SEM										
P _{Landrace}										

LAGB = live aboveground biomass; DAGB = dead aboveground biomass; RB = root biomass; STI = stress tolerance index

Appendix S3. Non-metric multidimensional scaling using Bray-Curtis dissimilarity coefficient to two-dimensionally visualize plant response to 0 mM and 75 mM NaCl. The analysis of similarity revealed that plants were more similar within a treatment than across treatments ($R=0.11$; $P<0.001$). Gray triangles represent individuals within the control treatment and black circles represent individuals within the 75 mM NaCl treatment.

Appendix S4. Relative decrease in plant biomass (RDPB) for each landrace. Data shown are means $\pm$ the standard error of RDPB values for each landrace. Different letters represent significant differences when comparing landraces ($P < 0.05$).

Landrace	RDPB _{Landrace}			
caudatum	0.35	$\pm$	0.04	bc
drummondii	0.66	$\pm$	0.01	ab
durra	0.15	$\pm$	0.04	c
durra-caudatum	0.18	$\pm$	0.04	bc
guinea-margaritifera	0.09	$\pm$	0.05	c
Intermediate (unknown)	0.40	$\pm$	0.10	abc
kafir	0.29	$\pm$	0.04	bc
subs. <i>propinquum</i>	0.97	$\pm$	0.00	a
subs. <i>verticilliflorum</i>	0.32	$\pm$	0.14	bc
SEM	0.06			
P _{Landrace}	<0.001			

RDPB = relative decrease in plant biomass

Appendix S5. (A) Landrace STI scores and (B) growth variation in response to NaCl. Data shown are means $\pm$ the standard error. Different letters represent significant differences when comparing landraces ($P < 0.05$).

A. Landrace	Stress Tolerance Index								
	STI _{LAGB}			STI _{DAGB}			STI _{RB}		
drummondii	0.18	$\pm$	0.01 ^c	0.99	$\pm$	0.13 ^{ab}	0.11	$\pm$	0.03 ^c
Intermediate (unknown)	0.57	$\pm$	0.05 ^b	0.32	$\pm$	0.05 ^b	0.20	$\pm$	0.03 ^{bc}
durra-caudatum	0.65	$\pm$	0.03 ^b	0.90	$\pm$	0.13 ^{ab}	0.24	$\pm$	0.04 ^{bc}
caudatum	0.69	$\pm$	0.06 ^b	0.42	$\pm$	0.04 ^b	0.24	$\pm$	0.01 ^{bc}
subs. <i>verticilliflorum</i>	0.81	$\pm$	0.17 ^b	1.46	$\pm$	0.23 ^a	0.27	$\pm$	0.08 ^{bc}
kafir	0.88	$\pm$	0.08 ^{ab}	0.75	$\pm$	0.09 ^{ab}	0.31	$\pm$	0.01 ^{bc}
durra	1.28	$\pm$	0.08 ^a	1.64	$\pm$	0.41 ^{ab}	0.55	$\pm$	0.05 ^b
subs. <i>propinquum</i>	0.01	$\pm$	0.00 ^d				0.57	$\pm$	0.00 ^b
guinea-margaritiflorum	0.75	$\pm$	0.05 ^b	2.08	$\pm$	0.40 ^a	1.69	$\pm$	0.20 ^a
SEM	0.04			0.76			0.03		
P _{Landrace}	p<0.001			p<0.001			p<0.001		

LAGB = live aboveground biomass; DAGB = dead aboveground biomass; RB = root biomass;
STI = stress tolerance index

B. Landrace	Control			75 mM NaCl		
	Total LAGB	Total DAGB	RB	Total LAGB	Total DAGB	RB
drummondii	2.49	0.41	1.18	0.83 ± 0.03	0.25	0.29 ± 0.09
Intermediate (unknown)	3.24 ± 0.27	0.18 ± 0.05	0.93 ± 0.10	1.89 ± 0.36	0.46 ± 0.07	0.56 ± 0.16
durra-caudatum	3.00 ± 0.14	0.24 ± 0.02	1.10 ± 0.08	2.54 ± 0.12	0.39 ± 0.07	0.54 ± 0.07
caudatum	3.65 ± 0.18	0.26 ± 0.04	1.25 ± 0.08	2.20 ± 0.17	0.26 ± 0.02	0.63 ± 0.05
subs. <i>verticilliflorum</i>	3.48 ± 0.25	0.48 ± 0.05	1.21 ± 0.13	2.87 ± 0.72	0.34 ± 0.05	0.60 ± 0.18
kafir	3.68 ± 0.48	0.27 ± 0.05	1.28 ± 0.21	2.48 ± 0.19	0.28 ± 0.03	0.65 ± 0.09
durra	3.86 ± 0.31	0.50 ± 0.11	1.90 ± 0.29	3.35 ± 0.13	0.30 ± 0.04	0.59 ± 0.07
subs. <i>propinquum</i>	2.01 ± 0.15	0.09 ± 0.02	1.04 ± 0.11	0.07 ± 0.01		1.41 ± 0.00
guinea-margaritifera	2.97 ± 0.18	0.77 ± 0.13	4.58 ± 0.56	3.24 ± 0.22	0.28 ± 0.07	1.24 ± 0.03
SEM						
P _{Landrace}						

LAGB = live aboveground biomass; DAGB = dead aboveground biomass; RB = root biomass; STI = stress tolerance index

Appendix S6. Mean proline content for control and NaCl conditions. Data shown are means $\pm$ the standard error of proline (gfw-1) for a subset of accessions. Different letters represent significant differences when comparing accessions and treatment ($p < 0.05$).

Landrace	Accession	Mean proline nmoles gfw ⁻¹							
		Treatment							
		Control				75 mM NaCl			
caudatum	Sb-1	0.09	$\pm$	0.01	ab	0.21	$\pm$	0.05	abc
durra	Sb-10	0.11	$\pm$	0.01	ab	0.09	$\pm$	0.03	a
durra-caudatum	Sb-15	0.07	$\pm$	0.01	a	0.13	$\pm$	0.02	ab
durra-caudatum	Sb-16	0.26	$\pm$	0.07	abc	0.32	$\pm$	0.13	abc
kafir-caudatum	Sb-17	0.19	$\pm$	0.01	ab	2.63	$\pm$	1.13	c
caudatum	Sb-7	0.10	$\pm$	0.02	a	1.35	$\pm$	0.76	bc
subs. <i>verticilliflorum</i>	V-1	0.15	$\pm$	0.00	ab	0.07	$\pm$	0.02	ab
	SEM	0.038							
	P _{Accession}	p<0.001							
	P _{Treatment}	p<0.001							
	P _{Accession*Treatment}	p<0.01							

Appendix S7. Pearson correlation matrix (75 mM NaCl).

Appendix S8. Correlation between (A) Na^+ accumulation and RDPB (B) Na^+ accumulation and live aboveground biomass STI

Appendix S9. A neighbor-joining tree constructed by Mace et al. (2013) using SNP data. (A) STI rank scores for live aboveground biomass overlaid on neighbor-joining tree (B) STI rank scores for dead aboveground biomass overlaid on neighbor-joining tree (C) STI rank scores for root biomass overlaid on neighbor-joining tree. Smaller STI rank scores (yellow) indicates sensitivity, whereas larger STI rank scores (red) indicates tolerance. Figure modified from Mace, E., Tai, S., Gilding, E. et al. Whole-genome sequencing reveals untapped genetic potential in Africa's indigenous cereal crop sorghum. Nat Commun 4, 2320 (2013). <https://doi.org/10.1038/ncomms3320>

CHAPTER 4

VARIATION IN THE EXPRESSION OF DETOXIFICATION GENES SUGGESTS THAT EARLY WATER ACQUISITION COMBINED WITH SODIUM EXCLUSION LEADS TO INCREASED SALT TOLERANCE IN *SORGHUM BICOLOR*

Ashley N. Henderson; Katelyn Delaney; Jennifer S. Hawkins

Abstract

Soil salinity hinders plant growth, development, and function. For agriculturally and economically important crops this directly results in decreased biomass and grain yield making it difficult to increase production on marginal soils. Reductions in crop production are a result of an initial osmotic stress followed by an ionic stress, making salinity tolerance a complex polygenic trait. In this work, two accessions of *Sorghum bicolor* were germinated and grown in optimal conditions. Once seedlings reached the fifth leaf stage of development, seedlings were treated with 150 mM NaCl and above- and belowground tissues were collected for gene expression analysis. Three genes (SOS1, NHX2, and HKT3) that have been previously shown to play a role in salt detoxification via Na⁺ extrusion, sequestration, and transport were assessed at eight time points. Upon the addition of non-saline water, we observed gene expression changes suggesting osmotic adjustment indicative of water acquisition in the tolerant accession. Further, in response to 150 mM NaCl, we observed a delayed response in the tolerant accession indicating Na⁺ exclusion at the root level. Taken together, our results suggest that the variation in tolerance observed between two accessions of *Sorghum bicolor* are a result of an initial osmotic adjustment followed by prolonged sodium exclusion and efficient extrusion, sequestration, and transport.

Keywords – *Sorghum bicolor*, SOS1, NHX2, HKT3, salt stress, Na⁺ extrusion, salinity stress tolerance, vacuolar Na⁺ sequestration, salt detoxification

Introduction

Soil salinity is a constraint for agriculture especially in arid and semi-arid regions where environmental conditions impose stress on plants. These regions are prone to drought due to a lack of consistent rainfall and therefore require irrigation for adequate crop production. Over-irrigation, however, results in increased soil salinity due to a combination of poor water quality and insufficient drainage (Munns & Tester, 2008; Food and Agriculture Organization (FAO), 2009; Parihar *et al.*, 2015; Singh, 2015; Munns *et al.*, 2019a). The resulting salt intensification impedes plant performance, both in terms of biomass accumulation and grain yield.

Reductions in plant performance on saline soils is hindered initially via osmotic stress, followed by a secondary ionic stress. Osmotic stress simulates drought-like conditions because the accumulation of salts in the soil alters the water potential. Consequently, when the soil water potential is lower than the plants osmotic potential, plants are unable to extract water and nutrients (Munns & Tester, 2008; Negrão *et al.*, 2017). Plants mitigate osmotic stress by accumulating and producing compatible solutes, such as amino acids, betaines, and sugars, that aid in osmotic adjustment, therefore improving the ability to take up water (Negrão *et al.*, 2017; Turner, 2018). During water uptake, ions enter the plant through high-affinity K^+ transporters (HKT) and non-selective cation channels (NSCC) (Tyerman & Skerrett, 1998; Munns & Tester, 2008; Deinlein *et al.*, 2014; Hanin *et al.*, 2016), and as Na^+ and Cl^- ions accumulate, an ion-dependent stress commences that disrupt normal metabolic function, resulting in further decreases in plant performance.

Salinity tolerance is a complex, polygenic trait that can arise from either osmotic or ionic stress resistance (Munns & Tester, 2008; Miranda *et al.*, 2017). Mechanistically, this resistance can be attained by continual K^+ uptake/transport, Na^+ sequestration, and/or Na^+ extrusion for which several genes families are known to play key roles (Maathuis & Amtmann, 1999; Tester & Davenport, 2003; Rus, 2004; Munns, 2005; Davenport *et al.*, 2007; Gao *et al.*, 2007; Munns & Tester, 2008; Olías *et al.*, 2009; Leidi *et al.*, 2010; Li *et al.*, 2011; Barragán *et al.*, 2012; Wang *et al.*, 2014; Miranda *et al.*, 2017; Baghour *et al.*, 2019). The HKT (high affinity potassium transporter) gene family has been extensively studied and is known to increase tolerance by unloading Na^+ from the xylem and loading it into the phloem (**Figure 1**) (Maathuis & Amtmann, 1999; Urao *et al.*, 1999; Tester & Davenport, 2003; Munns, 2005; Ren *et al.*, 2005; Sunarpi *et al.*, 2005; Tran *et al.*, 2007). This process excludes Na^+ from the leaves resulting in increased ionic tolerance, while also altering the osmotic potential. At the cytoplasmic level, mechanisms to maintain low Na^+ include ion extrusion and sequestration. The tonoplast-localized Na^+/H^+ antiporter (NHX) gene family is essential for the latter (**Figure 1**) (Deinlein *et al.*, 2014). Sequestration of Na^+ into vacuoles aids in both lower concentrations of toxic ions in the cytoplasm, while also contributing to osmotic adjustment (Yamaguchi *et al.*, 2013; Ahire *et al.*, 2018). Therefore, although accumulation of Na^+ is toxic to plants, if compartmentalized, it can be beneficial for osmotic adjustment resulting in increased tolerance (Ahire *et al.*, 2018), making the NHX gene family a pivotal player in both osmotic and ionic tolerance. The salt overly sensitive (SOS) signaling pathway is a well-defined pathway, crucial for Na^+ extrusion and ionic homeostasis. SOS1 is a plasma

membrane-localized Na⁺/H⁺ antiporter (Qiu *et al.*, 2002; Yamaguchi *et al.*, 2013) and has been the only Na⁺/H⁺ antiporter shown to efflux Na⁺ from the cytoplasm of plant cells (**Figure 1**) (Wu *et al.*, 2019). With three main roles, SOS1 extrudes Na⁺ from roots into surrounding medium, reduces the accumulation within the cytoplasm, and aids in unloading of Na⁺ from the xylem to prevent accumulation in the shoot (Shi *et al.*, 2000, 2003; Shi, 2002; Qiu *et al.*, 2002; Zhu, 2003; Batelli *et al.*, 2007; Olías *et al.*, 2009; Ji *et al.*, 2013; Lekshmy Sathee *et al.*, 2015; Miranda *et al.*, 2017; Baghour *et al.*, 2019; Wang *et al.*, 2019).

Sorghum bicolor (L.) Moench, commonly known as sorghum, is an important African grass used for food, fuel, and fiber. Compared to other important crops such as wheat, rice, and maize, *Sorghum bicolor* has been shown to have increased salt (Boursier & Läuchli, 1990; Almodares & Sharif, 2007) and drought tolerance (Mullet *et al.*, 2014; Fracasso *et al.*, 2016; McCormick *et al.*, 2018; Guo *et al.*, 2018) making it a valuable model system to understand the molecular mechanisms associated with the variation in response to these abiotic stressors. Here, we use two accessions of *Sorghum bicolor* that have previously been shown to vary in response to salt exposure (Henderson *et al.*, 2019). We focused on three key genes that play a role in ion uptake, transport (Munns, 2005; Parihar *et al.*, 2015), and detoxification (**Figure 1**) (Deinlein *et al.*, 2014). We found that the tolerant accession exhibited gene expression changes suggestive of early osmotic adjustment in response to the addition of water followed by a delay in salt specific sensing and signaling, indicating effective exclusion at the root level.

Materials and Methods

Plant Material

Two accessions of *Sorghum bicolor* that were previously described and ranked as salt tolerant (PI58574902SD) and salt sensitive (PI57112801SD) (Henderson *et al.*, 2019) were germinated and grown in a controlled growth chamber. The salt tolerant accession (PI58574902SD) is referred to as Sb-10, and the salt sensitive accession (PI57112801SD) is referred to as Sb-1 throughout this study, in accordance with previously used short-name designation in Henderson *et al.* (2019). All seeds were obtained from the USDA-Germplasm Resources Information Network (GRIN).

Experimental Design

Fifty-four seeds of each accession (three replicates per treatment and a total of two treatments at nine time points) were germinated in 5 cm x 5 cm x 25 cm tree pots (Stuewe and Sons, Tangent, OR, USA) filled with a 1:1 mix of silica sand #2 and #4. Growth chamber conditions were 12-hour days at 30.0°C and 12 hour nights at 25.0°C with 30% humidity. Plants were watered daily with non-saline tap water from sowing to the fifth leaf stage of development. Twice a week, all plants were treated with a 10-20-10 N-P-K fertilizer (Jacks water soluble fertilizer, Allentown, PA) diluted to 200 mg N L⁻¹. When 90% of the seedlings were at the fifth leaf stage of development, the treatment and tissue collections began.

A destructive harvest included collection of the roots, the second leaf, and the newest fully emerged leaf, referred to as the top leaf throughout this study (either 4th or 5th, youngest). Three biological replicates of both accessions were collected prior to treatment (time point 0). Half of the remaining replicates of each accession were treated with non-saline tap water (0 mM NaCl) and the other half were treated with saline water (150 mM NaCl). This concentration of sodium chloride was previously shown to inhibit growth and performance without causing death. Plants were watered to complete saturation. At 30 minutes post treatment, three replicates from the control group (tap water) and three replicates from the treatment group (150 mM NaCl) for each accession were collected. Additional collections were performed at 2 hours, 3 hours, 6 hours, 12 hours, 24 hours, 72 hours, and for each tissue. All samples were collected in RNase free tubes, immediately flash frozen, and transferred to -80°C to preserve RNA stability.

Gene Selection

The reference gene, PP2A (SORBI_3004G092500), was chosen as it has been shown to remain stable in various tissues under abiotic stress (Sudhakar *et al.*, 2016). The Sorghum genome encodes a single SbSOS1 gene (Miranda *et al.*, 2017), six members of the NHX gene family (Miranda *et al.*, 2017), and four orthologs of the single copy HKT1 gene in Arabidopsis (Wang *et al.*, 2014; Li *et al.*, 2019). Therefore, the target genes, SbSOS1 (SORBI_3008G192900), SbNHX2 (SORBI_3005G201000), and SbHKT3 (SORBI_3010G251700) were selected for qPCR. Primer sequences for SbPP2A (Sudhakar *et al.*, 2016), SbSOS1 (Miranda *et al.*, 2017), and SbNHX2 (Miranda *et al.*, 2017) were previously developed and published, while forward and reverse primers for SbHKT3 were newly designed and optimized in this experiment (**Table S1**). All primers were designed to span exon-exon junctions in order to target cDNA specifically and prevent genomic DNA amplification (**Supplementary Figure S1**, **Supplementary Figure S2**, **Supplementary Figure S3**, and **Supplementary Figure S4**).

Gene Verification

Conventional polymerase chain reaction (PCR) was used to amplify gene products from cDNA and amplicon specificity was verified by Sanger sequencing (**Supplementary Table S2**). PCR using genomic DNA template was performed to confirm that primers were designed to amplify cDNA only (**Supplementary Figure S5**). Each reaction contained 5 pmoles of F primer, 5 pmoles of R primer, 5 ng of template cDNA or DNA, 1X of Mg included Taq buffer, 0.2 mM dNTPs, and 1.25 units of Taq. Thermocycler conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 45 seconds, and a final extension of 72°C for 5 minutes. PCR products were visualized on a 1% agarose gel with a 1 Kb Plus DNA ladder (Invitrogen, Grand Island, NY, USA). PCR products were sephadex cleaned and directly sequenced at the West Virginia University Genomics and Bioinformatics Core Facility using the ABI 3130xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing results were compared with the *Sorghum bicolor* reference genome and transcriptome to ensure primer specificity to the specific SORBI gene of interest.

RNA Isolation and cDNA Synthesis

RNA safe handling was enforced from tissue collection through cDNA synthesis. Tissues were ground and RNA extracted following the MagMAX™ Plant RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) protocol. Following RNA extraction, samples were quantified on the nanodrop (Thermo Scientific NanoDrop 2000 Spectrophotometer, Waltham, MA, USA) and the quality was checked on the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). cDNA was synthesized following the SuperScript™ III First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA) protocol and quantified on the Qubit 2.0 Fluorometer (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA) with Qubit™ ssDNA assay kit reagents (Thermo Fisher Scientific, Waltham, MA, USA). Samples were diluted to 5 ng/ul for use in qRT-PCR.

qRT-PCR

Luna^R Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA), which uses SYBR^R/FAM fluorescence, was used to quantify transcript levels of mRNA. qPCR was performed on an iQ5 Real-Time PCR thermocycler (Bio-Rad, Hercules, CA, USA) where each reaction had 1X of Luna^R Universal qPCR Mastermix, 5 pmoles of F primer, 5 pmoles of R primer, and 5 ng of template cDNA. Thermocycler conditions were as follows: an initial denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 60°C for 15 seconds, and a final extension of 95°C for 10 seconds. Primer specificity was verified by melt curve analysis where temperatures ranged from 65°C to 95°C with 0.5°C increments for 5 seconds, and at the end of each temperature increment, the fluorescence of the plate was read. All biological replicates were performed in triplicate.

Fold Change

Relative expression was more appropriate than absolute quantification for our study given that we were interested in comparing the variation in two accessions that differ in long term salt tolerance rather than exact copy numbers of gene transcripts (Livak & Schmittgen, 2001). Gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Relative expression values for SbSOS1, SbNHX2, and SbHKT3 genes were normalized with SbPP2A (internal control), and by using an untreated control as the calibrator. Ct values for technical replicates were averaged when the standard deviation was less than 0.25.

Statistical Analysis

Relative expression was analyzed by a balanced two-way analysis of variance (ANOVA, significance assessed at $p < 0.05$), with accession and time point as factors and fold change as the response variable. If a significant difference was found, means were compared using Tukey's HSD test. Each gene of interest and tissue were independently assessed. Graphs were plotted in RStudio (ver.1.2.5019) (R Core Team, 2013) with the ggplot2 package (Wickham, 2016).

Results

Plant response to the addition of non-saline water

Plant response to the addition of non-saline water was tested by comparing gene expression at 0 minutes (pre-watering) to gene expression at 30 minutes (30 minutes after the addition of tap water). With the addition of non-saline water, we observed gene expression changes in both above- and belowground tissues suggesting greater uptake and transport of ions, indicative of osmotic adjustment. For example, in the roots, both accessions displayed decreased SbSOS1 expression; however, Sb-10 also experienced an increase in SbNHX2 expression, indicating greater osmotic potential in the tolerant accession (**Figure 2a**). Simultaneously, in the aboveground portion of the plants, we observed changes in expression of the Na⁺ transporter, SbHKT3. SbHKT3 is upregulated two- to four-fold in the second leaf of Sb-10, while it is downregulated approximately one- to two-fold in Sb-1, further supporting the results observed for roots, that there is increased transport in the tolerant accession (**Figure 2b**). In the top leaf, we observe an increase in expression of SbHKT3 in response to water for both accessions with a greater increase in expression in Sb-10 (**Figure 2c**). In addition, we observed increased expression of SbSOS1 and SbNHX2 in the top leaf, indicating increased extrusion and sequestration.

Plant response to the addition of NaCl

Two hours after salt exposure, we observed 1) a significant decrease in SbNHX2 expression in the root tips of Sb-10 (**Figure 3f**), 2) decreases in SbSOS1 expression in the second leaf of both accessions (**Figure 3e**), and 3) decreases in SbHKT3 expression in the second leaf of both accessions (**Figure 3e**). For the tolerant accession (Sb-10), these trends suggest exclusion of Na⁺ at the root level. For the sensitive accession (Sb-1), however, we observe almost a one and a half fold increase in SbSOS1 expression in the top leaf at this same time point (**Figure 3d**), and approximately a two-fold increase in SbSOS1 expression in the root tips at 30 minutes after exposure (**Figure 3c**). Increased expression levels of SbSOS1 at 30 minutes and two hours post salt exposure indicates the presence on Na⁺ ions.

Expression of SbSOS1 in the tolerant accession is stable until 3 hours post exposure, at which point SbSOS1 is significantly downregulated in the root tips, indicating the first salt specific response (**Figure 3i**). At this same time point, in the same tissue, we observe an increase in SbNHX2 expression for both accessions (**Figure 3i**) suggesting increased sequestration. With the increase in SbNHX2 expression and decrease in SbSOS1 expression, this suggests both the presence of ions and preferential sequestration of ions, a mechanism that would aid in osmotic adjustment. We also observe increased extrusion in the tolerant accession compared to the sensitive accession in the second leaf. At 6 hours, however, we observe increased extrusion in both accessions, suggesting the removal of salts from the aboveground portion of the plant (**Figure 3j,k**). Interestingly, following the significant increase in SbSOS1 expression in the second leaf at 6 hours, we observe decreases in SbSOS1 expression for both accessions (**Figure 4a,b**) and increases in SbHKT3 expression (**Figure 4b**). These results suggest that both accessions are attempting to transport ions belowground where they can be extruded into the soil

medium or sequestered into vacuoles. This is further supported by the significant increase in SbSOS1 and SbNHX2 expression, respectively, in the root tips at 72 hours (**Figure 4i**).

Discussion

It is well-known that salinity tolerance is associated with increased Na⁺ exclusion and/or sequestration (Munns & Tester, 2008; Munns & Gilliam, 2015; Munns *et al.*, 2016). While sequestration simply involves compartmentalization of excess sodium into vacuoles, Na⁺ exclusion can be achieved by four main mechanisms, including 1) reduction in Na⁺ uptake by the roots, 2) reduction in Na⁺ loading in the xylem, 3) increased Na⁺ extrusion, and 4) increased Na⁺ retrieval from the shoot (Wu *et al.*, 2019). Each of these mechanisms results in the exclusion of Na⁺ from the aboveground tissues. In this study, we delineate the variation in the onset of Na⁺ sensing and signaling in two accessions of *S. bicolor* that were previously shown to differ in long-term response to salinity stress, as indicated by expression variation of key detoxification genes.

Upon the addition of non-saline water, we observed variation in SbSOS1 and SbNHX2 expression in roots. SbSOS1, which codes for a plasma membrane-localized Na⁺/H⁺ antiporter, displayed a greater than two-fold decrease in expression in both the tolerant and sensitive accessions (**Figure 2**). At the same time and in the same tissue, we observed an increased expression of SbNHX2, which codes for a tonoplast-localized Na⁺/H⁺ antiporter, in the tolerant accession (Sb-10). With decreased extrusion (SbSOS1) and increased sequestration (SbNHX2), the osmotic potential within the plant would increase, assisting in the uptake of water by the roots. Interestingly, previous studies in barley have found higher correlations of tolerance with Na⁺ sequestration rather than extrusion (Wu *et al.*, 2019). Therefore, this initial sequestration mechanism identified in the tolerant accession may be responsible for some of the long-term tolerance observed (Henderson *et al.*, 2019). Further, we observed variation in SbHKT3 expression in the second and top leaf (**Figure 2b,c**). Specifically, SbHKT3 expression was downregulated in the second leaf of the sensitive accession (Sb-1) while expression was upregulated in the tolerant accession (Sb-10) (**Figure 2b**). Lower levels of expression suggest decreased transport and uptake of ions, which would result in decreased water absorption. Therefore, the increased expression observed in Sb-10 in both tissues (second leaf and top leaf) suggests the initiation of aboveground processes that alter the osmotic potential and promote the transport of water and nutrients. Together, these results indicate that the tolerant accession experiences the onset of osmotic adjustment at an earlier time point and in multiple above- and belowground tissues, even in the absence of excess Na⁺, promoting efficient uptake and transport water.

After 30 minutes of NaCl exposure, SbSOS1 expression in the roots of the sensitive accession increased, as would be expected in response to cytoplasmic sensing of salt (Olías *et al.*, 2009). This result aligns with previous findings demonstrating that sensitive accessions are generally poor excluders of Na⁺ and therefore experience an earlier onset of ionic stress. The delayed onset of SbSOS1 expression in Sb-10 until 3 hours post exposure suggests that the tolerant accession may be better at controlling Na⁺ uptake and exclusion (**Figure 3i**).

At 3 hours post-exposure, there is a shift in SbSOS1 and SbNHX2 expression in the roots, with SbSOS1 downregulated and SbNHX2 upregulated in both the tolerant and sensitive accessions (**Figure 3i**). This suggests that, in roots, ions are being sequestered into vacuoles, a process that acts as a cheap compatible solute that will aid in increased water uptake from the soil (Wu *et al.*, 2019; Munns *et al.*, 2019b). In contrast, significant differences between the tolerant and sensitive accession were observed in aboveground tissues at 3 hours post-exposure. For example, the tolerant accession shows increased levels of SbSOS1 expression, indicative of increased extrusion, in the second leaf, while Sb-1 experiences lower levels in expression at this time point. We also observed evidence for decreased transport in the top leaf for Sb-1 and increased extrusion in the top leaf for both accessions (**Figure 3g**). This indicates that, in aboveground tissues, the sensitive accession is less effective at transporting or extruding sodium ions, which may result in toxic Na⁺ accumulation in the cytosol.

SbSOS1 encodes a plasma membrane transporter that extrudes Na⁺ from the cytoplasm to the apoplast and aids in loading Na⁺ into the phloem to be exported to root tissue where it can be stored or effluxed (Munns, 2005; Sunarpi *et al.*, 2005; Munns & Tester, 2008; Lekshmy Sathee *et al.*, 2015). At 6 hours post-exposure in leaves, SbSOS1 expression in the tolerant accession is upregulated approximately two-fold, indicating increased extrusion, possibly due to excess cytoplasmic Na⁺ content (**Figure 3k**). Na⁺ accumulation is less harmful to belowground tissues compared to aboveground tissues because as ions become deposited into the aerial part of the plant following water loss during transpiration, vital metabolic processes such as photosynthesis are disrupted (Morton *et al.*, 2019). When water is lost through transpiration and salts are left behind, normal metabolic processes are disrupted. Therefore, it is reasonable to assume that extrusion of ions and loading of ions into the phloem is an effective mechanism of tolerance compared to aboveground compartmentalization. Interestingly, SbHKT3 expression remains down regulated in leaves until 12 hours post-exposure, when it becomes significantly upregulated (**Figure 4b**). This increase in transport is likely a downstream response to the increase in extrusion observed at 6 hours (increased SbSOS1 expression, **Figure 3k**).

At 72 hours, there was an increase in both SbSOS1 and SbNHX2 expression in the roots of both accessions, with the sensitive accession exhibiting greater transcript abundance. This suggests that, by this point after exposure, plants are overloaded with Na⁺ ions (ion dependent phase) and are attempting to both compartmentalize and extrude in order to avoid ionic disruption in the cytoplasm. The higher levels of transcript abundance in the sensitive accession (Sb-1) is likely a result of poor exclusion, extrusion, and sequestration (Wu *et al.*, 2019).

Finally, the top leaf of the sensitive accession experiences earlier increases in SbSOS1 expression relative to the tolerant accession, suggesting greater amounts of Na⁺ accumulation in the aboveground portions of the plant. Since SbSOS1 is specifically upregulated by salt stress and no other osmotic stressors such as drought, cold, or abscisic acid (Shi *et al.*, 2000; Shi, 2002; Wu *et al.*, 2019), this finding provides strong support for the presence of Na⁺ in leaf tissues of the sensitive accession. With Na⁺ ions present in the newest emerging leaves; normal metabolic processes would be disrupted, and new growth

would be limited. This finding is in accordance with previously reported results (Henderson *et al.*, 2019) where Sb-1 was limited in new growth over long term sodium exposure.

Conclusions

In the work presented here, we find early onset of sensing and signaling after salt exposure in a salt-sensitive accession (Sb-1) of *S. bicolor*. We also observe numerous changes in gene expression throughout the time course for the sensitive accession, which suggests that salt sensitivity in sorghum is a product of 1) the inability to exclude ions 2) the existence of biological chaos resulting in an unorganized response, and/or 3) a leaky tonoplast, therefore requiring increased effort to maintain ion sequestration in vacuoles (Wu *et al.*, 2019; Munns *et al.*, 2019b). Together, these processes are related to the availability of energy, and therefore provide an explanation for the decreased biomass production observed for Sb-1 in our previous study (Henderson *et al.*, 2019). We propose that the tolerant accession has higher tissue tolerance and is better at controlling uptake, exclusion, extrusion, and sequestration. With better control over these transport systems, fewer resources are diverted to the stress response, providing more available energy that can be utilized for new growth and development.

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FIGURES

Figure 1. Overview of SOS1, NHX2, and SbHKT3 genes and their role in sodium detoxification. SOS1, a plasma membrane-localized Na^+/H^+ antiporter, extrudes Na^+ from the cytoplasm of above- and belowground plant cells. NHX2, tonoplast-localized Na^+/H^+ antiporter, compartmentalizes Na^+ into the vacuoles of plant cells to maintain ion homeostasis in the cytoplasm of above- and belowground plant cells. SbHKT3 is a high affinity K^+ and Na^+ transporter that aids in uptake and unloading of Na^+ from the xylem. SbHKT3 is expressed only in aboveground plant cells.

Figure 2. Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 after the addition of non-saline water. Plants were watered with non-saline at the 5th or 6th leaf stage of development and relative expression of SbSOS1, SbNHX2, and SbHKT3 were calculated in the root tips, 2nd leaf, and top leaf 30 minutes after the addition of water. Boxplots display the interquartile range, with the black line representing the median. Whiskers represent the largest and smallest value within 1.5 times the interquartile range above and below the 75th and 25th percentile. No outliers were detected. (A) Expression fold change of SbSOS1 and SbNHX2 in the roots after the addition of H₂O. Both accessions experienced a decrease in SbSOS1 expression, however SbNHX2 expression increased in Sb-10. (B) Expression fold change of SbSOS1, SbNHX2, and SbHKT3 in the 2nd leaf after the addition of H₂O. SbSOS1 decreased for both accessions, while SbNHX2 decreased for Sb-10 and increased for Sb-1, and SbHKT3 increased for Sb-10. (C) Expression fold change of SbSOS1, SbNHX2, and SbHKT3 in the top leaf after the addition of H₂O. SbSOS1, SbNHX2, and SbHKT3 increased for both accessions with a greater increase in Sb-10 after the addition of non-saline water.

Figure 3. Gene expression changes for salt detoxification genes at 30 minutes, 2 hours, 3 hours, and 6 hours after salt exposure. Plants were watered with 150 mM NaCl at the 5th or 6th leaf stage of development and relative expression of SbSOS1, SbNHX2, and SbHKT3 were calculated in the root tips, 2nd leaf, and top leaf 30 minutes, 2 hours, 3 hours and 6 hours after exposure. Boxplots display the interquartile range, with the black line representing the median. Whiskers represent the largest and smallest value within 1.5 times the interquartile range above and below the 75th and 25th percentile. No outliers were detected. (A)-(C) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 30 minutes after the exposure to 150 mM NaCl in the top leaf, second leaf, and roots, respectively. (D)-(F) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 2 hours after the exposure to 150 mM NaCl the top leaf, second leaf, and roots, respectively. (G)-(I) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 3 hours after the exposure to 150 mM NaCl the top leaf, second leaf, and roots, respectively. (J)-(L) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 6 hours after the exposure to 150 mM NaCl the top leaf, second leaf, and roots, respectively.

Figure 4 Gene expression changes for salt detoxification genes at 12 hours, 24 hours, and 72 hours after salt exposure. Plants were watered with 150 mM NaCl at the 5th or 6th leaf stage of development and relative expression of SbSOS1, SbNHX2, and SbHKT3 were calculated in the root tips, 2nd leaf, and top leaf 12 hours, 24 hours, and 72 hours after exposure. Boxplots display the interquartile range, with the black line representing the median. Whiskers represent the largest and smallest value within 1.5 times the interquartile range above and below the 75th and 25th percentile. No outliers were detected. (A)-(C) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 12 hours after the exposure to 150 mM NaCl in the top leaf, second leaf, and roots, respectively. (D)-(F) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 24 hours after the exposure to 150 mM NaCl the top leaf, second leaf, and roots, respectively. (G)-(I) Gene expression changes for SbSOS1, SbNHX2, and SbHKT3 72 hours after the exposure to 150 mM NaCl the top leaf, second leaf, and roots, respectively. *Note: y-axis (expression fold change) values are different in panels G, H, and I.*

Supplementary Tables

Supplementary Table S1. Target gene and primer sequences. Primers SbPP2A, SbSOS1, and SbNHX2 used for qRT-PCR were previously published ^{7,40}. Primers for SbHKT3 were designed.

Target gene	Primer sequences	Amplicon size (bp)	Accessing number	Gene product
PP2A	Fwd 5' AACCCGCAAAACCCAGACTA 3' Rev 5' TACAGGTCGGGCTCATGGAAC 3'	138	SORBI_3004G092500	serine/threonine-protein phosphatase
SOS1	Fwd 5' GGTGTAGTAAAGTGGACGAGTC 3' Rev 5' ATCAGCACCTCATACAGTCCC 3'	98	SORBI_3008G192900	salt overly sensitive pathway, antiporter/ plasma membrane
NHX2	Fwd 5' CTTTtaggcagcaggttattg 3' Rev 5' GGTCAGTAGCCCAAAGACT 3'	179	SORBI_3005G201000	antiporter/vacuolar compartmentalization
HKT3	Fwd 5'CGT GAT CTT CGA GGT GAT CAG TG 3' Rev 5' TGT CAT GGC AGG CGG TGG TC 3'	105	SORBI_3010G251700	high affinity potassium transporter

Supplementary Table S2. Sequence data for SbPP2A, SbNHX2, and SbHKT3 PCR products. Sanger sequencing results for SbPP2A, SbNHX2, and SbHKT3 PCR products. Nucleotide sequences were BLASTN to verify amplification. SbSOS1 primer pair was published and the PCR product was not a high-quality sequence due to small amplicon size. SbSOS1 data was omitted.

Target gene	Accessing number	Sanger sequencing results
PP2A	SORBI_3004G092500	ATAATKCTGGTGGTGACCTTACTTACTGGTGTTGATGCTCCTCTCTTCTGCAACATCAGG GTCTGTAGATCTTCTGTCCTTAGATATGGGTTCCATGAGCCCGACCTGTAAAAAC
SOS1	SORBI_3008G192900	-
NHX2	SORBI_3005G201000	TKTCGGCYATGAGAGTGCTGTGTCATTGCGTTGGCTTACAATAAGTTCACAAGATCAGG CCATACTCAGCAACCTAGCAATGCTATCATGATCACAAGCACAATCACTGTTGTTCTTTT CAGCACAATTGTCTTTGGGCTACTGACCCAAATAAATTGTCTGAAAAAAAAAAGTGATG TKYRTYTRAWATGATACATTGYTKGTTGCTKAKTAGGKKYRTCTTGGAAMTTATTGTA GCCACGCTGGAACGCACTCCAGAMMCCCCMCCAAACATWACCTGCKCCCTAAAARG AWYS
HKT3	SORBI_3010G251700	CAWTTTGC GGKCGYGAGCAGCTGTAGCCAGTGGACAACCCACGTTTCCATAAGCAC TGATCACCTCGAAGATCACAACCWSTTKWRASARRASYCRSAGAGWTYYWKRARW KWCRA YTKMYCGWCARSAWCGGGGKGCGWSMGCSWCAGTGSSCAGCGYGAGGAGM AMGWWAT

Supplementary Table S3. Analysis of variance results. F-statistic for 2-way ANOVA. (***) represents a p-value < 0.001, (**) represents a p-value < 0.01, (*) represents a p-value < 0.05, and (+) represents a p-value < 0.1.

		SOS1	NHX2	HKT3
Root Tip	Genotype	4.7803 *	8.1063 **	
	Timepoint	56.9923 ***	25.9038 ***	
	G x T	2.6798 *	2.8878 *	
	RSS	0.7626	0.4952	
Leaf 2	Genotype	6.9813 *	0.6428	0.0048
	Timepoint	14.5000 ***	4.6725 ***	8.352 ***
	G x T	3.0827 *	2.6804 *	4.7009 ***
	RSS	0.66467	0.6316	0.78629
Top Leaf	Genotype	0.5622	0.4392	2.5632
	Timepoint	8.3479 ***	13.6257 ***	9.6173 ***
	G x T	3.6537 **	2.9417 *	0.8142
	RSS	1.37502	0.6678	0.79947
		0.001 ***	0.01 **	0.05 *

Supplementary Table S4. Effect of salt exposure on gene expression of SbSOS1 and SbNHX2 of *Sorghum bicolor* accessions in root tips. Plants were exposed to 150 mM NaCl at the 5th or 6th leaf stage of development and relative expression of SbSOS1 and SbNHX2 were calculated in the root tips at nine different time points after exposure. Means followed by different letters are significantly different at $P < 0.05$. Values are mean $\pm$ SE with $n=3$.

	SOS1								NHX2							
	Sb-10				Sb-1				Sb-10				Sb-1			
0 hr	-0.69	±	0.11	ab	-0.55	±	0.09	ab	0.21	±	0.08	cde	-0.02	±	0.08	bcd
0.5 hr	-0.17	±	0.09	bcd	0.22	±	0.09	d	-0.19	±	0.08	bc	0.09	±	0.08	bcd
1 hr	0.02	±	0.09	cd	-0.04	±	0.09	cd	-0.21	±	0.14	abcd	-0.09	±	0.10	bcd
2 hr	0.01	±	0.09	cd	0.17	±	0.09	d	-0.69	±	0.10	a	-0.12	±	0.10	bcd
3 hr	-0.78	±	0.11	a	-0.34	±	0.09	abc	0.40	±	0.10	def	0.49	±	0.14	def
6 hr	0.13	±	0.09	cd	0.04	±	0.09	cd	-0.16	±	0.08	bc	-0.16	±	0.08	bc
12 hr	-0.17	±	0.09	bcd	-0.40	±	0.15	abcd	-0.33	±	0.10	ab	-0.09	±	0.14	abcd
24 hr	-0.06	±	0.09	cd	-0.16	±	0.09	bcd	-0.23	±	0.08	abc	-0.08	±	0.08	bcd
72 hr	1.04	±	0.09	e	1.17	±	0.09	e	0.57	±	0.08	ef	0.66	±	0.08	f
SEM	0.095				0.096											
P _{Accession}	0.036				0.009											
P _{Timepoint}	2.00E-16				2.93E-10											
P _{Accession*Timepoint}	0.022				0.020											

Supplementary Table S5. Effect of salt exposure on gene expression of SbSOS1, SbNHX2, and SbHKT3 of *Sorghum bicolor* accessions in the second leaf. Plants were exposed to 150 mM NaCl at the 5th or 6th leaf stage of development and relative expression of SbSOS1, SbNHX2, and SbHKT3 were calculated in the second leaf at nine different time points after exposure. Means followed by different letters are significantly different at $P < 0.05$. Values are mean $\pm$ SE with $n=3$.

	SOS1		NHX2		HKT3	
	Sb-10	Sb-1	Sb-10	Sb-1	Sb-10	Sb-1
0 hr	-0.21 ± 0.09^{bcde}	-0.37 ± 0.11^{abcde}	-0.08 ± 0.08^{abc}	0.13 ± 0.10^{abcd}	0.42 ± 0.10^{de}	-0.18 ± 0.12^{abc}
0.5 hr	-0.29 ± 0.11^{abcde}	-0.10 ± 0.09^{bcdef}	-0.15 ± 0.10^{abc}	-0.20 ± 0.08^{abc}	-0.27 ± 0.12^{abc}	-0.03 ± 0.10^{abcde}
1 hr	-0.01 ± 0.09^{def}	0.05 ± 0.09^{def}	0.15 ± 0.08^{bcd}	-0.07 ± 0.08^{abc}	-0.01 ± 0.10^{abcde}	-0.01 ± 0.10^{abcde}
2 hr	-0.32 ± 0.11^{abcde}	-0.54 ± 0.09^{abc}	-0.22 ± 0.08^{ab}	-0.28 ± 0.08^a	-0.24 ± 0.10^{ab}	-0.46 ± 0.10^a
3 hr	0.07 ± 0.11^{def}	-0.19 ± 0.09^{bcdef}	-0.13 ± 0.08^{abc}	-0.09 ± 0.08^{abc}	-0.10 ± 0.12^{abcde}	-0.14 ± 0.12^{abcde}
6 hr	0.28 ± 0.09^f	0.21 ± 0.11^{ef}	0.10 ± 0.08^{abcd}	-0.03 ± 0.08^{abcd}	-0.15 ± 0.12^{abcd}	-0.27 ± 0.10^{ab}
12 hr	-0.42 ± 0.11^{abcd}	-0.74 ± 0.09^a	-0.03 ± 0.08^{abcd}	-0.01 ± 0.10^{abcd}	0.18 ± 0.10^{bcde}	0.20 ± 0.12^{bcde}
24 hr	-0.07 ± 0.09^{cdef}	-0.62 ± 0.11^{ab}	-0.08 ± 0.08^{abc}	0.41 ± 0.10^d	-0.11 ± 0.10^{abc}	0.50 ± 0.12^c
72 hr	-0.10 ± 0.09^{bcdef}	0.04 ± 0.09^{def}	0.11 ± 0.10^{abcd}	0.24 ± 0.10^{cd}	0.19 ± 0.12^{bcde}	0.31 ± 0.10^{cde}
SEM	0.095		0.090		0.106	
P _{Accession}	0.013		0.429		0.945	
P _{Timepoint}	2.94E-08		8.70E-04		9.93E-06	
P _{Accession*Timepoint}	0.012		0.024		0.001	

Supplementary Table S6. Effect of salt exposure on gene expression of SbSOS1, SbNHX2, and SbHKT3 of *Sorghum bicolor* accessions in the second leaf. Plants were exposed to 150 mM NaCl at the 5th or 6th leaf stage of development and relative expression of SbSOS1, SbNHX2, and SbHKT3 were calculated in the top leaf at nine different time points after exposure. Means followed by different letters are significantly different at $P < 0.05$. Values are mean $\pm$ SE with $n=3$.

	SOS1								NHX2								HKT3							
	Sb-10				Sb-1				Sb-10				Sb-1				Sb-10				Sb-1			
0 hr	0.62	$\pm$	0.12	d	0.33	$\pm$	0.12	cd	0.60	$\pm$	0.08	e	0.44	$\pm$	0.08	de	0.51	$\pm$	0.11	c	0.26	$\pm$	0.09	bc
0.5 hr	-0.17	$\pm$	0.12	abc	-0.41	$\pm$	0.12	ab	-0.08	$\pm$	0.08	abc	0.01	$\pm$	0.08	abc	0.08	$\pm$	0.09	abc	0.03	$\pm$	0.09	abc
1 hr	-0.21	$\pm$	0.15	abc	0.09	$\pm$	0.12	abcd	0.26	$\pm$	0.10	cde	0.14	$\pm$	0.08	bcd	0.01	$\pm$	0.09	abc	0.04	$\pm$	0.09	abc
2 hr	-0.29	$\pm$	0.12	abc	0.16	$\pm$	0.15	bcd	-0.28	$\pm$	0.08	a	0.07	$\pm$	0.08	abcd	-0.18	$\pm$	0.09	ab	-0.13	$\pm$	0.09	ab
3 hr	0.27	$\pm$	0.12	cd	0.21	$\pm$	0.12	bcd	-0.22	$\pm$	0.08	ab	-0.11	$\pm$	0.08	abc	-0.09	$\pm$	0.09	ab	-0.35	$\pm$	0.09	a
6 hr	0.07	$\pm$	0.12	abcd	0.31	$\pm$	0.12	cd	0.11	$\pm$	0.08	abcd	-0.13	$\pm$	0.08	abc	-0.22	$\pm$	0.09	ab	-0.27	$\pm$	0.09	a
12 hr	-0.24	$\pm$	0.15	abc	-0.26	$\pm$	0.15	abc	-0.06	$\pm$	0.08	abc	-0.02	$\pm$	0.08	abc	-0.22	$\pm$	0.11	ab	-0.22	$\pm$	0.09	ab
24 hr	0.14	$\pm$	0.12	abcd	0.17	$\pm$	0.15	bcd	0.09	$\pm$	0.08	abcd	-0.03	$\pm$	0.08	abc	-0.28	$\pm$	0.09	a	-0.44	$\pm$	0.11	a
72 hr	0.23	$\pm$	0.12	bcd	-0.54	$\pm$	0.15	a	-0.04	$\pm$	0.08	abc	-0.30	$\pm$	0.10	a	-0.08	$\pm$	0.09	ab	-0.07	$\pm$	0.11	abc
SEM	0.133								0.083								0.096							
P _{Accession}	0.459								0.512								0.119							
P _{Timepoint}	6.84E-06								1.32E-08								1.13E-06							
P _{Accession*Timepoint}	4.42E-03								0.013								0.596							

Supplementary Figures

Supplementary Figure S1. SbPP2A gene sequence and primer location.

Nucleotide sequence for SbPP2A gene. Forward primer indicated by cyan highlighting. Reverse primer indicated by magenta highlighting. Exons indicated by orange highlighting. Introns indicated by white highlighting.

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1 AGTTATAATTTTAAAGTTATTTTAAATAATTTACTTAAAGTTAGGCCCTTTTGTACCG 60
61 TTCTGCTTCATCGACGAACTTTTAAATTTTGTAGCTATTTAGAAAAATGTTTGACAAA 120
121 AAAAATTCATGCATGACTAGGAGGAAAAATGAAAAAGAAAGTTATGAAAACTACTTTT 180
181 TTTTAGTTTACCTGCTCAATTTTGTGATGAGAACATACAAAAGAGCTTCGTACCAC 240
241 GAGCTATTTTAAAAAGATATTTAATAAAAGAAACCCAGTTTACAAACCGCTTATAAACT 300
301 GTGCCAAATAGATCTTAAAGTTTGATCATATCAAATCAAATATAAAATACATTATTTT 360
361 TTTTGATGGAGTATTTGCCGTTTGGAGATGCTCGAAACAACATCCTCTTTGAAAATTGA 420
421 AACGCTGGCATGCCACCTACGACTACGAAGTCTTTGTTGACGAGCTCCCGTGGCCCCCCC 480
481 TGGCAATTACAATTTCCATCCCTCCAAACACGAAACACACACGACCCCGGAGCCGAC 540
541 CTGCCCCCTCTCTGCACACCTGGAACGAGGTGTTACCGTCCCGAGAAAGAAGAGCCGAGG 600
601 AAAAATCTCGCACTCGCAAAACAAACAAAGCAGCGTGCCCCCAAAAAATGGAGGCATGA 660
661 GCTCCGCTCTCTCCCGCTCTCTCGCGATCTCCCCCAACCCCTAGCCCGGACCTGACCGC 720
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1201 GTGATATGGGACGTTTGGTGCCGCTGAAGTAGGGGAGTTTGTAGATCATGCGGTATACCTG 1260
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8041	CAATTGGATTGTTATCATGAAAAAGTACACTAAATTTGTGAATATAATCTTTGTAGTTCCT	8100	
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8161	CTCCCTTCGTTCCAAATATAAGATGTTTTGGCTTCTCTAGATACATTGTTTTTGCAAT	8220	
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8341	TATCCCACTGGAAGCTTAAATGTTTATTGTTATAATTTTGCAGGATAAGAAATGTAGTCA	8400	
8401	CAGTCTTCAGTGCGCCTAACTATTGTTACCGATGTGGTAACATGGCTGCTATTCTTGAAA	8460	
8461	TTGGGGAAAACATGGACCAGAACTTCCTTCAATTTGACCCGGCACCTCGGCCAAATTGAGC	8520	
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8581	CTGGTGTTGATGCTCCTCTCATCCGCAACATCAGGGTCTGTAGATCTTCTGTCCTTAGAT	8640	
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8701	TGCTTTCTCGATCTTTATACAAGATGTTAAAAGTTAAGTGCCAGTGGATTTTCTTTTCG	8760	
8761	TTTCTGTTGTACGTCGACTTTGTATTATTTGTAGACAGTGAATGGAAGTCACAGTAGCAA	8820	
8821	CGCCCTGCTATGTTTTTATTTTCGGCTTAGGGATGTGTATCCGCGTGGCTGAATAAATT	8880	
8881	CTGTCAGATTTTAGTCGCGGAATCCCAATCTGATTGCTGTTTCATTCTACATTCATGTG	8940	
8941	ATGCTAAAAATGGTTTCACGATCTAATGTGCGTATAATGTTGGTTGGCGCCTTGCCGTTTC	9000	
9001	ATTCTACATACATGTGATGGCTACAATAGTTTCGCAGTGTTCCTAGTACCATTATGGTA	9060	
9061	CATCCAGATCATTGATGATGTACAGATGAGATGACGCATAATCCACAGCTCTATTCAATT	9120	
9121	TTAAAAATCCACTGGGTGAAAAGTACAGGACAAAGATTTTCGTTATCTCAACCGCACAGTAGA	9180	
9181	TGATGACGTTTAAATCACGAGAAGAAATCAGAACACATGAAATCATAAGCGTGAACAGGAT	9240	
9241	GTTTGGCCTTGATCGTTTCTCTTGGTTAGCAGTGGGACATCGAACGCTCAGTTCAGTTC	9300	
9301	CAGCGTCACATGTCCTGGCTTTACAACATGCAAAATGGGCATGTCAGTGCACATCTAACTT	9360	
9361	CGATTGATCAGGCTTCGTACCATGTGTCAGTGAACGCGATCAACAGCCTGCTGGTAAGT	9420	
9421	CCCCGGGCAAGGTGCAATGTACGTGAGTTCCTTCTAGAAATGTAGAATGTTAGTTCTTG	9480	
9481	GTGCACACTGATAATCTAAAACTCGCATATTAGCGCTGTCTCCTAAG	9528	

Supplementary Figure S2. SbSOS1 gene sequence and primer location.
Nucleotide sequence for SbSOS1 gene. Forward primer indicated by cyan highlighting.
Reverse primer indicated by magenta highlighting. Exons indicated by orange highlighting.
Introns indicated by white highlighting.

```

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421  GAGGTGCTCTCACAACCTCCATCTTTTCTCGAACTGAGTGCCTGGAGTTAAAACTGT 480
481  TTGGCTAAAAAACATAGAGCAAAGCTAAAAAACATAGAGCAGATCAGTCCCAAACACC 540
541  CCCTAAACCAGGCCAAAATATAAGCTGCTGCTGTCTTGTCTTGGAAACCGATCCATACCC 600
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83

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19261 CCACCTGGTTAGGGTTGGCCCAAGTTAAGGAAAACCTAACACCTTTGTGAAAAAATAA 19320
19321 AAAAATTTGGTGGGCTAAAGAAAAAATCTAGTTTTTGACATGTGGTGTCCCATGTTTCATC 19380
19381 ATAGTGAATGCATAAAAGGCTGGTATTTATACAACATGTTAATGAGCTATATATAAAG 19440
19441 ATAATGTTGAACTAACCCCTGTATTAGATCCCTCTGCCTGCTTCTGAATTTTAGTGCCG 19500
19501 TAGGGAACCTCTGATTCTACTTCAGTTAGCCTATACTTCAAACACTTGGCATTGATTAT 19560
19561 GAAAAACATATGCTTGAACGTTTCATCTTGGGTAGATATCATGTGACAGCAAAATCATG 19620
19621 TTTTGTATATGATTTATATATAAAATTTGATAACACTATTTGAAGTTTGGTATGAGCT 19680
19681 TTTGTTGAGATTAACCTATTAATCTTGTGTATGCACATTGATTTTATATCTTGCTC 19740
19741 TTGTAG**GCAGTTCATGGGGCTTCCTTCTGCTGCTCTATGTCTTTGTACAGATATCTCGAC** 19800

STRT-F
END-F
R

23761 GAAAGCCATATATCTGTGACATGACCACGGATTAGTGGCACACTGTTTCTTCATCGAAA 23820
23821 CTGAAAAGATAGAAAGAGCTGCGCCATTAGATCCTTCGATTGAGGTTTTCTGTGGCAGG 23880
23881 TAATACATAATTACTCCTACTAGTAAATAAAATAAAATAAAATAAAATGGTACTCGATG 23940
23941 GATGAATGTTGGCAGGAAAGCGCTCTGGTTCTTGCCAGGCTTTTGCTCCCTCGCATATTC 24000
24001 GAGAAAAATGGGAATGCATGAGATGAGGGTTCTCATTGCTGAAAGGTCGACCATGAACATA 24060
24061 TACATCAAGGGAGAAAGACTTGAAGTGAGGAGAAATGCATTGGCATTCTTGCTGGAAGGA 24120
24121 TTCTTGAAGACCGACAACCTGACTCTAATCACGCCTCCAGCGGTGCTTCTGCCATCCGAT 24180
24181 GCTGACTTGGGTCTCGAGTCCTCAGGTCTCTTTATTTATTATAGTTATAATTATAATATA 24240
24241 TACTATATGTTTTTTTGCTAATATATTTATAGATAATAATGAAGAAATAAAGAGTCAACAA 24300
24301 AAGGCTCTGATTTGACTCGCTCTCTCTGGTATAGACTACTGTCATACTGCACCTAGGTAT 24360
24361 CAGGTGGAAGCTAGAGCACGGATCATCTTCTTGAGGAGGCAGAGGCCATCTTCATCGA 24420
24421 AGTGCGTCGCGGTGCTGCTGCTGCCCCAAGGGCAAGGAGGAGGTCATGAGCCAACACGGAGC 24480
24481 ATGAGCAAGGAGCACAGCGGCTTGCTCAGCTGGCCCGAGAGCTTCCGGAGGTCCCGTGGC 24540
24541 AACCTAGGCCTAGCAGCTGAGATGCTGCCGGCGGCTTGCTTCCAGGGCCCTGCAACTG 24600
24601 AGCATGTACGGCAGCATGGTGATCTCTCTCCGGCCAGGGCCACTCTCACGGCGGCGAG 24660
24661 GGGCGTCAATCGCGTGCAGCGACGCAACAGACCAGAAGCAAAGCTCGTCTTACCCAAGG 24720
24721 ATGCCATCCATATCCAAAGAACGGCTCTGCTCTCCGTGCAATCAGAGGGCTCCAACATG 24780
24781 AAGCGAGTGGCAGTCTTACCTCTCAGAGACGACGCTGCTGAGGTTGAGGCTCCGGCAGCC 24840
24841 CAGCAGCGGCGGCGGCGGAAGGCTATGCACTTGCAAGGAGGCAACTCAAGCGACGACTCC 24900
24901 GCCGGCGAAGAAGTCATTGTCAGAGTTGACTCGCCAGCATGCTCTCTTCCGTGAGTCA 24960
24961 GCCGCCGACCTCCGCCGAGGACAGTAGTCGTCGCTCTCTCTTTGCGCTGTCATACAA 25020
25021 ATATATCATATCCATTTCTCTTGATTTGTATTTGTACAGAAAAATAAAATAAACACATTC 25080
25081 TCTGCTCTATATTTCTCTTCATAATCACTGAATAAAAAACAGAATTCTCTTAAACATG 25140
25141 ATGCAGAAAAATGCACTGCAAAAGCCACATTACAGACTCGTTTTATTGGATCGGACGGGTA 25200
25201 CGTTACGGCTGATCAGTAGCTACTACGCTATTCTGTCTGCACCCACCTCTCCCGATTAAAC 25260
25261 TTACGAGACCAACCTAGAAATCAGGTTAGGTACATACATCGGAATTCATTTCATCTTCTT 25320
25321 CCCATGGACTAGGACACTGCTCCCTGTACCTGTGGCTCATGCTGGTAGCAGCTGATGAA 25380
25381 AACTCAAAAAATATACATCAAAACACAGCTAGGTGAGCAACCGACGATGTTAGGCCACGGT 25440
25441 TCGCCCATAGCATATCCCAGCAAGGTAGAGATTCTTGGCCCACTTATCTGCGACAGCCC 25500
25501 AGAGGAACAAGTAAGGAAAAAACACTGGTCAAGCTAGAGAGAGAGAGAGAGAGAGAGAG 25560
25561 AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACAGGCTCCCGTGTCTTACCTTGACATGT 25620
25621 TGACTGGGAAACTGTGTGGTCCGCAGAGTCTCTTGCTGCTCGTCTCTCGGACGACGCCTT 25680
25681 GGCTTGCTTAGGATCAGCGCAGACTGATCCATTGTGGCAGCCATTGCAGTGATACGAAAG 25740
25741 CCATTGTCCAGCGTCTATGGATGCCCTCACTTGGGTACAAGAAGTCCAACTCCACAACC 25800
25801 TGCAAGAATTGCAATGCTAAGTCCATCAAGTCAAGGCACCCCATCTTTGTTTCATCAG 25860
25861 TACACGTAGTAGCACATTCTCATCTTCAAGGCTATCCCGCAAATAAAATACCAGACCTA 25920
25921 ACAATCAATGAAGATGCACCTGAACATTTAGAAAATCACATTGCTACTAAAAACATTTTC 25980
25981 TACTGTCCTTTGCAACAGTGACGATGAAAGAAAATATTAACCTCTAGCATAAGACA 26040
26041 CTTGGCTTTTTTCTTTT 26058

Supplementary Figure S3. SbNHX2 gene sequence and primer location.
Nucleotide sequence for SbNHX2 gene. Forward primer indicated by cyan highlighting. Reverse primer indicated by magenta highlighting. Exons indicated by orange highlighting. Introns indicated by white highlighting.

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1  GTCCCCACGCCCGGAACGCGCCGCCCGGACCAGTCACTGACGCGTGGGGCCACGCTTAC  60
61  CCGGTGGGCCCGGTGGGTCCCGGGTTAACCGACCGTCCGAGAGCGACCTGCTGCCCGGTG  120
121  GGCCCAACGTGTAGCGCTTTTCTGGCCCCACGTGTTACCCCACTGGCCTGTGGCCACT  180
181  CCCCTCTCCCGCGCCGCGCGGAGCAGCAACCGCCCCCGTGCCACCGACACGTGGGCCA  240
241  TCCCCACCAAAGCCCGTCCAGTGTCTACCTCCCCCTCCCTTCGCGTCACTTCCGACCTCA  300
301  CCCCAAATATAAAAAGTTTTCAAAATTACTATCGCATCGAATATTACGATATATACATA  360
361  AAATATTAATATAGATAAAAAACAAACTAATCATACAGTTTGACTGTAAATTGCGAGA  420
421  CGAATCTTTTAACCTAGTTAGTCTATGATTGGACAATATTTTTTTTATCACAACAAAC  480
481  GAGTGAAATTTAAAAAATAACATCTAAATAAGGCTGATATTTTGTGTTTATTAT  540
541  TTAATTATTATTAATTATTAATTTCCGCACAAAATCCCGTTCGAATCGTATCTCCTCCTCC  600
601  TCCTCCTCGCCTACGACTACGACGCGGTGGCGGCCACCCGCCACAGGCCCGCCGAACC  660
661  CTACGCCCCGTCCCCGAATCGGAGGAGCAACCGCCTCGCGGAGATTCCCTCCGATACCCG  720
721  CCGCCCCACGCCGGGATCTCCGCATCCGGATCCGACTTGCCTCATTTGCCCCCTGATATC  780
781  TCCTCCACGCACACAGTCCACCCCGGCTTCCAGAGTCCAGAGTCTGACCACGGGCGAGC  840
841  GCACGAGCTCTCGCGCGCGTAGAGGCGCCGGATTTCGTCGATTCCGGCGACATAGCAGAT  900
901  CCATTGCCTTTCTCCGATACAGTCCGGGCTGGGTGGGGGACTCCTTTTCCGCCTAGCA  960
961  GGGTTGCGTCCGGGCGCGGGGTTGCTTGAAGGCAAGCCGAGGTGGGAATCGGGTGT  1020
1021  AGGGGCGCGGTGGGTAAAGGTACTAGGTTGGGGCGGTGATTAGTGGAGCTGCGA  1080
1081  GGAGGTCGATAGAGAGGTGGGGAACGATGGGGCTGATGGCGCTGGGCGACCCGCCGGCG  1140
1141  ACTACGCGTCCATCGCGCGGTGGGGCTGTTCGTGGCGCTCATGTGCGTCTGCATCATCG  1200
1201  TCGGCCACCTCTCGAGGAGAACCGATGGATGAACGAGTCCATCACCGCGCTTTCATCG  1260
1261  TGAGTCCATCCCAACCGCCCCCTTTCTCGCTTTTCATGACCTGAGTTTATGCTCTCTCTCT  1320
1321  CTCACCCCTGTGGTATTGGTATTGCAATGTTGTCTGTGCGGAATTGAGGGGTAATTGTG  1380
1381  GCCCTGTTGTTTATGTGCTGTGGCTTTGCAGGGGCTGGGAAGTGGAGCGGTGATCCTG  1440
1441  TTCGGCTCCAGCGGGAAGCACTCGCGCGTGTCCAGTTCAGCGAGGACCTCTTCTTTATT  1500
1501  TACCTGCTGCCGCCATCATATTTAATGCAGGATATGGAATCTTGCAATTGATGCTATA  1560
1561  TGTTCTAGCGTTATGGTGCCATTATTTATTTATTTGTTGCAATTTATCGGTTTTTCTTAC  1620
1621  ATAAAGGATTATGTTACAATTTATCAGTTTTTCTACATAATAAAGGACAAATAAAGAT  1680
1681  GATTCTTCTGACAAATTAAGAAGTTGAGTTGGGCCACACATTGGAGATTGAGAACTTGCT  1740
1741  ATGATTTTAAAGAACTGTAGTTCAAGAGAGCTGTTGTACCATCCCTGTGCTTGAAT  1800
1801  TTCTCCTTCTTTCTTCTAATTAAGACAGGAGAATCCTTAATTGGAAGGCAGAGCTCCT  1860
1861  TCTGTTTACGTTAAACAGAGCAATACATGTAATTTGTTACTGTTTATAGACCTCTT  1920
1921  TGGATAGGTTTTGTCTTTGGAGCTTTCTGAGCTAAAATCCAAAAAGATTCCCTCTTCTA  1980
1981  AACACTTTGGTAGCAAGCACTACGGTGTGCTATTAATAATAAATCTGCAATTATTCC  2040
2041  TCTGAGTATTCGCATTAAGAGTGTAGGTGATATGCCATGTATAATTTAGTGAATATCTG  2100
2101  GACTAGGAACCTATATGTATAACTCACTCATAATTTCTCCATTTTCTGTGTAAGCCAGG  2160
2161  TTCGAAGTGAAGAAAGAACAGTTCTTTCGAAACTTCATTACTATTACACTGTTTGGTGCA  2220
2221  GTTGGCACCTTGATCTCTTTTACTGTAATATCCCTTGTAAGTTTGTCTCCAAATTTTCAT  2280
2281  TTTTACCATGATTTGTCAATTTCTGGGTTTCTGCCTGCCTTAACCTCACATTTGATTTCAC  2340
2341  GGTGCTCTAGGACTAGTATCAAGGCTTAATATTGGCGCACTTGAGCTTGGGGACTATCTC  2400
2401  GGTAATTCATTACCCAAAGTATAAGCACTTCCATTGCCCATATACAGTTCTTTCTTTTCT  2460
2461  GACTCATCTATACCTTAAATTTTCAGCACTTGGGGCAATATTCTCGGCCACAGACTCGGTG  2520
2521  TGCACCTTCGAGGTGTTAAGCCAAGATGAGACACCCTTCTTGTACAGCCTCGTGTGTTGGT  2580
2581  GAAGGTGTTGTCAATGACGCACTTCTGTTGTGTTGTTCAATGCAATCCAGAATTTGAT  2640
2641  CTTGGAATATCAGTGGTGCCAAATTACTTAACCTTATTTGGCAGTTTCTTTATCTATTT  2700
2701  GGCACAGCACCATTCTTGGAGTAGCTGTAAGTTATATGATGCTTCCGCCTCTAGCTTTG  2760
2761  CCTAAGTTTATTCTTTACTGACATGTCTTTGGCTGCAGTCTGGACTTCTTAGTGCTTAT  2820
2821  ATCATCAAGAAGTTGTACTTTTGGCAGGTTTGTAAGTTGACACACACGATTTGTTTGATTG  2880
2881  AAATTGATGACATTTCTGTAACCTATGTTCTCTGATTGACATGCACCTCAGGCACTCA  2940
2941  ACCGATCGTGAAGTTTCCATTATGATGCTAATGGCTTATTTATCTTACATGCTAGCCGAA  3000
3001  GTGAGCACCTGTACTCTTAACCTCTGTAGATATTGTGATTTTCAGAAAAGAAAATACAG  3060
3061  CTGTAAATTGTTATTGACGGGTGTCCTTTTATGGTAGTTGCTTGATTGAGTGGCATTCT  3120
3121  TCACAGTGTCTTCTCGCGGTATCGTAATGTCCCACTATACCTGGCACAATGTAACAGAAA  3180
3181  GTTCCAGGGTCACAACCAAGTAAGTAGCTTCGTAGCTTCTGTGGACATTTCTCTTTGA  3240
3241  TCATGCATCCTTTTGCTAATGCATATACTAATATACCATTGGATGGATCACAGACATGCCT  3300
3301  TTGCAACATTGTGCTTTATCGCCGAGACTTTTCTGTTTCTCTATGTTGGCATGGATGCA  3360
3361  TGGATATAGAGAAGTGAAGATTGTGAGTCAAACCTACAGGCATGCTCTCTCCCTCTTTC  3420
3421  TCTCTCTCTTCTTCTGCTCAAGTAAGTTTTTTTTTCTGAAATCTGTATGTTCTGTCATGT  3480
3481  GCAGCCAGTAAATCTGTTGCTTTGAGCTCCACTATTTTAGCTTTGGTGTGCTTTCAA  3540
3541  GAGCTGCTTTCTGTTTCCCATTTATCTATCTATCAAATTTGACCAAAAAAAGTCCAAATG  3600
3601  GAAAGATATCTTTTAGCAGCAGGTGAGTCTTTAAGTACATCTTAAGTCTTTATG  3660 FOR
3661  TCCTCTTGAACAGTTATGTTCTTACTTGATAGTGATCATTGCTTATGCTTTGGTGG  3720 FOR
3721  CGCGGTCTATAGAGGTGCTGTGTCATTGCGTGGCTTACAATAAGGTAATCTTGTG  3780
3781  TTTGGTCCATCTGTTTGAATTAATTTGTTCACTAGTGGTTGATTTTCTTTCTTATAT  3840
3841  ATGCTTGAATACACTAGCCTCAAAGATCTGTAGTGTAAATGTTCAATTGCTGTGTT  3900
3901  AATGCCTTTTATCTAACCAGTGGTTTTCTATTGTGCAGTTTCAAGATCAGGCCATACTC  3960

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3961	AGCAACCTAGCAATGCTATCATGATCACAAGCACAATCACTGTTGTTCTTTTCAGCACAA	4020	REV
4021	TTGTAAGTTTTTCCTACCTAGTACACACAATATAACACCTAGGTTGATTTTCAAACCT	4080	
4081	ATATTGCAGACAAATACCAACATAAGAACATGACGTGATAGGGGCATTCTTTTCTGC	4140	
4141	TACAACATGGTTCTGAATAGAACAAATTATCCTCTGTTCTTGCAGGTCTTTGGGCTACTGA	4200	REV
4201	CCAAACCGCTGATCAGGCTCTTGATCCCACCAAGGCACCTATCCAGGGAAGCCAGCGCCC	4260	REV
4261	TTTCTGAACCATCCAGCCCCAAGTCTTTCTTTGAGCAGCTGGCCACGAACAGCCCTGGTC	4320	
4321	TCCCTGATATTGAGAACGGCATAAGCCTACGCCGCCCGACAAGCCTCCGTTTGCTCCTAG	4380	
4381	CGAGCCCGGCACGGTCGGTCCACTATTACTGGCGCAAGTTTGACGACGGTTTCATGCGGC	4440	
4441	CGGTTTTTGGCGGCCGTGGCTTCGTCCCCTTCCACCCTGGTTCACCGACTGAGAGCAGTG	4500	
4501	TGCCGCTGCTTGCTGGCAATGGGAAGTGAACACAGCATACCGTGGTGACTGGGTTTCAGCA	4560	
4561	AAAGTTTTGTAAATTAACCTTCGCTTATTAGGAATAATGAATTGGTGTAATATAGGTAGT	4620	
4621	AGATTCACCTTGCTAAGCTTGACATGCTGATGTAGGCTAGTTGCCTGACCAGAGACCACA	4680	
4681	CGGCTCTTGATGAATTTGTATTTATGAATTACGTACTCTACATATTGGATCACAAAATAT	4740	
4741	GTATCATCTCCGAATTAATAGATATATGTAAATTATCAATCCATTTTGGAGTGAATACTA	4800	
4801	TGCTTGAAATATTCACGGGAGGATCTTTTGATTGTATTTCATCCAAACACATCGAACGT	4860	
4861	TTTTCAGGAAGGACGTCTCTTCCCAAGTAGCTAATCTATGAGGAATTTATTGGCTATTA	4920	
4921	ATGACAATTTACAAATAGCTAGTAAGATGACATATATAAATTAAAAAATAAACACTATA	4980	
4981	GTGGAGAGTTATGTGAGAGATATTTGCAGGAGATAGTGCTAGATTAGTTATTTGTAGTGG	5040	
5041	AGAGTTATGTGAGAGAGATTTCCAGGAGATAGTGGTAGATTAGTTATTTGGAAGTCGCTA	5100	
5101	GCTATTTGATCTCTCTTTTATAGTCATTGGAAGTGTTACTTTTTTATTATTCTATACC	5160	
5161	TCATCTAGCTAGCTATTTATACGTTATCTTTAAGACATTTTATAGCTAATAGATTTT	5220	
5221	TTTTCTAAACTAACTATTTGATCTAAAGATTGTCTCTCTTGATTTGCACCCTGAAGGCT	5280	
5281	GAAATCTCACCACCTCACATCTTTCGGTATCCGTGACTGCAAGACGTATTTACAAAACC	5340	
5341	TCTCCATTTACGCCTTCTCATTCCTTCTTCGTGGCCCGTCGTCGGTCCGACCCACGC	5400	
5401	ATCCCGACTGGCAG	5414	

Supplementary Figure S4. SbHKT3 gene sequence and primer location.

Nucleotide sequence for SbHKT3 gene. Forward primer indicated by cyan highlighting.

Reverse primer indicated by magenta highlighting. Exons indicated by orange highlighting.

Introns indicated by white highlighting.

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1  GAACCCGTGTCCATGGAGATGCAGATGATTGGTCTGCTCCTGCAGCAGCGCGGTCTCT 60
61  CCGGCAGCCTCGCTATCACAAAGCAATGGCCGGTCTCTGCCTCTCTGAAGTGGCCACCGT 120
121  TTCATTTGCTGGCGATCTCGACGCCGCCGGCGTATCATATCAGTATCACTCGCCGGAAGC 180
181  TTTCACTTACTACTAGTGGCTGGGGTTGGCCTGCCTCTAGGAGCAGCCGGCTGCCACCCC 240
241  TGCCTTTCCGTTTTGCCTGATCTGGCCGTGGTTGCCCTCGGCCCATGGCAGACGGTTGG 300
301  GCAAAGAGAGGCTCACCATTACACACGCACGCATGTGCACAAGGCTCTGAACCTCTAACT 360
361  GAATTTTCTCCAGCCTTCTCTGTGCTGTGCGTAGTAGTTTCGCGCGTGTGTATTTCAGTCA 420
421  TCTCATCTCACTCACGGCCGGCGTGTCCATCTGCAGGCGCGCATGCCATTAAAGTTCTGA 480
481  AAGCTGCCCTGATGAGCCATGCTACTACCTTGCACTTGACACATGTCTGCTCCTCCACCCA 540
541  CTAATCTCTCTAAAGCCAATGCCATGGGCTGGCTGAAACAGTTACTTGCATTTCCATATG 600
601  ATCACTGCTCTGTCAAGCTAAGTTGTATATATAGTTATATTATATACACCCATTGAGATC 660
661  TCAGTAATCACTTGTACTAGGTTCCATATGTATTAGTACCAGTAAAAGTAAGTAGTTTTC 720
721  CATTGAAACAAGAGCTAGCTAGATCATCAGGCACTGGTCATTGCCCTCTCTAGCTACCAC 780
781  CACCATCTCCAATGCCATATTCGGTTCATGTCTTGGCCAGCGCCGCGAGACACGCCGCTCA 840
841  GCTCATCGTGCTGCTGCTGCCGGCTCATCGCCTACCACCTCACCCTGCTGCTCCACC 900
901  TGACCTACTTCTCGCCCTCGACCTGCTCGGCTTCTCGCCTTGGTCTCTCTCAAGCCAA 960
961  GCAACCCCGGGTACCGTCCGCGCTACATCGACGTGTTCTTCATGTGACGTCCGCGGTCA 1020
1021  CCGTCACCGGACTAGCCACCGTCGAGATGGAGGACCTCTCCAGCGCCAGCTCGTCTGCTCC 1080
1081  TCACCCCTCCTCATGCTCCTCGGCAGCGAGATGTTGCTCTCCTTGTCTCGGTCTCGTCTCG 1140
1141  AGTCGTCGCGCAACCGAGCAGCAGCAGCAGCGGACCATCAAGATCAGCAGACGAGGTCA 1200
1201  TGGCGGCGGCCGCGCTCCGCGCAGCAGCGGATCTCGAGGAGGCGAATGGTCCGCGCAGCAG 1260
1261  CTCGCTCTGCGGATTCGTCGCGCGATGGTGGTGATCGCAATCGCAAGGAGAGCTCCCGCG 1320
1321  ACGTCAGAAAGTTTAGCGCTTGTGGTGTGCGCCTACATGGCCGCGATCCTCGTCTGCTCG 1380
1381  CCGTGTGCTGGTGTTCGCGTACGTGCGCCACCGTCCCAGCCGCGCGCAGCTGCTGGCGAGGA 1440
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1501  GCCTGCTCCCGACAAACGAGAGCATGGCGGTGTTGCGCCCGAACCGTGGTCTGCTCCTGC 1560
1561  TGCTCGCGGCACAGATCCTCGCCGCGCAGCAGCTGCTGCGGTGTTCTCAGGCTGGTGG 1620
1621  TGAGCGCCGACGAGGGGTGGCGAGGGCGTTGCTCTGTTTCAACGGGCGAGGAGGGTCCG 1680
1681  TCGAGGAGCTCGTGCCCATGGACATGGAGAAGAGCGCCGCGCGCGGGGTTTCGGCCACC 1740
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1981  ACGTCAGTCACGATGCTCCTCCGCGCGAAATGAAGCATACTACGCAACAATATGTTGTT 2040
2041  CGATCATTTGACAGATTTTGGCATGCGGACCGAGATTTCAGGTGCAATCCCGCGCTCGGC 2100
2101  GACATTGTTGTGCGGTGCGAGCAGCAGCGCGCGGAAAAGACAAGGAGCGCGCGGAGAAC 2160
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2221  GCTGTACCCGCTGGCCTGCAACGCGCGCGGTAATGCTCGCCTGCATCACCAGAGAGGCG 2280
2281  ATCGATCGCCGCGGACCGCTCAACTTCTCCACGTTCAAAGTATCTTCGAGGTGATCAG 2340 FOR
2341  GTGAGGTGCACATGCACGATGCAGCAACATCACAGTTTCAAAACCATCTCATCAGAAG 2400
2401  CTTGCTTCTCTGAACTGACATATGATTCCTGCATGTGTGCACTGCTTATGGAACGTTG 2460 FOR
2461  GGGTGTCTCACTGGCTACAGCTGCTCGAGGCTGCGCGCGCGCGCGGCAACCCGCTGCT 2520 REV
2521  CATGACAAGCCATACAGCTTCTCCGGTGGTGGAGCGACAGGGGAAACTGCTCCTTGT 2580 REV
2581  CTCCTCATGCTCTACGGGAGGCTCAAGGCTTCCATGGACAGCGACGAGGAGGTGAAC 2640
2641  TTATTTGGTGATCGATGACCGATGATGAAGGCAATGCCGGGATGGTCTGGGGATCAAC 2700
2701  TTGAAATCTCTCAAGATTCATGGGCGATAAGAATGAAATTCAGAGTGAATGCGATTG 2760
2761  GAAGAAGGATACACACTCCGTGGAGAAATATATATAGAATATAGATTGTAAGTTTTCTA 2820
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2881  ACAACAATAGGAATTAGGAAAAAGTCTATTGACCCCTCAACTTTCACGGTCATCTGAT 2940
2941  TTCTCTTCTTCATCTACAAAAGTAGATTTTGTGTCATCTTAACTTTTATATAAACGTTT 3000
3001  GTTTGGTCAATTGGCACATGTCACTGCTCCTATGTGGCGCCATGATAACCATGAGCGGG 3060
3061  TTAATTCGAATAATTGATGGTGGTGGGTTTGCATATCAGACTATAAAATATTTTCTC 3120
3121  AAATAAATACACAAATATTTTCCATAGAAAAGTTAAATCCTAACTTGTAAATCGGG 3180
3181  TTTAATCATAGAAAATATTTTAGCATAAATTTTCTTAAATCATGATGTATCTTATAGTG 3240
3241  ACTAACTTGAACACTTTGAGTCTTATATGTACCCATGTCCATTTTGTGTTTATTTAC 3300
3301  CGGTAGATGCCCTTACTATCTATTACAGTTAGTCTACATTGAAGACTCGAACTTTTATAA 3360
3361  GGTTCAAATGACTTTGGCGACAACACAATTTCCATGTAACATAGCATACTGATTTCTAT 3420
3421  ATATGATCATCTTAGTATGAAAAAGTATGGAGGGTGAAGTGTGGATAACAAGACCCACA 3480
3481  GTATATCAATGAAAGAACAAAGTAATGCACAACAATTTTCATGAAACTAGAGTATGTGT 3540
3541  CACACTGGCCTTTTTATGTGTTCTGGTGGCCAAAGTTGACTAATTATAGATAACAAGAG 3600
3601  CATATACTAGAAAATAAACACAAAAAGGGT 3630

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Supplementary Figure S5. DNA and cDNA PCR for gene verification.

Agarose gel (1.0%) electrophoresis verifying PCR amplification with 1 Kb plus DNA ladder.

(A) PCR run with DNA. Primers span introns.

(B) PCR runs with cDNA. Sizes are expected for genes listed.

A.

B.

CHAPTER 5

DOMESTICATION & DETOXIFICATION: EARLY SENSING, SIGNALING, AND OSMOTIC ADJUSTMENT AID IN THE INCREASED SALINITY TOLERANCE OF SORGHUM BICOLOR

Ashley N. Henderson; Rajanikanth Govindarajulu; Jennifer S. Hawkins

Abstract

Soil salinity is an intensifying problem affecting lands used to produce agronomically important crops. Salt stress impedes crop production via both osmotic and ionic stress. Such stress slows plant growth and disrupts normal metabolic processes, resulting in decreased biomass and increased leaf senescence. Therefore, the ability to maintain biomass in response to salt exposure is indicative of tolerant genotypes. We used a recombinant inbred line (RIL) population derived from *Sorghum propinquum* and *Sorghum bicolor* (Tx7000), two genotypes shown to differ for tolerance to salt exposure, to map regions of the genome associated with salt tolerance. Low coverage Illumina sequencing was used to generate a high-density genetic map that covered the 10 *Sorghum* chromosomes with 1692 Mendelian segregating markers. Eighteen total QTL were detected for dead aboveground biomass, live aboveground biomass, total aboveground biomass, root biomass, total biomass, rank score, and height in two environmental conditions (control and salt), and when stress tolerance index (STI) values were mapped. Candidate genes associated with salinity tolerance include genes associated with osmotic and ionic stress. Most interestingly, however, was the enrichment of aquaporins in salt specific QTL suggesting that better water acquisition and transport may be responsible for the greater salinity tolerance observed in *S. bicolor*.

Keywords – abiotic stress, aquaporins, bin mapping, genetic architecture, recombinant inbred line, salt stress, *Sorghum bicolor*, *Sorghum propinquum*, stress tolerance index, quantitative trait loci

Introduction

Saline soils are an intensifying problem that directly affects the production of agriculturally important crops. Soil salinity is an abiotic stressor where soluble ions, such as Na⁺ and Cl⁻, accumulate in the soil surrounding root rhizospheres. These ions cause a decrease in the soil water potential, making it difficult for plants to absorb water without altering its own potential. With salinity affecting more than 6% of the total land and 20% of the irrigated land used for crop production, it is imperative to identify ways to maintain and even increase crop productivity in saline environments in order to meet population demands (Rengasamy, 2006; Food and Agriculture Organization (FAO), 2008, 2009; Hasegawa, 2013; FAO, 2017; Yang *et al.*, 2020). Research over the last 20 years has focused on unraveling the key mechanisms of tolerance, and the results suggest that tolerance is a result of: 1) ion exclusion, 2) tissue tolerance, and 3) osmotic tolerance (shoot ion-independent tolerance) (Munns & Tester, 2008; Carillo *et al.*, 2011; Fan *et al.*, 2015; Genc *et al.*, 2016; Negrão *et al.*, 2017; Munns *et al.*, 2019). Ion exclusion is the absence of salt ions from the shoot of the plant, whereas tissue tolerance is the sequestration of ions into specific tissues, cells, and subcellular organelles. Lastly, osmotic tolerance is defined as the ability to maintain water uptake and growth despite lower water potential.

Salinity stress negatively affects many economically important crops. Sorghum (*Sorghum bicolor* L. Moench), which is naturally drought and salt tolerant (Boursier & Läuchli, 1990; Almodares & Sharif, 2007; Almodares *et al.*, 2007, 2008b,a; Mullet *et al.*, 2014; Fracasso *et al.*, 2016; McCormick *et al.*, 2018; Guo *et al.*, 2018; Henderson *et al.*, 2019), ranks fifth for food, fuel, and feed production after wheat, maize, rice, and barely (Doggett, 1970, 1988). Compared to other C4 cereal crops, Sorghum requires minimal resources and delivers high yields (biomass, grain) making it a model species to dissect the various tolerance mechanisms. In a recent study, 18 diverse genotypes of *Sorghum bicolor* and four wild relatives were assessed for performance following salt exposure (Henderson *et al.*, 2019). Genotypes ranged in maintaining as little as 30% to as much as 95% of their biomass after 12 weeks of salt exposure. Most of the individuals from the landrace durra ranked in the top 5 for most tolerant. Further, the most sensitive genotype, in terms of live aboveground biomass production, was *Sorghum propinquum*, which lost 95% of its biomass in response to stress. This wide range in biomass retention indicates continuous variation in salinity tolerance and suggests that tolerance is a quantitatively inherited trait.

Quantitative trait loci (QTL) mapping is a powerful tool that enables the association of quantitatively inherited phenotypes to candidate genomic loci (Kearsey, 1998; Lynch & Walsh, 1999; Broman & Sen, 2009). Given recent results that demonstrate extensive variation in response to salinity stress among sorghum genotypes and their close relatives (Henderson *et al.*, 2019), the recombinant inbred line (RIL) population used in this study (*S. propinquum* by *S. bicolor* (Tx7000 - landrace durra) serves as an invaluable resource to dissect the genetic underpinnings of salinity tolerance. Here, we provide a high-density genetic map from 177 F_{3.5} lines and identify QTL associated with numerous biomass-related traits that are indicative of salinity tolerance, such as dead aboveground biomass, live aboveground biomass, total aboveground biomass, root biomass, total biomass, rank score, and height. Eighteen QTL and 140 candidate genes previously shown to aid in osmotic and ionic stress alleviation were identified.

Materials and Methods

Plant Material

A recombinant inbred line (RIL) mapping population derived from an interspecific cross of *Sorghum propinquum* (William Rooney, Texas A&M University, College Station, TX) and *Sorghum bicolor* (inbred Tx7000, landrace durra) was used to investigate the genetic underpinnings associated with variation in salinity tolerance. The RIL population consists of 177 F_{3.5} lines with 75% (132 RILs) of the individuals being F₃, 18% (31 RILs) of the individuals being F₄, and 7% (14 RILs) of the individuals being F₅. Each line was derived by the single seed descent method (Brim, 1966; Snape & Riggs, 1975).

Experimental Conditions

In a controlled greenhouse room, five seeds of each RIL were sown in 5 cm x 5 cm x 5 cm planting plugs filled with metromix soil. Conditions during germination were: 21°C, 75% humidity, and 4.5 vapor pressure deficit (VPD). During germination, seedlings were misted regularly with non-saline tap water and watered with a 20-10-20 N-P-K (J.R. Peters, Inc., Allentown, PA, USA) fertilizer diluted to 200 mg N L⁻¹ every 4th day. Once all plants reached at least the third leaf stage (V3) of development (approximately 32 days post sowing), soil plugs were transplanted into 5 cm x 5 cm x 25 cm treepots (Stuewe and Sons, Tangent, OR, USA) filled with silica sand #4. Treatment room conditions were: 27°C/23°C (day/night) with 16 hours of daylight and 75% humidity. Following transplant, seedlings were watered to saturation with non-saline tap water daily for two weeks to provide a period of establishment. All plants were fertilized twice weekly with a 20-10-20 N-P-K (J.R. Peters, Inc., Allentown, PA, USA) fertilizer diluted to 200 mg N L⁻¹ for the remainder of the study. Following establishment, three of the five biological replicates were randomly assigned to a 75 mM NaCl salt treatment and two of the five biological replicates were randomly assigned to a 0 mM NaCl control treatment. Seedlings were watered once daily, in accordance with their assignment, to complete saturation for the duration of the experiment. Treatment began 51 days after planting and plants were treated for a total of 45 days.

Phenotypic Measurements

The following measurements were recorded at various developmental stages for each of the 177 lines: height (cm), tiller count, rank score, root biomass (g), dead aboveground biomass (g), live aboveground biomass (g), total aboveground biomass (g), total biomass (g), and the shoot to root ratio. Height was taken from the base of the stem to the tip of the newest emerged leaf. Tiller count was the total number of tillers attached to the base of the stem. Rank score was a qualitative score that described overall leaf ‘greenness’, leaf health, and mortality, where plants that displayed no signs of stress received a low rank score, and plants that were extremely stressed or had died received a high rank score (**Table 1**). Rank score was assessed by the same person throughout the entirety of study to minimize bias. All biomass measurements were taken on tissue collected from a destructive harvest and dried in 65°C for a minimum of 72 hours. Root biomass was the total belowground biomass collected. Roots were rinsed in water to remove all dirt and sand. Dead aboveground biomass was all biomass (leaves, tillers, and/or stem) attached to the plant where more than 50% of the tissue was brown; whereas, live aboveground biomass was all biomass attached to the plant that was more than 50% green suggesting it was alive. Total

aboveground biomass was the sum of live and dead aboveground biomass, while total biomass included live, dead, and root biomass. The shoot to root ratio was the total aboveground biomass divided by the root biomass. Mortality was scored as 1 if plants were alive and 0 if dead.

Phenotypes were measured at three time points: 0 days (51 days after planting, 0 days of treatment, referred to as pre-treatment), then 15 (short term exposure), and 45 (long term exposure) days after treatment began. Height and tiller count were taken at 0, 15, and 45 days after treatment began, with 0 days indicating immediately before treatment. Rank score was taken at 15 and 45 days after treatment began. All biomass was collected between 45-50 days after treatment and was immediately dried.

The stress tolerance index (STI) value for each trait was calculated using the following formula, where Y is the phenotypic trait, control is the trait measurement in 0 mM NaCl conditions, salt is the trait measurement in 75 mM NaCl conditions, and control average is the population average of the trait in control conditions (Negrão *et al.*, 2017):

$$\text{Stress Tolerance Index} = \left(\frac{Y_{\text{control}}}{Y_{\text{control average}}} \right) \times \left(\frac{Y_{\text{salt}}}{Y_{\text{control average}}} \right)$$

For each RIL, the control value was averaged across control replicates and an STI value was calculated for each salt treated plant. The STI value accounts for the overall performance of the population and compares each RIL's ability to maintain performance under stress conditions. RIL's with large STI values indicate larger phenotypic values for a given trait and are often considered tolerant depending on the phenotype.

Statistical analysis of phenotypic values

All statistical analyses were performed on the control population, the salt treated population, and on stress tolerance index (STI) values. Three biological replicates of each RIL in the salt population and two biological replicates of each RIL in the control population were considered for QTL analysis. There were three biological replicates for STI values. Least square means for each phenotype in each population (control, salt, STI) were calculated for each RIL. Normality was assessed using both a Shapiro-Wilk test in RStudio (ver.1.2.5019) and Q-Q plots from the *car* (Fox & Weisberg, 2019) package of R (R Core Team, 2013). Traits that were not normally distributed were transformed (**Supplementary Table S1**). Transformed values were used in statistical tests and in QTL analysis. Correlations of phenotypes within each treatment (control or salt) were assessed via a Pearson's Correlation analysis in R (R Core Team, 2013) using the *PerformanceAnalytics* (Peterson & Carl, 2019) package (**Figure S1**)(R Core Team, 2013).

To determine if there was a treatment effect, both a non-metric multidimensional scaling (NMDS) analysis (Julkowska *et al.*, 2019) and an analysis of variance (ANOVA) was performed. The NMDS ordinated RILs based on similarity/dissimilarity when all phenotypes were considered. The NMDS was generated using the *vegan* (Oksanen *et al.*, 2019) package in RStudio (ver.1.2.5019)(R Core Team, 2013). The NMDS was paired with an analysis of similarity (ANOSIM) to statistically test if individuals cluster within a treatment or among treatments (significance assessed at $\alpha = 0.05$). An analysis of variance (ANOVA) was used to

test if control and salt populations differed for individual phenotypes (significance assessed at $\alpha = 0.05$). All statistics were completed using RStudio ver.1.2.5019.

Genetic map construction and QTL analysis

A high-quality genetic map for the *S. propinquum* by *S. bicolor* (Tx7000) RIL population was previously constructed as described in Govindarajulu *et al.* (2020). In summary, using high-quality nuclear DNA, the parent plants (*S. propinquum* and *S. bicolor*) were sequenced at 18x depth, while the RILs were sequenced at 2x depth. SNP data were aligned to the masked *Sorghum bicolor* reference genome ver 3.1 (Paterson, 2008). Loci were called as *S. propinquum* (A), *S. bicolor* (B), or *heterozygous* (H) when SNPs were analyzed with the GenosToABHPlugin in Tassel ver 5.0 (Bradbury *et al.*, 2007). The ABH formatted SNP data file was then used as input to SNPbinner (Gonda *et al.*, 2019), which calculated breakpoints (Govindarajulu *et al.*, 2020). Breakpoints were merged if they were shorter than 0.2% of the chromosome length. After removing heterozygous bin markers, duplicate bin markers, and markers that deviated significantly from the expected Mendelian ratio (1:1) at $P < 10^{-5}$ after Bonferroni correction, the kosambi map function in R/qtl (Broman *et al.*, 2003) was used to construct a high density genetic map. A second map was constructed including all markers that deviated from the expected Mendelian ratio (1:1).

QTL analysis was performed in RStudio (ver.1.2.5019) using the *qtl* (Broman *et al.*, 2003) package. QTL were first identified by a single interval mapping QTL model. Significant LOD peak scores were determined by comparing LOD peak scores after a 1,000 permutation test ($\alpha = 0.05$) (Churchill & Doerge, 1994). If QTL were detected by interval mapping (IM), phenotypes were assessed via a multiple QTL model. The multiple QTL model (MQM) tested for additional additive QTL, refined QTL positions, and tested for epistasis. Following MQM, a type III analysis of variance assessed the significance of the fit for the final model, the proportion of variance explained, and the additive effect. QTL with a negative additive value indicated that the trait was negatively influenced by *S. bicolor* alleles, whereas a positive additive value indicates the trait was positively influenced by *S. bicolor* alleles. Genes (*Sorghum bicolor* ver. 3.1) within a 1.0 logarithm of the odds (LOD) confidence interval for each QTL were identified.

Results

High quality genetic map

The resequencing and bin calling of the 177 RILs generated 4055 total bin markers (**Figure S2**); however, after removing distorted markers (**Figure S3**) and duplicate markers (**Figure S4**), our map covered the 10 *Sorghum* chromosomes with 1692 bin markers (**Supplementary Table S2**) and was 888.7 cM in length (**Supplementary Table S3**). Chromosomes 7 and 8 contained no distorted regions and chromosomes 3 and 9 contained less than 10 distorted markers; however, the distorted region of 61.09 Mb to 70.23 Mb on chromosome 2 and the distorted region of 6.37 Mb to 59.45 Mb on chromosome 5 indicate preferential transmission bias of *S. propinquum* alleles. Chromosomes 1, 4, 6, and 10 contain distorted regions favoring *S. bicolor* alleles (**Supplementary Table S3**).

Treatment Effect

The control and salt population summary statistics of phenotypes are listed in **Supplementary Table S4**. With the exception of mortality, all other phenotypes were significantly different between control and salt treated populations (short term and long term exposure)(**Supplementary Table S5**). In response to salt exposure, plants were shorter (**Figure 1a**) and had less live aboveground biomass, root biomass, total aboveground biomass, total biomass, and percent of live aboveground biomass (**Figure 1b**). In contrast, plants had a larger rank score, more dead aboveground biomass, and a larger shoot to root ratio (**Figure 1b**). These significant differences between treatment and control conditions, in addition to the clear and significant clustering ($p < 0.001$, ANOSIM $R = 0.16$) of treatments in the NMDS analysis (**Figure 2**), are indicative of a decrease in performance in response to treatment.

QTL Results and Phenotypic Responses

Although variation among the control and salt population was observed in response to treatment, no QTL were detected for short term salt exposure (DAT=15). Therefore, the remaining sections will focus on QTL detected for long-term salt exposure (DAT=45). All genes detected in QTL when only considering Mendelian segregating markers are listed in **Supplementary Table S6**. All genes detected in QTL when including all markers that deviated from the expected Mendelian ratio are listed in **Supplementary Table S7**.

Total biomass (TB)

In control conditions, plants ranged from 1.98 g to 12.18 g of total biomass, with a mean of 7.49 g; however, in salt conditions, a 32% decrease in total biomass was observed (0.66 g - 9.42 g with a mean of 5.11 g, **Supplementary Table S4**). Three QTL were detected for total biomass with a common QTL detected on chromosome 4 in both salt conditions and when STI values were mapped (qTB45_4.S and qTB45_4.STI, respectively) (**Figure 3a**). An additional unique QTL was detected on chromosome 5 in the salt population (qTB45_5.S). Each QTL had a positive additive effect indicating that *S. bicolor* alleles positively influenced the total biomass and explained greater than 10 percent of the phenotypic variation. The QTL on chromosome 4 that was detected when STI values were mapped (qTB45_4.STI), explained the greatest amount of phenotypic variation (PVE = 13.02) (**Table 2**). Of the 595 genes within qTB45_4.STI, candidate genes were associated with aquaporins, oxidative stress, salt tolerance, LEA (late embryogenesis abundant) proteins, and transporters (**Supplementary Table S8**). When including distorted markers, the peaks for the two QTL detected on chromosome 4 (qTB45_4S and qTB45_4STI) were not affected (**Supplementary Table S9, Figure 3b**).

Total aboveground biomass (TAGB)

In control conditions, plants accumulated an average of 5.00 g of total aboveground biomass (1.34 g - 8.56 g), however, in response to salt treatment, there was a 20% decrease in total aboveground biomass (average = 3.98 g)(**Supplementary Table S5**). A single QTL (qTAGB45_4.STI) was detected when STI values were mapped (**Figure 3a**). The QTL was located on chromosome 4 between 62.17 Mb - 66.68 Mb with a peak at 62.29 Mb (**Table 2**). qTAGB45_4.STI explained 12.34 percent of the phenotypic variation and had a positive additive effect indicating that *S. bicolor* alleles positively influence the total amount of aboveground biomass. qTAGB45_4.STI co-localized with qTB45_5.S and qTB45_4.STI. Therefore, the same

595 total genes and candidate genes identified in the total biomass QTL were also identified for total aboveground biomass. When including distorted markers, the QTL detected on chromosome 4 for total aboveground biomass when STI values were mapped remained at approximately the same location, however, an additional QTL was detected for the salt population, explaining 9.49 percent of the phenotypic variation and having a larger additive effect of 0.40 (**Figure 3b**).

Dead aboveground biomass (DAGB)

In control conditions, dead aboveground biomass ranged from 0.06 g to 1.61 g (mean of 0.55 g), whereas in salt conditions there was an increase of 45% with a mean of 0.80 g (**Supplementary Table S4**). Two QTL (qDAGB45_2.C and qDAGB45_2.STI) were detected for dead aboveground biomass when control and STI values were mapped (**Figure 3a**). The QTL were both detected on chromosome 2 and explained 9.14 and 12.03 percent of the phenotypic variation, respectively (**Table 2**). RILs with *S. propinquum* alleles in this region positively influenced the amount of dead aboveground biomass. In qDAGB45_2.STI, there were 739 genes. Of the 739 genes, candidates included genes that encode aquaporins, Na⁺/H⁺ transporters, LEA-like proteins, and CLB (calcium-dependent lipid-binding) proteins (**Supplementary Table S8**). When including all distorted markers, the two QTL on chromosome 2 remained, however, an additional QTL was detected on chromosome 9 in the control population. The range of the QTL detected on chromosome 2 when control values were mapped (qDAGB45_2.C) shifted and decreased significantly when distorted markers were included (64.40 Mb - 67.54 Mb, peak @ 65.42), however, when STI values were mapped the QTL peak shifted and the range increased (13.85 Mb - 67.54 Mb, peak @ 62.28 Mb) (**Figure 3b**). The new QTL, qDAGB45_9.C, was detected between 55.47 and 57.91 Mb with a peak at 56.32 Mb and explained 9.71 percent of the phenotypic variation with an additive effect of -0.06.

Live aboveground biomass (LAGB)

In control conditions, live aboveground biomass ranged from 1.20 g to 6.95 g with an average of 4.45 g (**Supplementary Table S4**). In response to salt treatment, there was a decrease of 28% in live aboveground biomass with a range from 0.32 g to 6.22 g and an average of 3.18 g (**Figure 1b**). Two QTL were detected for live aboveground biomass (qLAGB45_4.STI and qLAGB45_5.STI) when STI values were mapped (**Figure 3a**). Both QTL had a positive additive effect indicating that *S. bicolor* alleles positively influenced live aboveground biomass. The QTL detected on chromosome 4 explained 15.35 percent of the phenotypic variation and the QTL detected on chromosome 5 explained 10.40 percent of the phenotypic variation (**Table 2**). In the 4.38 Mb region where qLAGB45_4.STI was identified, there were 577 genes from which we identified several candidate genes. Candidate genes include genes associated with aquaporins, stress induced proteins, cation efflux proteins, and a calcineurin B subunit protein. In the region where qLAGB45_5.STI was identified, there were 1356 genes from which we identified candidate genes. Candidate genes included genes that encode proteins associated with photosynthesis, auxin signaling and response, stress response, potassium channels, and growth regulation (**Supplementary Table S8**). When distorted markers were included, the QTL on chromosome 4 (qLAGB45_4.STI) was still detectable, however there was a slight shift in the range and peak (**Supplementary Table S9**).

Root biomass (RB)

Root biomass for plants grown in control conditions ranged from 0.48 g to 6.38 g with a population average of 2.50 g; however, root biomass for plants grown in salt conditions was reduced by 55% and ranged from 0.09 g to 2.48 g with a population average of 1.13 g (**Supplementary Table S4**). A single QTL was detected for root biomass when STI values were mapped (qRB45_4.STI) (**Figure 3a**). qRB45_4.STI explained 9.09 percent of the phenotypic variation and had an additive effect of 0.08 indicating that individuals with *S. bicolor* alleles in this region positively influenced root biomass. There were 812 genes within the 6.26 Mb region (**Supplementary Table S6**). Of these, there were several notable genes for their role in stress response, specifically genes that have been annotated as salt tolerance homologs (**Supplementary Table S8**). When including all distorted markers, the QTL detected on chromosome 4 was retained in approximately the same location (peak @ 62.46 Mb), however, the range of the QTL was reduced (62.17 Mb - 62.54 Mb)(**Figure 3b**).

Rank Score (RS)

Rank score was a qualitative measure used to describe overall plant health (**Table 1**). There was a 58% increase in the rank score in response to treatment indicating that there was an overall decrease in the health of plants exposed to NaCl (**Figure 1b**). In control conditions, the average rank score of the population was 2.01 (0.72-3.20) indicating that most of the individuals in the population were beginning to show signs of leaf tip curling; however, some individuals had dead leaves present but still were able to produce new leaves. In contrast, the rank score of the salt treated population averaged 3.19 with a range of 1.68 to 4.55 (**Supplementary Table S4**). This increase suggests that the production of new leaves was halted, most leaves were dead or began dying, and all individuals were displaying signs of stress. When STI values were mapped, a single QTL (qRS45_7.STI) was detected on chromosome 7 (**Figure 3a**). qRS45_7.STI explained 9.00 percent of the phenotypic variation and had a negative additive effect indicating that *S. bicolor* alleles are associated with a lower the rank score. Of the 134 genes located within qRS45_7.STI, there are genes that encode aquaporins and cation transporters (**Supplementary Table S8**). When all distorted markers were included a new QTL was detected on chromosome 4 (qRS45_4.STI). qRS45_4.STI ranged from 62.46 Mb to 63.95 Mb with a peak near 63.67 Mb (**Figure 3b**). qRS45_4.STI explained 10.77 percent of the phenotypic variation and also had a negative additive effect of 0.19 indicating that *S. bicolor* alleles are associated with a smaller rank score.

Height (HT)

At the final recording of height, the control population ranged from 52.92 cm to 122.37 cm with a population average of 87.81 cm. In response to treatment, the salt population displayed a 16.38% decrease in height, ranging from 46.32 cm to 112.47 cm with a population average of 75.46 cm (**Figure 1a**). Six QTL were detected for height with two common QTL in the control (qHT45_7.C and qHT45_9.C) population and when STI values were mapped (qHT45_7.STI and qHT45_9.STI) (**Table 2**). qHT45_7.S was also detected in the salt population; however, a unique QTL (qHT45_1.S) was detected in the salt population, explaining 12.07 percent of the phenotypic variation and having an additive effect of -4.36. Of the 3644 total genes within qHT45_1.S, several candidate genes were identified, including genes associated with osmotic and ionic stress alleviation, such as SOS3- and CBL-interacting proteins, and vacuolar cation exchangers (**Supplementary Table S8**). When including distorted markers, all six previously detected QTL were retained, however, an additional two QTL were detected when STI values

were mapped (**Figure 3b**). With the exception of qHT45_1.S, the positions of all other QTL (qHT45_7.C, qHT45_9.C, qHT45_7.S, qHT45_7.STI, and qHT45_9.STI) were unchanged. The range of qHT45_1.S significantly decreased (76.22 Mb to 80.57 Mb); however, the peak remained close to its original location (peak @ 75.72 Mb). The two additional QTL detected when STI values were mapped (qHT45_1.STI and qHT45_4.STI) explained an average 9.82 percent of the phenotypic variation. Interestingly, qHT45_1.STI had a negative additive effect indicating that *S. bicolor* alleles negatively influenced plant height while qHT45_4.STI had a positive additive effect indicating that *S. bicolor* alleles positively influenced height. Further, qHT45_1.STI co-localized with the previously detected qHT45_1.S, which was only detected when salt values were mapped.

Discussion

In the present study, we screened 177 F_{3.5} RILs derived from a cross between an inbred *Sorghum bicolor* (Tx7000; landrace durra) and its wild relative, *Sorghum propinquum* for performance in saline conditions. Because of Sorghum's importance in biofuel and forage production, salinity tolerance was assessed as the ability of the plant to maintain traits related to growth and performance in response to salt treatment. This tolerance can be achieved by various mechanisms including osmotic adjustment, Na⁺ exclusion from the aerial organs of the plant, and overall tissue tolerance; however, Na⁺ exclusion can also result from reduced Na⁺ uptake, increased Na⁺ extrusion to the roots and/or soil media, or increased retrieval from the shoot (Wu *et al.*, 2019). Ultimately, each of these tolerance mechanisms results in the maintenance of plant vigor similar to those plants grown in optimal conditions. In this study, we identified QTL associated with total biomass, total aboveground biomass, height, dead aboveground biomass, live aboveground biomass, root biomass, and rank score, in a control environment, a saline environment, and from STI values. Among the 18 QTL detected, 11 were either 1) unique to the STI values, 2) unique to the saline environment, and/or 3) explained more than 10 percent of the phenotypic variation (**Table 2**).

The data presented here, in combination with previous findings (Henderson *et al.*, 2019), collectively demonstrates that there is increased tolerance to salinity stress in *Sorghum bicolor* compared to *S. propinquum*. For dead aboveground biomass, live aboveground biomass, total aboveground biomass, and total biomass, *S. bicolor* alleles were associated with tolerance. For example, the negative additive effect for the QTL detected for dead aboveground biomass indicates that *S. bicolor* alleles were associated with less accumulation of dead aboveground biomass. Similarly, the QTL detected for live aboveground biomass, total aboveground biomass, and total biomass all have positive additive effects indicating that *S. bicolor* alleles promote continued growth in stressful conditions. It is important to note that, in optimal conditions, *S. propinquum* produces more aboveground biomass compared to *S. bicolor* (Govindarajulu *et al.*, 2020). Therefore, in response to salt, the ability for the lines with *S. bicolor* alleles to perform favorably supports the conclusion that *S. bicolor* possesses greater tolerance to salinity stress. Further, these results suggest that *S. bicolor* is better at handling both osmotic and ionic stress, because with osmotic adjustment, increased water can be taken up by the plant to support the production of new biomass and to limit necrosis due to cell dehydration, while increased ionic tolerance results in decreased leaf senescence resulting in overall greater aboveground growth.

Because salinity stress is the product of both osmotic and ionic factors, their respective causes and consequences are often difficult to distinguish; however, these two stresses are often temporal in their action. When salts initially begin to accumulate in the soil, the osmotic potential of the soil water decreases, resulting in decreased water extraction by plant roots. This osmotic stress causes a sudden, short term loss of water, cell volume, and turgor from leaf cells. Plants that are tolerant to stress during the osmotic phase are better able to modify long distance signaling, limit stomatal closure, osmotically adjust, and continue cell expansion/lateral bud development, resulting in the continuation of both above and belowground growth (Munns & Tester, 2008). One important mechanism that plants utilize to overcome the osmotic phase of salinity stress is via the production and accumulation of compatible solutes such as amino acids (i.e. proline), amines, betaines, organic acids, sugars, and polyols (Parihar *et al.*, 2015) which aid in water acquisition and maintenance of cell turgor. For the QTL detected in this study, we identified various genes whose products are related to osmotic adjustment, including genes involved in proline production, aquaporins, CDPKs (calcium-dependent protein kinases), sensing and signaling, cell division, Na⁺/Ca²⁺ exchanger, leaf senescence, early response to dehydration, heat shock proteins, SAPK3 (stress-activated protein kinases), vacuolar proton exchangers, potassium antiporters, and stress response proteins.

Perhaps most striking was the presence of a large number of genes encoding aquaporins (**Supplementary Table S6**). Aquaporin genes are well known for their role in water transport (Sakurai *et al.*, 2005; Alexandersson *et al.*, 2005; Maurel *et al.*, 2008; Liu *et al.*, 2015; Reddy *et al.*, 2015; Kadam *et al.*, 2017; Hasan *et al.*, 2017). In a previous study that characterized the aquaporin (AQP) gene family in *Sorghum bicolor*, SbAQP transcript abundance was affected by both salt and drought stress (Reddy *et al.*, 2015). In response to salt stress specifically, most of the SbAQP genes were significantly upregulated. Reddy *et al.* (2015) hypothesized that increased SbAQP expression may be important in not only water uptake, but also in sodium dilution in root cells. In our study we identified 14 unique aquaporin genes, specifically, Sobic.001G208500, Sobic.001G389900, Sobic.002G124700, Sobic.002G125000, Sobic.004G295100, Sobic.007G039500, and Sobic.005G091600, which encode TIP3;1, SIP2;1, PIP2;2, PIP2;6, TIP2;1, NIP3;2, and SIP1;1, respectively (**Supplementary Table S6**). Given that osmotic stress is a result of water limitation due to the decreased water potential within the plant cells, we predict that the various genes encoding aquaporins detected in our QTL are important for osmotic tolerance.

Following osmotic stress, as soil salinity levels rise, plants begin to accumulate Na⁺ and Cl⁻ ions, which if not properly handled will become toxic within the leaves. The most common phenotype associated with ionic stress is increased leaf necrosis. Therefore, we used dead aboveground biomass and rank score as a proxy for ionic toxicity. RILs with greater amounts of dead aboveground biomass or larger rank scores were considered sensitive to ionic stress, whereas tolerant genotypes exhibit less leaf necrosis. For qDAGB45_2.STI, *S. propinquum* alleles positively correlated with greater dead aboveground biomass, possibly as a result of increased Na⁺ accumulation in the aerial plant tissue. Larger amounts of dead aboveground biomass is often a result of the inability to exclude ions resulting in toxic accumulation, ionic disequilibrium, or tissue sensitivity (Munns & Tester, 2008). Similarly, qRS45_7.STI also had a negative additive effect, indicating that *S. propinquum* alleles positively influenced the rank score, suggestive of greater susceptibility to ionic toxicity (**Table 1**). Candidate genes associated

with ionic stress in these QTL include: calcium-dependent protein kinases, LEA-like proteins, aquaporins, heat shock proteins, Na⁺/H⁺ antiporters, WRKY transcription factors, K⁺ uptake, and cation transporters (**Supplementary Table S8**). Most interestingly, there were numerous genes within the two QTL that we considered most informative of ionic stress, specifically genes associated with Ca²⁺ sensing/signaling and Na⁺ transport, which are important in limiting cytoplasmic ion toxicity. A notable candidate gene associated with ionic sensing and signaling identified in qDAGB45_2.STI is CDPK (calcium-dependent protein kinase) (Sobic.002G114800). CDPKs are a class of calcium sensors that, in response to most environmental stresses, have been previously shown to mediate abiotic stress via calcium waves that signal various physiological responses (Urao *et al.*, 1994a,b; Knight *et al.*, 1997; Cheng *et al.*, 2002; Delormel & Boudsocq, 2019). Further, two genes that encode Na⁺/H⁺ transporters were identified as candidate genes. Na⁺/H⁺ transporters are especially important in sodium exclusion from areas of the plant such as the cytoplasm and aerial organs. Lastly, genes associated with potassium uptake and distribution were identified. Potassium (K⁺) is an essential nutrient for plant growth and development (Maathuis, 2009; Ahmad & Maathuis, 2014; Morton *et al.*, 2019). Because of the similarity in size and structure of Na⁺ and K⁺, both ions often share transport systems. K⁺, however, is essential for protein synthesis (Jones *et al.*, 1979; Blaha *et al.*, 2000), enzymatic reactions (Bhandal & Malik, 1988), and signaling (Shabala, 2017), whereas Na⁺ is not. Therefore, maintaining high K⁺/Na⁺ ratios is important for salinity tolerance (Chen *et al.*, 2005, 2007; Cuin *et al.*, 2008; Shabala, 2013; Wu *et al.*, 2018; Morton *et al.*, 2019).

Conclusions

In a previous study comparing salt tolerance rankings among a diverse group of *Sorghum* genotypes and species, we concluded that the most parsimonious explanation for the phylogenetic distribution of variation in plant response to salinity is that tolerance was acquired early during domestication, specifically in the durra landrace, and then lost in improved lines in a lineage-specific manner (Henderson *et al.*, 2019). The results presented here, specifically with respect to the enrichment of aquaporins in salt responsive QTL, indicate that the greater salt tolerance in *Sorghum bicolor* is likely a result of efficient water acquisition under stressful conditions. Therefore, since drought stress and salt stress share a common osmotic component, and since *S. bicolor* was domesticated and improved in arid environments, we hypothesize that salt tolerance in *Sorghum* is a byproduct of the drought tolerance acquired during domestication. As we also observed genes associated with sensing, signaling, and transport of Na⁺, it is possible that sorghum is able to maintain ionic homeostasis, while also using Na⁺ as a cheap compatible solute. One such example is SAPK3 (qHT45_1.S), a serine/threonine-protein kinase that has been previously shown to be associated with increased salinity tolerance through its role in signal transduction in response to hyperosmotic stress (Huai *et al.*, 2008; Basu & Roychoudhury, 2014; Guan *et al.*, 2019). By using Na⁺ as a cheap compatible solute, plants would be able to avoid ionic toxicity and alleviate osmotic stress, while also conserving energy that can be used for biomass production (Munns *et al.*, 2019, 2020). When considering the QTL and candidate genes identified here, we propose that the aquaporin gene family provides a promising avenue of research aimed at the improvement of sorghum for growth on saline soils. Additionally, while there were several other genes identified in this study that have previously been shown to contribute to increased abiotic stress and salinity tolerance, there were a significant number of unannotated genes. These genes remain attractive for future studies, as they may be pivotal in the maintenance of plant health and biomass production in marginal environments.

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Tables

Table 1. Rank scoring parameters of plant vigor. Plant vigor was assessed on a scale of 0 to 5 with 0 indicating no signs of stress and 5 indicating plant death.

Score	Observation
0	No leaf signs
1	Some leaf and leaf tip curling Severe leaf and leaf tip curling, few leaves
2	elongated
3	Most leaves dead but still producing new leaves
4	Plant still alive but no new growth
5	Plant dead

Table 2. QTLs identified in the RIL population using transformed least square means in control conditions, salt conditions, and with stress tolerance index values. The QTLs reported were identified when mapping using Multiple QTL Mapping (MQM) in control conditions (0 mM NaCl), salt conditions (75 mM NaCl), and with stress tolerance index (STI) values. QTLs are named using the following system: q[Trait][DAT]_[Chr].[Treatment]

Trait	Trt	QTL Name	DAT	Chr	Position (cM)	Bin (Max LOD)	Lod score	p-value	PVE	Additive	Start Mb	Peak Mb	End Mb
DAGB	Control	qDAGB45_2.C	45	2	52.64	56.26	3.33	1.02E-04	9.14	-0.06	13.85	56.26	58.70
DAGB	STI	qDAGB45_2.STI	45	2	52.64	56.26	4.42	7.53E-06	12.03	-0.17	13.85	56.26	56.74
HT	Control	qHT45_7.C	45	7	57.64	59.01	4.04	1.16E-04	9.64	-4.65	58.66	59.01	60.24
HT	Control	qHT45_9.C	45	9	64.00	55.07	4.75	2.35E-05	11.45	-5.19	54.37	55.07	56.91
HT	Salt	qHT45_1.S	45	1	125.00	77.03	4.93	1.58E-05	12.07	-4.36	18.46	77.03	80.57
HT	Salt	qHT45_7.S	45	7	63.29	60.17	4.13	9.52E-05	9.99	-3.86	59.01	60.17	61.46
HT	STI	qHT45_7.STI	45	7	58.80	59.35	3.80	1.99E-04	9.34	-0.04	58.68	59.35	60.24
HT	STI	qHT45_9.STI	45	9	64.00	55.07	3.61	3.00E-04	8.87	-0.04	54.37	55.07	56.68
LAGB	STI	qLAGB45_4.STI	45	4	73.00	66.68	6.26	1.30E-01	15.35	0.00	62.29	66.68	66.68
LAGB	STI	qLAGB45_5.STI	45	5	56.00	64.81	4.37	6.16E-02	10.40	0.00	6.15	64.81	66.57
RB	STI	qRB45_4.STI	45	4	67.00	62.29	3.29	1.12E-04	9.09	0.08	60.42	62.29	66.68
RS	STI	qRS45_7.STI	45	7	22.53	3.02	3.25	1.23E-04	9.00	-0.16	2.55	3.02	4.01
TAGB	STI	qTAGB45_4.STI	45	4	73.00	62.29	4.55	5.60E-06	12.34	0.12	62.17	62.29	66.68
TB	Salt	qTB45_4.S	45	4	73.00	62.29	4.90	1.67E-05	12.12	0.69	62.17	62.29	66.68
TB	Salt	qTB45_5.S	45	5	63.04	66.80	4.34	5.90E-05	10.64	0.39	64.50	66.80	67.30
TB	STI	qTB45_4.STI	45	4	71.00	62.29	4.82	2.97E-06	13.02	0.10	62.17	62.29	66.68

DAGB-dead aboveground biomass, LAGB-live aboveground biomass, RB-root biomass, RS-rank score, HT-height, TB-total biomass, TAGB-total aboveground biomass; Trt-treatment, STI-stress tolerance index, C-control, S-salt; P-*Sorghum propinquum*, B-*Sorghum bicolor*; DAT-days after treatment; Chr-chromosome, PVE-percent variance explained

Figures

Figure 1. Phenotypic averages in control (0 mM NaCl) and treatment (75 mM NaCl) conditions. Phenotype averages for the control population and the salt population for height (cm) (HT), dead aboveground biomass (DAGB) (g), live aboveground biomass (LAGB) (g), mortality, root biomass (RB) (g), rank score (RS) (0-5), shoot to root ratio (SRR), total aboveground biomass (TAGB) (g), total biomass (TB) (g), and tiller number (TN). In response to salt exposure, dead aboveground biomass, rank score, and shoot to root ratio increased, whereas height, live aboveground biomass, root biomass, total aboveground biomass, and total biomass decreased.

Figure 2. Non-metric multidimensional scaling (NMDS) analysis paired with an analysis of similarity (ANOSIM) reveals treatment clustering. A NMDS paired with an ANOSIM reveals individuals were more similar within a treatment than between treatments (ANOSIM R=0.16, $p<0.001$).

Figure 3. Sorghum genetic map with QTL locations for 14 traits from 177_{F3:F5} RILs. (A) High-density genetic map with Mendelian segregating markers. QTL identified only when including distorted markers at $p < 10^{-5}$ after Bonferroni correction. Empty regions are regions that were removed because bins were called heterozygous or markers were distorted or duplicates. (B) High-density genetic map that includes markers that deviated significantly from the Mendelian ratio (1:1). Empty spaces are regions that were removed because bins were either heterozygous or duplicate markers. The genetic map position is shown on the y-axis. Horizontal lines represent bins used as markers. Colored vertical lines show the physical position of each QTL for each trait in control conditions, salt conditions, or when STI values were mapped. *Note: the genetic distance on chromosome 6 is inflated due to segregation distortion.

Supplementary Tables

Supplementary Table S1. Transformations of phenotypes. Phenotypes were transformed to meet normality. Transformed data was used in an analysis of variance and QTL mapping.

Trait	Transformation	DAT	Treatment
Height (HT)		0	Control
Height (HT)		15	Control
Rank Score (RS)		15	Control
Stem Diameter (SD)		29	Control
Height (HT)		45	Control
Rank Score (RS)		45	Control
Tiller Number (TN)		45	Control
Root Bimass (RB)	sqrt	45	Control
Dead aboveground biomass (DAGB)	sqrt	45	Control
Live aboveground biomass (LAGB)		45	Control
Total aboveground biomass (TAGB)		45	Control
Total biomass (TB)		45	Control
Percent alive aboveground biomass (PAAGB)		45	Control
Shoot to root ratio (SRR)	log	45	Control
Height (HT)		0	Salt
Height (HT)		15	Salt
Rank Score (RS)		15	Salt
Stem Diameter (SD)		29	Salt
Height (HT)		45	Salt
Rank Score (RS)		45	Salt
Tiller Number (TN)		45	Salt
Root Bimass (RB)		45	Salt
Dead aboveground biomass (DAGB)		45	Salt
Live aboveground biomass (LAGB)		45	Salt
Total aboveground biomass (TAGB)		45	Salt
Total biomass (TB)		45	Salt
Percent alive aboveground biomass (PAAGB)		45	Salt
Shoot to root ratio (SRR)	log	45	Salt
Height (HT)	sqrt	0	STI
Height (HT)	sqrt	45	STI
Rank Score (RS)		45	STI
Root Bimass (RB)	sqrt	45	STI
Dead aboveground biomass (DAGB)	sqrt	45	STI

Live aboveground biomass (LAGB)	sqrt	45	STI
Total aboveground biomass (TAGB)	sqrt	45	STI
Total biomass (TB)	sqrt	45	STI
Percent alive aboveground biomass (PAAGB)		45	STI
Shoot to root ratio (SRR)	log	45	STI

Supplementary Table S2. Summary of markers retained following bin calling, removal of distorted regions, and removal of duplicate markers.

Chromosome	Markers Present on each Chromosome										Total
	1	2	3	4	5	6	7	8	9	10	
Markers retained after bin calling	610	485	457	430	331	365	364	285	374	354	4055
Markers retained after removing distorted markers	332	396	454	301	254	169	364	285	374	277	3206
Markers retained after removing duplicate markers	163	214	230	145	151	94	174	170	209	142	1692

Supplementary Table S3. Genetic map summary. (A) The genetic map used in this study to map salinity tolerance consisted of 10 total chromosomes spanning 888.7 cM with a total of 1692 bin markers. On average markers were 0.5 cM apart with a maximum spacing of 62.29 cM of any marker being on chromosome 1. (B) Bin markers that deviated from the expected mendelian ratio (1:1) were removed from the analysis. A total of 866 bin markers were distorted across the 10 *Sorghum* chromosomes with 172 displaying preferential bias towards *S. propinquum* and 694 displaying preferential bias towards *S. bicolor*.

A.

Genetic Map Information				
Chromosome	Marker Distribution			
	Marker Total	Length (cM)	Acg. Spacing (cM)	Max Spacing (cM)
1	163	132.9	0.8	62.9
2	214	110.4	0.5	26.9
3	230	119.9	0.5	8.7
4	145	87.8	0.6	19.3
5	151	83.1	0.6	9.7
6	94	38.4	0.4	1.2
7	174	84.6	0.5	2.2
8	170	78.3	0.5	1.6
9	209	87.7	0.4	2.1
10	142	65.7	0.5	4.1
Total	1692	888.7	0.5	62.9

B.

Distorted Marker Information							
Chromosome	Number of distorted markers	Marker Type			Region (Mb)		
		<i>S. propinquum</i>	<i>S. bicolor</i>	Average marker ratio (SP:SB)			
1	280		280	20:116	18.60	75.83	
2	91	91		112:40	61.09	70.23	
3	3		3	39:110	43.51	44.58	
4	133		133	35:105	55.96	66.64	
5	81	81		107:34	6.37	59.45	
6	198		198	16:115	0.09	50.15	
7							
8							
9	1		1	48:102	1.01	1.01	
10	79		79	29:113	54.47	61.09	
Total	866	172	694				

Supplementary Table S4. Summary statistics of phenotypic values for control and salt treated populations.

Days after treatment (DAT)	Trait	Treatment	Min	Max	Mean	S.D.	S.E.	Kurtosis	Skew
0	HT	Control	18.75	71.38	44.50	9.66	0.73	0.17	-0.27
		Salt	21.67	63.12	42.57	8.50	0.64	-0.49	-0.10
15	HT	Control	34.17	99.76	63.75	13.19	0.99	-0.10	-0.13
		Salt	29.95	83.38	55.30	10.01	0.75	-0.25	0.00
	RS	Control	-0.49	2.10	0.45	0.48	0.04	0.05	0.71
		Salt	0.88	3.72	2.18	0.53	0.04	-0.44	0.03
45	HT	Control	52.92	122.37	87.81	14.36	1.08	-0.43	0.04
		Salt	46.32	112.47	75.46	11.50	0.86	0.12	0.12
	RS	Control	0.72	3.20	2.01	0.53	0.04	-0.06	-0.19
		Salt	1.68	4.55	3.19	0.44	0.03	0.83	-0.46
	TN	Control	-0.16	2.98	0.18	0.54	0.04	9.39	3.07
		Salt	-0.35	5.14	0.36	0.89	0.07	10.11	2.99
	M	Control	1.00	1.00	1.00	0.00	0.00		
		Salt	0.67	1.04	1.00	0.03	0.00	144.19	-11.53
	RB	Control	0.48	6.38	2.50	1.05	0.08	0.16	0.05
		Salt	0.09	2.48	1.13	0.45	0.03	0.21	-0.32
	DAGB	Control	0.06	1.61	0.55	0.28	0.02	0.12	0.08
		Salt	-0.30	2.80	0.80	0.42	0.03	0.71	0.58
	LAGB	Control	1.20	6.95	4.45	1.31	0.10	-0.58	-0.19
		Salt	0.32	6.22	3.18	1.01	0.08	0.00	0.01
	TAGB	Control	1.34	8.56	5.00	1.49	0.11	-0.48	-0.16
		Salt	0.57	7.11	3.98	1.19	0.09	-0.31	-0.23
	TB	Control	1.98	12.18	7.49	2.21	0.17	-0.51	-0.22
		Salt	0.66	9.42	5.11	1.51	0.11	-0.12	-0.24
	SRR	Control	0.10	9.38	2.40	1.09	0.08	11.86	-1.64
		Salt	1.80	10.92	4.09	1.42	0.11	0.80	0.23
	PAAGB	Control	0.75	0.96	0.89	0.04	0.00	0.15	-0.68
		Salt	0.41	1.10	0.78	0.10	0.01	2.02	-0.64

HT-height, RS-rank score, TN-tiller number, M-mortality, RB-root biomass (g), DAGB-dead aboveground biomass (g), LAGB-live aboveground biomass (g), TAGB-total aboveground biomass (g), TB-total biomass (g), SRR-shoot to root ratio, PAAGB-percent alive aboveground biomass

Supplementary Table S5. Analysis of variance summary.

Phenotype	Days After Treatment	Control	S.D.	Salt	S.D.	RDPB	P-value	Significant
						(Control-Salt)/Control		
HT	15	63.75	13.19	55.30	10.01	0.13	4.69E-11	***
RS	15	0.45	0.48	2.18	0.53	-3.81	1.19E-105	***
TN	15	0.57	0.94	0.79	1.20	-0.39	1.62E-02	*
DAGB	45	0.55	0.28	0.80	0.42	-0.45	4.29E-13	***
HT	45	87.81	14.36	75.46	11.50	0.14	2.30E-17	***
LAGB	45	4.45	1.31	3.18	1.01	0.28	2.03E-21	***
Mortality	45	1.00	0.00	1.00	0.03	0.00	2.12E-01	
PAAGB	45	0.89	0.04	0.78	0.10	0.13	1.82E-36	***
RB	45	2.50	1.05	1.13	0.45	0.55	5.74E-47	***
RS	45	2.01	0.53	3.19	0.44	-0.58	1.26E-71	***
SRR	45	2.40	1.09	4.09	1.42	-0.71	1.50E-34	***
TAGB	45	5.00	1.49	3.98	1.19	0.20	8.63E-12	***
TB	45	7.49	2.21	5.11	1.51	0.32	2.06E-27	***
TN	45	0.18	0.54	0.36	0.89	-1.05	1.73E-02	*

Significant Codes: (*) 0.05 (**) 0.01 (***) 0.001

HT-height, RS-rank score, TN-tiller number, M-mortality, RB-root biomass (g), DAGB-dead aboveground biomass (g), LAGB-live aboveground biomass (g), TAGB-total aboveground biomass (g), TB-total biomass (g), SRR-shoot to root ratio, PAAGB-percent alive aboveground biomass; RDPB-relative decrease in plant biomass

Supplementary Table S6. Genes within a 1.0 logarithm of the odds (LOD) confidence interval for each QTL when markers that deviated significantly from the expected Mendelian ratio (1:1) were removed. *(omitted from the document)*

Supplementary Table S7. Genes within a 1.0 logarithm of the odds (LOD) confidence interval for each QTL when markers that deviated significantly from the expected Mendelian ratio were included. *(omitted from the document)*

Supplementary Table S8. Candidate genes identified in QTL windows when markers that deviated significantly from the expected Mendelian ratio (1:1) were removed.

QTL	Sorghum Gene ID	Sorghum Annotation	Arabidopsis Annotation	Rice Annotation
qDAGB45_2.STI	Sobic.002G113900	similar to Putative CLB1 protein (Calcium-dependent lipid binding) protein	Calcium-dependent lipid-binding (CaLB domain) family protein	lipid binding protein, putative, expressed
qDAGB45_2.STI	Sobic.002G114800	similar to Calcium-dependent protein kinase	calcium-dependent protein kinase 16	CAMK_CAMK_like.32 - CAMK includes calcium/calmodulin depeident protein kinases, expressed
qDAGB45_2.STI	Sobic.002G118300	similar to Hydrophobic LEA-like protein	AWPM-19-like family protein	AWPM-19-like membrane family protein, putative, expressed
qDAGB45_2.STI	Sobic.002G124700	similar to Aquaporin PIP2-2	plasma membrane intrinsic protein 2	aquaporin protein, putative, expressed
qDAGB45_2.STI	Sobic.002G125000	similar to Aquaporin PIP2-6	plasma membrane intrinsic protein 2	aquaporin protein, putative, expressed
qDAGB45_2.STI	Sobic.002G125200	similar to Aquaporin PIP2-6	plasma membrane intrinsic protein 2	aquaporin protein, putative, expressed
qDAGB45_2.STI	Sobic.002G125300	similar to Aquaporin PIP2-6	plasma membrane intrinsic protein 2	aquaporin protein, putative, expressed
qDAGB45_2.STI	Sobic.002G125700	similar to Aquaporin PIP2-6	plasma membrane intrinsic protein 2	aquaporin protein, putative, expressed
qDAGB45_2.STI	Sobic.002G136300	similar to Calmodulin binding heat shock protein	alpha/beta-Hydrolases superfamily protein	calmodulin-binding heat-shock protein, putative, expressed
qDAGB45_2.STI	Sobic.002G141900	similar to Similarity to Na ⁺ /H ⁺ antiporter	sodium:hydrogen antiporter 1	Citrate transporter protein, putative, expressed
qDAGB45_2.STI	Sobic.002G156000	similar to Sodium/hydrogen exchanger	Na ⁺ /H ⁺ antiporter 6	transporter, monovalent cation:proton antiporter-2 family, putative, expressed
qDAGB45_2.STI	Sobic.002G174200	weakly similar to Transcription factor WRKY74	WRKY DNA-binding protein 46	WRKY74, expressed
qDAGB45_2.STI	Sobic.002G174300	weakly similar to Transcription factor WRKY74	WRKY family transcription factor	WRKY74, expressed
qDAGB45_2.STI	Sobic.002G130800	similar to Putative uncharacterized protein	K ⁺ uptake permease 11	potassium transporter, putative, expressed
qDAGB45_2.STI	Sobic.002G157800		solute:sodium symporters;urea transmembrane transporters	urea active transporter, putative, expressed
qDAGB45_2.STI	Sobic.002G159100	similar to Putative uncharacterized protein	Chlorophyll A-B binding family protein	chlorophyll A-B binding protein, putative, expressed
qDAGB45_2.STI	Sobic.002G162400	similar to Os09g0299400 protein	Ca ²⁺ activated outward rectifying K ⁺ channel 6	potassium channel protein, putative, expressed
qDAGB45_2.STI	Sobic.002G168550		cold shock domain protein 1	RNA recognition motif containing protein, expressed
qDAGB45_2.STI	Sobic.002G180300	similar to Voltage-dependent anion channel protein 1b	voltage dependent anion channel 1	isochorismate synthase 1, chloroplast precursor, putative, expressed
qDAGB45_2.STI	Sobic.002G178000		Leucine-rich repeat transmembrane protein kinase protein	senescence-induced receptor-like serine/threonine-protein kinase precursor, putative, expressed
qDAGB45_2.STI	Sobic.002G178101		Leucine-rich repeat transmembrane protein kinase protein	senescence-induced receptor-like serine/threonine-protein kinase precursor, putative, expressed
qHT45_1.S	Sobic.001G379600	similar to CIPK-like protein 1, putative, expressed	SOS3-interacting protein 1	CAMK_KIN1/SNF1/Nim1_like.16 - CAMK includes calcium/calmodulin depeident protein kinases, expressed
qHT45_1.S	Sobic.001G523200	similar to CIPK-like protein 1, putative, expressed	CBL-interacting protein kinase 9	CAMK_KIN1/SNF1/Nim1_like.15 - CAMK includes calcium/calmodulin depeident protein kinases, expressed
qHT45_1.S	Sobic.001G212900	similar to DNA damage signaling and repair protein	nijmegen breakage syndrome 1	FHA domain containing protein, putative, expressed

qHT45_1.S	Sobic.001G236300	similar to Cell division control protein 48 homolog D	ATPase, AAA-type, CDC48 protein	cell division control protein 48 homolog E, putative, expressed
qHT45_1.S	Sobic.001G240300	similar to Sodium/calcium exchanger protein, expressed	calcium exchanger 7	sodium/calcium exchanger protein, putative, expressed
qHT45_1.S	Sobic.001G242100	similar to Leaf senescence protein-like	TRICHOME BIREFRINGENCE-LIKE 19	expressed protein
qHT45_1.S	Sobic.001G251700	similar to Serine/threonine-protein phosphatase PP2A-4 catalytic subunit	protein phosphatase 2A-3	OsPP2Ac-4 - Phosphatase 2A isoform 4 belonging to family 2, expressed
qHT45_1.S	Sobic.001G265900	similar to Proline-rich protein precursor	proline-rich protein 4	POEI13 - Pollen Ole e I allergen and extensin family protein precursor, expressed
qHT45_1.S	Sobic.001G266100	similar to Proline-rich protein precursor	proline-rich protein 2	POEI11 - Pollen Ole e I allergen and extensin family protein precursor, expressed
qHT45_1.S	Sobic.001G266200	similar to Proline-rich protein precursor	proline-rich protein 4	POEI14 - Pollen Ole e I allergen and extensin family protein precursor, expressed
qHT45_1.S	Sobic.001G266300	similar to Proline-rich protein, putative, expressed	proline-rich protein 2	POEI3 - Pollen Ole e I allergen and extensin family protein precursor, expressed
qHT45_1.S	Sobic.001G267100	similar to Auxin transporter-like protein 3	like AUXIN RESISTANT 2	transmembrane amino acid transporter protein, putative, expressed
qHT45_1.S	Sobic.001G282700	similar to Early-responsive to dehydration protein, putative, expressed	early-responsive to dehydration stress protein (ERD4)	early-responsive to dehydration protein-related, putative, expressed
qHT45_1.S	Sobic.001G294400	similar to Serine/threonine-protein kinase SAPK3	Protein kinase superfamily protein	CAMK_CAMK_like.41 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
qHT45_1.S	Sobic.001G313100	similar to Calcium-dependent protein kinase isoform 1	Calcium-dependent protein kinase family protein	CAMK_CAMK_like.8 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
qHT45_1.S	Sobic.001G333500	similar to Heat shock protein	casein lytic proteinase B3	chaperone protein clpB 1, putative, expressed
qHT45_1.S	Sobic.001G346500	similar to Vacuolar cation/proton exchanger 2 (Ca(2+)/H(+))	cation exchanger 2	sodium/calcium exchanger protein, putative, expressed
qHT45_1.S	Sobic.001G346500	similar to Vacuolar cation/proton exchanger 2 (Ca(2+)/H(+))	cation exchanger 5	sodium/calcium exchanger protein, putative, expressed
qHT45_1.S	Sobic.001G389000	similar to WRKY DNA binding domain containing protein, expressed	WRKY family transcription factor	WRKY55, expressed
qHT45_1.S	Sobic.001G389900	similar to Aquaporin SIP2-1	small and basic intrinsic protein 2;1	aquaporin SIP2-1, putative, expressed
qHT45_1.S	Sobic.001G512300	similar to Putative voltage-dependent ion channel	voltage dependent anion channel 4	outer mitochondrial membrane porin, putative, expressed
qHT45_1.S	Sobic.001G522100	similar to Putative potassium/proton antiporter-like protein	K ⁺ efflux antiporter 5	transporter, monovalent cation:proton antiporter-2 family, putative, expressed
qHT45_1.S	Sobic.001G535900	similar to Aquaporin TIP3-1	tonoplast intrinsic protein 1;3	aquaporin protein, putative, expressed
qHT45_1.S	Sobic.001G209800	similar to Putative uncharacterized protein	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	harpin-induced protein 1 domain containing protein, expressed
qHT45_1.S	Sobic.001G307800	similar to Harpin-induced protein 1	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	harpin-induced protein 1 domain containing protein, expressed

qHT45_1.S	Sobic.001G330100	containing protein, expressed similar to C2H2-type zinc finger protein ZFP36	salt tolerance zinc finger	ZOS3-12 - C2H2 zinc finger protein, expressed
qHT45_1.S	Sobic.001G330200	similar to C2H2-type zinc finger protein ZFP36	salt tolerance zinc finger	ZOS3-12 - C2H2 zinc finger protein, expressed
qHT45_1.S	Sobic.001G444600	similar to Transposon protein, putative, unclassified, expressed	proline-rich family protein	outer mitochondrial membrane porin, putative, expressed
qHT45_1.S	Sobic.001G465800	similar to PgPOR29	voltage dependent anion channel 2	stress responsive A/B Barrel domain containing protein, expressed
qHT45_1.S	Sobic.001G535700		Stress responsive alpha-beta barrel domain protein	
qHT45_1.S	Sobic.001G278100	similar to Putative uncharacterized protein OSJNBa0071K19.13	roline-rich extensin-like receptor kinase 4	serine/threonine-protein kinase, putative, expressed
qHT45_1.S	Sobic.001G305000		hydroxyproline-rich glycoprotein family protein	stress responsive protein, putative, expressed
qHT45_1.S	Sobic.001G505100	similar to Aquaporin TIP1-1	gamma tonoplast intrinsic protein	aquaporin protein, putative, expressed
qHT45_1.S	Sobic.001G541166			sodium/potassium/calcium exchanger 1, putative, expressed
qLAGB45_4.STI	Sobic.004G288700	similar to Aquaporin PIP1-3/PIP1-4	plasma membrane intrinsic protein 1;4	aquaporin protein, putative, expressed
qLAGB45_4.STI	Sobic.004G298400	similar to WRKY-like DNA-binding protein similar to Putative stress-induced protein sti1	WRKY family transcription factor	WRKY36, expressed
qLAGB45_4.STI	Sobic.004G301700		stress-inducible protein, putative	heat shock protein STI, putative, expressed
qLAGB45_4.STI	Sobic.004G315400	similar to Cation diffusion facilitator 8	Cation efflux family protein	cation efflux family protein, putative, expressed
qLAGB45_4.STI	Sobic.004G323900		stress-inducible protein, putative	protein kinase, putative, expressed
qLAGB45_4.STI	Sobic.004G333700	similar to Putative calcineurin B subunit	Calcium-binding EF-hand family protein	EF hand family protein, putative, expressed
qLAGB45_4.STI	Sobic.004G295100	similar to Probable aquaporin TIP2-1	tonoplast intrinsic protein 2;3	aquaporin protein, putative, expressed
qLAGB45_4.STI	Sobic.004G295900	similar to Anaphase-promoting complex subunit 8-like protein	anaphase-promoting complex subunit 8	cell division cycle protein 23, putative, expressed
qSD29_5.S	Sobic.005G145950		zinc knuckle (CCHC-type) family protein	photosynthetic reaction center protein, putative, expressed
qTB45_4.STI	Sobic.004G295100	similar to Probable aquaporin TIP2-1	tonoplast intrinsic protein 2;3	aquaporin protein, putative, expressed
qTB45_4.STI	Sobic.004G312200	similar to WRKY transcription factor 32	WRKY DNA-binding protein 9	WRKY32, expressed
qTB45_4.STI	Sobic.004G279100		Calcium-dependent protein kinase family protein	CAMK_CAMK_like.15 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
qTB45_4.STI	Sobic.004G279700	similar to Putative uncharacterized protein	oxidative stress 3	expressed protein
qTB45_4.STI	Sobic.004G288400	similar to Putative uncharacterized protein	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	harpin-induced protein 1 domain containing protein, expressed
qTB45_4.STI	Sobic.004G301000	similar to Putative uncharacterized protein	salt tolerance homolog2	B-box zinc finger family protein, putative, expressed
qTB45_4.STI	Sobic.004G290900		phosphate starvation-induced gene 3	transporter, major facilitator family, putative, expressed
qTB45_4.STI	Sobic.004G295900	similar to Anaphase-promoting complex subunit 8-like protein	anaphase-promoting complex subunit 8	cell division cycle protein 23, putative, expressed
qTB45_5.S	Sobic.005G171200	similar to Sugar transporter family protein, expressed	Major facilitator superfamily protein	transporter family protein, putative, expressed
qTB45_5.S	Sobic.005G169600	similar to CRM family member 3	CRM family member 3A	chloroplastic group IIA intron splicing facilitator CRS1,

				chloroplast precursor, putative, expressed
qLAGB45_5.STI	Sobic.005G087000	similar to Chlorophyll a/b-binding apoprotein CP26 precursor	light harvesting complex of photosystem II 5	chlorophyll A-B binding protein, putative, expressed
qLAGB45_5.STI	Sobic.005G098400	weakly similar to AUX/IAA family protein, expressed	AUX/IAA transcriptional regulator family protein	OsIAA27 - Auxin-responsive Aux/IAA gene family member, expressed
qLAGB45_5.STI	Sobic.005G117400	similar to WRKY transcription factor 72	WRKY DNA-binding protein 75	WRKY72, expressed
qLAGB45_5.STI	Sobic.005G132000	similar to Auxin response factor 23	auxin response factor 2	auxin response factor, putative, expressed
qLAGB45_5.STI	Sobic.005G150900	similar to Putative growth-regulating factor 2	growth-regulating factor 2	growth regulating factor protein, putative, expressed
qLAGB45_5.STI	Sobic.005G114000		Outward rectifying potassium channel protein	potassium channel protein, putative, expressed
qLAGB45_5.STI	Sobic.005G116400		photosynthetic electron transfer A	apocytochrome f precursor, putative, expressed
qLAGB45_5.STI	Sobic.005G126600	similar to F-box domain containing protein, expressed	auxin signaling F-box 3	OsFBL55 - F-box domain and LRR containing protein, expressed
qLAGB45_5.STI	Sobic.005G060100	similar to Os12g0174100 protein	O-fucosyltransferase family protein	growth regulator related protein, putative, expressed
qLAGB45_5.STI	Sobic.005G072900			stress responsive protein, putative, expressed
qLAGB45_5.STI	Sobic.005G073000			stress responsive protein, putative, expressed
qLAGB45_5.STI	Sobic.005G097400	similar to HSR203J, putative, expressed	carboxyesterase 17	cell death associated protein, putative, expressed
qLAGB45_5.STI	Sobic.005G103050		zinc knuckle (CCHC-type) family protein	photosynthetic reaction center protein, putative, expressed
qLAGB45_5.STI	Sobic.005G106432		zinc knuckle (CCHC-type) family protein	photosynthetic reaction center protein, putative, expressed
qLAGB45_5.STI	Sobic.005G108601		phragmoplastin interacting protein 1	photosynthetic reaction center protein, putative, expressed
qLAGB45_5.STI	Sobic.005G110415			photosynthetic reaction center protein, putative, expressed
qLAGB45_5.STI	Sobic.005G114500		O-fucosyltransferase family protein	growth regulator related protein, putative, expressed
qLAGB45_5.STI	Sobic.005G145950		zinc knuckle (CCHC-type) family protein	photosynthetic reaction center protein, putative, expressed
qRS45_7.STI	Sobic.007G039500	similar to Aquaporin NIP3-2	NOD26-like intrinsic protein 5;1	aquaporin protein, putative, expressed
qRS45_7.STI	Sobic.007G039600	similar to Aquaporin NIP3-2	NOD26-like intrinsic protein 5;1	aquaporin protein, putative, expressed
qRS45_7.STI	Sobic.007G037900	similar to Organic cation transporter-like protein	Oxidoreductase family protein	oxidoreductase family, NAD-binding Rossmann fold containing protein, expressed
qRS45_7.STI	Sobic.007G030900	similar to Putative uncharacterized protein	Ca(2)-dependent phospholipid-binding protein (Copine) family	copine-6, putative, expressed
qRS45_7.STI	Sobic.007G038500	similar to Putative nodulin	NOD26-like intrinsic protein 5;1	aquaporin protein, putative, expressed
qRB45_4.STI	Sobic.004G260500	similar to Peptide transporter-like	nitrate transporter 1.5	peptide transporter PTR2, putative, expressed
qRB45_4.STI	Sobic.004G271800	weakly similar to WRKY transcription factor 66	WRKY DNA-binding protein 14	WRKY66, expressed
qRB45_4.STI	Sobic.004G272700	similar to 4-coumarate--CoA ligase 2	4-coumarate:CoA ligase 3	AMP-binding domain containing protein, expressed
qRB45_4.STI	Sobic.004G284700	similar to Aluminum-activated malate transporter-like	Aluminium activated malate transporter family protein	aluminum-activated malate transporter, putative, expressed
qRB45_4.STI	Sobic.004G286700	similar to Putative anter-specific proline-rich protein APG	GDSL-like Lipase/Acylhydrolase superfamily protein	GDSL-like lipase/acylhydrolase, putative, expressed
qRB45_4.STI	Sobic.004G295100	similar to Probable aquaporin TIP2-1	tonoplast intrinsic protein 2;3	aquaporin protein, putative, expressed

qRB45_4.STI	Sobic.004G298400	similar to WRKY-like DNA-binding protein	WRKY family transcription factor	WRKY36, expressed
qRB45_4.STI	Sobic.004G298900	similar to Chaperone protein dnaJ-like		OR, putative, expressed
qRB45_4.STI	Sobic.004G299700	similar to Iron transport protein 2	YELLOW STRIPE like 3	
qRB45_4.STI	Sobic.004G300600	similar to Putative serine/threonine-specific protein kinase	Protein kinase superfamily protein	protein kinase domain containing protein, expressed
qRB45_4.STI	Sobic.004G301700	similar to Putative stress-induced protein sti1	stress-inducible protein, putative	heat shock protein STI, putative, expressed
qRB45_4.STI	Sobic.004G301800	similar to Vacuolar processing enzyme 1	beta vacuolar processing enzyme	vacuolar-processing enzyme precursor, putative, expressed
qRB45_4.STI	Sobic.004G302200	similar to Auxin-responsive protein-like	SAUR-like auxin-responsive protein family	OsSAUR11 - Auxin-responsive SAUR gene family member, expressed
qRB45_4.STI	Sobic.004G312200	similar to WRKY transcription factor 32	WRKY DNA-binding protein 9	WRKY32, expressed
qRB45_4.STI	Sobic.004G315400	similar to Cation diffusion facilitator 8	Cation efflux family protein Double Clp-N motif-containing P-loop nucleoside triphosphate hydrolases superfamily protein	cation efflux family protein, putative, expressed
qRB45_4.STI	Sobic.004G325000	similar to 101 kDa heat shock protein-like	plasma membrane intrinsic protein 1;4	expressed protein aquaporin protein, putative, expressed
qRB45_4.STI	Sobic.004G288700	similar to Aquaporin PIP1-3/PIP1-4	Chaperone DnaJ-domain superfamily protein	heat shock protein DnaJ, putative, expressed
qRB45_4.STI	Sobic.004G275000	similar to DnaJ protein-like		CAMK_CAMK_like.15 - CAMK includes calcium/calmodulin dependent protein kinases, expressed
qRB45_4.STI	Sobic.004G279100		Calcium-dependent protein kinase family protein	dehydrin, putative, expressed
qRB45_4.STI	Sobic.004G286600	similar to Dehydrin	cold-regulated 47 hydroxyproline-rich glycoprotein family protein	expressed protein
qRB45_4.STI	Sobic.004G296100	weakly similar to Os02g0656100 protein	cationic amino acid transporter 9	amino acid permease, putative, expressed
qRB45_4.STI	Sobic.004G296300	similar to Amino acid permease-like	cationic amino acid transporter 9	amino acid permease, putative, expressed
qRB45_4.STI	Sobic.004G296400	similar to Amino acid permease-like	Vacuolar iron transporter (VIT) family protein	integral membrane protein, putative, expressed
qRB45_4.STI	Sobic.004G301500		Vacuolar iron transporter (VIT) family protein	integral membrane protein, putative, expressed
qRB45_4.STI	Sobic.004G301600	similar to Nodulin-21-like	Vacuolar iron transporter (VIT) family protein	integral membrane protein, putative, expressed
qRB45_4.STI	Sobic.004G301650		Heavy metal transport/detoxification superfamily protein	expressed protein
qRB45_4.STI	Sobic.004G317900	similar to Os05g0337400 protein	Calcium-dependent phosphotriesterase superfamily protein	SMP-30/Gluconolactonase/LRE-like region containing protein, expressed
qRB45_4.STI	Sobic.004G318800	similar to Putative uncharacterized protein	Calcium-dependent phosphotriesterase superfamily protein	SMP-30/Gluconolactonase/LRE-like region containing protein, expressed
qRB45_4.STI	Sobic.004G329600	similar to Putative uncharacterized protein	Calcium-dependent phosphotriesterase superfamily protein	SMP-30/Gluconolactonase/LRE-like region containing protein, expressed
qRB45_4.STI	Sobic.004G330200	similar to Putative uncharacterized protein	Calcium-dependent phosphotriesterase superfamily protein	expressed
qRB45_4.STI	Sobic.004G332700	similar to Putative monosaccharide transporter 6	sugar transporter 1	transporter family protein, putative, expressed
qRB45_4.STI	Sobic.004G261400	similar to Putative cysteine proteinase	xylem cysteine peptidase 1	xylem cysteine proteinase 2 precursor, putative, expressed
qRB45_4.STI	Sobic.004G264100	similar to Putative uncharacterized protein	cation/hydrogen exchanger 15	ATCHX15, putative, expressed
qRB45_4.STI	Sobic.004G264200	similar to Os02g0710300 protein	ROOT HAIR DEFECTIVE6	helix-loop-helix DNA-binding domain containing protein, expressed
qRB45_4.STI	Sobic.004G269900		growth-regulating factor 2	growth-regulating factor, putative, expressed

qRB45_4.STI	Sobic.004G269900		growth-regulating factor 5	growth-regulating factor, putative, expressed
qRB45_4.STI	Sobic.004G279700	similar to Putative uncharacterized protein	oxidative stress 3	expressed protein
qRB45_4.STI	Sobic.004G288400	similar to Putative uncharacterized protein	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	harpin-induced protein 1 domain containing protein, expressed
qRB45_4.STI	Sobic.004G301000	similar to Putative uncharacterized protein	salt tolerance homolog2	B-box zinc finger family protein, putative, expressed
qRB45_4.STI	Sobic.004G308700	similar to Putative light-harvesting chlorophyll-a/b protein of photosystem I	photosystem I light harvesting complex gene 5	chlorophyll A-B binding protein, putative, expressed
qRB45_4.STI	Sobic.004G333700	similar to Putative calcineurin B subunit	Calcium-binding EF-hand family protein	EF hand family protein, putative, expressed
qRB45_4.STI	Sobic.004G295800	similar to DnaJ-like protein	DNAJ homologue 2	chaperone protein dnaJ, putative, expressed
qRB45_4.STI	Sobic.004G298000		Major facilitator superfamily protein	transporter, major facilitator family, putative, expressed

Supplementary Table S9. QTLs identified in the RIL population using transformed least square means in control conditions, salt conditions, and with stress tolerance index values when markers that deviated from the expected Mendelian ratio (1:1) were included. The QTLs reported were identified when mapping using Multiple QTL Mapping (MQM) in control conditions (0 mM NaCl), salt conditions (75 mM NaCl), and with stress tolerance index (STI) values. QTLs are named using the following system: q[Trait][DAT]_[Chr].[Treatment]

Trait	Trt	QTL Name	DAT	Chr	Position (cM)	Bin (Max LOD)	Lod score	p-value	PVE	Additive	Start Mb	Peak Mb	End Mb
DAGB	C	qDAGB45_2.C	45	2	72.7	65.42	4.91	1.63E-05	12.15	-0.08	64.40	65.42	67.54
DAGB	C	qDAGB45_9.C	45	9	69.1	56.32	3.98	1.32E-04	9.71	-0.06	55.47	56.32	57.91
DAGB	STI	qDAGB45_2.STI	45	2	73	62.28	4.21	1.24E-05	11.63	-0.20	13.85	62.28	67.54
LAGB	C	qLAGB45_4.C	45	4	71.5	64.27	3.32	1.05E-04	9.22	0.45	62.17	64.27	67.29
LAGB	STI	qLAGB45_4.STI	45	4	73	63.41	4.22	1.22E-05	11.65	0.10	62.06	63.41	67.49
HT	C	qHT45_7.C	45	7	57.7	59.01	4.72	2.50E-05	11.18	-5.00	58.66	59.01	60.24
HT	C	qHT45_9.C	45	9	63.4	55.07	5.32	6.57E-06	12.71	-5.46	54.37	55.07	56.91
HT	S	qHT45_1.S	45	1	108	75.72	5.17	9.14E-06	12.57	-4.44	76.22	75.72	80.57
HT	S	qHT45_7.S	45	7	63.5	60.17	4.95	1.51E-05	11.99	-4.25	59.01	60.17	61.46
HT	STI	qHT45_1.STI	45	1	107.8	77.00	5.45	3.37E-03	10.63	-0.04	75.67	77.00	80.57
HT	STI	qHT45_4.STI	45	4	82.2	66.96	4.66	1.16E-02	9.00	0.05	66.42	66.96	67.49
HT	STI	qHT45_7.STI	45	7	63.5	60.17	7.16	1.93E-04	14.34	-0.05	59.01	60.17	60.24
HT	STI	qHT45_9.STI	45	9	63.4	55.07	4.43	1.66E-02	8.51	-0.04	54.70	55.07	56.68
TAGB	S	qTAGB45_4.S	45	4	83.4	67.29	3.40	8.65E-05	9.49	0.40	61.70	67.29	68.41
TAGB	STI	qTAGB45_4.STI	45	4	64	60.23	4.18	1.34E-05	11.55	0.09	61.91	60.23	67.44
TB	S	qTB45_4.S	45	4	63.7	62.29	3.73	3.90E-05	10.37	0.51	61.70	62.29	68.41
TB	STI	qTB45_4.STI	45	4	64.6	62.46	4.91	2.41E-06	13.40	0.09	62.06	62.46	64.38
RS	STI	qR.S45_4.STI	45	4	69.1	63.67	3.89	2.71E-05	10.77	-0.19	62.46	63.67	63.95
RB	STI	qRB45_4.STI	45	4	64.6	62.46	4.13	1.53E-05	11.40	0.08	62.17	62.46	62.54

DAGB-dead aboveground biomass, LAGB-live aboveground biomass, RB-root biomass, RS-rank score, HT-height, TB-total biomass, TAGB-total aboveground biomass; Trt-treatment, STI-stress tolerance index, C-control, S-salt; P-*Sorghum propinquum*, B-*Sorghum bicolor*; DAT-days after treatment; Chr-chromosome, PVE-percent variance explained

Supplementary Figures

Figure S1. Pearson correlations on raw phenotypes and transformed phenotypes for control and salt populations at 15 days and 45 days after treatment. (A) Control population 15 days after treatment (B) Salt population 15 days after treatment (C) Control population 45 days after treatment (D) Transformed control data 45 days after treatment (E) Salt population 45 days after treatment

B.

C.

D.

E.

A.

B.

Figure S2. Sorghum genetic map after using a sliding window method to call bin markers as AA (*S. propinquum*), BB (*S. bicolor*), or AB. (A) Black horizontal lines represent bins on each of the 10 *Sorghum* chromosomes. (B) X-axis represents bin markers, Y-axis represents each of the 177 lines, colors represent the genotype data for each RIL at each marker. AA=Red, BB=Green, AB=Blue

Supplementary Figure S3. Sorghum genetic map after removing markers that deviated significantly from the expected mendelian ratio (1:1). Black horizontal lines represent bins on each of the 10 *Sorghum* chromosomes.

Supplementary Figure S4. Final Sorghum genetic map used to map phenotypic traits after removing duplicate bins. Black horizontal lines represent bins on each of the 10 *Sorghum* chromosomes

CHAPTER 6

GENERAL CONCLUSIONS

Soil salinity is becoming increasingly prevalent in areas that are typically used for agriculture due to various natural and anthropogenic factors. As reviewed in Chapter 2, the overabundance of salts in the soil leads to both osmotic and ionic stress. In response to osmotic stress plants are unable to extract water and nutrients from the soil without altering their own osmotic potential, and plants experience decreased above- and belowground biomass accumulation due to reduction in cell elongation rates (Yeo *et al.*, 1991; Passioura & Munns, 2000; Fricke & Peters, 2002; Munns & Tester, 2008). Plants are able to overcome the ion-independent stress by producing and accumulating solutes that aid in osmotic adjustment. With water acquisition, however, plants acquire Na^+ and Cl^- ions from the predominant NaCl . During ionic stress, the overabundance of ions in the cytoplasm becomes toxic and disrupts ionic homeostasis. Disruption of ionic homeostasis results in the inactivation of enzymes, oxidative stress, and, ultimately leads to leaf senescence (Munns & Tester, 2008; Iqbal *et al.*, 2014; Parihar *et al.*, 2015); however, if salts can be sequestered into vacuoles or extruded to the belowground biomass and soil, plants can continue to photosynthesize and produce enough carbon for continued growth and development.

Within my dissertation, I addressed the questions outlined in Chapter 1 relating to the morphological, physiological, and genetic variation of salinity tolerance in *Sorghum bicolor*. Specifically, in Chapter 3 I assessed the standing variation in morphological and physiological responses to salt exposure for a diverse panel of *Sorghum* genotypes. Significant variation was observed for live aboveground biomass, dead aboveground biomass, and root biomass. Accessions were first rank ordered for tolerance and then a subset of individuals were analyzed for proline content and ion profiles. Although much of the literature suggests proline accumulation is an indicator of tolerant genotypes due to its role in osmotic adjustment (Delauney & Verma, 1993; Iqbal *et al.*, 2014; Surender Reddy *et al.*, 2015; Amini *et al.*, 2015; de Freitas *et al.*, 2019), we did not observe this correlation. Further, when ion profiles were analyzed, we observed an increase in Na^+ in response to treatment, but we did not observe lower accumulation of Na^+ in tolerant accessions compared to sensitive accessions. In fact, one of our most tolerant accessions (Sb-10) and one of our most sensitive accessions (Sb-1) showed similar ion profiles. When tolerance rankings were evaluated within the phylogenetic framework presented in Mace *et al.* ((Mace *et al.*, 2013), I observed tolerant individuals clustering within the basal clade of the phylogenetic tree. Given the known drought tolerance of *Sorghum bicolor*, I hypothesized that the increased salinity tolerance observed (especially in the landrace durra) was a byproduct of the drought tolerance acquired during domestication.

In Chapter 4 I used two accessions that varied in response to salt exposure from Chapter 3. I dissected the spatial and temporal variation in gene expression of three genes known to play key roles in salt detoxification (Henderson *et al.*, 2019). SOS1, NHX2, and HKT3 gene products aid in tolerance due to their role in extrusion, sequestration, and transport, respectively. Upon the addition of non-saline water, I observed expression changes suggesting osmotic adjustment in the tolerant accession. Further in the tolerant accession upon the addition of 150 mM NaCl , I observed a delayed onset of gene expression suggesting Na^+ exclusion at the root level.

Numerous gene expression changes in the sensitive accession across the time course indicate biological chaos and the inability to exclude ions. Additionally, recent research suggests that sensitive genotypes and species may experience a significant amount of Na⁺ back-leak into the cytoplasm after sequestration, thus requiring increased gene expression of antiporters and transporters (Wu *et al.*, 2019; Tyerman *et al.*, 2019; Munns *et al.*, 2020). The energy requirement associated with ion extrusion, sequestration, and transport comes with a significant cost. Therefore, the decreased biomass observed in the sensitive accession in Chapter 3 may be a result of energy limitations due to poor ionic control.

In Chapter 5, I used a recombinant inbred line (RIL) mapping population derived from wild, weedy, *Sorghum propinquum* (sensitive) and domesticated *Sorghum bicolor* (tolerant) to identify regions of the genome responsible for increased salinity tolerance. A high-density genetic map covering the 10 *Sorghum* chromosomes was constructed with bin markers from SNP data. Phenotypes associated with biomass production were mapped and a total of 146 candidate genes were identified from 18 QTL. Given that dead aboveground biomass and live aboveground biomass are key indicators of salinity tolerance, we focused on candidate genes in these QTL windows. We observed *Sorghum propinquum* loci positively affecting dead aboveground biomass, whereas we observed *Sorghum bicolor* loci positively affecting live aboveground biomass. Large amounts of dead aboveground biomass are often indicative of ionic toxicity whereas large amounts of live aboveground biomass are often indicative of osmotic tolerance. Further, candidate genes identified in these two QTL windows included genes associated with both osmotic and ionic stress alleviation. Altogether, I report that the increased tolerance observed in *Sorghum bicolor* is a result of superior osmotic adjustment, which is likely due to the drought tolerance acquired during domestication. With osmotic adjustment, roots can absorb water and nutrients to support new growth.

In summary, this dissertation work contributes to the field of salinity stress in C4 crops. While it is accepted that tolerance can be achieved by various mechanisms, much of the research over the past two decades specifically focuses on osmotic adjustment or ionic exclusion independently. From the findings of my dissertation, however, I suggest salinity tolerance in bicolor is a complex network of both. To elaborate, we concluded that: (1) tolerant accessions are located in the basal clade of Mace *et al.*'s (2013) phylogenetic tree, (2) tolerant accessions do not differ from sensitive accessions in proline production or Na⁺ accumulation, (3) tolerant accessions experience an earlier osmotic response to aid in water acquisition, (4) tolerant accessions experience a delayed salt specific response, (5) genes that aid in osmotic adjustment aid in live aboveground biomass production, and (6) genes that aid in sensing, signaling and transport of Na⁺ ions assist in ionic tolerance. In combination with recent literature, this work suggests that the ability to efficiently acquire water is the main mechanism associated with increased tolerance in *Sorghum bicolor*. Further, *Sorghum bicolor* may be effectively transporting and sequestering Na⁺, therefore, using sodium as a cheap osmolyte. This would adjust the osmotic pressure, reduce energy expenditures, and decrease ionic toxicity.

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Last, but not least, below you will find a figure I made and planned to use in Chapter 4, however it did not make the cut. Regardless, I felt the image warranted a place in my dissertation given the significant amount of time it took me to draw in Adobe Illustrator. This figure points out the important tissues sampled, *in multiple research projects*, within my dissertation. Don’t be fooled, it may look like a *Zea mays* seedling, but it is in fact a *Sorghum* seedling.