QTL mapping in an interspecific sorghum population uncovers candidate regulators of salinity tolerance

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Abstract

Salt stress impedes plant growth and disrupts normal metabolic processes, resulting in decreased biomass and increased leaf senescence. Therefore, the ability of a plant to maintain biomass when exposed to salinity stress is critical for the production of salt tolerant crops. To identify the genetic basis of salt tolerance in an agronomically important grain crop, we used a recombinant inbred line (RIL) population derived from an interspecific cross between domesticated *Sorghum bicolor* (inbred Tx7000) and a wild relative, *Sorghum propinquum*, which have been shown to differ in response to salt exposure. One-hundred seventy-seven $F_{3:5}$ RILs were exposed to either a control or salt treatment and seven traits related to plant growth and overall health were assessed. A high-density genetic map that covers the 10 *Sorghum* chromosomes with 1991 markers was used to identify nineteen total QTL related to these traits, ten of which were specific to the salt stress response. Salt-responsive QTL contain numerous genes that have been previously shown to play a role in ionic tolerance, tissue tolerance, and osmotic tolerance, including a large number of aquaporins.

Introduction

Soil salinity imposes abiotic stress on plants when soluble ions, such as Na^+ and Cl^-, accumulate in the soil surrounding the root rhizosphere. Saline soils have been found to affect more than 6% of total land and 20% of irrigated land (Rengasamy, 2006; Food and Agriculture Organization (FAO), 2008, 2009; Hasegawa, 2013; FAO, 2017; Yang *et al.*, 2020). Although most of the lands with elevated salinity have arisen from natural causes, anthropogenic factors have recently led to the increase of salinity in lands cultivated for agriculture (Munns and Tester, 2008).

The level of salt in the soil directly affects the production of agriculturally important crops, yet crop tolerance varies depending on species and genotype (Munns and Tester, 2008). Previous research has identified the key mechanisms of salt tolerance as: 1) ion exclusion, which results in the absence of salt ions from the shoot of the plant, 2) tissue tolerance, achieved by the sequestration of ions into specific tissues, cells, and subcellular organelles, and/or 3) osmotic tolerance, defined as the ability to maintain water uptake and growth despite lower water potential (Munns & Tester, 2008; Carillo *et al.*, 2011; Fan *et al.*, 2015; Genc *et al.*, 2016; Negrão *et al.*, 2017; Munns *et al.*, 2019). In order to incorporate tolerant genotypes into breeding programs, it is first necessary to determine if tolerance is a result of ionic tolerance, tissue tolerance, and/or osmotic tolerance; however, the genetic basis of these mechanisms remains unknown.

Sorghum (*Sorghum bicolor* L. Moench) is a staple crop for food, fuel, and feed production (Doggett, 1970, 1988), and is notable for varieties that are 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.*, 2020). In a previous study, we measured the variation in salinity tolerance across a diverse panel of *Sorghum* genotypes that included wild species, domesticated *S. bicolor* landraces, and improved *S. bicolor* lines (Henderson et al., 2020). Salinity tolerance was assessed as the maintenance of biomass following 12 weeks of 75 mM salt exposure (sodium chloride, NaCl). After long-term exposure to salinity, *S. bicolor* genotypes ranged from 30% - 95% in biomass maintenance when compared to genotypes in non-saline (control) conditions. The genotype that had the greatest reduction in biomass upon exposure to salt was the wild species *S. propinquum*, which maintained only 5% of its aboveground biomass (Henderson et al., 2020). Additionally, we also measured the accumulation of sodium and potassium in genotypes that spanned the tolerance rankings. The results showed that sodium and potassium accumulation was accession dependent and did not correlate tolerance ranking, suggesting that the salt response in sorghum is reliant on multiple mechanisms. We concluded that sorghum serves as a valuable resource for dissecting the various underlying genetic controls of salinity tolerance.

The observed variation in biomass retention upon exposure to saline conditions (Henderson et al., 2020) indicates that there is quantitative genetic variation in salinity tolerance in *Sorghum*. In the work presented here, a recombinant inbred line (RIL) population constructed from a cross between *S. propinquum* (95% biomass reduction) and *S. bicolor* (Tx7000 - landrace *durra*; 5% biomass reduction) was used to dissect the

genetic underpinnings of salinity tolerance. We developed a high-density genetic map from 177 $F_{3:5}$ RILs and identified quantitative trait loci (QTL) associated with biomass-related traits during salt exposure. These findings and this population establish a resource that can be used to further dissect the underlying genetic basis of ionic tolerance, tissue tolerance, and osmotic tolerance.

Materials and Methods

Plant Material

A RIL mapping population derived from an interspecific cross of *S. propinquum* and *S. 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 F_5, 18% (31 RILs) of the individuals being F_4, and 7% (14 RILs) of the individuals being F_3. Each line was derived by the single seed descent method (Brim, 1966; Snape & Riggs, 1975) as described in Govindarajulu et al. (2020).

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. Target germination conditions 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 fertilizer (J.R. Peters, Inc., Allentown, PA, USA) 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. Target growth conditions were 27°C day/23°C night with 16 h ambient 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 fertilizer (J.R. Peters, Inc., Allentown, PA, USA) 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 phenotypes were measured for each of the 177 lines: height (cm), rank score, root biomass (g), dead aboveground biomass (g), live aboveground biomass (g), total aboveground biomass (g), and total biomass (g). Height (cm) was taken from the base of the stem to the tip of the newest emerged leaf. 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 h. Root biomass (g) was the total belowground biomass collected. Roots were rinsed in water to remove all dirt and sand. Dead aboveground biomass (g) included all biomass (leaves, tillers, and/or stem) attached to the plant where more than 50% of the tissue was brown; whereas live aboveground biomass (g) included all biomass attached to the plant that was more than 50% green suggesting it was alive. Total aboveground biomass (g) was the sum of live and dead aboveground biomass, while total biomass (g) included live, dead, and 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), 15 days (short term exposure), and 45 days (long term exposure) after treatment began. Height was 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 is an valuable metric when comparing genotypic tolerance within a population (Negrão et al., 2017). The 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):

$$Stress\ Tolerance\ Index\ = \left(\frac{Ycontrol}{Y\ control\ average}\right) \times \left(\frac{Ysalt}{Y\ control\ average}\right)$$

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. RILs 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 STI values. Three biological replicates of each RIL in the salt population, three biological replicates for STI values, and two biological replicates of each RIL in the control population were considered for QTL analysis. All statistics and graphing were completed using R version 4.0.2 (R Core Team, 2013).

Least square means for each phenotype in each population (control, salt, STI) were calculated for every RIL. Normality was assessed using both a Shapiro-Wilk test in R and Q-Q plots from the *car* package version 3.0.10 (Fox & Weisberg, 2019). 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 using the *PerformanceAnalytics* package 2.0.4 (Peterson & Carl, 2019) (**Figure S1**).

To determine if there was a treatment effect, both a nonmetric multidimensional scaling (NMDS) analysis (Julkowska *et al.*, 2019) and an analysis of variance (ANOVA) was performed. The NMDS ordination clustered individuals based on Bray–Curtis dissimilarity measures when all phenotypes were considered. The dimcheckMDS function in the *goeveg* package version 0.4.2 was used to generate stress values for each dimension; two dimensions was deemed appropriate. The NMDS was generated using the *vegan* package version 2.5.6 (Oksanen *et al.*, 2019) in R. The NMDS was paired with an analysis of similarity (ANOSIM) to statistically test the ordination results from the NMDS. Here, we tested if RILs were more similar within or between 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 $\alpha = 0.05$).

Genetic Map Construction and QTL analysis

The RIL population used in this study was generated as previously described in Govindarajulu et al. (2020). This population has been successful in identifying genes that are key regulators of tiller elongation in sorghum (Govindarajulu et al., 2020); however, in this study, advanced lines were included, therefore requiring a new genetic map. A new genetic map for the *S. propinquum* by *S. bicolor* (Tx7000) RIL population was constructed as previously 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 version 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 and duplicate bin markers, the kosambi map function in R/qtl (Broman *et al.*, 2003) was used to construct a high density genetic map.

QTL analysis was performed in R using the *qtl* package version 1.46.2 (Broman *et al.*, 2003). 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 (MQM). The 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 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 indicated that 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.

Aquaporin Enrichment Analysis

To determine if there was an enrichment of aquaporin genes (SbAQP) located within detected QTL, we randomly sampled the *Sorghum bicolor* (version 3.1) genome 50 times in R (R Core Team, 2013). Starting positions were determined by first randomly selecting a chromosome followed by selecting a random starting position on that chromosome. Chromosome selection was determined by multiplying the total number of chromosomes (2n = 10) by a randomly generated number [runif(1)] between 0 and 1. The resulting number was truncated to an integer and represented the starting chromosome number. This same process was repeated to determine the starting location on the chromosome; however, instead of multiplying a random number by the total number of chromosomes, the random number (between 0 and 1) was multiplied by the size of the corresponding chromosome. The result was again truncated and represented the starting location in the genome (in Mb). Genes within a 5 Mb window (5 Mb downstream from the starting location) were extracted (*Sorghum bicolor* version 3.1)

Results

A high density genetic map covers 10 *Sorghum* chromosomes with 1991 makers

The resequencing, SNP calling, and bin calling of the 177 RILs generated 4055 total bin markers (**Figure S2**). After removing duplicate markers, the map covered the 10 *Sorghum* chromosomes with 1991 markers (**Supplementary Table S2**) and was 913.71 cM in length (**Supplementary Table S3**).

S. bicolor and *S. propinquum* exhibit a differential response to salt exposure

Previous work from our lab described the variation in biomass retention following 12 weeks of 75 mM NaCl in the domesticated, *S. bicolor*, and the wild progenitor, *S. propinquum* (Henderson et al., 2020). In response to salt treatment *S. bicolor*, landrace *durra*, maintained 95% of its biomass, had high STI values for live aboveground biomass, and had low STI values for dead aboveground biomass, whereas *S. propinquum* maintained only 5% of its biomass, ranked low for live aboveground biomass, and ranked high for dead aboveground biomass. Variation in tolerance in *S. bicolor* was genotype dependent, and when tolerance rankings were evaluated within a phylogenetic framework, all of the accessions from the landrace durra ranked as tolerant (~95% biomass retained). The findings from our previous study (e.g., *S. bicolor*, specifically the landrace durra, was shown to be tolerant to salinity stress whereas *S. propinquum* was shown to be sensitive) set the foundation for the parental genotypes of the RIL population used here.

Overall plant health decreased in response to salt exposure

In both the control and salt treated populations, the following phenotypes were recorded: height (cm), rank score, root biomass (g), dead aboveground biomass (g), live aboveground biomass (g), total aboveground biomass (g), total biomass (g), and mortality. With the exception of mortality, all phenotypes were significantly different between the control and salt treated populations (in both short term and long-term exposure) (**Table 2**). Generally, the response to salt exposure was characterized as shorter plants with reduced live aboveground biomass, root biomass, total aboveground biomass, total biomass (**Table 2, Supplementary Tables S4**). Further, plants had larger rank scores and more dead aboveground biomass (**Table 2, Supplementary Tables S4**). These significant differences between RILs in control conditions and salt treated conditions, in addition to the clear and significant clustering ($p < 0.001$, ANOSIM R=0.16) of treatments in the NMDS analysis (**Figure 1**), are indicative of an overall decrease in plant health in response to salt.

In response to long-term salt exposure, QTL were identified as important regulators of salt tolerance

Although there was variation in plant response among RILs in the control and salt treated populations after short term salt exposure (DAT=15) (**Table 2**), there were no QTL detected. Therefore, the following analyses focus on QTL detected after long-term salt exposure (DAT=45). QTL analysis was performed on RILs in the control treated population, RILs in the salt treated population, and on the stress tolerance index (STI) values. The individual QTL analysis performed on the RILs in the control and salt treated populations was to determine QTL that were shared. QTL that were shared between the control and salt treated populations are related to trait architecture and not the salt specific response. All genes detected in QTL are listed in **Supplementary Table S5**.

<u>Total biomass (TB)</u>

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 response to salt treatment, the total biomass decreased on average 32% (0.66 g - 9.42 g with a mean of 5.11 g, **Table 2**, **Supplementary Table S4**). There were no QTL detected when the control population was mapped. This is likely due to limited variation in architecture and biomass between *S. bicolor* and *S. propinquum* when plants are grown in space restricted pots (data not shown); however, because *S. bicolor* is tolerant and *S. propinquum* is sensitive to salt exposure (Henderson et al., 2020), we suspect that salt specific QTL for total biomass were identified (qTB45_4.S and qTB45_4.STI) (**Figure 2**) because genotypes with *S. propinquum* alleles had a greater reduction in biomass in response to treatment, whereas genotypes that contain *S. bicolor* alleles maintained biomass. Both QTL (qTB45_4.S and qTB45_4.STI) were detected on chromosome 4 and both had positive additive effects indicating the *S. bicolor* alleles positively influence the total biomass (**Table 3**). This indicates that RILs with *S. bicolor* alleles in the 61.70 Mb - 68.41 Mb region on chromosome 4 have more overall biomass (live aboveground biomass, dead aboveground biomass, and root biomass) after long-term exposure to NaCl. The QTL detected when STI values were mapped (qTB45_4.STI) explained the greatest amount of phenotypic variation (PVE = 13.02) (**Table 3**). Of the

genes within qTB45_4.STI, candidate genes were associated with aquaporins, stress response proteins, salt tolerant proteins, and transporters (**Supplementary Table S6**).

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 an average 20% decrease in total aboveground biomass (average = 3.98 g) (**Table 2, Supplementary Table S4**). Similar QTL were detected when the total biomass and the total aboveground biomass were mapped as would be expected given the high correlation between total biomass and total aboveground biomass (**Supplementary Figure S1**). There were no QTL detected in the control population; however the same two QTL (qTB45_4.S and qTB45_4.STI) were detected in the salt population (qTAGB45_4.S) and when STI values (qTAGB45_4.STI) were mapped (**Figure 2**). qTAGB45_4.STI co-localized with qTB45_4.STI and qTAGB45_4.S co-localized with qTB45_4.S. Therefore, the same candidate genes identified in the total biomass QTL were also identified for total aboveground biomass.

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 average increase of 45% with a mean of 0.80 g (**Table 2**, **Supplementary Table S4**). Genotypes that accumulate more dead aboveground biomass, like *S. propinquum* (Henderson et al., 2020), are sensitive compared to genotypes that do not. When dead aboveground biomass was mapped, two QTL were detected on chromosome 2 (qDAGB45_2.C and qDAGB45_2.STI) and one QTL was detected on chromosome 9 (qDAGB45_9.C) (**Figure 2**). RILs with *S. propinquum* alleles positively influenced the amount of dead aboveground biomass. Of the genes within qDAGB45_2.STI, candidate genes were associated with aquaporins, sodium transporters, potassium transporters, salt tolerant proteins, and leaf senescence (**Supplementary Table S6**).

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 an average 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 (**Table 2**). Two QTL were detected (qLAGB45_4.C and qLAGB45_4.STI) on chromosome 4 (**Figure 2**). Both QTL had positive additive effects, indicating that *S. bicolor* alleles positively influence live aboveground biomass. The QTL detected when STI values were mapped (qLAGB45_4.STI) explained 11.65 percent of phenotypic variation (**Table 3**).

Root biomass (RB)

The root biomass of plants grown in control conditions ranged from 0.48 g to 6.38 g with a population average of 2.50 g; however, in salt conditions, the root biomass was reduced by an average of 55% and ranged from 0.09 g to 2.48 g with a population average of 1.13

g (**Table 2**, **Supplementary Table S4**). A single QTL was detected when STI values were mapped (qRB45_4.STI) (**Figure 2**). qRB45_4.STI explained 11.40 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.

Rank score (RS)

Rank score was a qualitative measure used to describe overall plant health (**Table 1**). There was an average 58% increase in rank score in response to treatment, indicating that there was an overall decrease in the health of plants exposed to NaCl (**Table 2**). 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 with dead leaves continued 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 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 was detected on chromosome 4 (qRS45_4.STI). qRS45_4.STI is located at 62.46 Mb - 63.95 Mb with a peak near 63.67 Mb (**Figure 2**). 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 overall better plant health in stress conditions. Of the genes located within qRS45_4.STI, there are several genes that encode aquaporins, ion channels, and chaperone proteins (**Supplementary Table S6**).

Height (HT)

At the final recording, height in the control population ranged from 52.92 cm to 122.37 cm with a population average of 87.81 cm (**Supplementary Table S4**). In response to treatment, there was an average of 16.38% decrease in height, ranging from 46.32 cm to 112.47 cm with a population average of 75.46 cm (**Table 2**, **Supplementary Table S4**). Eight QTL were detected for height (**Figure 2**). Three QTL (qHT45_7.C, qHT45_7.S, and qHT45_7.STI) were detected on chromosome 7 in approximately the same region (58.66 Mb - 61.46 Mb) (**Table 3**). Two QTL (qHT45_9.C and qHT45_9.STI) were detected on chromosome 9 in approximately the same region (54.37 Mb - 56.91 Mb). Since these five QTL were detected in the control population and the salt population, we suspect that these QTL are important in plant height in the absence of stress. Two additional salt specific QTL were detected on chromosome 1 (qHT45_1.S and qHT45_1.STI) (**Figure 2**). Further, a QTL was detected on chromosome 4 (qHT45_4.STI) when STI values were mapped (**Figure 2**). Several candidate genes were identified within the salt-specific QTL, including genes associated with aquaporins, potassium transporters, and stress response proteins (**Supplementary Table S6**).

Enrichment Analysis

A total of 4276 unique genes were located within 1.0 logarithm of the odds (LOD) confidence interval of the QTL identified in this study (**Table 3**, **Supplementary Table S5**). Of these, we observed numerous genes (13) that encode aquaporins. In order to determine if this constitutes an enrichment of aquaporin genes, we performed a random

sampling of 50 independent 5 Mb segments (34% of the genome) from the *S. bicolo*r reference genome and recorded the number of aquaporins found in each segment. A total of 9098 genes were identified, of which only 9 encode aquaporins.

Discussion

In the present study, we screened 177 $F_{3:5}$ RILs derived from a cross between the inbred *S. bicolor* (Tx7000; landrace *durra*) and its wild relative, *S. propinquum,* for performance-related traits 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 Na^+ exclusion from the aerial organs of the plant, overall tissue tolerance, and osmotic adjustment; 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 population, a saline treated population, and from STI values. Among the 19 QTL detected, ten were either 1) unique to the STI values, 2) unique to the saline environment, and/or 3) explained more than ten percent of the phenotypic variation (**Table 3**).

The data presented here, in combination with previous findings (Henderson *et al.* 2020), collectively demonstrates that there is increased tolerance to salinity stress in *S. 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 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. 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 disentangle; 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 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^+/Ca^{2+} exchanger, leaf senescence, early response to dehydration, heat shock proteins, vacuolar proton exchangers, potassium antiporters, and stress response proteins.

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. 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. Similarly, qR.S45_4.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 S5**).

Most interestingly, there were numerous genes within the two dead aboveground biomass QTL that we considered informative of ionic stress, specifically genes associated with Ca^{2+} 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).

Further, in a previous study that characterized the aquaporin (AQP) gene family in *S. bicolor*, SbAQP transcript abundance was affected by both salt and drought stress (Reddy *et al.*, 2015). Here, we identified 13 unique aquaporin genes, which encode TIP1;1, TIP2;1, TIP3;1, PIP1;3, PIP1;4, PIP2;2, PIP2;6, PIP2;7, and PIP1;5 (**Supplementary Table S5**). Aquaporins are well known for their role in the transport of water and other neutral solutes (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). Emerging evidence indicates that some aquaporins are also capable of coupling water and ion transport, resulting in osmotic adjustment (Byrt *et al.*, 2017). Specifically, the *Arabidopsis* plasma membrane intrinsic proteins AtPIP2;1 (AT3G53420) and AtPIP2;2 (AT2G37170) have been shown to co-transport water and Na^+, suggesting that they play dual roles in nutrient transport and osmotic adjustment (Byrt *et al.*, 2017; Kourghi *et al.*, 2017). In the presence of salts, PIP2 is transported from the plasma membrane, resulting in significant reductions in root hydraulic conductance (Boursiac *et al.*, 2005; Sutka *et al.*, 2011; Byrt *et al.*, 2017). In addition, the ionic conductance of AtPIP2;1 has been shown to be inhibited by divalent cations, specifically Ca^{2+}, which is known to play an essential role in intracellular signaling in plants, particularly in response to abiotic stress (Maurel *et al.*, 2008; Verdoucq *et al.*, 2008; Byrt *et al.*, 2017). Therefore, AtPIP2;1 may constitute a mechanism of sensing and signaling during the salt stress response in plants. In our study, we identified the tandemly arranged Sobic.002G125000, Sobic.002G125200, Sobic.002G125300, and Sobic.002G125700, which encode four copies of SbAQP2;6, lie within the salt-specific QTL (qDAGB_2.STI), and share ~85% sequence similarity with AtPIP2;1 (**Supplementary Table S5**). Given their presence in salt specific QTL, known response to abiotic stress, and high sequence similarity to AtPIP2;1, we propose that these aquaporins play a critical role in maintaining water balance, controlling ion transport, and possibly in sensing and signaling, as mechanisms of salinity tolerance in sorghum.

Conclusions

In our previous study, which compared salt tolerance rankings among a diverse group of *Sorghum* genotypes and species, we concluded that salinity 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.*, 2020). Here, we detected numerous genes associated with sensing, signaling, and transport of Na^+ in salt specific QTL. Further, we identified numerous genes that encode aquaporins detected within salt responsive QTL. The results of this study provide insights into QTL important for each of the tolerance mechanisms (ionic, tissue, and osmotic tolerance). This is the first study where individuals of each tolerance category (1-tolerant to ionic and osmotic stress; 2-tolerant to ionic stress but sensitive to osmotic stress; 3-sensitive to ionic stress and tolerant to osmotic stress; or 4-sensitive to ionic and osmotic stress) are identified in a common genetic background. Therefore, these findings and this population provide a foundation for future studies aimed at the dissection of the genetic basis of salinity tolerance.

Data Availability Statement

Phenotype data and the binned genotype data used for QTL mapping can be found in Supplementary Tables S7-S8. All data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

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Author Contributions

A.N.H and J.S.H designed experiments; A.N.H and J.S.H managed the project; A.N.H., prepared the samples; A.N.H. and R.G. lead the data analysis; A.N.H, and J.S.H wrote the manuscript with contributions from R.G.

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Figure Legends

Figure 1. Non-metric multidimensional scaling (NMDS) analysis paired with an analysis of similarity (ANOSIM) reveals treatment clustering. A NMDS paired with an ANOSIM reveals that individuals were more similar within a treatment than between treatments (ANOSIM $R=0.16$, $p<0.001$).

Figure 2. Sorghum genetic map with QTL locations from $177_{F3:F5}$ RILs. QTL detected from a high-density genetic map. Empty spaces are regions that were removed because bins were either heterozygous or neighboring markers were identical (duplicate markers). The genetic map position is shown on the y-axis. Horizontal lines represent bins used as markers. Colored vertical lines show the position of each QTL for each trait in control conditions, salt conditions, or when STI values were mapped.

Supplemental 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

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) After the resequencing, SNP calling, and bin calling, 4055 total bins were detected across 10 sorghum chromosomes. (B) The 4055 total bins are illustrated on the x-axis and the 177 RILs are illustrated on the y-axis. The red regions illustrate bins that were called *S. propinquum* (AA); the green regions illustrate bins that were called *S. bicolor* (BB); the blue regions illustrate regions that were called heterozygous (AB).

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
2	Severe leaf and leaf tip curling, few leaves elongated
3	Most leaves dead but still producing new leaves
4	*Plant still alive but no new growth*
5	Plant dead

Table 2. Summary of summary statistics and phenotypic averages for RILs in the control population and salt population. An analysis of variance (ANOVA) was used to test if there was significant variation in response to salt treatment. In response to salt exposure plants were shorter, had less live aboveground biomass, root biomass, total aboveground biomass, and total biomass. Additionally, in response to salt exposure plants had larger rank scores and more dead aboveground biomass. Mortality was not affected in response to salt treatment.

Phenotype	Days After Treatment	Control	S.D.	Salt	S.D.	RDPB (Control-Salt)/Control	Significant
HT	15	63.75	13.19	55.30	10.01	0.13	***
RS	15	0.45	0.48	2.18	0.53	-3.81	***
HT	45	87.81	14.36	75.46	11.50	0.14	***
LAGB	45	4.45	1.31	3.18	1.01	0.28	***
RB	45	2.50	1.05	1.13	0.45	0.55	***
TAGB	45	5.00	1.49	3.98	1.19	0.20	***
TB	45	7.49	2.21	5.11	1.51	0.32	***
RS	45	2.01	0.53	3.19	0.44	-0.58	***
DAGB	45	0.55	0.28	0.80	0.42	-0.45	***
Mortality	45	1.00	0.00	1.00	0.03	0.00	

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

HT-height, RS-rank score, 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), RDPB-relative decrease in plant biomass

Table 3. 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 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
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
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
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
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
RS	STI	qRS45_4.STI	45	4	69.1	63.67	3.89	2.71E-05	10.77	-0.19	62.46	63.67	63.95
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

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

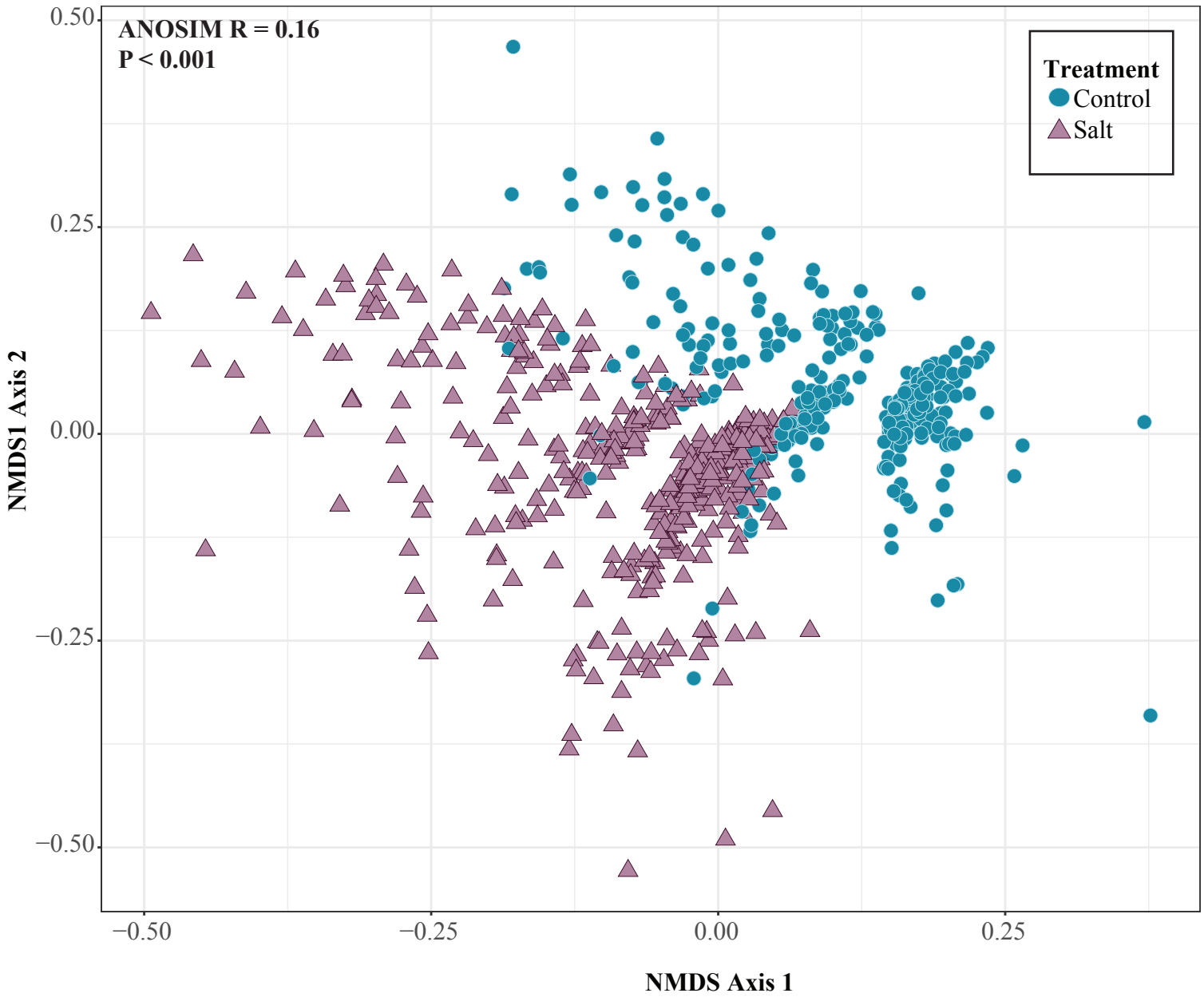

Figure 1. Non-metric multidimensional scaling (NMDS) analysis paired with an analysis of similarity (ANOSIM) reveals treatment clustering.

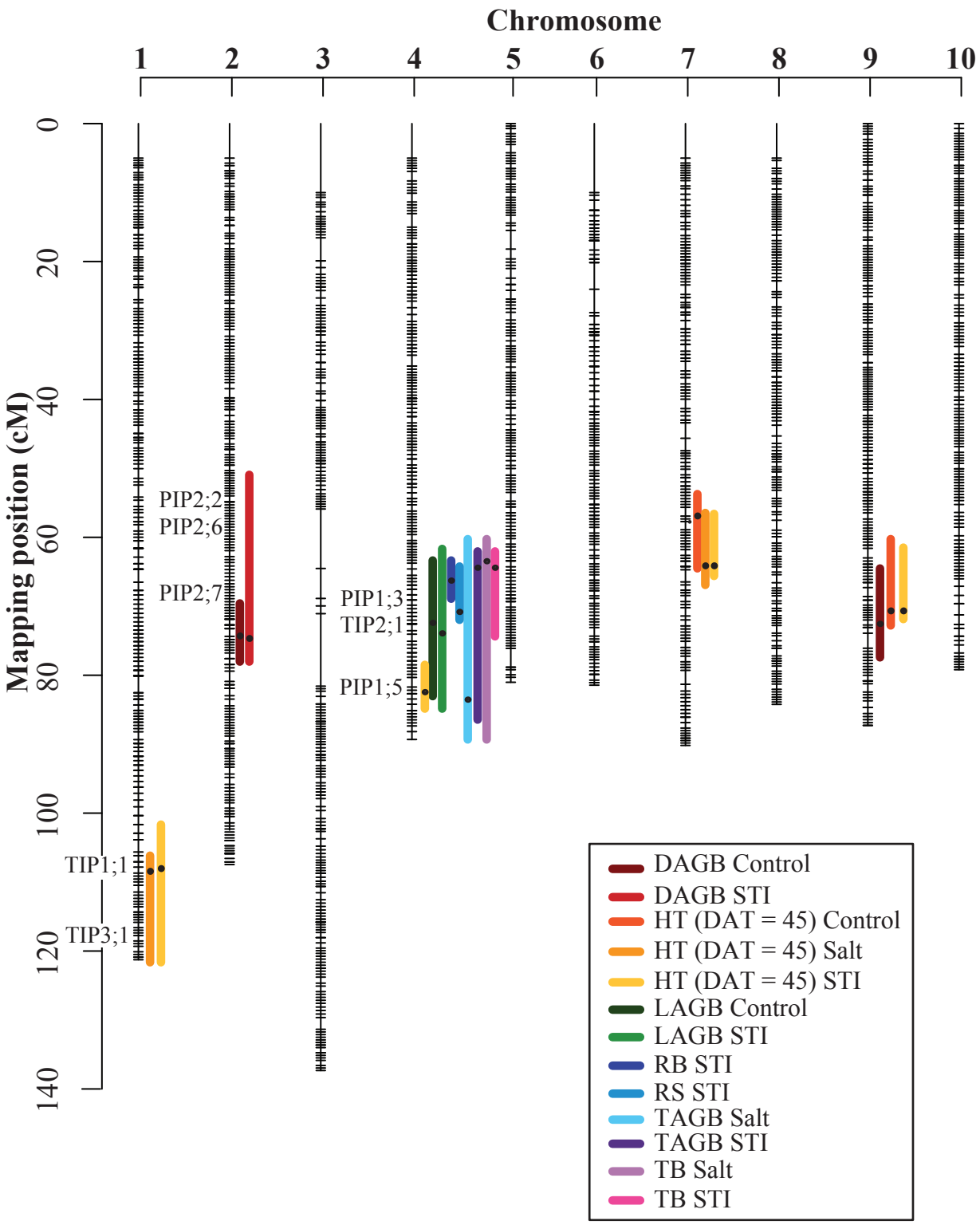

Figure 2. Sorghum genetic map with QTL locations from $177_{F3:F5}$ RILs.