PHENOTYPING AND QUANTITATIVE TRAIT LOCUS MAPPING FOR SALINITY STRESS RELATED TRAITS AT THE PRE-EMERGENCE SEEDLING STAGE OF RICE (*Oryza sativa* L.).

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

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January 2017

ABSTRACT

Salinity stress is one of the major abiotic stresses affecting rice (*Oryza sativa* L.) production, as rice is the most susceptible cereal crop to salt stress. A population of 174 $F_{10} - F_{12}$ recombinant inbred lines derived from an IR64 (*indica*) x Azucena (*japonica*) cross was evaluated under control and salt stress conditions at pre-emergence seedling stage using a novel phenotypic platform. The phenotypic results indicated that the abscisic acid and sugar content in shoot meristem region increased due to salt stress whereas the growth related traits were negatively affected. The mapping study successfully identified 27 QTLs under control conditions and 25 QTLs under salt stress conditions for shoot meristem weight, shoot meristem glucose, sucrose and total sugar content, shoot length, primary root length, lateral root count and length traits with LOD scores ranging from 3.7 to 9.9. Many of the QTL locations matched with previous studies and some novel QTLs were also identified in this study.

BIOGRAPHICAL SKETCH

Parthiban was born in a small town situated in the state of Tamil Nadu, India. Frequent visit to farmland with his dad was a major factor for his interest to learn biology. The interest in the subject made him understand things better and helped him fetch a good score in his high school. He went on to pursue his undergraduate degree at Tamil Nadu Agricultural University which is one of the leading institutions for agricultural research in the country. It was there he broadened his knowledge about different crops and learned the basics in the courses like breeding, genetics, physiology and molecular biology. He always aspired to do graduate studies in order to deepen his knowledge and his dream came true when he got selected for the Cornell Sathguru Foundation's scholarship for the MS program in Plant Breeding and Genetics at Cornell. His masters research work is focused on "Salinity tolerance in rice" which includes phenotyping and identification of QTLs for salt tolerance at seedling stage. The committee for his research includes Dr. Margaret Smith and Dr. Tim Setter. He is more of an outdoor person with interests in ecological camps, trekking and also plays tennis.

Dedicated to God and Nature

ACKNOWLEDGMENTS

First I would like to thank my mom, dad and brother and all my family and friends for supporting and motivating me to pursue my field of interest. I am very grateful to my wife who is with me throughout my academic journey. Thanks to the God for giving me good health, mind and spirit for everything I do.

The most important person to thank is Dr Tim Setter who has been a wonderful guide to me since day one I had gotten into Cornell. His sound knowledge, humility and helpfulness have always inspired me. Special thanks to my adviser Dr Margaret Smith for all the valuable inputs and for serving on my committee. I would also acknowledge Dr Susan McCouch for providing me the rice population to work on. I would also like to thank all my Cornell faculties who have taught me good science.

My sincere thanks to Dr Joshua Cobb for his collaboration with mapping studies and for all the meaningful meetings and discussions. Thanks to Dr Randy Clark for his help in image processing.

I would also acknowledge Cornell Sathguru Foundation for Development for funding my graduate program.

Finally, thanks to all who have helped directly or indirectly to achieve this feat.

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LIST OF ABBREVIATIONS

Treatment

C – Control condition where only water is applied

N – Salt Stress condition -0.4 MPa of NaCl stress is applied

Material

Azu – Azucena

Traits

gdw - grams dry weight

SM dw - Shoot meristem (dry weight), g $shoot^{-1}$

ABA $pmol\_gdw^{-1}$ - Shoot meristem Abscisic acid, picomoles gdw^{-1}

Glu $umol\_gdw^{-1}$ - Shoot meristem Glucose, micromoles gdw^{-1}

Suc $umol\_gdw^{-1}$ - Shoot meristem Sucrose, micromoles gdw^{-1}

Tot sug, umol gdw^{-1} - Shoot meristem Total sugar, micromoles gdw^{-1}

RL D6 - Primary root length at 6 days after germination, cm

RL D8 - Primary root length at 8 days after germination, cm

SL D6 - Shoot length at 6 days after germination, cm

SL D8 - Shoot length at 8 days after germination, cm

RGR 6-8d - Root growth rate from Day 6 to Day 8, cm

SGR 6-8d - Shoot growth rate from Day 6 to Day 8, cm

LRC D6 - Number of lateral roots at Day 6 (count)

LRC D8 - Number of lateral roots at Day 8 (count)

LRL D6 - Total Lateral root length at Day 6 (cm)

LRL D8 - Total Lateral root length at Day 8 (cm)

LRC 6-8d - Difference in the lateral root number from Day 6 to Day 8 (count)

LRL 6-8d - Difference in the total lateral root length from Day 6 to Day 8 (cm)

Traits and QTL nomenclature

qSMD - Shoot meristem (dry weight), g shoot^{-1}

qGLU - Shoot meristem Glucose, micromoles gdw^{-1}

qSUC - Shoot meristem Sucrose, micromoles gdw^{-1}

qSUG - Shoot meristem Total sugar, micromoles gdw^{-1}

qRL - Primary root length at 6 & 8 days after germination, cm

qSL - Shoot length at 6 & 8 days after germination, cm

qRGR - Root growth rate from Day 6 to Day 8, cm d^{-1}

qSGR - Shoot growth rate from Day 6 to Day 8, cm d^{-1}

qLRC - Number of lateral roots at Day 6 & 8 (count)

qLRL - Total Lateral root length at Day 6 & 8 (cm)

qLRCR - Difference in the lateral root count from Day 6 to Day 8, count

qLRLR - Difference in the total lateral root length from Day 6 to Day 8, cm

CHAPTER 1

INTRODUCTION

Asian civilization was built upon the domestication of *Oryza sativa* L., making it one of the most important crops in the world. The domestication of rice dates back to 8000 to 9000 years ago from its closely related wild relative *Oryza rufipogon* Griff. Currently rice feeds more than half of the global population (Gross and Zhao, 2014) and the demand will be higher than present as the population is growing. It is believed that the continuous selection for higher yield and pest resistance has led to the reduction of diversity in rice and therefore the modern rice is more vulnerable for adverse effects of climate change (Callaway, 2014).

Salinity is one of the major abiotic stresses affecting more than 6% of the global area (Munns and Tester, 2008). Salinity in soil is caused by sea water intrusion in coastal regions either by gradual gradient movement or by natural disasters such as storms and tsunamis. There is also secondary salinity caused by improper irrigation methods. Therefore breeding for salinity tolerance is very important.

Among the cereals , rice is more sensitive to salinity stress (Munns and Tester, 2008) and the degree of salinity tolerance varies with growth stages. Rice is more susceptible in its early seedling stage and reproduction stage of its growth (Flowers and Yeo, 1981; Lutts et al., 1995).

Salinity stress affects crops in two phases. Firstly the osmotic phase, which is due to the low water potential at the roots, and secondly the ionic phase, where the Na+ ions accumulate in the shoot region causing damage (Mostek et al., 2015). Very little is known about the osmotic phase and the tolerance mechanisms associated with it (Roy et al., 2014) and thus further research is needed to understand this better.

ABA

Abscisic acid is a 15-carbon sesquiterpenoid compound that regulates numerous growth and developmental activities and is synthesized in response to environmental stresses such as drought, salinity, and high temperature. Abscisic acid is well known for its role as a signaling molecule that mediates expression of numerous cellular genes during salinity and water deficit stress. Previous studies have reported that shoot level changes are possible with the signals originated from roots which are directly exposed to salinity stress (Davies and Zhang, 1991). One of the studies reported that ABA accumulation in the shoot region could be due to the transport of ABA synthesized from roots as a result of salt stress (Jia et al., 2002). The ABA accumulated in the shoot meristem region will regulate the various physiological responses in the shoot (Jia et al., 2002). In the present study, the ABA was quantified from 8-day old salt-stressed seedlings that had not yet developed any leaves and the entire experimental setup was performed in a closed tub without any light exposure. These two conditions exclude the possibility of transpiration and thereby exclude any ABA signal for stomatal closure to control transpiration.

The increase of ABA concentrations in shoot meristem during salt stress could be from ABA transported from roots, or it could be from ABA synthesis directly in the shoot tissues. In studies involving root-shoot grafts of *Arabidopsis thaliana* (L.) Heynh wildtype and ABA-synthesis mutants, the direct synthesis of ABA in leaves is well established (Christmann et al., 2007). Some reports suggest that the ABA accumulation is higher and less transient in tolerant plants and, lower in susceptible plants (Moons et al., 1995). Rice shoots accumulate more ABA during NaCl stress

than roots and the ABA content keeps on increasing in tolerant cultivars (Kang et al., 2005). Traditionally ABA is considered to be a growth inhibiting hormone in healthy and well-watered conditions but this condition changes for plants under water (osmotic) stress. During water stress in transpiring plants, ABA closes stomata, thereby decreasing water loss and preventing desiccation. It has been reported that young healthy tissues have high ABA content whereas stunted growth is observed in ABA mutants (Finkelstein, 2013). Studies of germinating seedlings indicate that in growing zones, ABA growth sensitivity is greater in shoots than roots, such that ABA results in an increase in root:shoot growth ratio during stress (Sharp et al., 2004).

Sugars

In contrast to salts, which damage cells by disrupting protein and membrane structure, sugars are compatible osmolytes that can be tolerated at relatively high concentration without harm to cells. Sugar accumulation in cells contributes toward creating a more negative solute component of water potential during salinity, and thereby helps to maintain the osmotic balance of cells either through increased influx of water or by reducing the efflux of water, thereby protecting the turgidity of the cell (Hasegawa et al., 2000). This limits water loss such that cells are able to maintain a positive turgor in the face of low water potential, or at least avoid extreme tissue shrinkage. In cases of extreme salt concentration, which destabilizes proteins and membrane systems, sugars are capable of interacting with proteins and membrane systems to stabilize their structure and lessen salinity damage (Hoekstra et al., 2001). Sucrose is a non-reducing sugar, and as such is somewhat more stable than reducing sugars such as glucose and fructose. In addition to sucrose, salinity has been reported

to increase glucose concentrations in *Zygophyllum album* L. (El-Shourbagy and Kishik, 1975). One of the studies in rice reported that salinity increased glucose levels in leaves and root tissues in both tolerant and susceptible genotypes (Cha-Um et al., 2009). They also reported that the tolerant genotype accumulated more glucose than the susceptible genotype. Many studies have reported accumulation of sugars in plant tissues during salinity stress (El-Shourbagy and Kishik, 1975; Dubey and Singh, 1999; Kerepesi and Galiba, 2000). For example, in wheat, osmotic and salinity stress resulted in accumulation of water soluble carbohydrates including sucrose, glucose and fructose in shoots (Kerepesi and Galiba, 2000; Amirjani, 2011). Even though many studies have been done related to salinity tolerance in rice, there is still little information on carbohydrate accumulation in response to salinity stress (Thitisaksakul and Maysaya, 2008).

Shoot length

Plant cells grow by turgor induced expansion. Such growth is among the most sensitive processes in response to slight decreases in tissue water potential, which is one component of salt stress. Shoot length is an easily measured trait, and many studies have indicated that salinity negatively affects shoot length (Wang et al., 2011; Bimpong et al., 2014). Salinity often reduces shoot growth by 50 to 60% (Albacete et al., 2008).

Root architecture

The importance of roots for nutrient and water acquisition is well acknowledged, though less well understood than above-ground shoot processes. Some

researchers believe that improvement of root function will be the key for the second green revolution (Virginia, 2010). However, for decades breeding for root traits has received little attention due to the time, expense and phenotyping complexity for root related traits (Toyofuku et al., 2015). Many earlier studies focus only on primary root growth in response to stress conditions but in reality 90% of total root length is composed of lateral roots (Yamauchi et al., 1987). Researchers have proposed different root system architectures for better water and nutrient acquisition (Banoc et al., 2000; Lynch, 2013; Zhan et al., 2015). This emphasizes how important it is to understand more about root architecture, including primary and lateral root growth, branching patterns, and response to different stresses.

From the previous literature it is known that rice is an important staple crop, perhaps the most susceptible cereal to salinity stress especially at the early seedling stage; a species for which little is known about osmotic tolerance of salinity stress. Therefore this study focuses on identifying quantitative trait loci related to early seedling stage tolerance to the osmotic component of salinity stress. Seedling stage salinity tolerant varieties will also enable the direct seeding of rice in salt affected areas, which would be very valuable in the agriculture context.

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CHAPTER 2

MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES AT PRE-EMERGENCE SEEDLING STAGE DUE TO SALINITY STRESS IN A RICE POPULATION

Introduction

There are three major salinity tolerance mechanisms in plants – ion exclusion, osmotic tolerance and tissue tolerance (Munns and Tester, 2008). Among all three mechanisms, ion exclusion and tissue NaCl tolerance mechanisms have been fairly well studied, whereas osmotic tolerance mechanisms in relation to salinity are poorly understood (Roy et al., 2014). Osmotic effects are observed soon after a plant is exposed to a salinity treatment, before significant accumulation of salt ions, and are attributed to the lower water potential represented by the saline medium (Munns and Tester, 2008). While such effects are expected to be the same as those due to lowered tissue water potential in drought stress and desiccation, the evidence is not always clear-cut. In many cases, salinity responses due to osmotic effects have been clearly separated experimentally from those due to ionic effects and can be evaluated (Munns et al., 1995). In other cases, Na ions are taken up apoplastically and accumulated quickly in the leaf tissues, thereby confounding the osmotic and ionic effects (Yeo et al., 1987). Another factor is that when leaves transpire, a large flux of water containing NaCl moves from the soil pores to the root surface. Given that exclusion is only partial, NaCl eventually enters the plant and is transported via the xylem to leaves, where it progressively accumulates to high levels. The current study avoided this additional confounding factor by growing seedlings in non-transpirational conditions where both root and shoot tissues were exposed to the saline test medium.

The objective of this study was to determine whether two rice genotypes (Azucena and IR64) that are generally considered sensitive to salinity, differ in their seedling growth characteristics and associated metabolic traits such as accumulation of ABA and sugars in the shoot meristem region. The relationships between these traits are also evaluated in a population of 174 progeny of Azucena X IR64.

The objectives of this chapter include: 1) testing a newly developed phenotypic platform to screen for pre-emergence seedling stage of rice and 2) identifying how salinity stress affects different morphological and physiological traits.

Materials and Methods

Germplasm

A recombinant inbred line (RIL) population consisting of 174 genotypes from the cross Azucena (japonica) x IR 64 (indica) was evaluated at the pre-emergence seedling stage in response to control and salt stress treatments. Advantages of phenotyping the plants at this early stage were that 1) the seedlings utilize stored nutrient reserves in the seed so stress effects on photosynthesis, which is a factor at later stages, do not confound the results, and 2) the shoot tissues had not yet emerged from the wetted medium and begun transpiration, thus avoiding complications that are faced in salinity studies that involve salt transport to and deposition in transpiring tissues.

Procedure for seed treatment

All the seeds were imbibed in distilled water for one day prior to seed treatment in partially submersed 96-well 0.3-mL plates (one seed per well) which was modified by boring holes at the base of each well. After imbibition, the plates were removed from the submersion water and the solutions were allowed to pass through, while seeds were retained. Then the plates were placed in a tray containing 0.8 % (w/v) sodium hypochlorite (mixed up by diluting commercial-grade sodium hypochlorite (Clorox, which has 5.25% [w/v] NaClO) by 1:10 with distilled water; Clorox Company, Oakland, CA, USA) and soaked for 5 minutes. The plates with the seeds were rinsed twice by placing them in a tray containing distilled water to remove the Na-hypochlorite on the seed surface. After washing, a slurry of captan (fungicide)

mixed with water was pipetted into each well containing the seed. The seeds were then used for sowing.

Apparatus

The seed were sown onto an apparatus constructed with the following materials.

1. Corrugated plastic sheet (twinwall, fluted polypropylene sheets) 61 x 33 cm, 0.4 cm thick
2. Clear thermoplastic polycarbonate (Lexan, General Electric Company, Schenectady, New York) sheet 61 X 33 cm, X 0.3 cm thick
3. Corduroy fabric (100% cotton) 66 x 41 cm, black, no wale, finely spaced tufts (2 mm)

Solutions

Control - Distilled water

Test solution - NaCl solution (-0.4 MPa) 97.35 millimolal (mmol/kg H_2O)

The corduroy fabric was spread on the corrugated plastic plate and then wetted with the respective test solution using a squeeze bottle. Then the seeds (12 seeds each plate) were placed in a straight line 10 cm from the top separated by equal distance (4.45 cm). Once the seeds were placed on the wet fabric, it was then covered by the transparent polycarbonate sheets and the entire sandwich was clamped together using binder clips (Staples, Framingham, MA, USA) near the four corners. The entire setup was then placed in a plastic tub with the bottom 5 cm immersed in each respective solution allowing the fabric to wick the solution and thereby supply nutrients and water to the roots.

Experimental procedure

Plates containing the seeds were placed in the plastic flat-lid tub with the respective solution and the tub covered with a lid to prevent evaporation and covered with a black cloth to exclude light. This apparatus was designed to mimic the seeds growing under soil during germination. After 6 days the plates were taken out from the tubs and the outer polycarbonate sheet was removed carefully without disturbing the seedlings for photography. Once the images were captured the plates were covered and placed back into the tub. Then using a squeeze bottle, solution (water) was delivered onto all plates which compensated for any evaporative loss during the growth and photography. At the 8th day after sowing the same method was followed for photography but after imaging, the tissues were harvested. Three tissues, namely root tip, shoot meristem and remaining shoot parts were harvested and stored separately.

Root and shoot length measured using photographic technique

The plates were placed on the base of a copy stand and photos were taken from above using a digital SLR camera (Canon Rebel). These images were used for the evaluation of morphological traits of the seedlings using an image analyzing software named Root Reader 2D, which was developed at Cornell University (Clark et al., 2013). Photos were taken at two different time periods of growth, one at the sixth day and the other at the eighth day after sowing.

ABA analysis

From each seedling, the basal portion of the shoot initiating from the seed was excised to a length of 0.5 cm and immersed into 300 uL of ice cold 80% (v/v) methanol; after harvesting, samples were stored at -20^{o} C. Later tissues were incubated at room temperature and then placed in a shaker for one hour to extract the metabolites into the 80% methanol. C18 reverse phase flash chromatography was used to fractionate the ABA from the methanol extract (Setter et al., 2001). The extracted ABA was measured using indirect enzyme linked immunosorbent assay (ELISA) (Setter et al., 2001). Following are the steps for conducting the ELISA (in brief). First, the 96 well ELISA plates are coated with a constant amount of ABA-bovine serum albumen antigen reagent. Second, the sample containing an unknown quantity of ABA and the anti-ABA primary antibody are added to the wells. The relative amount of primary antibody binding to the immobilized ABA compared to the freely dissolved ABA will correspond to the amount of test ABA concentration. Using a sandwich assay, the ABA reagent is quantified with the help of an enzyme (alkaline phosphatase) labelled secondary antibody which is directed against the primary antibody. The bound secondary antibody is quantified using an enzyme assay.

Carbohydrate assay

A peroxidase/glucose oxidase (PGO) coupled enzyme method (Setter et al., 2001) was used to quantify the glucose and sucrose present in the 80% methanol extract. To measure the glucose content, 200 µL of PGO reagent was added to an aliquot of the methanol extract and when the reaction was completed the absorbance

was measured at 490 nm using a plate reader (model 750, Cambridge Technology, Watertown, MA, USA). In order to measure the sucrose present in the extract, the enzyme invertase was first added to the aliquot which hydrolyzed the sucrose into glucose and fructose, then PGO was added to quantify the amount of total glucose from sucrose hydrolysis + original free glucose in the aliquot.

Results

Growth, morphology and metabolite traits are presented in Table 2.1 for the main effect of salinity treatment, using ANOVA to compare control and salt stress treatments averaged over all genotypes. Salinity decreased shoot length by 53, and 49% compared to controls at day 6 and day 8, respectively. In contrast, salinity decreased primary root length by 2.0 % at day 6 and 6.6% at day 8. The growth increment from 6 to 8 days after germination also showed that shoot growth rate was decreased by salinity to a greater extent that root growth rate: 41 versus 12%, respectively. While primary root growth was relatively insensitive to salt stress, lateral root growth was quite responsive. Salinity decreased the number of lateral roots at day 6 by 36%, and it decreased lateral root length by 60% at day 6. Lateral root count appeared to be more sensitive during the first 6 days of germination, than at 8 days after germination. The rate of increase in lateral root count between day 6 and 8 was 8.6% higher in the salinity treatment than in control.

Given the observed higher sensitivity of shoot growth than primary root growth to salinity stress, it was of interest to explore shoot tissues further. At young pre-emergence stages the shoot meristem region controls the growth, development and morphology of the shoot and hence any changes in that region would result in modifications in shoot growth. For this work, shoot meristems were dissected to include only the shoot meristem ± about 2 mm of surrounding leaf tissue. Total sugars, glucose and sucrose concentration on a dry weight basis in the shoot meristem region were 93, 74 and 104% higher, respectively, in salt stress conditions than control

Table 2.1 Comparison of physiological traits of shoot meristems, and root morphology of 174 recombinant inbred lines of rice from the Azucena x IR 64 population measured under control versus salt stress conditions

Traits	Trait explanation	Control treatment, Mean	NaCl treatment, Mean	p Value	Significance between treatments ANOVA [1]	Percent change from control
Shoot Meristem						
SM dw	Shoot meristem (dry weight), g shoot^{-1}	0.00055	0.00044	< 2.2e-16	***	-20.0
Metabolites in Shoot Meristem						
ABA	Shoot meristem abscisic acid, picomoles gdw^{-1}	217.38	320.18	< 2.2e-16	***	47.3
Glu	Shoot meristem glucose, micromoles gdw^{-1}	27.90	48.50	< 2.2e-16	***	73.8
Tot sug	Shoot meristem total sugar, micromoles gdw^{-1}	109.90	212.57	< 2.2e-16	***	93.4
Suc	Shoot meristem sucrose, micromoles gdw^{-1}	82.16	167.63	< 2.2e-16	***	104.0
Glu: suc ratio	Shoot meristem glucose:sucrose molar ratio	0.41	0.42	1.98E-13	***	2.7
Shoot length						
SL D6	Shoot length at 6 days after germination, cm	2.21	1.05	< 2.2e-16	***	-52.7
SL D8	Shoot length at 8 days after germination, cm	4.07	2.08	< 2.2e-16	***	-48.9
SGR 6-8d	Shoot growth rate from day 6 to day 8, cm	1.90	1.11	< 2.2e-16	***	-41.3
Primary root length						
RL D6	Primary root length at 6 days after germination, cm	5.92	5.80	0.13754	NS	-2.0
RL D8	Primary root length at 8 days after germination, cm	8.70	8.12	1.80E-07	***	-6.6
RGR 6-8d	Root growth rate from day 6 to day 8, cm	2.89	2.54	4.92E-10	***	-11.8
Lateral root count						
LRC D6	Number of lateral roots at day 6 (count)	10.09	6.50	< 2.2e-16	***	-35.6
LRC D8	Number of lateral roots at day 8 (count)	32.78	30.61	0.007007	**	-6.6
LRC 6-8d	Difference in the lateral root number from day 6 to day 8 (count)	22.32	24.24	0.000892	***	8.6
Lateral root length						
LRL D6	Total lateral root length at day 6 (cm)	27.12	10.94	< 2.2e-16	***	-59.6
LRL D8	Total lateral root length at day 8, (cm)	129.33	94.97	< 2.2e-16	***	-26.6
LRL 6-8d	Difference in the total lateral root length from day 6 to day 8 (cm)	101.95	84.28	< 2.2e-16	***	-17.3

[1] "**" significant at 0.01 "***" significant at 0.001 "NS" Not significant

(Table 2.1). Salinity increased ABA levels by 47% in shoot meristems, which is consistent with its involvement in stress responses.

Genotypic differences

The parent lines were compared under control versus salt stress treatment in Figure 2.1. While IR64 and Azucena root lengths were similar on day 6, at day 8, IR64 had longer primary root length (RL8) and its root growth rate between day 6 and day 8 (RGR 6-8d) was about twice that of Azucena under both treatment conditions. Similarly, in controls, shoot lengths in the two genotypes were about the same on day 6, but shoot growth rate between day 6 and 8 (SGR 6-8d) in IR64 was about twice that of Azucena. However, under salt stress both genotypes had low shoot growth rates, consistent with the substantial inhibition of shoot growth that was observed in the population as a whole (Table 2.1). Also consistent with the population, both Azucena and IR64 root growth was relatively insensitive to salt stress (Figure 2.1).

In the shoot meristem tissue, ABA levels were higher in Azucena than IR64 in both control and NaCl condition, and ABA level in Azucena was significantly higher than IR64 under salt stress (Figure 2.1) In NaCl treatment, sucrose and total sugar content were significantly higher in both Azucena and IR64, whereas glucose content was significantly higher only in IR64. Previous studies have reported that salt tolerant varieties accumulate sugars to higher concentration under salinity stress (Cha-Um et al., 2009). Increases in sugar concentration under salt stress may reflect the smaller growth rate and decreased accumulation of cell wall and other cellular constituents, while prioritizing the accumulation of sugar, which serves as osmoticum and as a

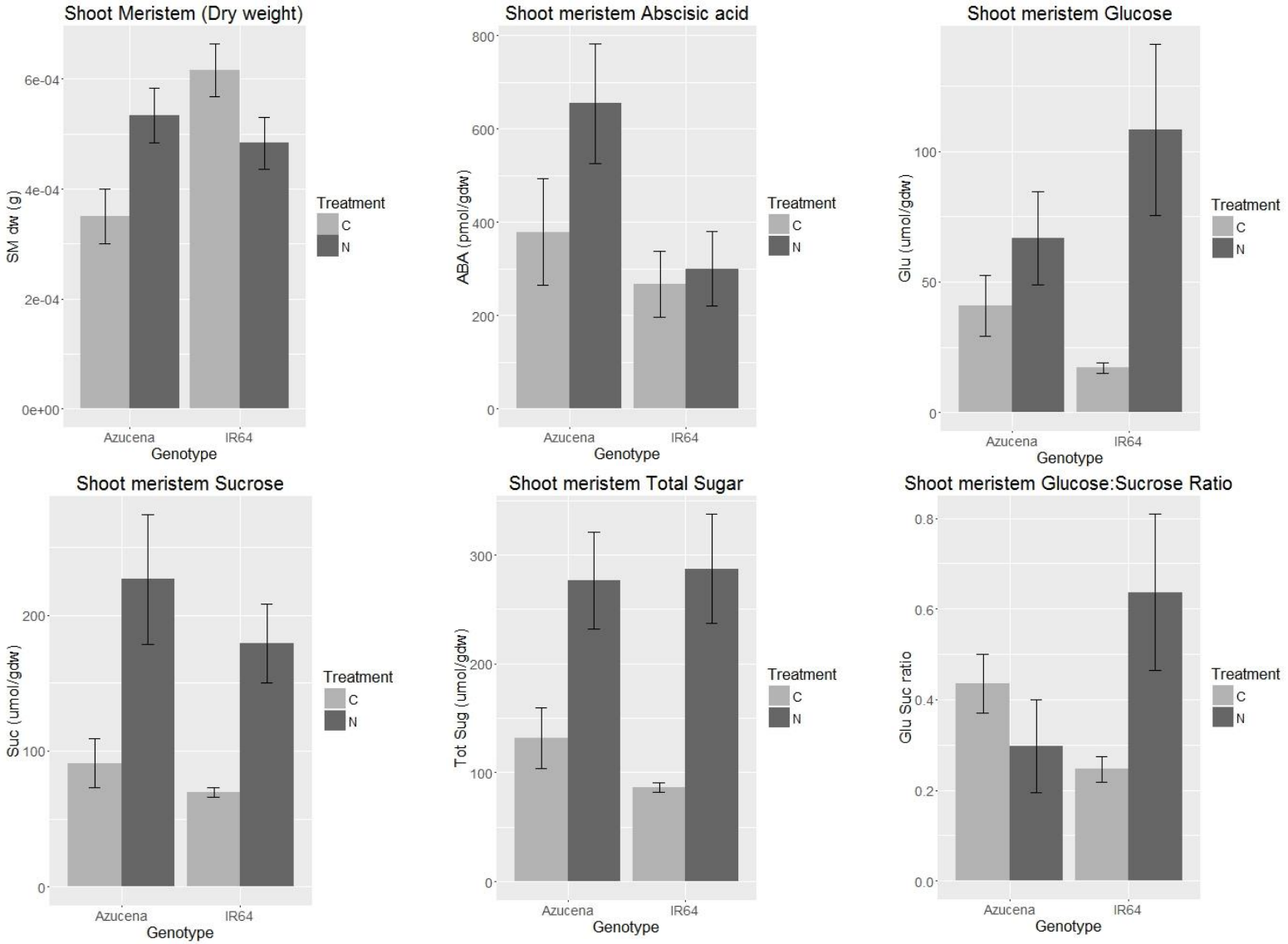


Figure 2.1. Shoot meristem dry weight, levels of abscisic acid and sugars in shoot meristem, shoot length, root length and lateral root traits in two of the parents, Azucena and IR 64, measured under control (C) and NaCl stress (N) condition. Light grey bars represent control measures and the dark grey represents salinity stress measures. Means ± SEM are shown.

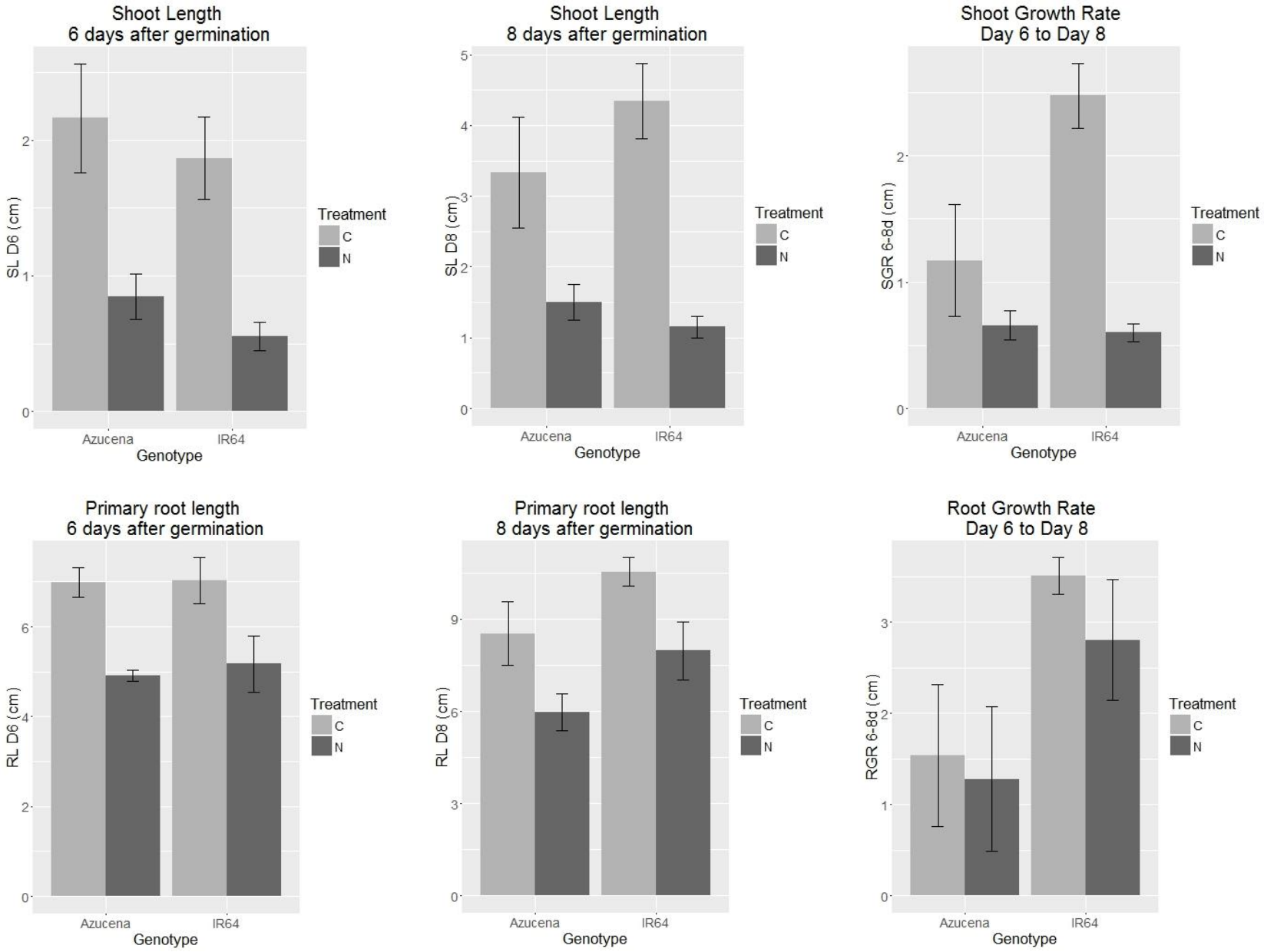
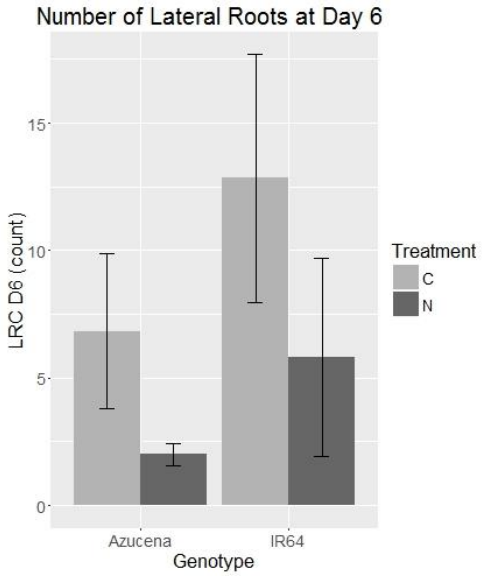
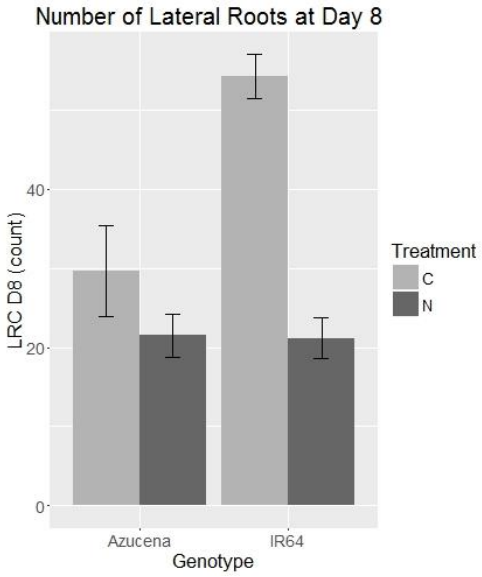
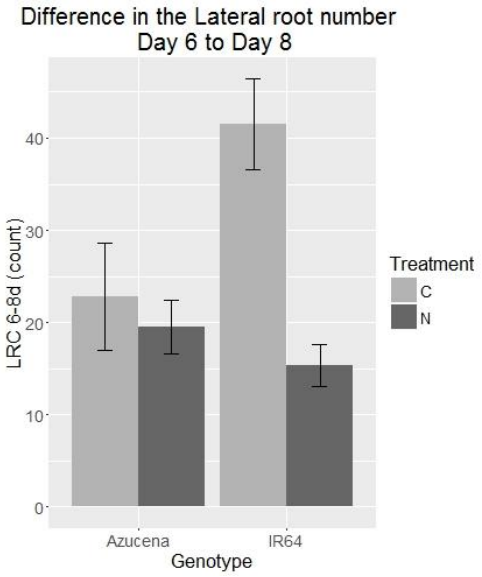
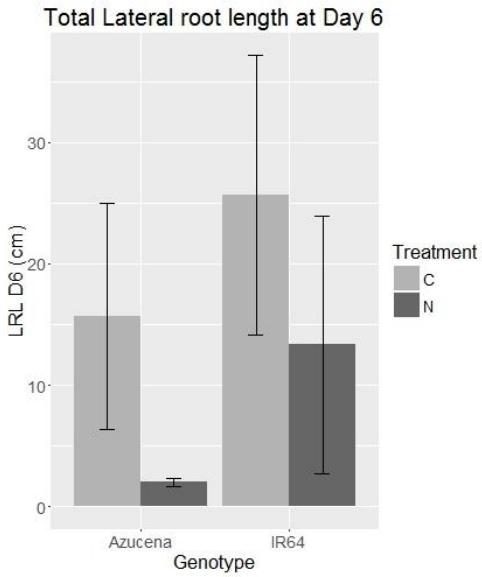


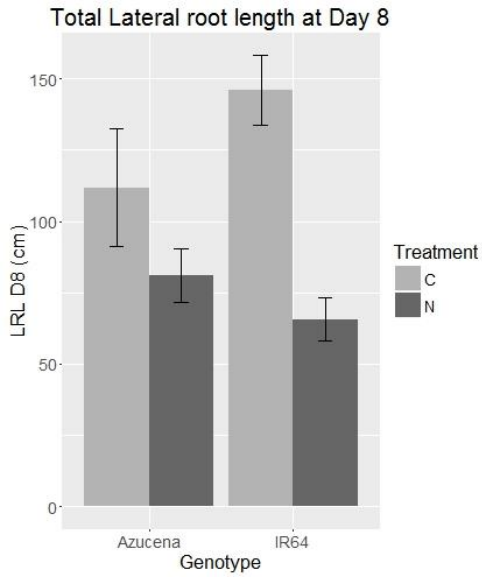
Figure 2.1 (continued)











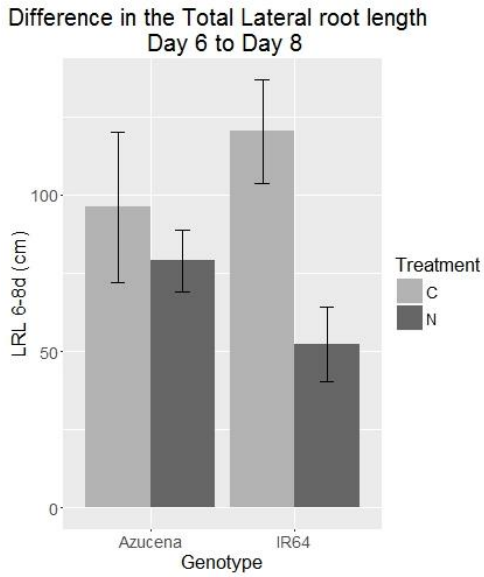


Figure 2.1 (continued)

stabilizer of protein and membrane structure (Hasegawa et al., 2000; Hoekstra et al., 2001).

Histogram results

To evaluate the distribution of responses in the population, histograms for different traits under control and salt stress were constructed with the phenotypic value of the trait in the x-axis and the frequency of genotypes in the y-axis (Figure 2.2). The arrow marks indicate the phenotypic value of each parent in the distribution. Histograms depict the variation in the RIL lines in comparison with that of the parents. Under salt stress, parental means for root and shoot growth traits tended to be in the lower half or one third of the distribution, while the population as a whole displayed considerable transgressive segregation with values shifted towards the upper half of the frequency distribution. In contrast, under control watering, root growth of parental lines tended to be on the upper half of the frequency range. The root and shoot growth rates between day 6 and 8 for the parents tended to span the range of the population and there was little evidence for transgressive segregation.

Under control watering, lateral root counts (LRC) and lateral root lengths (LRL) were considerably higher for IR64 than Azecena; however, under salt stress lateral root counts in IR64 declined substantially while Azecena declined slightly so that the two parental lines converged on about the same values (Figure 2.1). The lateral root counts of the RIL population showed considerably more spread than the parental lines (Figure 2.2).

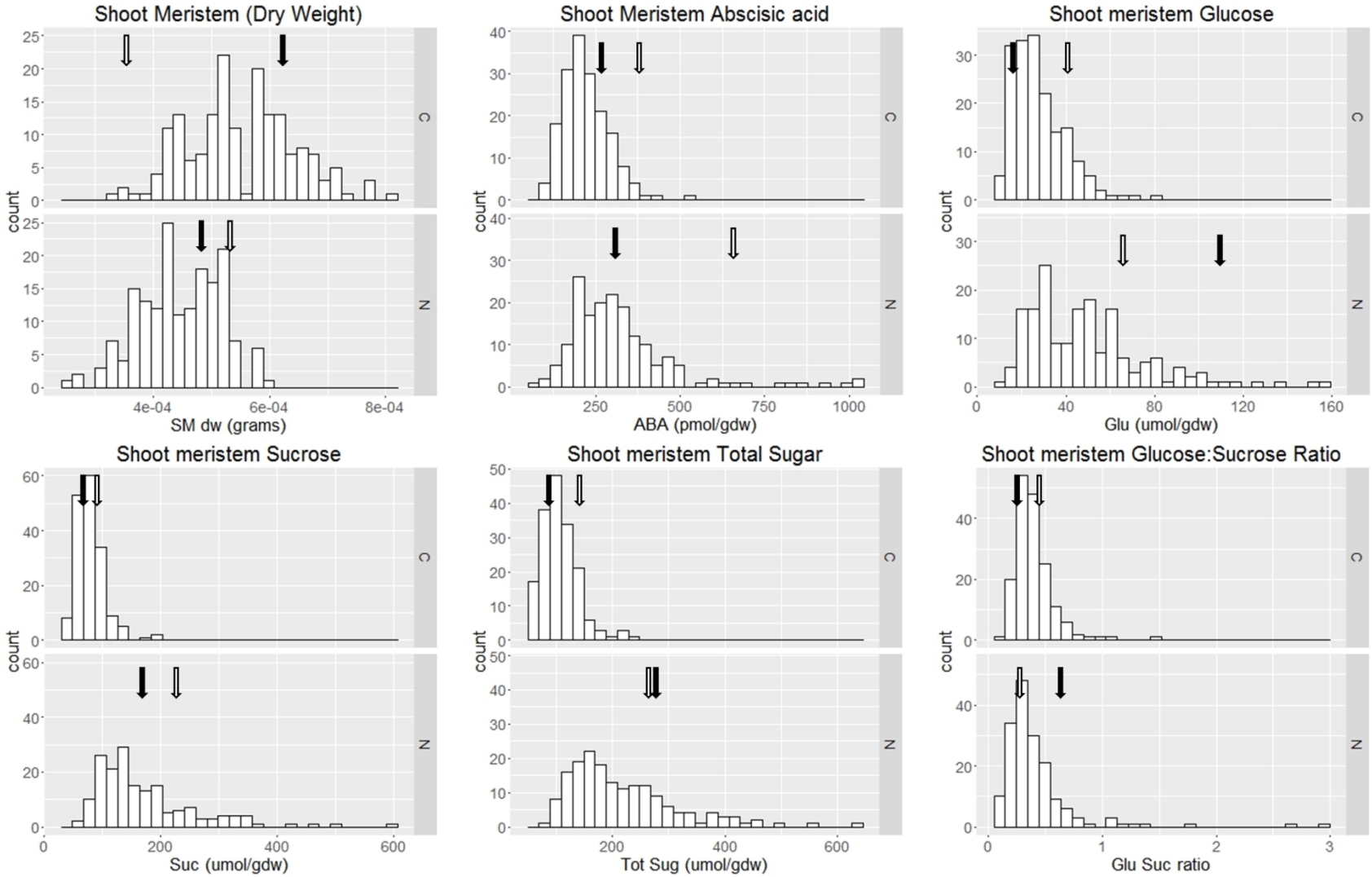


Figure 2.2 Frequency distribution of physiological traits under control watering (C) and salt stress (N) in 174 recombinant inbred lines of rice from the Azucena x IR64 population. Arrows indicate the mean of traits for the two parents, Azu (Azucena) and IR 64

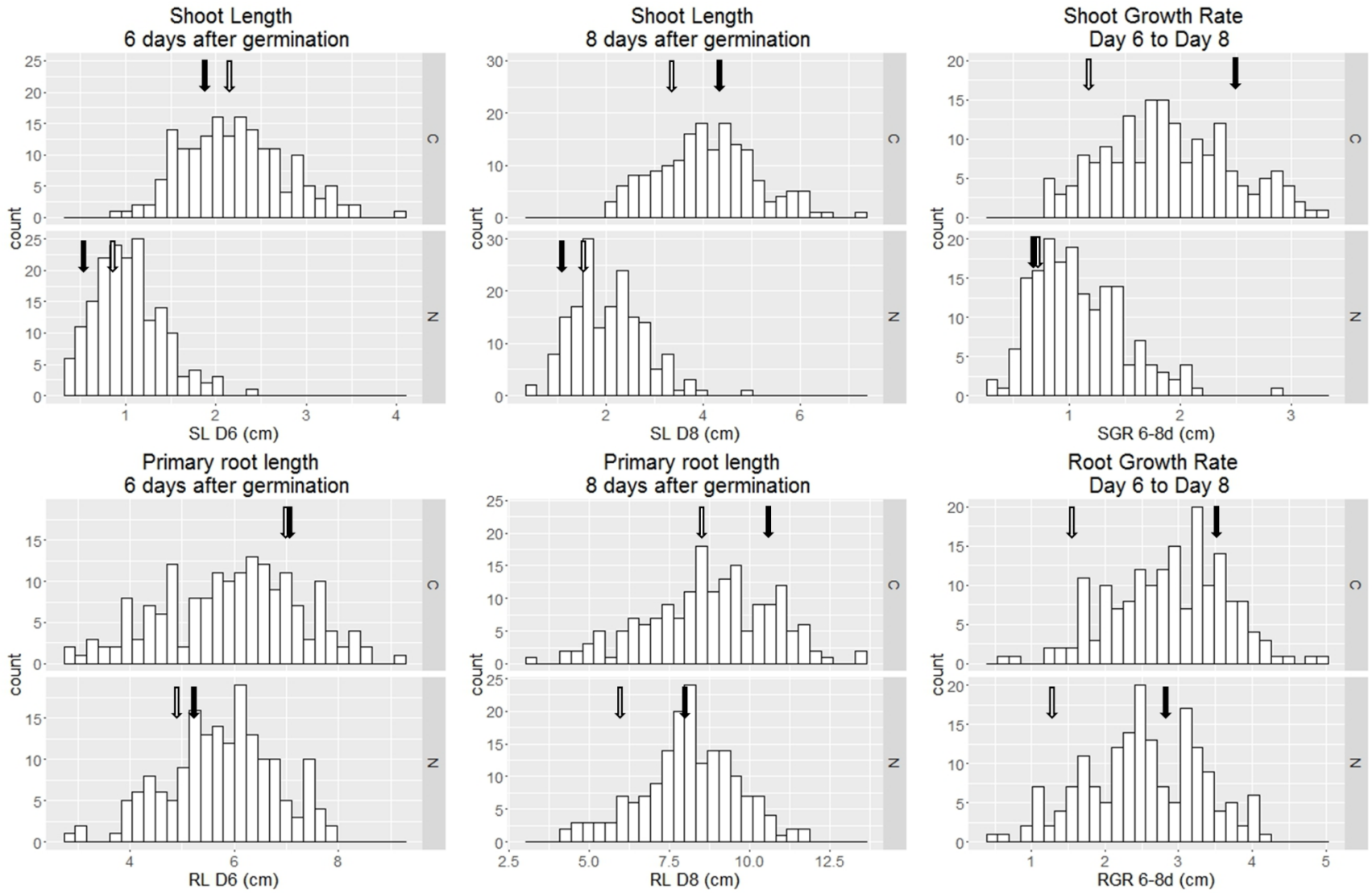


Figure 2.2 (continued)

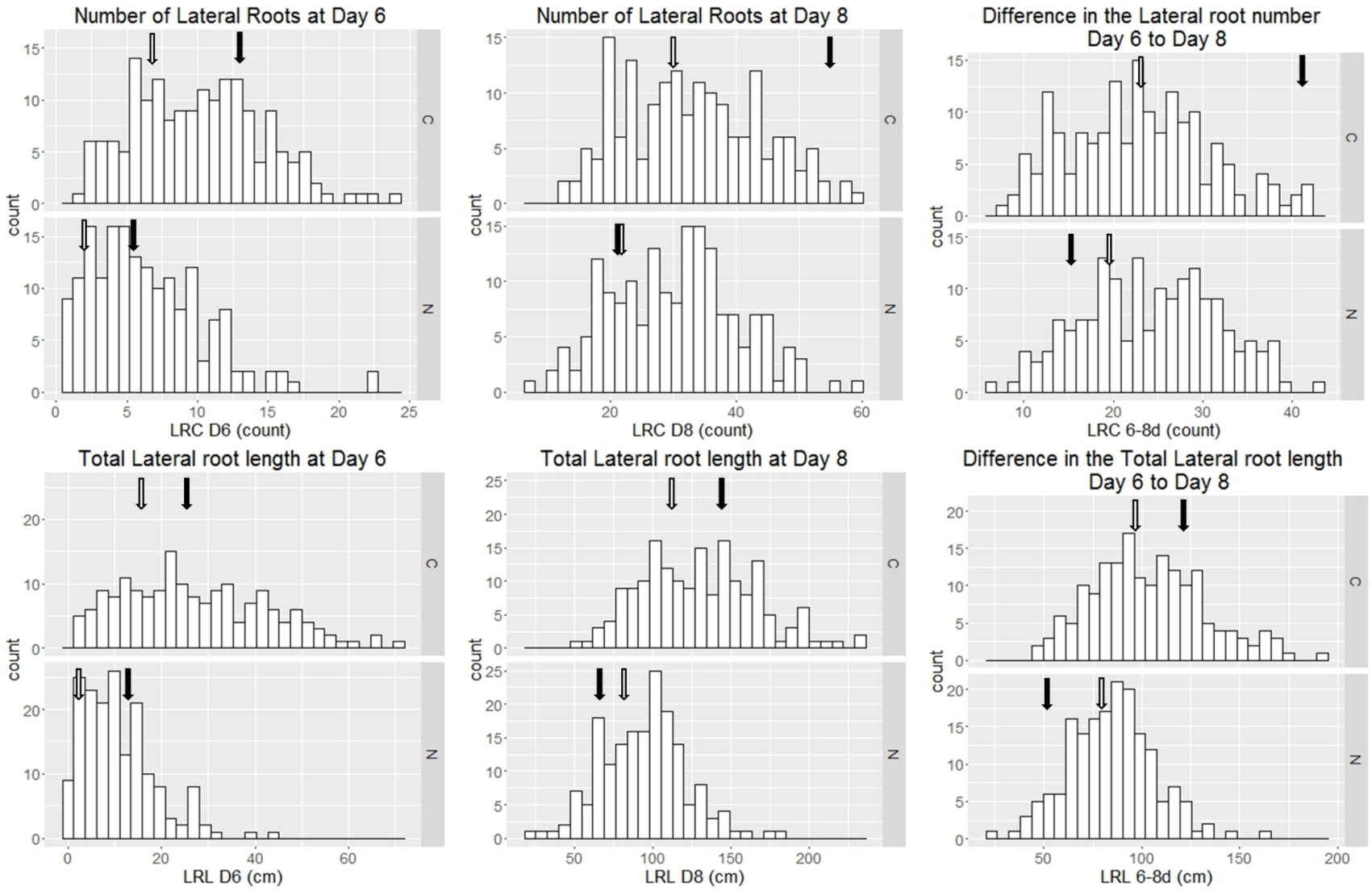


Figure 2.2 (continued)

In the salt stress treatment the parental means for glucose, sucrose and total sugars were also towards the higher end of the frequency distribution. Both these conditions indicate that there are many lines that fall outside the range of the parents confirming the presence of transgressive segregation for the traits.

Scatter and box plots

Scatter plots were constructed to relate the values of each genotype in the population under control vs salt stress (Figure 2.3). Scatter plots for shoot meristem abscisic acid, glucose, sucrose and total sugar content exhibits that data points were skewed towards the NaCl treatment (N) axis which indicated that many genotypes have higher values under salt stress. Scatter plots of shoot length and total lateral root length related traits exhibit that data points were skewed towards the control treatment (C) axis which indicated that many genotypes have lower values under salt stress. All other traits scattered similarly on both control (C) and NaCl (N) axes. The box plots were constructed (Figure 2.4) for the phenotypic traits to observe the positions of the genotypes with respect to the mean and median. Under salt stress, sugar concentrations in the population had a more skewed distribution, with some genotypes displaying extremely high values.

Correlation matrix

To understand the correlation between the various phenotypic traits, a correlation matrix was constructed using Pearson's correlation coefficients (Figure 2.5). The primary root length was positively correlated (ρ between 0.50 and 0.66)

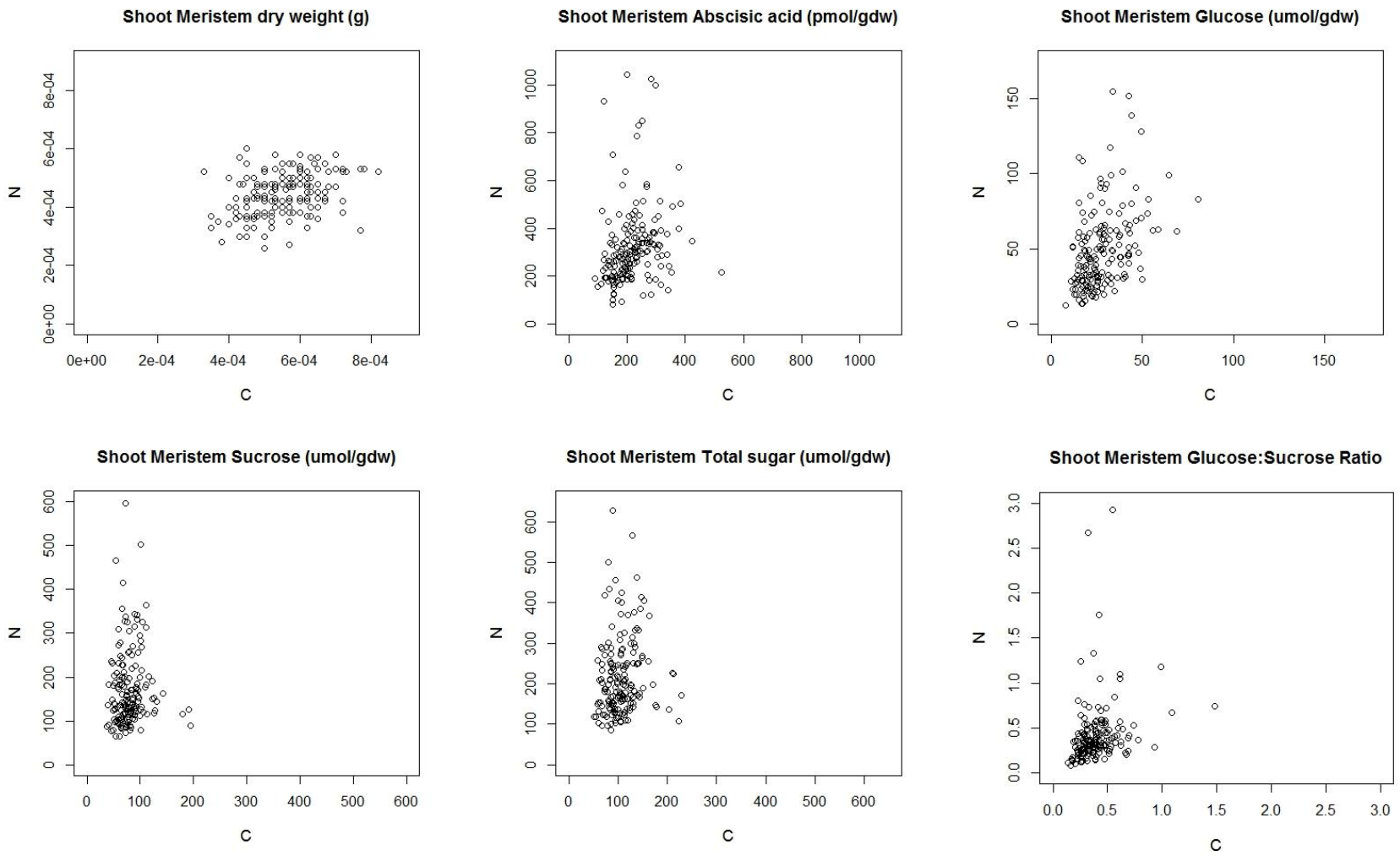


Figure 2.3 Scatter plots for phenotypic traits of 174 recombinant inbred lines of rice from the Azucena x IR64 population where values of genotypes under control (C) and salinity stress (N) are plotted against each other

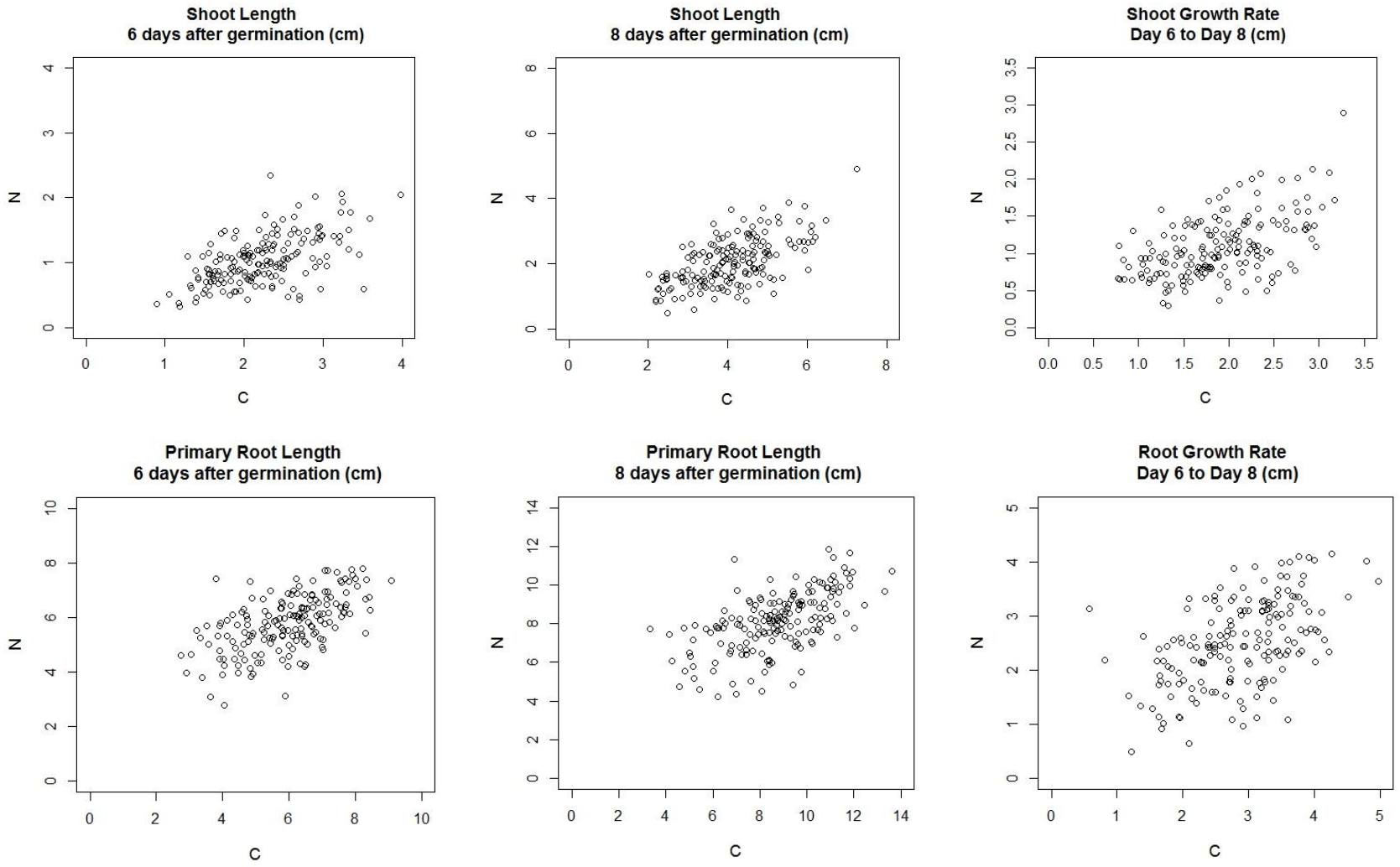


Figure 2.3 (continued)

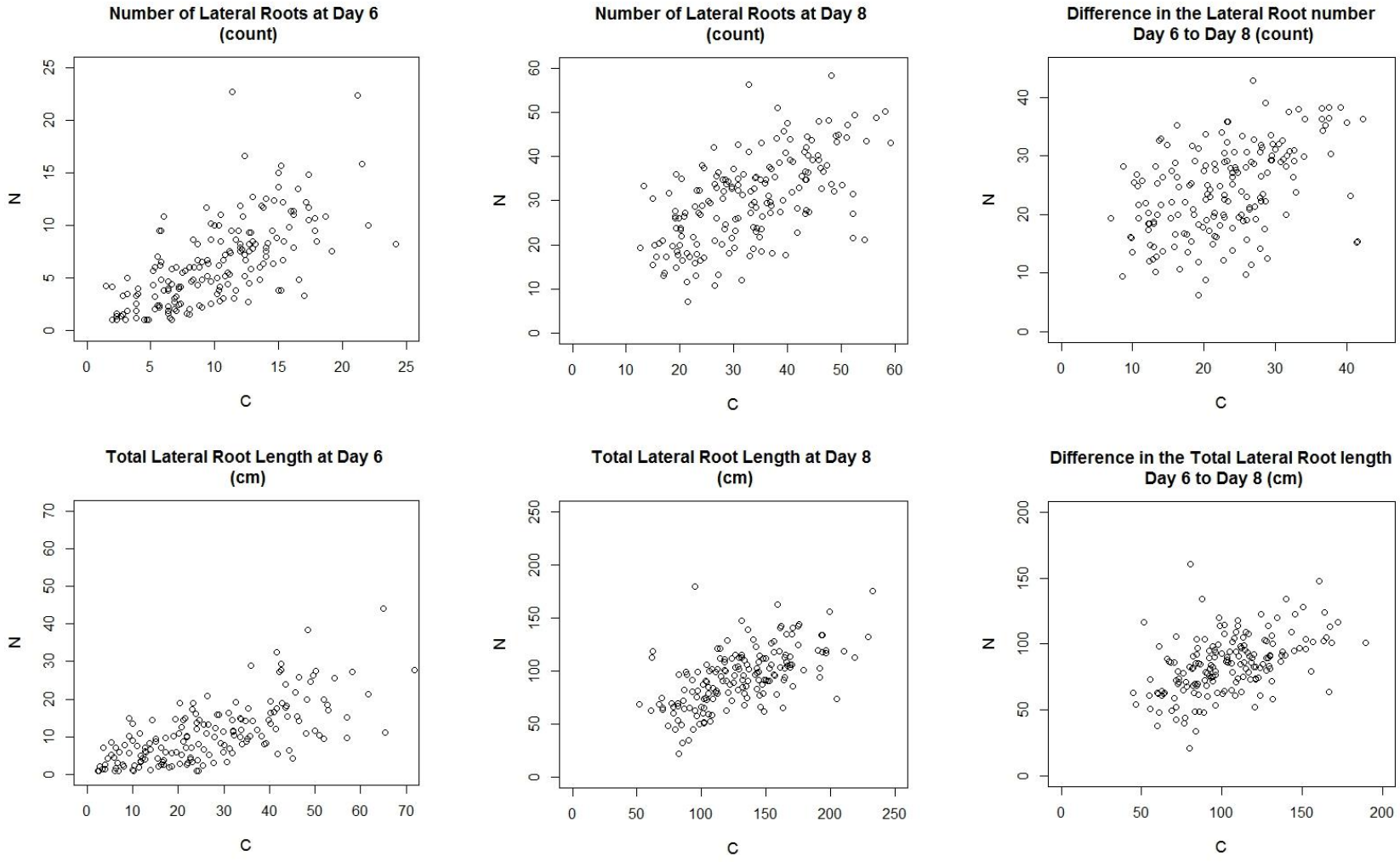


Figure 2.3 (continued)

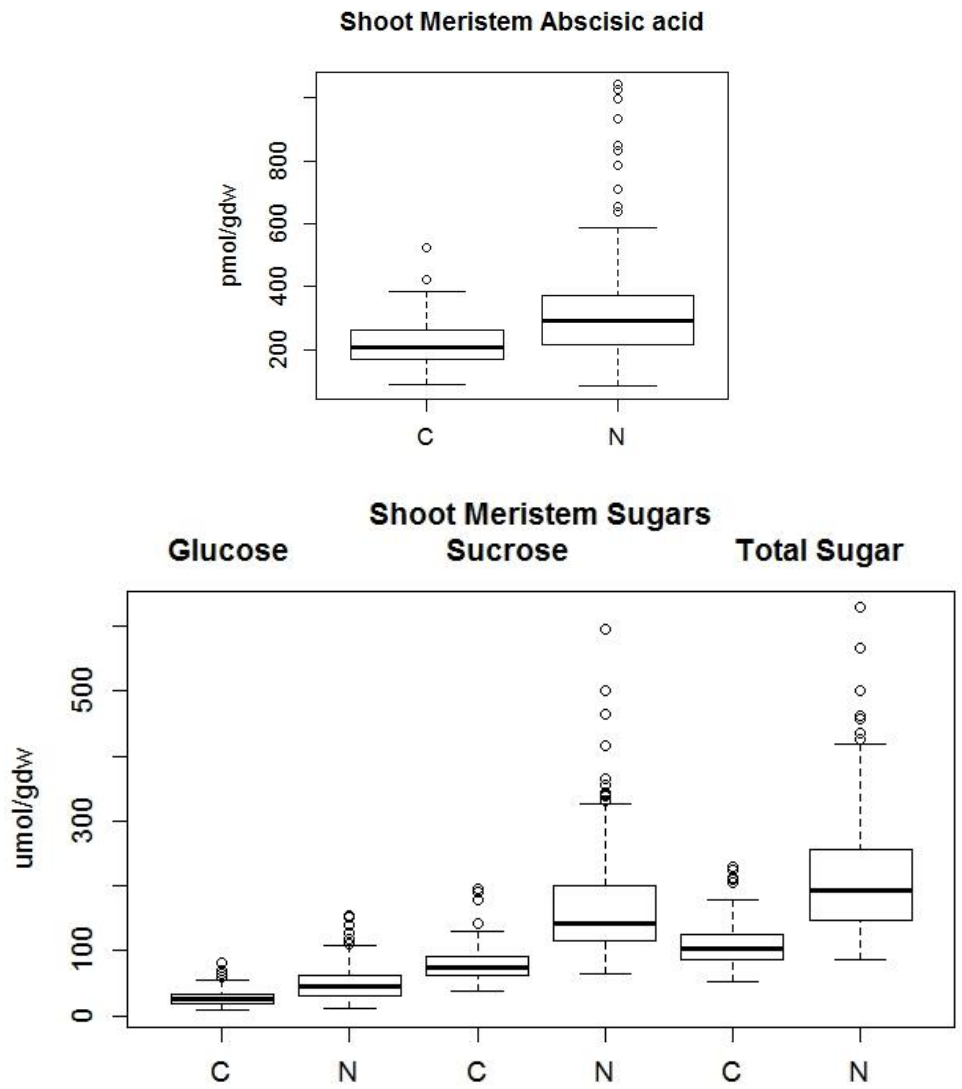


Figure 2.4 Boxplots of 174 recombinant inbred lines of rice from the Azucena x IR64 population for different phenotypic traits under control condition (C) and salinity stress condition (N)

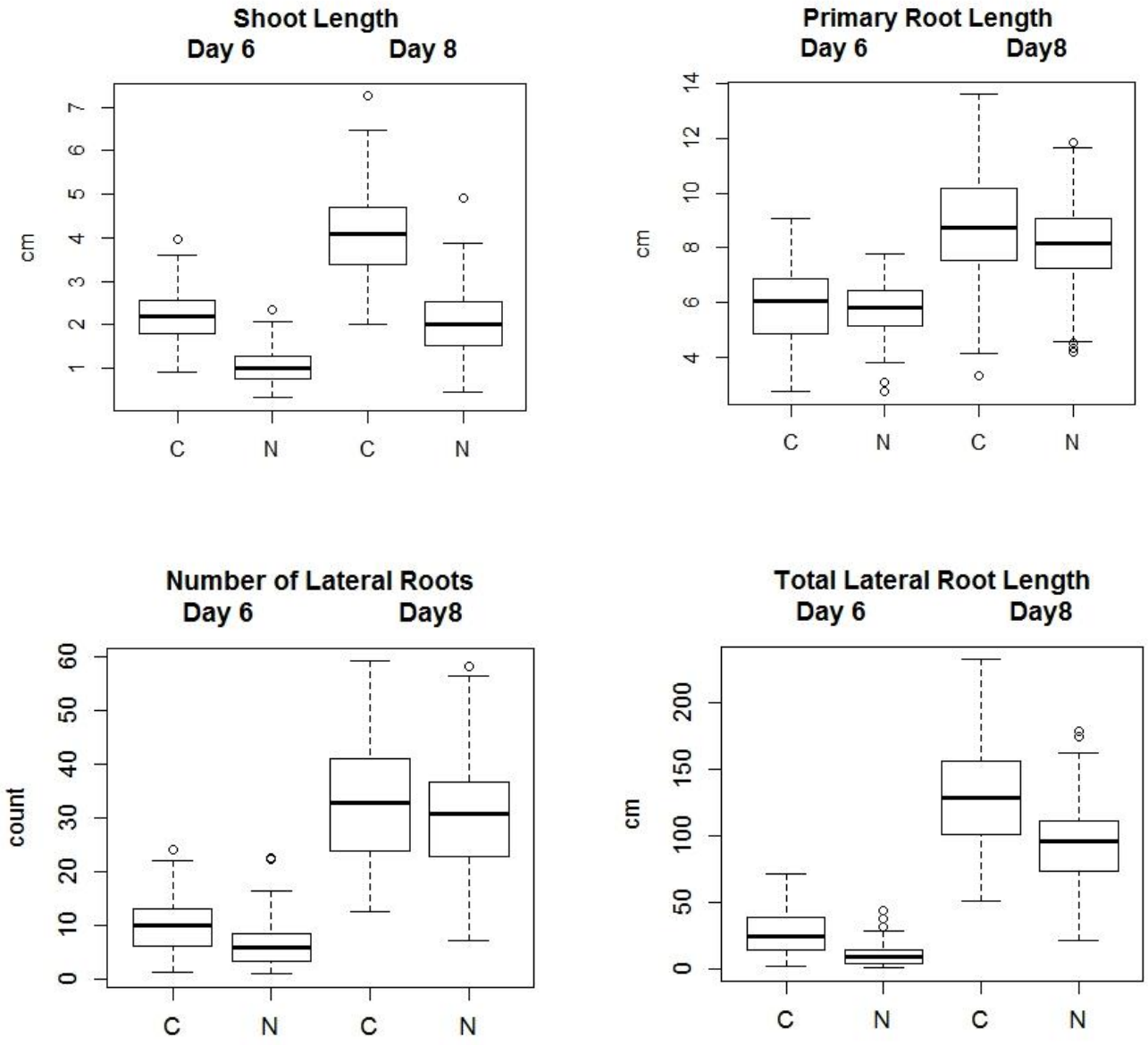
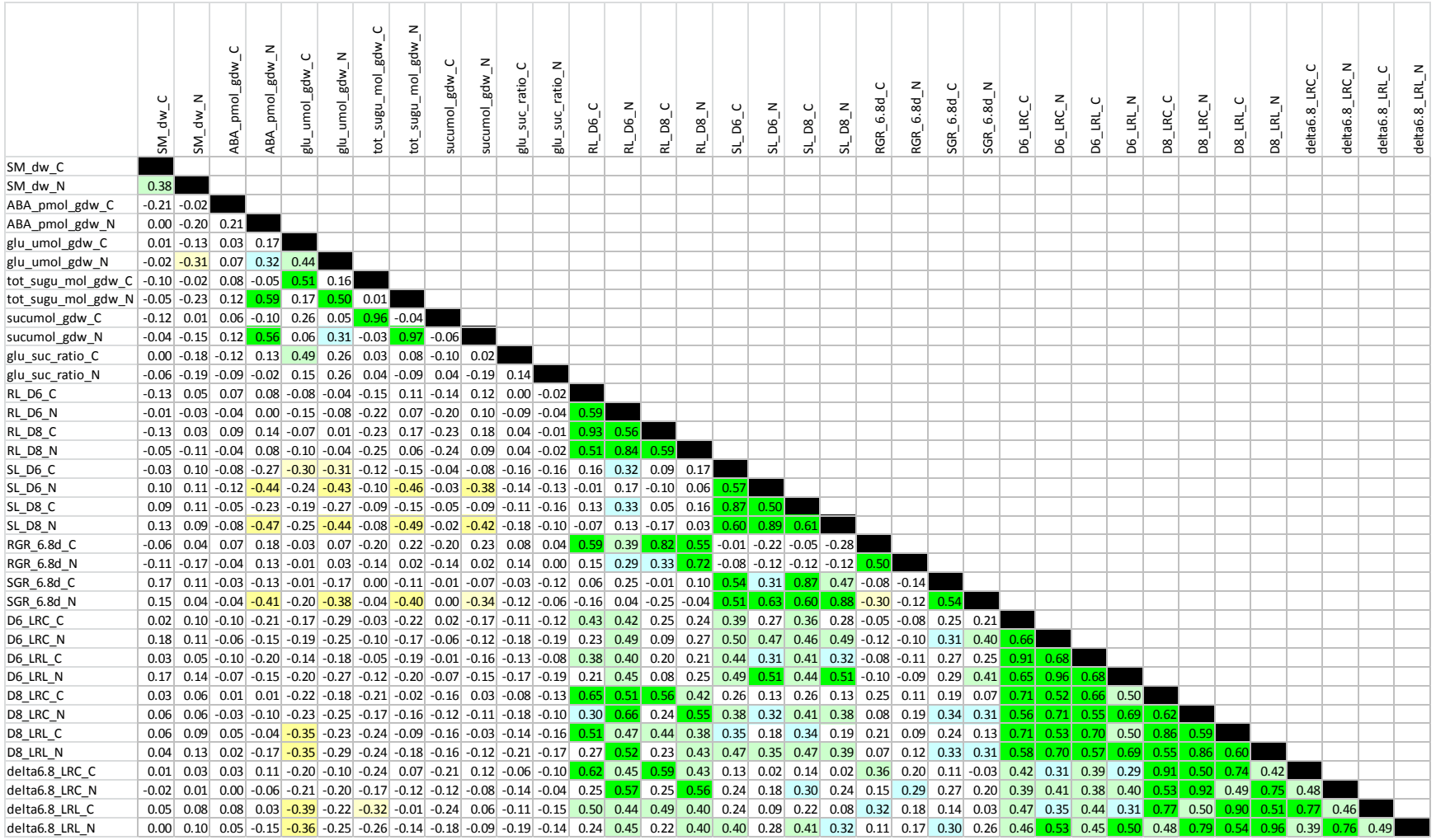


Figure 2.4 (continued)



	SM_dw_C	SM_dw_N	ABA_pmol_gdw_C	ABA_pmol_gdw_N	glu_umol_gdw_C	glu_umol_gdw_N	tot_sugu_mol_gdw_C	tot_sugu_mol_gdw_N	sucumol_gdw_C	sucumol_gdw_N	glu_suc_ratio_C	glu_suc_ratio_N	RL_D6_C	RL_D6_N	RL_D8_C	RL_D8_N	SL_D6_C	SL_D6_N	SL_D8_C	SL_D8_N	RGR_6.8d_C	RGR_6.8d_N	SGR_6.8d_C	SGR_6.8d_N	D6_LRC_C	D6_LRC_N	D6_LRL_C	D6_LRL_N	D8_LRC_C	D8_LRC_N	D8_LRL_C	D8_LRL_N	delta6.8_LRC_C	delta6.8_LRC_N	delta6.8_LRL_C	delta6.8_LRL_N
SM_dw_C																																				
SM_dw_N	0.38																																			
ABA_pmol_gdw_C	-0.21	-0.02																																		
ABA_pmol_gdw_N	0.00	-0.20	0.21																																	
glu_umol_gdw_C	0.01	-0.13	0.03	0.17																																
glu_umol_gdw_N	-0.02	-0.31	0.07	0.32	0.44																															
tot_sugu_mol_gdw_C	-0.10	-0.02	0.08	-0.05	0.51	0.16																														
tot_sugu_mol_gdw_N	-0.05	-0.23	0.12	0.59	0.17	0.50	0.01																													
sucumol_gdw_C	-0.12	0.01	0.06	-0.10	0.26	0.05	0.96	-0.04																												
sucumol_gdw_N	-0.04	-0.15	0.12	0.56	0.06	0.31	-0.03	0.97	-0.06																											
glu_suc_ratio_C	0.00	-0.18	-0.12	0.13	0.49	0.26	0.03	0.08	-0.10	0.02																										
glu_suc_ratio_N	-0.06	-0.19	-0.09	-0.02	0.15	0.26	0.04	-0.09	0.04	-0.19	0.14																									
RL_D6_C	-0.13	0.05	0.07	0.08	-0.08	-0.04	-0.15	0.11	-0.14	0.12	0.00	-0.02																								
RL_D6_N	-0.01	-0.03	-0.04	0.00	-0.15	-0.08	-0.22	0.07	-0.20	0.10	-0.09	-0.04	0.59																							
RL_D8_C	-0.13	0.03	0.09	0.14	-0.07	0.01	-0.23	0.17	-0.23	0.18	0.04	-0.01	0.93	0.56																						
RL_D8_N	-0.05	-0.11	-0.04	0.08	-0.10	-0.04	-0.25	0.06	-0.24	0.09	0.04	-0.02	0.51	0.84	0.59																					
SL_D6_C	-0.03	0.10	-0.08	-0.27	-0.30	-0.31	-0.12	-0.15	-0.04	-0.08	-0.16	-0.16	0.16	0.32	0.09	0.17																				
SL_D6_N	0.10	0.11	-0.12	-0.44	-0.24	-0.43	-0.10	-0.46	-0.03	-0.38	-0.14	-0.13	-0.01	0.17	-0.10	0.06	0.57																			
SL_D8_C	0.09	0.11	-0.05	-0.23	-0.19	-0.27	-0.09	-0.15	-0.05	-0.09	-0.11	-0.16	0.13	0.33	0.05	0.16	0.87	0.50																		
SL_D8_N	0.13	0.09	-0.08	-0.47	-0.25	-0.44	-0.08	-0.49	-0.02	-0.42	-0.18	-0.10	-0.07	0.13	-0.17	0.03	0.60	0.89	0.61																	
RGR_6.8d_C	-0.06	0.04	0.07	0.18	-0.03	0.07	-0.20	0.22	-0.20	0.23	0.08	0.04	0.59	0.39	0.82	0.55	-0.01	-0.22	-0.05	-0.28																
RGR_6.8d_N	-0.11	-0.17	-0.04	0.13	-0.01	0.03	-0.14	0.02	-0.14	0.02	0.14	0.00	0.15	0.29	0.33	0.72	-0.08	-0.12	-0.12	-0.12	0.50															
SGR_6.8d_C	0.17	0.11	-0.03	-0.13	-0.01	-0.17	0.00	-0.11	-0.01	-0.07	-0.03	-0.12	0.06	0.25	-0.01	0.10	0.54	0.31	0.87	0.47	-0.08	-0.14														
SGR_6.8d_N	0.15	0.04	-0.04	-0.41	-0.20	-0.38	-0.04	-0.40	0.00	-0.34	-0.12	-0.06	-0.16	0.04	-0.25	-0.04	0.51	0.63	0.60	0.88	-0.30	-0.12	0.54													
D6_LRC_C	0.02	0.10	-0.10	-0.21	-0.17	-0.29	-0.03	-0.22	0.02	-0.17	-0.11	-0.12	0.43	0.42	0.25	0.24	0.39	0.27	0.36	0.28	-0.05	-0.08	0.25	0.21												
D6_LRC_N	0.18	0.11	-0.06	-0.15	-0.19	-0.25	-0.10	-0.17	-0.06	-0.12	-0.18	-0.19	0.23	0.49	0.09	0.27	0.50	0.47	0.46	0.49	-0.12	-0.10	0.31	0.40	0.66											
D6_LRL_C	0.03	0.05	-0.10	-0.20	-0.14	-0.18	-0.05	-0.19	-0.01	-0.16	-0.13	-0.08	0.38	0.40	0.20	0.21	0.44	0.31	0.41	0.32	-0.08	-0.11	0.27	0.25	0.91	0.68										
D6_LRL_N	0.17	0.14	-0.07	-0.15	-0.20	-0.27	-0.12	-0.20	-0.07	-0.15	-0.17	-0.19	0.21	0.45	0.08	0.25	0.49	0.51	0.44	0.51	-0.10	-0.09	0.29	0.41	0.65	0.96	0.68									
D8_LRC_C	0.03	0.06	0.01	0.01	-0.22	-0.18	-0.21	-0.02	-0.16	0.03	-0.08	-0.13	0.65	0.51	0.56	0.42	0.26	0.13	0.26	0.13	0.25	0.11	0.19	0.07	0.71	0.52	0.66	0.50								
D8_LRC_N	0.06	0.06	-0.03	-0.10	-0.23	-0.25	-0.17	-0.16	-0.12	-0.11	-0.18	-0.10	0.30	0.66	0.24	0.55	0.38	0.32	0.41	0.38	0.08	0.19	0.34	0.31	0.56	0.71	0.55	0.69	0.62							
D8_LRL_C	0.06	0.09	0.05	-0.04	-0.35	-0.23	-0.24	-0.09	-0.16	-0.03	-0.14	-0.16	0.51	0.47	0.44	0.38	0.35	0.18	0.34	0.19	0.21	0.09	0.24	0.13	0.71	0.53	0.70	0.50	0.86	0.59						
D8_LRL_N	0.04	0.13	0.02	-0.17	-0.35	-0.29	-0.24	-0.18	-0.16	-0.12	-0.21	-0.17	0.27	0.52	0.23	0.43	0.47	0.35	0.47	0.39	0.07	0.12	0.33	0.31	0.58	0.70	0.57	0.69	0.55	0.86	0.60					
delta6.8_LRC_C	0.01	0.03	0.03	0.11	-0.20	-0.10	-0.24	0.07	-0.21	0.12	-0.06	-0.10	0.62	0.45	0.59	0.43	0.13	0.02	0.14	0.02	0.36	0.20	0.11	-0.03	0.42	0.31	0.39	0.29	0.91	0.50	0.74	0.42				
delta6.8_LRC_N	-0.02	0.01	0.00	-0.06	-0.21	-0.20	-0.17	-0.12	-0.12	-0.08	-0.14	-0.04	0.25	0.57	0.25	0.56	0.24	0.18	0.30	0.24	0.15	0.29	0.27	0.20	0.39	0.41	0.38	0.40	0.53	0.92	0.49	0.75	0.48			
delta6.8_LRL_C	0.05	0.08	0.08	0.03	-0.39	-0.22	-0.32	-0.01	-0.24	0.06	-0.11	-0.15	0.50	0.44	0.49	0.40	0.24	0.09	0.22	0.08	0.32	0.18	0.14	0.03	0.47	0.35	0.44	0.31	0.77	0.50	0.90	0.51	0.77	0.46		
delta6.8_LRL_N	0.00	0.10	0.05	-0.15	-0.36	-0.25	-0.26	-0.14	-0.18	-0.09	-0.19	-0.14	0.24	0.45	0.22	0.40	0.40	0.28	0.41	0.32	0.11	0.17	0.30	0.26	0.46	0.53	0.45	0.50	0.48	0.79	0.54	0.96	0.39	0.76	0.49	

Figure 2.5 Pearson's correlation matrix of 174 recombinant inbred lines of rice from the Azucena x IR64 population of all phenotypic traits. The colors indicate the correlation coefficient ranges where dark green color represents 0.5 to 1.0, the light green represents 0.36 to 0.49, the light blue represents 0.29 to 0.35, the light yellow represents -0.31 to -0.34 and the dark yellow represents -0.35 to -0.5.

with the lateral root count and length under both treatment conditions. There was no correlation observed between shoot lengths and root lengths. However there were some significant positive correlations observed between the shoot lengths and the lateral root count and lateral root length under both treatment conditions and time periods with correlation values reaching 0.51.

ABA was positively correlated with glucose ($\rho = 0.32$), sucrose ($\rho = 0.59$) and total sugars ($\rho = 0.56$) under salinity stress but not correlated under control condition. It was observed that the shoot length was negatively correlated with ABA under salinity stress condition ($\rho = -0.47$), glucose ($\rho = -0.44$), sucrose ($\rho = -0.42$), and total sugars ($\rho = -0.49$).

Discussion

The objective of the study was to evaluate different phenotypic traits under salinity stress at the pre-emergence seedling stage of rice. The novel phenotyping method used in this study worked fine as we could see a clear difference between the control and salt stress among the genotypes. This study was focused on the pre-emergence seedling stage. Salt stress was imposed right from the start, as pre-emergence seedling stage salinity tolerance will benefit direct seeding in salt affected areas (Wang et al., 2011). Another novel feature of this study was to analyze ABA and sugar content in the shoot meristem region of the rice seedling. Effect of salinity stress on the primary root of rice has been reported by many studies but the lateral root response to salt tolerance is still not well studied. In this study, we have measured the lateral root length and count using non- destructive methods at two different time points.

Phenotyping for root system architecture is challenging and requires carefully designed phenotyping strategies (de Dorlodot et al., 2007). Root studies are often conducted using hydroponic techniques where the seedlings are grown in a tub of nutrient solution (Price and Tomos, 1997; Bimpong et al., 2014). Most root studies report how salinity affects the primary root length (Ghomi et al., 2013; Bimpong et al., 2014). None of the previous studies investigated lateral root growth under salinity stress. Also the earlier methods of quantifying root structures from fields and greenhouses were destructive and could capture information only at one point in time. This is due to the complexity and unavailability of phenotyping platforms for

quantifying lateral root growth under salt stress. The plant growth system used here involved a wetted fabric surface with a rough texture which provided a mechanical stimulus for growth. This was intended to more closely resemble the mechanical stimuli that roots experience in soil, and contrasts with the situation in hydroponics. The phenotypic setup and image processing software used in the study enabled us to quantify primary and lateral root traits in two dimensions at multiple time points non-destructively.

Root architecture

From this study we found that salinity causes significant decrease in lateral root length and lateral root number at the pre-emergence stage, which is in accordance with previous observations (Julkowska et al., 2014). The primary root length was the least affected trait due to salinity stress at pre-emergence stage. Interestingly, shoot length and lateral root length were moderately positively correlated with each other, with values of the correlation coefficient ranging up to 0.51 under the control and salinity stress conditions, whereas the correlations between the shoot length and primary root length were low. Therefore more attention has to be paid to the lateral root architecture rather than focusing only on the primary root length to develop salinity tolerant varieties. Lateral root growth was decreased by salt stress to a much greater extent than primary root growth. Similar results were observed in a study reporting that endodermal ABA signaling inhibits lateral root growth under salt stress in Arabidopsis (Duan et al., 2013). It is also possible that such a response might help plants conserve resources so that with limited carbohydrate for growth, more resources

will be directed to primary root growth, thus providing a better opportunity to grow deeply where more water supply might be found.

Shoot growth

Studies have reported that under mild osmotic stress, the root length tends to elongate whereas the shoot growth is inhibited (Westgate and Boyer, 1985; Wu and Cosgrove, 2000). Similar trends were observed in this study and shoot length was the most affected trait due to salt stress at pre-emergence stage. As found in other systems (Sharp et al., 2004), salt stress inhibited root growth relatively little, while shoot growth was substantially inhibited. This response has been interpreted as an adaptation that will partition carbohydrates away from shoots and toward roots, thus enhancing deep root growth and access to water at depth.

ABA in shoot meristem

ABA concentration in the shoot meristem was significantly ($P \leq 0.05$) increased in the population due to salinity stress. In a previous study, ABA increased to a greater extent and the increase was more prolonged in tolerant compared to susceptible plants (Moons et al., 1995). Exogenous ABA application helps in combatting the osmotic effects of salinity stress in rice (Sripinyowanich et al., 2013) and wheat (Gurmani et al., 2013). These reports suggest that genotypes with higher ABA levels will be more tolerant to salinity stress. However, it is also possible that ABA accumulation is a symptom of stress, and that the most severely stressed plants will accumulate ABA to the greatest extent.

Sugars in shoot meristem

Results from this study suggest that the sugar content in the shoot meristem region increased two-fold, which matches the findings from previous studies where researchers have quantified sugar content in the shoot region (Dubey and Singh, 1999; Kerepesi and Galiba, 2000; Amirjani, 2011) . One of the previous studies in wheat seedlings reported a similar increase in sucrose concentration in stems (non photosynthetic tissues) due to salinity stress and also reported that the rate of increase was higher in salinity tolerant varieties (Kerepesi and Galiba, 2000). This encourages us to propose the hypothesis that the reduction of the shoot length might be due to the accumulation of sugars at the meristem, lessening the carbon source for the seedlings to grow. This hypothesis may be supported by the negative correlation values of shoot length and sugar content in the shoot meristem region. The positive correlation of ABA and sugars indicates that both ABA and sugars are together responsible for combatting the salinity stress. This idea is contrary to a study which reported a positive correlation for plumular length and soluble sugars under salinity and high pH in switchgrass (*Panicum vitagatum* L.) (Liu et al., 2014). But in the previous study, the sugar content was estimated from entire shoot and not from shoot meristem region.

Finally, most of the traits analyzed in this study exhibited transgressive segregation. Presence of transgressive segregation has been previously reported for multiple salinity-related traits in a mapping population developed between Gharib (indica) and Sepidroud (indica) rice (Ghomi et al., 2013). Another study also reported strong presence of transgressive segregation in a rice population developed between IR29 (indica) and Hasawi (landrace) for plant height and root length under salinity

stress (Bimpong et al., 2014). The two parents used to develop the population in this study come from two different subpopulations of rice with variable genetic architecture. This is a primary reason for the population to express transgressive segregation although the parents were not significantly different for the suite of traits measured in this study. The additional variation due to transgressive segregation is a beneficial result as breeders do not typically cross extreme parents. This might also reveal new tolerance mechanisms distinct from those previously identified.

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CHAPTER 3

QUANTITATIVE TRAIT LOCUS MAPPING FOR PRE-EMERGENCE SEEDLING STAGE SALINITY TOLERANCE IN RICE

Introduction

Salinity tolerance is a quantitatively inherited and polygenic in nature making it a genetically complex trait (Jones and Qualset, 1984; Zhu, 2000). Traditional plant breeding and selection for salinity tolerance is limited by its genetically complex and polygenic nature (Roy et al., 2014). Thus molecular markers have the potential to assist breeding by identifying the salt tolerance regions and efficiently transferring the underlying genes or QTLs into high yielding cultivars (Munns et al., 2002).

The QTLs identified and used in rice breeding programs had previously come from Pokkali and Nona Bokra background and also most of the reported QTLs are related to ionic (Na^+ and/or Cl^-) tolerance (Gregorio, 1997; Ren et al., 2005). The identified QTLs for ionic component of salinity tolerance have also been successfully introgressed into elite cultivars (Luu TN et al., 2012; Ali et al., 2013; Huyen et al., 2013; Islam and Gregorio, 2013). Limited research has been done on identifying QTLs related to osmotic tolerance (tolerance to osmotic phase of salinity stress which occurs initially before ion accumulation) and so far no specific candidate gene has been identified for osmotic tolerance (Roy et al., 2014). By growing seedlings under non-transpirational conditions that do not promote ion accumulation in tissues, and by focusing on the initial 8 days after imbibition, when osmotic factors are thought to predominate, the current study is set up to identify QTLs for traits related to osmotic tolerance in the pre-emergence seedling stage of rice.

Identification of Saltol in Pokkali

The first major salt tolerance QTL was identified in rice at chromosome 1 by Gregorio (1997). In that study, an F8 recombinant inbred line (RIL) population was developed by crossing IR29, a salt susceptible improved rice variety (*indica*), and Pokkali, a salt tolerant traditional tall rice variety (*indica*). Around 206 AFLP markers were used in the genotyping with a map length of 181.4 cM and an interval size of 10.53 cM. Multiple QTLs related to salinity tolerance were identified. For example, QTL were found for high K^+ absorption, low Na^+ absorption and low Na/K absorption ratio on chromosomes 1, 3, 4, 10 and 12. A common QTL for all the three traits was located on chromosome 1 with a LOD score of 14.5 accounting for 64 – 80 % of the phenotypic variation. This QTL was named Saltol, which is the most important QTL for salinity tolerance. Pokkali (*Oryza sativa* ssp. *indica*) is the donor for the salinity tolerance allele at the Saltol locus on chromosome 1.

Following the discovery of Saltol, many salt tolerant QTL have been reported by using mapping populations and various molecular markers. In the last 20 years, research on mapping QTL for salt tolerance in rice has developed significantly (Zhang et al., 1995; Flowers et al., 2000; Prasad et al., 2000; Koyama et al., 2001; Sabouri and Sabouri, 2008; Islam et al., 2011). Different studies have used different mapping populations such as recombinant inbred lines (RILs), doubled haploids or $F_{2:3}$ derived from crossing two contrasting parents for salinity stress from the same or different subpopulations of rice (for example, *indica* x *japonica or indica* x *indica*).

The degree of salt tolerance varies with different growth stages and various mechanisms are responsible for salinity tolerance at specific growth stages. In rice the

major growth stages are: 1) germination stage, for which few studies have reported QTLs (Wang et al., 2011), 2) seedling stage, for which salinity tolerance QTLs were reported in many studies (Prasad et al., 2000; Lin et al., 2004; Islam et al., 2011) and, 3) mature stage, for which a few QTL have been reported (Khan et al.; Tiwari et al., 2016).

Similar studies on rice during germination and seedling stages

A seedling stage salt stress study in rice used doubled haploid lines developed from IR64 and Azucena (Prasad et al., 2000). They considered IR64 as a moderately tolerant parent and Azucena as a susceptible parent in the study. QTL mapping was done using 76 doubled haploid lines and AFLP markers. In total, 7 QTLs were mapped for traits such as seed germination (Chr 6 and 7), seedling root length (Chr 6), seedling dry matter (Chr 5, 6 and 10) and seedling vigor (Chr 6). The current work is the only similar study that has used the same two parents and their population to map QTLs for salinity tolerance at the pre-emergence seedling stage. The only common phenotypic trait between this study and that of Prasad et al. (2000) is seedling primary root length. Another study detected 16 QTLs related to salinity at the germination stage on chromosomes 2, 3, 4, 7, 8, 9 and 10 for imbibition rate at 12 and 24 hours, and germination percentage at 5 and 10 days (Wang et al., 2011). These investigators identified different QTLs for the same traits at different stages of development.

In most studies, it has been reported that the indica subspecies is generally more salt tolerant than the japonica subspecies (Prasad et al., 2000; Lee et al., 2003).

One of the studies compared morpho-physiological response of seven rice varieties which included Azucena and IR64 at 21 and 42 days after salinity stress. The authors of this study concluded that Moroberekan variety accumulated the highest Na content, Azucena variety was the most affected in shoot fresh and dry weights, and IR64 was neither tolerant nor susceptible (Haq et al., 2009). Another study rated 57 rice genotypes for seedling stage salinity and IR64 was placed in the moderately tolerant group (Krishnamurthy et al., 2014). Interestingly, one of the studies conducted by Wang et al. (2011) showed that the japonica rice Jiucaiqing was more tolerant to salt stress than indica IR26.

Root system architecture has been compared between IR64 and Azucena during the first 10 days of growth under gellan gum without any salinity treatment (Clark et al., 2011). The same study compared a number of root-related traits in gellan gum versus in other media such as hydroponic solution and sand. The researchers noticed that root architecture of the two genotypes responded differently to the rooting medium. IR64 had higher primary root length, lateral root number and average lateral root length in sand medium but not under hydroponics or the gellan gum system when compared to Azucena.

Salinity QTL study with SNP markers

In studies reported to date, the mapping of salinity tolerance genes was done only with AFLP, RFLP and SSR markers. Recently one of the first studies reported QTLs for salinity tolerance at the seedling stage using SNPs for QTL mapping (Bimpong et al., 2014). These authors used 142 F_5 RILs derived from an IR29 (indica) x Hasawi (indica) cross for screening with 384 SNP markers developed for the indica x indica background. The screening was done in the seedling stage for plant height, root length, fresh weight and dry weight. In total, seven QTLs were identified for three out of four traits on chromosome 1, 2 and 6.

The objective of the current study was to use a newly-developed high-density marker system for the Azucena X IR64 RIL population to identify QTL for root and shoot growth in normal water and salt stress conditions during pre-emergence seedling stage. In addition, we sought to identify QTL for metabolic traits: the levels of sugars and the stress hormone abscisic acid (ABA) in shoot apical meristems.

Materials and Methods

Mapping Population

The RIL population IR64 ×Azucena consisting of 176 $F_{10} – F_{12}$ RILs was developed through single seed descent in greenhouse conditions at IRD, Montpellier, France (Spindel et al., 2013). IR64 is a semi-dwarf variety that belongs to *O.sativa* ssp. *indica* subpopulation and Azucena is a tall variety that belongs to *O.sativa* ssp. *japonica* (Zhao et al. 2011). The two different subpopulations have contrasting traits and phenotypes and the population developed from the two parents contained a good amount of variation, which is very useful for mapping studies.

Morphological, metabolite, and ABA phenotyping

The population of 176 RILs from the cross of IR64 × Azucena, and their parents, were phenotyped as described in Chapter 1.

Marker Analysis and QTL Identification

The population was mapped by Spindel et al. (2013). These authors describe the process as follows. Young leaf tissue was collected from each of the 176 IR64 × Azucena RILs and the two parents (IR64 and Azucena) and DNA was extracted using the Qiagen 96-plex DNeasy kit as per the Qiagen fresh leaf tissue 96-plex protocol (http://www.qiagen.com/HB/DNeasy96Plant). The high density SNP markers in rice for IR64 (*indica*) x Azucena (*japonica*) were developed using 384 plex GBS protocol (Elshire et al., 2011). A total of 30,984 markers were added to the 176 recombinant

lines between IR64 x Azucena (Spindel et al., 2013). QTL mapping was performed using the R/QTL package (R version 2.15.1, R/qtl package 1.24.9).

QTL nomenclature

In this report we have followed the naming approach described by McCouch et al.(1997) where we used two to four capital letters denoting the measured phenotypic trait followed by a hyphen and the chromosome number and then followed by a hyphen and an additional number if more than one QTL was found on the same chromosome.

The phenotypic analysis was performed using 172 lines as a few of the lines were removed due to missing samples.

Results

In the current study a total of 52 QTLs were identified. Almost all traits exhibited some QTLs, except abscisic acid, and QTLs were mapped on all chromosomes except 5 and 6. Among the 52 QTLs mapped, 27 were identified in control conditions and 25 in stress conditions. Many QTLs were specific to either control or salinity conditions: 17 out of the 27 QTLs were only found in the control condition and 18 out of 25 QTLs were only found in the stress condition. Almost all of the traits exhibited a continuous distribution (See Chapter 1, Figure 2.2), which indicates that the traits are polygenic in nature.

QTLs in control condition

The 27 QTLs identified under control conditions were located on chromosomes 1, 3, 4, 7, 8, 9, 10, 11 and 12 with phenotypic variance of each individual QTL ranging from 7.2 to 18.5 % and LOD scores ranging from 3.7 to 9.9 (Table 3.1). Out of the 27 QTLs identified under control conditions, one QTL was identified for shoot meristem dry weight on chromosome 4; eight QTLs for shoot growth on chromosomes 1, 3, 8, 11 and 12; 13 QTLs for root growth traits on chromosomes 1, 4, 8, 9, 10 and 11; and five QTLs for sugar related traits on chromosome 1, 3 and 7.

For shoot length, different QTLs were mapped at different time points, indicating the presence of time-dependent QTLs. Shoot length when measured at six days of growth mapped QTLs on chromosomes 1 and 12 whereas shoot length at eight

Table 3.1 Putative QTLs for different traits under control conditions in the SSD RIL population derived from Azucena and IR64. QTLs marked in bold are unique to control conditions

No	Trait class	Trait	QTL name	Chr	Peak markers	Peak position (cM)	Peak Lod	Left Marker	Right marker	Left Position (cM)	Right position (cM)	% Phenotypic variance
1	Meristem	Shoot meristem (dry weight)	qSMD-4	4	S4_27848186	221.29	3.72	S4_27571156	S4_31157231	216.58	259.27	9.7
2	**Shoot**	**Shoot Length at 6 days after germination**	**qSL-1**	**1**	**S1_37922333**	**376.53**	**4.68**	**S1_35135733**	**S1_38642939**	**344.70**	**383.03**	**11.1**
3	**Shoot**	**Shoot Length at 6 days after germination**	**qSL-12**	**12**	**S12_21417101**	**143.39**	**3.86**	**S12_17910476**	**S12_24587723**	**114.56**	**180.01**	**9.1**
4	Shoot	Shoot Length at 8 days after germination	qSL-3-2	3	S3_36170560	418.95	6.58	S3_34908292	S3_36398607	396.46	420.12	13.2
5	Shoot	Shoot Length at 8 days after germination	qSL-8	8	S8_27220957	229.08	4.97	S8_26200666	S8_27854290	213.07	239.68	9.8
6	**Shoot**	**Shoot Length at 8 days after germination**	**qSL-11-1**	**11**	**S11_1852930**	**21.36**	**4.76**	**S11_1368001**	**S11_6196082**	**17.22**	**80.24**	**9.4**
7	Shoot	Shoot growth rate from Day 6 to Day 8	qSGR-3	3	S3_36170595	418.95	9.95	S3_35801182	S3_36398607	407.86	420.12	18.5
8	Shoot	Shoot growth rate from Day 6 to Day 8	qSGR-8	8	S8_27220957	229.08	5.71	S8_26267476	S8_27854290	214.55	239.68	10.0
9	**Shoot**	**Shoot growth rate from Day 6 to Day 8**	**qSGR-11**	**11**	**S11_2421109**	**28.43**	**7.14**	**S11_1609419**	**S11_2704193**	**20.77**	**37.29**	**12.8**
10	Root	Primary root length at 6 days after germination	qRL-1	1	S1_41298501	427.64	4.39	S1_40871639	S1_43248714	418.77	452.01	11.3
11	Root	Primary root length at 8 days after germination	qRL-1	1	S1_41271407	427.05	6.81	S1_40910413	S1_43248714	420.53	452.01	17.0
12	Root	Root growth rate from Day 6 to Day 8	qRGR-1	1	S1_41266450	427.05	7.52	S1_40910413	S1_43248714	420.53	452.01	16.2
13	**Root**	**Root growth rate from Day 6 to Day 8**	**qRGR-9**	**9**	**S9_21630557**	**243.90**	**5.39**	**S9_21383102**	**S9_22903307**	**239.14**	**253.99**	**11.3**
14	Root	Number of lateral roots at Day 8	qLRC-1	1	S1_41297743	427.64	3.74	S1_40706234	S1_41561988	413.37	431.20	7.3
15	**Root**	**Number of lateral roots at Day 8**	**qLRC-4**	**4**	**S4_9603569**	**58.55**	**3.70**	**S4_5611551**	**S4_15974198**	**43.78**	**89.25**	**7.2**
16	**Root**	**Number of lateral roots at Day 8**	**qLRC-10**	**10**	**S10_21591429**	**174.66**	**4.87**	**S10_21012536**	**S10_21721090**	**166.40**	**177.02**	**9.7**
17	**Root**	**Number of lateral roots at Day 8**	**qLRC-11**	**11**	**S11_9092189**	**104.54**	**3.76**	**S11_4362683**	**S11_17413632**	**64.90**	**166.50**	**7.4**
18	Root	Difference in the lateral root number from Day 6 to Day 8	qLRCR-1	1	S1_41217072	424.66	4.78	S1_40706234	S1_41387973	413.37	430.02	9.2
19	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR-4**	**4**	**S4_9603569**	**58.55**	**3.77**	**S4_5771830**	**S4_15974198**	**44.37**	**89.25**	**7.2**
20	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR-8**	**8**	**S8_19583597**	**131.63**	**4.43**	**S8_17155648**	**S8_22196154**	**122.80**	**160.74**	**8.5**
21	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR-11**	**11**	**S11_9092189**	**104.54**	**4.37**	**S11_8828792**	**S11_15888819**	**100.99**	**142.39**	**8.4**
22	**Root**	**Difference in the total lateral root length from Day 6 to Day 8**	**qLRLR-1**	**1**	**S1_40468832**	**410.43**	**4.23**	**S1_35154329**	**S1_41306236**	**345.29**	**428.23**	**10.9**
23	**Sugar**	**Shoot meristem Glucose**	**qGLU-1**	**1**	**S1_36543675**	**365.35**	**3.90**	**S1_4826553**	**S1_37116197**	**47.42**	**367.70**	**8.4**
24	**Sugar**	**Shoot meristem Glucose**	**qGLU-7**	**7**	**S7_13200749**	**118.54**	**5.90**	**S7_6061910**	**S7_15241600**	**96.69**	**124.44**	**13.1**
25	**Sugar**	**Shoot meristem Total Sugar**	**qSUG-1**	**1**	**S1_35764131**	**355.92**	**4.01**	**S1_10687413**	**S1_36972925**	**131.93**	**365.94**	**10.4**
26	**Sugar**	**Shoot meristem Glucose : sucrose molar ratio**	**qGSR-3**	**3**	**S3_27693150**	**294.67**	**3.87**	**S3_25325717**	**S3_31780606**	**262.65**	**340.72**	**8.8**
27	**Sugar**	**Shoot meristem Glucose : sucrose molar ratio**	**qGSR-7**	**7**	**S7_14648106**	**122.08**	**6.80**	**S7_11237073**	**S7_15243694**	**113.83**	**125.02**	**16.0**

days mapped QTLs on 3, 8 and 11. Root growth did not exhibit any time-dependent QTLs as both the time points mapped the same QTL on chromosome 1. Among the 13 QTLs mapped for root growth traits, four QTLs were for primary root length and nine QTLs were for lateral root count and rate of increase in lateral root count. None of the QTLs were mapped for lateral root length under control conditions. Two QTLs were mapped for glucose-sucrose ratio on chromosomes 3 and 7. Earlier studies have reported QTLs for fructose-glucose ratio in peaches (*Prunus persica* L. Batsch) (Desnoues et al., 2014). All the QTLs mapped for sugar related traits under control conditions were unique and not found under stress conditions.

QTLs in salt stress conditions

The 25 QTLs identified under stress conditions were located on chromosome 1, 2, 3, 4, 8, 9 and 11 chromosomes with phenotypic variance of each individual QTL ranging from 6.3 to 22.7 % and LOD scores ranging from 3.7 to 9.5 (Table 3.2). Out of the 25 QTLs identified under stress conditions, six QTLs were identified for shoot growth on chromosomes 2, 3, 9 and 11; 17 QTLs for root growth traits on chromosomes 1, 2, 3, 4, 8 and 11, and two QTLs for sugar related traits on chromosome 9.

Similar to the control condition, different QTLs were mapped for the shoot length at different time points, indicating the presence of time dependent QTLs. One QTL was mapped for shoot length at day six on chromosome 2, and three QTLs were

Table 3.2 Putative QTLs for different traits under stress conditions in the SSD RIL population derived from Azucena and IR64. QTLs marked in bold are unique to stress conditions.

No	Trait Class	Trait	QTL name	Chr	Peak marker	Peak position (cM)	Peak Lod	Left Marker	Right marker	Left Position (cM)	Right position (cM)	% Phenotypic variance
1	**Shoot**	**Shoot Length at 6 days after germination**	**qSL-2**	**2**	**S2_30620406**	**310.02**	**4.2**	**S2_28647152**	**S2_32611229**	**291.76**	**338.35**	**10.9**
2	**Shoot**	**Shoot Length at 8 days after germination**	**qSL-3-1**	**3**	**S3_30027906**	**321.82**	**4.2**	**S3_26674733**	**S3_35305848**	**282.87**	**403.73**	**9.1**
3	**Shoot**	**Shoot Length at 8 days after germination**	**qSL-9**	**9**	**S9_19586243**	**217.19**	**4.0**	**S9_18957443**	**S9_20970299**	**209.50**	**233.78**	**8.6**
4	**Shoot**	**Shoot Length at 8 days after germination**	**qSL-11-2**	**11**	**S11_16917661**	**157.65**	**5.0**	**S11_16359828**	**S11_17353900**	**146.99**	**164.71**	**11.0**
5	Shoot	Shoot growth rate from Day 6 to Day 8	qSGR-3	3	S3_36170560	418.95	5.2	S3_34473882	S3_36398607	388.05	420.12	11.8
6	**Shoot**	**Shoot growth rate from Day 6 to Day 8**	**qSGR-9**	**9**	**S9_20887955**	**232.59**	**4.7**	**S9_18872082**	**S9_21390653**	**207.73**	**240.33**	**10.5**
7	Root	Primary root length at 6 days after germination	qRL-4	4	S4_28566103	234.89	4.6	S4_28358016	S4_30963977	231.34	256.90	11.8
8	Root	Primary root length at 8 days after germination	qRL-1	1	S1_41271407	427.05	8.5	S1_40874958	S1_42054294	419.36	435.92	18.2
9	Root	Primary root length at 8 days after germination	qRL-4	4	S4_28412458	233.71	6.4	S4_28358016	S4_29962287	231.34	244.32	13.3
10	Root	Root growth rate from Day 6 to Day 8	qRGR-1	1	S1_41266450	427.05	7.7	S1_40889641	S1_42054294	419.95	435.92	19.0
11	**Root**	**Number of lateral roots at Day 8**	**qLRC-2**	**2**	**S2_25157864**	**251.64**	**5.2**	**S2_24522747**	**S2_26128229**	**244.57**	**260.47**	**9.9**
12	**Root**	**Number of lateral roots at Day 8**	**qLRC-4**	**4**	**S4_28566103**	**234.89**	**7.8**	**S4_28391215**	**S4_29962287**	**233.13**	**244.32**	**15.5**
13	**Root**	**Number of lateral roots at Day 8**	**qLRC-11**	**11**	**S11_16710793**	**154.10**	**7.5**	**S11_13634029**	**S11_17353900**	**125.76**	**164.71**	**14.9**
14	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR-2**	**2**	**S2_25157864**	**251.64**	**4.7**	**S2_22630888**	**S2_25752911**	**225.67**	**255.76**	**9.5**
15	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR4**	**4**	**S4_28580138**	**236.07**	**5.4**	**S4_28210778**	**S4_31564453**	**225.42**	**265.78**	**11.0**
16	**Root**	**Difference in the lateral root number from Day 6 to Day 8**	**qLRCR-11**	**11**	**S11_13725875**	**127.53**	**6.4**	**S11_9060264**	**S11_17298572**	**102.16**	**163.54**	**13.3**
17	Root	Total Lateral root length at Day 6	qLRL-4	4	S4_28224415	227.19	3.8	S4_27571156	S4_32487335	216.58	276.60	9.9
18	**Root**	**Total Lateral root length at Day 8**	**qLRL-3**	**3**	**S3_2726045**	**38.40**	**3.7**	**S3_1146306**	**S3_3965462**	**10.05**	**57.97**	**7.2**
19	**Root**	**Total Lateral root length at Day 8**	**qLRL-4**	**4**	**S4_31270184**	**261.04**	**8.2**	**S4_30865838**	**S4_31969528**	**253.95**	**267.55**	**17.0**
20	**Root**	**Difference in the total lateral root length from Day 6 to Day 8**	**qLRLR-1**	**1**	**S1_36132222**	**361.22**	**4.0**	**S1_35500430**	**S1_41225838**	**351.79**	**427.05**	**6.3**
21	**Root**	**Difference in the total lateral root length from Day 6 to Day 8**	**qLRLR-3**	**3**	**S3_1677034**	**18.32**	**5.4**	**S3_1498891**	**S3_3832943**	**14.78**	**55.59**	**8.6**
22	**Root**	**Difference in the total lateral root length from Day 6 to Day 8**	**qLRLR-4**	**4**	**S4_31270184**	**261.04**	**5.0**	**S4_30735893**	**S4_31969528**	**249.68**	**267.55**	**8.0**
23	Root	Difference in the total lateral root length from Day 6 to Day 8	qLRLR-8	8	S8_27185048	226.72	5.8	S8_26762931	S8_28389376	222.00	244.43	9.3
24	**Sugar**	**Shoot meristem Sucrose**	**qSUC-9**	**9**	**S9_19017581**	**213.06**	**9.5**	**S9_18957443**	**S9_20082826**	**209.50**	**218.96**	**22.7**
25	**Sugar**	**Shoot meristem Total Sugar**	**qSUG-9**	**9**	**S9_19589056**	**217.78**	**5.0**	**S9_18920921**	**S9_22503375**	**208.32**	**252.22**	**12.8**

mapped on chromosomes 3, 9 and 11 for shoot length at eight days. One QTL for shoot growth rate (qSGR-3) was mapped under both control and stress conditions.

Two QTLs for root length (qRL-1 and qRL-4) were mapped on chromosome 1 and 4 under salt stress conditions. The QTL qRL-4 was co-localized with shoot meristem dry weight on chromosome 4 under control conditions. Two root length QTLs under salt stress (qRL-1 and qRGR-1) mapped on chromosome 1 were also mapped under control conditions. Two unique QTLs (qLRC-2 and qLRC-4) for salt stress conditions were mapped for lateral root count on chromosomes 2 and 4, and the same two QTLs were also mapped for lateral root count growth (qLRCR-2 and qLRCR-4) . One QTL for lateral root count on chromosome 11 was mapped under both control and stress conditions. However, due to its larger interval size, the peak positions differ and it is hard to determine if they are the same QTL or two peaks that are close to each other. QTLs for lateral root length were mapped only under salt stress conditions on chromosomes 1, 3, 4 and 8. Two of the QTLs (qLRL-4 and qLRLR-8) for lateral root length on chromosome 4 and 8 were mapped very close to the shoot meristem dry weight and shoot length QTL, respectively, under control conditions. One unique QTL on chromosome 9 was mapped for both sucrose (qSUC-9) and total sugars (qSUG-9) under salt stress and the same QTL co-localized with a shoot length QTL (qSL-9). Overall, many unique QTLs that were mapped only under salt stress conditions were identified in this study.

Discussion

This study has reported that different QTLs are responsible for shoot length measured at different time points six and eight days after sowing. Similarly, one of the previous salinity studies in rice also reported different QTLs for imbibition rate at 24 hours and 48 hours (Wang et al., 2011). This emphasizes the point of QTLs being dynamic and time dependent. The same study by Wang et al. (2011) has reported a *japonica* variety to be more salt tolerant than an *indica* variety at the germination stage.

In our study, we found that Azucena performs slightly better than IR64 under salt stress during the pre-emergence seedling stage. Given that IR64 is not a highly tolerant variety and with the evidence that genotypes respond differently to salinity at different growth conditions, it is less surprising that Azucena performs better under pre-emergence seedling stage salinity stress than IR64. Although IR64 and Azucena have not been observed to differ substantially for salinity tolerance, and tend to be sensitive (Haq et al., 2009; Zhang et al., 2011; Krishnamurthy et al., 2014), the RILs exhibited transgressive segregation and a significant number of RILs had values higher or lower than either of the two parents.

A comparative root architecture study between IR64 and Azucena was conducted by Clark et al.(2011) in different growth media such as gellan gum, hydroponics and sand. The results of our study showed that IR64 has higher primary root length, lateral root number and average lateral root length than Azucena. The results of our study under well-watered control conditions most closely match those of

Clark et al. (2011) in the sand medium. This study reported that QTLs for shoot length and sugar content in the shoot meristem were co-localized on chromosome 9. These two traits were negatively correlated and also occupy the same loci which suggests that these two traits may be linked with each other. The loci also match with QTLs reported in previous studies for traits such as imbibition rate and shoot Na concentration (Wang et al., 2011; Ghomi et al., 2013). The evidence of four different phenotypic traits being mapped to the same region of the chromosome indicates that this region in chromosome 9 plays an important role in salinity stress.

QTLs matching with previous studies

One of the advantages of this study is that it involved saturating the mapping population with SNPs, giving us the ability to detect more QTLs compared to previous studies using fewer SSR markers and SNPs. For instance, two additional QTLs for the same population were identified for aluminium tolerance when 30,984 SNPs were used compared to earlier mapping with 200 SSRs and 1464 SNP markers (Spindel et al., 2013).

Many studies have been conducted to map QTLs for salinity tolerance at different growth stages in rice (Prasad et al., 2000; Koyama et al., 2001; Haq et al., 2010; Thomson et al., 2010; Wang et al., 2010; Alam et al., 2011; Wang et al., 2011; Ghomi et al., 2013; Bimpong et al., 2014). Often times these studies have used recombinant inbred lines (RILs) ranging from selfing for six to nine generations (F6 – F9) and Doubled Haploid populations. Almost all the studies have used either AFLP, RFLP or SSR molecular markers and only one of the studies used SNPs for QTL

identification (Bimpong et al., 2014). Therefore the current study is one of the few studies that have mapped QTLs using high density SNP markers with high mapping resolution.

Almost all of the QTLs reported in this study matched or were close to QTLs mapped for different phenotypic traits from earlier studies on salinity stress in rice. This strengthens the case that the QTLs identified in this study are real and encourages further investigation. A few loci on chromosomes 8 and 11 were not reported in previous studies. Three QTLs, namely qSL-8 and qSGR-8 under control conditions and qLRLR-8 under stress conditions, did not have any matching QTLs with previous studies. Two other QTLs (qSL-11 and qLRC-11) under stress conditions also did not match with any of the previously reported QTLs. From all the salinity tolerant QTL studies, it is inferred that some QTLs are repeatedly reported on chromosomes 1, 4, 6 and 7, no QTLs are reported on chromosomes 8 and 11, and few QTLs are reported on chromosomes 2, 3, 5, 9, 10 and 12 (Negrão et al., 2011). Only two to three studies including the current study have reported QTLs on chromosome 8 and 11.

Two of the mapping studies from Prasad et al. (2000) and Wang et al. (2011) were performed at germination and the very young seedling stage. These two studies matched well with the current study in terms of the stage of phenotyping. Some of the QTL positions reported by Wang et al. (2011) matched with the present study, although the two studies did not have any common phenotypic traits.

One of the major objectives of the current study was to find QTLs that are responsible for pre-emergence seedling stage salinity tolerance in rice that can aid in the direct seeding approach in saline affected areas. The QTLs identified in this study

can be introgressed into elite cultivars that are saline tolerant in the vegetative and reproductive stages of growth. Most of the breeding programs focus on the introgression of the Saltol locus which will provide ionic tolerance, as discussed below, but not necessarily osmotic tolerance. Saltol is the first major QTL identified for salt tolerance from Pokkali landrace in rice on chromosome 1 (Gregorio, 1997). After that discovery, the same region was mapped in Nona Bokra (*indica*) for salt tolerance and the molecular basis of the QTL was studied (Ren et al., 2005). The researchers cloned a candidate gene *SKC1* (shoot K concentration 1) and found that it acts as a regulator of K^+/Na^+ homeostasis and its proteins act as Na^+ selective transporters (Ren et al., 2005). Therefore plants carrying Saltol loci will be tolerant to the ionic component of salt stress by maintaining the ion concentration. In reality, having both ionic and osmotic tolerance will be doubly beneficial for breeding programs. By introgressing multiple genes we can save time and ensure faster breeding progress. Thus by using high density SNPs, which is a recent genomics approach, we could potentially identify novel and important missing links and better enhance salinity tolerance in rice.

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