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Physiological Assessment of Chenopodium quinoa to Salt Stress

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PHYSIOLOGICAL ASSESSMENT OF *CHENOPODIUM QUINOA* TO SALT STRESS

by

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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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ABSTRACT

PHYSIOLOGICAL ASSESSMENT OF

CHENOPODIUM QUINOA TO SALT STRESS

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Master of Science

The physiological responses to salt stress were measured in *Chenopodium quinoa*. In a greenhouse experiment, salt water was applied to the quinoa varieties, Chipaya and KU-2, and to the model halophyte *Thellungiella halophila* to assess their relative responses to salt stress. Height and weight data from a seven-week time course demonstrated that both cultivars exhibited greater tolerance to salt than *T. halophila*. In a growth chamber experiment, three quinoa cultivars, Chipaya, Ollague, and CICA 17 were hydroponically grown and physiological responses were measured with four salt treatments. Tissues collected from the growth chamber treatments were used to obtain leaf succulence data, tissue ion concentrations, compatible solute concentrations, and RNA for real-time PCR. Stomatal conductance and fresh weight were measured to determine the degree of stress and recovery. The expression profiles of SOS1, NHX1, and TIP2, genes involved in salt stress, showed constitutive expression in root tissue and up-regulation in leaf tissue in response to salt stress. These data suggest that quinoa tolerates salt through a combination of exclusion and accumulation mechanisms.

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TABLE OF CONTENTS

CHAPTER 1: PHYSIOLOGICAL ASSESSMENT OF *CHENOPODIUM QUINOA* TO SALT STRESS

Introduction

Due to irrigation and poor resource management, soil salinity has been gradually increasing on agricultural land and having adverse effects upon crop production (Maas, 1986). Increases in soil salinity can cause decreases in yield of as much as 100% in salt sensitive crops such as rice, corn, and peanut (Ayers and Westcot, 1985). Furthermore, the amount of agricultural land affected by salinity is increasing. It is estimated that 900×10^6 hectares (Flowers, 2004) or 20% of the earth's arable land (Mühling and Läuchli, 2002) is currently affected by high soil salinity.

Most crops are classified as glycophytes. Glycophytes are plants that only tolerate low levels of soil salinity (<50 mM) without showing signs of reduced growth and do not accumulate high concentrations of salt in growing tissue (Orcutt and Nilsen, 2000). Halophytes are defined as plants that can cope with saline environment, typically around 300 mM, without being adversely affected (Orcutt and Nilsen 2000). When grown on saline soils, crops that are considered to be tolerant do not show the same degree of tolerance as seen in halophytes. One halophyte, *Thellungiella halophila* (salt cress), has been established as a physiological model for abiotic stress and it has since been extensively characterized (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005; Vera-Estrella et al., 2005; Yiyue Zhang, 2008). One justification of its characterization is that perhaps mechanisms of *T. halophila* salt tolerance could eventually be applied to crop plants.

Chenopodium quinoa is a crop grown throughout South America in a variety of environments ranging from the altiplano to coastal and valley climates. Varieties from the southern altiplano of South America grow near salt flats where soil salinity is much higher than soils typically

found in agricultural regions of the United States. Despite the high salinity, cold temperatures, and low water supply, altiplano ecotypes thrive in these conditions and perform better in high salt soil than in low salt soil (Sanchez et al., 2003). Kancolla, an altiplano cultivar, had a germination rate of 75% at a concentration of 57 mS cm^{-1} (Christiansen et al., 1999; Jacobsen et al., 1999) where 50 mS cm^{-1} is the electroconductivity of seawater, or 600 mM NaCl. Perhaps quinoa has unique physiological properties that allow it to tolerate such harsh abiotic stresses, especially salt.

Because of its role as a model halophyte, a comparison between *T. halophila* and quinoa may elucidate some of the underlying adaptive traits of this hearty pseudo-cereal. *T. halophila* has been well characterized as a halophyte and its response to salt was carefully quantified relative to *A. thaliana* (Inan et al., 2004). This quantification represents an excellent benchmark for comparison of other less studied halophytes such as quinoa. While research has routinely identified quinoa as a salt tolerant crop (Wilson et al., 2002; Jacobsen et al., 2003; Trognitz, 2003; Koyro and Eisa, 2008), a benchmark comparison between quinoa and *T. halophila* will quantify relative salt tolerance and may provide clues underlying the unique physiological mechanisms of quinoa's salt tolerance. For example, *T. halophila* generally avoids salt toxicity by actively pumping salt ions out of the plant. Halophytes tolerate high salinity conditions primarily through mechanisms that aid in water acquisition and facilitate salt avoidance. Salt avoidance mechanisms can be further classified into mechanisms of exclusion, secretion, shedding, and succulence (Cronk and Fennessy, 2001). Exclusion mechanisms remove or prevent salt from entering tissues or areas that would otherwise be damaged. Secretion mechanisms remove salt from a plant by expelling it through glands. Shedding mechanisms sequester salt into plant organs which are then shed from the plant. Succulence mechanisms

increase the water content per unit area of the leaf, thereby diluting the salt and minimizing its impact. During salt stress, succulent plants retain water by reducing stomatal aperture (Lovelock and Ball, 2002).

Genes underlying molecular mechanisms that regulate salt content have been found in model plants. Using an *A. thaliana* microarray, Vera-Estrella et al. (2005) identified several genes associated with salt tolerance in *T. halophila*, including SOS1, NHX1, and TIP2, genes that had been independently characterized for their functionality. The SOS1 gene has been shown as a key component of salt exclusion mechanisms by encoding for an H^+/Na^+ antiporter to control sodium efflux (Shi et al., 2000) at the plasma membrane, functioning through exclusion. Turner (2007) identified the gene sequence and genomic context of a homolog of *A. thaliana* SOS1 in quinoa although the function of the quinoa SOS1 homolog has not been fully demonstrated. Shi et al. (2000) observed differences in root and shoot expression of SOS1. Roots exhibited low expression during control followed by a marked increase during salt stress and shoots exhibited no expression during control and very low expression during salt stress. NHX1 aids in salt accumulation by coding for an antiporter located in the tonoplast that sequesters salt into the central vacuole (Apse et al., 1999). TIP2 codes for an aquaporin that increases water uptake from the vacuole to the cytoplasm in response to salt stress, thus increasing succulence, in *A. thaliana* (Boursiac et al., 2005) and *T. halophila* (Yiyue Zhang, 2008). It is possible that homologs of these genes also have a role in molecular mechanisms that regulate salt in quinoa.

Compatible solutes or osmoprotectants are compounds involved in osmoregulation during salt stress and have been shown to be involved in salt stress in many plants (McNeil et al., 1999; Trinchant et al., 2004; Chen et al., 2007). Osmoprotectants buffer the effects of salt in several ways. When accumulated in high amounts, osmoprotectants can offset the osmotic imbalance

caused by a high accumulation of salt in the intercellular space. As salt is excluded from the cell it builds up in the intercellular spaces and creates an osmotic potential across the cell membrane and cell wall. Compatible solutes are accumulated in the cytoplasm in response to high salt concentrations outside the cell and prevent cellular water loss by balancing the osmotic potential (Yancey, 1994). High salinity can cause the water potential in the soil to become lower than the water potential in the plant, also leading to plant water loss. Compatible solutes accumulated in high concentrations can also lower the water potential in the plant below that of the adjacent soil and restore the movement of water from the soil to the plant (Orcutt and Nilsen, 2000). Osmoprotectants can also provide enzyme protection and maintain membrane integrity under salt stress (Sakamoto and Murata, 2002). One type of osmoprotectants is glycine betaine, also known as and heretofore referred to as betaine. Tobacco exhibited improved salt tolerance when transgenically modified to produce betaine (Holmstrom et al., 2000). Betaine and betaine derivatives such as trigonelline have also been identified, though not quantified, in quinoa seeds (Dini et al., 2006) and they may act as an important component of salt tolerance in chenopodium species. Trigonelline has also been identified as a compatible solute in *Glycine max* (Cho et al., 1999) and tomato (Rajasekaran et al., 2001). Other identified osmoprotectants include pinitol (Adams et al., 1998), sorbitol, and trehalose (Rontein et al., 2002). Proline has also been shown to accumulate under high-salt conditions in some plant species (Ashraf and Foolad, 2007). Inan et al. (2004) found that proline accumulated in large enough quantities (150 µmol g^{-1} DW) to alter osmotic balance and identified proline as the principle compatible solute responsible for osmoprotection while the other compatible solutes accumulated in quantities too small to have a significant impact. While this may be true for *T. halophila* and *A. thaliana*, some of these osmoprotectants may play a role in salt tolerance in other organisms including quinoa.

In this study we first compared two cultivars of quinoa to *T. halophila* in a greenhouse in a large replicated design carried out over a seven-week period and compared their height, weight, and soil electroconductivity under various salt concentrations. Both quinoa cultivars performed better than *T. halophila* and the results suggested that quinoa implements mechanisms not used by *T. halophila*. A closer examination of potential tolerance mechanisms was performed by evaluating the salt response of three quinoa cultivars in a hydroponic growth chamber. We compared root and shoot fresh weight, measured stomatal conductance over time, and examined salt and compatible salt accumulation through ICP and HPLC techniques. Intriguing responses to salt were further investigated via candidate genes using real-time PCR.

Materials and Methods

Greenhouse Plant Materials

A total of four quinoa ecotypes were used in the two studies. Chipaya and Ollague are altiplano salares ecotypes and CICA 17 and KU-2 are valley types (Mason et al., 2005; Christensen et al., 2007). In the greenhouse study, Chipaya and KU-2 cultivars were planted in 36-cell flats, germinated in Sunshine Basic Mix 2 soil (Sun Gro, Vancouver, British Columbia, Canada) under ambient greenhouse conditions. After one week of growth, successfully germinated plants were transferred to four-inch square pots for the duration of treatment. *T. halophila*, Shangdong variety, was grown using the growth protocol provided by the University of Illinois, Urbana-Champaign (www.thellungiella.org) and then transferred to 3.5-inch propagation pots. *T. halophila* required a cold treatment of 10-14 days followed by two weeks of germination and growth and then a vernalization stage of three to four weeks to allow for flowering to occur (Inan et al., 2004). Growth of quinoa and *T. halophila* was synchronized by commencing salt treatments at the eight-leaf stage of development for all plants. All plants were grown at latitude 40.233N and longitude ¯111.657W at an elevation of 4,551 feet with an average daily maximum temperature of 37 ºC, an average daily minimum temperature of 16.5 ºC, and an average day length of 14 hours in soil supplemented with Osmocote (Scotts, Marysville, OH) slow release fertilizer and Marathon 1% granular insecticide (OHP Mainland, PA) after three weeks of growth.

Salinity Treatments and Data Collection in Soil

At the eight-leaf stage of development, quinoa (Chipaya, and KU-2 cultivars) and *T. halophila* were randomly assigned a position in a greenhouse room and one of five salt treatments. Treatments consisted of tap water with NaCl added to reach the concentrations of 150 mM, 300

mM, 450 mM, and 600 mM with a 0 mM concentration serving as the control. To avoid over-watering, plants were only watered as needed. Five plants from each treatment-ecotype combination were harvested every seven days and height, weight, and stomatal conductance measured. Soil electro-conductivity was also measured by the Brigham Young University Plant and Soil Analysis Lab (Provo, UT) for three samples randomly chosen from the original five plants harvested. A total of 700 plants (175 of each cultivar) were used for each replicate. Three replicates of the experiment were carried out.

Growth Chamber Salinity Treatments and Tissue Collection in Hydroponics

Three cultivars of quinoa (Ollague, CICA17, and Chipaya) were also grown in a hydroponic growth chamber using a previously described protocol (Camp et al. 1987) with a day temperature of 29.5 ºC, a night temperature of 19 ºC, and 13 hour days. This allowed for root tissue to be obtained and to better control the salt concentration and environment. A randomized block split-plot design with two paired treatments, high salt/low salt and recovery/low salt recovery, was setup with four blocks. Each treatment bucket contained one representative from each ecotype studied. All treatments consisted of a combination of Hoagland's growth solution (Camp et al. 1987) and NaCl. All salt treatments were increased in 50 mM daily increments until the desired salt concentration was reached including the high salt treatment of 450 mM. The low salt control maintained a concentration of 50 mM throughout the experiment. The high salt and low salt control were simultaneously harvested. The recovery treatment followed the same pattern as the high salt treatment but was then followed by an incremental decrease to 50 mM. The recovery treatment was then harvested with its corresponding low salt control. All plants were harvested 48-72 hours after reaching their final treatment concentration. Stomatal conductance was

measured every 3-4 days and root and shoot fresh weights measured at the time of harvest. Upon harvest all tissue was flash frozen using liquid nitrogen and stored at -80ºC until use.

Phenotypic Measurements

Stomatal conductance was measured for all quinoa plants from the greenhouse trials and the hydroponic trials using a steady state leaf porometer (Decagon Devices, Pullman, WA) which calculated stomatal conductance by measuring vapor concentration at two distinct points in the diffusion path. Stomatal conductance in *T. halophila* was not measured because there was insufficient leaf area for the sensor head to measure accurately. Measurements were taken over a 30 second period and reported in mmol $m^{-2}s^{-1}$. Electro-conductivity measurements were taken using Oakton's Conductivity/TDS/ Meter (Vernon Hills, IL) and were reported in mS cm^{-1}. Sodium, potassium, and calcium tissue concentrations were obtained by desiccation of frozen tissue using a Thermo Savent ModulyoD-115 Freeze Drier followed by nitric-perchloric acid tissue digestion (Johnson and Ulrich, 1959) and inductively coupled plasma (ICP) analysis on an IRIS Intrepid II XSP (Thermo Electron Corporation, Franklin, MD) by the Plant and Soil Analysis Lab, Brigham Young University (Provo, UT). Water content was obtained by weighing tissue before and after 2-3 days of desiccation and dividing the fresh weight by the dry weight.

Compatible Solute Accumulation

Compatible solute concentrations were determined using the extraction and high performance liquid chromatography (HPLC) protocol developed by Naidu (1998). HPLC was performed on an Agilent 1100 HPLC platform (Agilent, Santa Clara, CA) using RI detection and a Waters Sugar-Pak I 6.5 X 300mm column (Waters, Milford, MA) maintained at 80° C with a mobile phase of 5 mg L^{-1} Ca-EDTA (Sigma-Aldrich, St. Louis, MO) and a flow rate of 0.6 ml min^{-1} and 20 µl injection volume. The standards used were obtained through Sigma Aldrich and include

Betaine (#61962), Pinitol (#441252), Proline (#81710), Sorbitol (#S1876), Trehalose (#T9531), and Trigonelline (#T5509).

RNA Extraction and Quantitative PCR

Plant tissue was prepared for extraction by flash freezing at the time of harvest. Tissue was then ground using liquid nitrogen and RNA was extracted from leaf and root tissue using an RNeasy® Plant Mini Kit (Qiagen, Valencia, CA). DNA was removed from the RNA samples using a TURBO DNase Kit (Ambion, Austin, TX). The RNA was quantified using a Quant-iT RiboGreen Assay Kit (Invitrogen, Carlsbad, CA) and a TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA) and then run on a Bioanalyzer using an RNA Nano Chip (Agilent, Santa Clara, CA) to verify RNA quality. Primers were designed for SOS1 using sequences known to include the SOS1 gene in *C. quinoa* (Maughan et al, 2009). NHX1 primers were created in conserved domains using a consensus sequence generated by accessions AM746985 (*Mesembryanthemum crystallinum),* AB038492 (*Atriplex gmelini*), and AY371319 (*Chenopodium glaucum*). TIP2 primers were created using a consensus sequence created from accessions AF118381 (*Brassica napus*), NM_113559 (*A. thaliana*), AY821911 (*Gossypium hirsutum*), and D25534 (*Oryza sativa*). All consensus sequences were made using Geneious version 4.5.1 (Biomatters, New Zealand). GAPDH primers reported previously (Balzotti et al., 2008) were used. Supplemental Table 1 summarizes the primer attributes. Gene expression of SOS1, NHX1, and TIP2 was quantified using a High Capacity RNA-to-cDNA Master Mix Reagents Kit (ABI, Foster City, CA) and a Fast SYBR Green Master Mix (Roche, Indianapolis, IN) on a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) using the provided standard protocol with GAPDH serving as an endogenous control.

Results

Halophyte Height and Weight

Chipaya, a salares-type cultivar, and KU-2, a valley-type cultivar, were compared to *T. halophila* in a greenhouse experiment. Total plant height and weight, averaged across all time-points, were evaluated at each treatment concentration (*i.e.* salt concentration). When comparing the plant height of each of the three plant types at the various salt concentrations all the plant types had an adverse decline in response to salt at all of the treatment levels. While each plant type was affected, *T. halophila* showed a more rapid decrease in plant height across salt treatments than quinoa after adjusting for life cycle by comparing the height of treated plants relative to their control (Fig. 1). Quinoa height began to plateau at approximately 30% of the control height at high salt levels while *T. halophila* reached a plateau approaching 0%. This suggests that salt concentrations were adversely affecting *T. halophila* more than quinoa. Most of the *T. halophila* treated with 300 mM NaCl or more died within three weeks and caused the rapid decline in height and weight. We were concerned that the high greenhouse temperature during the comparison would negatively impact *T. halophila*. However, the performance of the control showed that, while Shandong is grown in a temperate climate, high temperatures likely did not trigger stress nor decrease growth. Quinoa was able to withstand 600 mM NaCl for three to four weeks and 450 mM NaCl for five to six weeks, the approximate life cycle of the control plants, although chlorosis of the bottom leaves was pronounced in high-salt treated plants (Supplemental Fig. 1). No difference in height was observed between the two quinoa cultivars. Similar trends were also observed for relative plant weight (Fig. 1).

Analysis of plant height and weight over time also distinguished quinoa as a halophyte (Supplemental Fig. 2). We expected to find some measurable difference in salt response

between quinoa cultivars since saline conditions are prevalent in the southern altiplano where Chipaya is grown but not in the valley region where KU-2 is grown. However, both quinoa cultivars behaved similarly. When treated with varying concentrations of salt over time, quinoa exhibited an increase in height for the control, 150 mM, and 300 mM treatments and maintenance of height at 450 mM and 600 mM (Supplemental Fig. 2 B,D). *T. halophila* also followed this pattern but the height in *T. halophila* control plants continued to increase past the last week of data collection, creating a larger difference in height between stressed plants and control plants by the end of the study than in quinoa which had reached a plateau prior to the end. For example, at week 7, control *T. halophila* was four times larger than *T. halophila* treated with 150 mM NaCl (Supplemental Fig. 2 E) while control KU-2 was twice as large as quinoa treated with 150 mM NaCl (Supplemental Fig. 2 C). Under these circumstances, one would expect a greater response from *T. halophila* than was observed given that after seven weeks of growth the control had still not reached a plateau or decrease in height associated with senescence as was the case with both quinoa cultivars (KU-2 and Chipaya height began to plateau at three and five weeks, respectively, see Supplemental Figure 2 A,C). The continued growth in the *T. halophila* control may, in part, be due to varying flowering time among the control plants. Quinoa also retained its weight under stress. Quinoa plants grown in the 150 mM and 300 mM treatments increased or maintained weight while plants grown in the 450 mM and 600 mM treatments maintained plant weight until week four when the plants began to die (Supplemental Fig. 2 B,D). *T. halophila*, in contrast, only maintained weight at 150 mM and while under 300, 450, and 600 mM NaCl treatments weight was quickly lost (Supplemental Fig. 2 F).

Soil Electroconductivity

Soil electroconductivity was measured to determine if salt was accumulating in the soil in amounts greater than the treatment concentration. Each treatment accumulated salt in excess of the treatment concentration, many times by as much as 2-fold (Fig. 2). An unexpected trend was also present. As seen in Figure 2 C, the salt concentration in soil of the *T. halophila* pots continued to increase in a linear fashion over time while the salt concentration in soil of quinoa pots (Fig. 2 A,B) showed a plateau three weeks after treatment. Because *T. halophila* was a smaller plant and required less water, one would expect to see a lower EC in *T. halophila* soil because less salt was applied, but little difference was observed. The observation of an EC plateau in quinoa-pot soil suggests that quinoa has a mechanism for managing salt within the plant in addition to salt exclusion. *T. halophila*, in contrast, appeared to tolerate salt primarily through exclusion (Fig. 2). It is also possible that the larger mass of quinoa allowed it to manage a greater amount of salt than *T. halophila* which is much smaller. If this were the case one would expect to see a plateau at the same concentration of salt. However, the plateau begins at the same time for each salt concentration treatment, suggesting that a mechanism of salt tolerance is time dependent rather than concentration dependent. Leeching of salts could have also occurred, although since both plant types were watered as needed, leeching should have been similar between plants when the numerous technical and biological replicates are considered. Because water amounts were not precisely quantified, further evidence is needed to conclusively quantify the difference in salt accumulation between quinoa and *T. halophila*.

Stomatal Conductance of Soil-grown Quinoa

To further characterize quinoa's response to salt, stomatal conductance was measured concurrently with height and weight measurements. A stress response commonly observed in

green plants is decreased stomatal apertures to limit water loss. The decrease of stomatal aperture is detected by estimating stomatal conductance based on measured vapor concentration (Lovelock and Ball, 2002). An incremental decrease in stomatal conductance was observed at every salt treatment after one week of treatment (Supplemental Figure 3A). This pattern was also observed to a greater extent in subsequent weeks (Supplemental Figure 3B). This response suggests a linear physiological response that is dependent upon the degree of stress applied. When jointly considered with the fresh weight and height data, we see that quinoa was stressed by the salt treatments and that it tolerated high salt in the greenhouse environment. It is important to note that stomatal conductance decreased in the control plants over time which is to be expected as the plant matures and eventually senesces.

Physiology Measurements of Hydroponically-grown Quinoa

Further characterization of quinoa as a halophyte and analysis of potential mechanisms was also performed using plants grown in a hydroponic system in a growth chamber by applying one of the four treatments designated high salt, control, recovery, and recovery control. *T. halophila* was not amendable to this hydroponic growth system. At a salt concentration of 450 mM, all three stressed cultivars exhibited less than half the weight of their corresponding control (Fig. 3). Stomatal conductance measurements of salt treated plants were also significantly lower than their corresponding controls (Table 1). Similar to the greenhouse study, these data indicated that the quinoa plants were under stress, but in the growth chamber study there were no other visible signs of stress such as leaf chlorosis or wilting.

Quinoa also exhibited a robust ability to recover from high salt stress. The recovery treatment restored NaCl concentration from high levels (450 mM) to low levels (50 mM). Once the salt stress was reduced, root and shoot fresh weight resumed growth to normal levels (Fig. 3). It is

likely that the high concentration of salt caused quinoa to initiate a dormant state of growth. This would permit the plant to survive its challenging environment rather than continue to grow with the risk of unbalanced metabolism and membrane leakage (Orcutt and Nilsen, 2000). Chipaya and Ollague, the salt tolerant salares cultivars, had root and shoot weights similar to their control and CICA 17, the valley cultivar suspected to be less tolerant, had surpassed the height and weight of its control (Fig. 3). All three cultivars exhibited a recovery of stomatal conductance to levels similar to their controls by the end of treatment although CICA 17 was not as close to its untreated control as Chipaya and Ollague were to their respective untreated controls (Table 1).

At high salt treatment, quinoa leaves remained morphologically unchanged and their water content (*i.e.* succulence) warranted investigation. Quinoa water content under high salt stress exhibited a modest decrease. We observed that quinoa FW:DW ratios were 10:1 in the control and 7:1 in the 450 mM treatment (Fig. 4). The recovery treatment also had a FW:DW ratio of 10:1 and its corresponding control had a FW:DW ratio of 7:1. The return to a normal FW:DW ratio indicated successful recovery once salt stress was removed.

Tissue Sodium Concentration

ICP analysis of Chipaya leaf tissue revealed that the sodium concentration in the high salt treatment was almost twice that of the low salt treatment, increasing from 26.69 mg g DW^{-1} to 43.68 mg g DW^{-1}. Analysis of Chipaya root tissue revealed a similar pattern with a 2.6-fold increase from 15.95 mg g DW^{-1} in the low salt treatment to 42.05 mg g DW^{-1} in the high salt treatment (Fig. 5). Similar results were observed in Ollague and CICA 17 leaf and root tissue. Interestingly, the leaf sodium content did not significantly decrease in any of the three cultivars in the recovery treatment despite the decrease in sodium concentration in the treatment solution.

Root tissue under recovery treatment exhibited a decrease in sodium concentration to normal levels.

Compatible Solutes

The compatible solutes betaine, pinitol, proline, sorbitol, trehalose, and trigonelline were measured in quinoa tissue grown in hydroponics. HPLC detected negligible, inconsistent quantities of sorbitol, pinitol, and proline. However, betaine, trehalose, and trigonelline showed significant changes in response to salt (Fig. 6). Trigonelline accumulated in greatest abundance, ranging from 800 to 7000 µmol g^{-1} DW (Fig. 6 A,B). Betaine accumulation ranged from 40 to 425 µmol g^{-1} DW (Fig. 6 C,D) and trehalose accumulation ranged between 35 and 95 µmol g^{-1} DW (Fig. 6 E,F).

Trigonelline was present in quinoa in amounts far exceeding amounts reported in other plants (Cho et al., 1999; Wood, 1999; Inan et al., 2004) including the control plants and it showed a marked increase between control and treated plants. In quinoa, trigonelline quantities increased 2.8-fold from 1718 µmol g DW^{-1} in low salt to 4845 µmol g DW^{-1} in high salt Ollague leaf tissue (Fig. 6 A). Chipaya and CICA-17 also exhibited similar increases in leaves. In roots, all three cultivars had similar accumulation under salt stress (approximately 6500 µmol g^{-1} DW), but varying accumulation under normal conditions (Fig. 6 B). Low-salt treated Ollague roots accumulated trigonelline in amounts five times greater than CICA-17 and 1.4 times greater than Chipaya roots. Trigonelline also increased three-fold from its control in recovery-treated Chipaya leaves and was equivalent to the amount present in tissue harvested at 450 mM NaCl (Fig. 6 A). Ollague leaf tissue also showed a similar pattern although the difference was not as extreme. CICA 17 trigonelline content returned to levels similar to its corresponding control in recovery-treated leaf tissue, although the CICA 17 data points were only represented by a single

data point and hence a standard error cannot be calculated to determine the statistical significance. In roots, all three cultivars retained approximately 60% of the trigonelline accumulated during salt stress (Fig. 6B).

Betaine accumulated in roots in concentrations less than half that of leaf tissue and only slightly higher than the control, suggesting that betaine may not play a vital role in stress response in roots. In roots, high salt was the only treatment with a significant increase of betaine ($p=0.05$) and was only significant in Chipaya (Fig. 6 D). In leaf tissue, betaine increased three-fold from 139 to 431 µmol g DW^{-1} in Ollague and 1.75-fold from 203 to 356 µmol g DW^{-1} in Chipaya (Fig. 6C). Quinoa under the recovery treatment showed a decrease in betaine to levels similar to the control by the end of treatment. While there were subtle, isolated differences in betaine accumulation between cultivars, general betaine accumulation patterns were very similar.

Trehalose accumulated at slightly higher levels in roots than leaves in Ollague and at similar levels in Chipaya. Trehalose increased 1.5-fold from 48 µmol g DW^{-1} in low salt to 75 µmol g DW^{-1} in high-salt treated Chipaya leaves and 2.4-fold from 40 µmol g^{-1} DW to 95 µmol g^{-1} DW in high-salt treated Chipaya roots (Fig. 6 E,F). In recovery treated quinoa, trehalose returned to levels equal to the recovery control in both roots and leaves for all cultivars.

Gene Expression

The expression of SOS1, NHX1, and TIP2 was also measured in quinoa using real-time PCR to indirectly quantify the role of each salt-coping mechanism in quinoa. Expression analysis showed no statistical significance between treatments in roots for SOS1 (Fig. 7). Rather, constitutively high expression was observed for SOS1 and suggests a preemptive counter-mechanism is present in quinoa roots. Statistically significant up-regulation of SOS1 was

observed in leaf tissue, suggesting a mechanism for removing cytoplasmic Na^+, thus minimizing the effects of Na^+ that may have been transported by the plant vasculature. NHX1 followed the same expression pattern as SOS1 with constitutive expression in roots and up-regulation in leaves. This is in contrast to expression in *T. halophila* which had low root expression of SOS1 and NHX1 under control conditions and significant up-regulation under salt stress conditions (Vera-Estrella et al., 2005). Vera-Estrella et al. also observed up-regulation of SOS1 and no expression of any members of the NHX gene family in leaf tissue, although sequestration was still observed in leaf vacuoles indicating that another gene product may function in a similar manner. TIP2 was expressed at constitutively high levels in quinoa roots and up-regulated only during salt stress in *T. halophila* (Yiyue Zhang, 2008). TIP2 did not exhibit differential expression in quinoa leaf tissue. Rather, low expression levels are maintained throughout stress with no statistically significant up-regulation. Perhaps it has additional molecular functions besides the regulation of cellular salt concentrations.

Discussion

Halophyte Comparison

Our comparison of *C. quinoa* and *T. halophila* showed that *C. quinoa,* a South American staple crop, exhibited greater salt tolerance than its model counterpart. The negative effects of increased salt concentration were more pronounced in *T. halophila* than in quinoa, as seen in the statistical difference in slopes of relative height which were -0.1067, -0.1095, and -0.1505 for KU-2, Chipaya, and *T. halophila*, respectively ($p=0.01$). The significant difference in slopes was evident given that approximately 30% of the initial relative height and weight were retained in both quinoa cultivars at a treatment level of 600 mM compared to *T. halophila* which reached a point approaching 0%, signifying that the majority of plants had died, in both categories at the same treatment level (Fig. 1). Quinoa was also able to cope with salt stress over time as well as, if not better than, *T. halophila* (Supplemental Fig. 2). Throughout the majority of the time-course, *T. halophila* and quinoa both increased in growth over time in the control, 150 mM, and 300 mM treatments (although more gradually in the 150 and 300 mM treatments), and simply maintained height in 450 and 600 mM treatments. In weeks six and seven, however, *T. halophila* exhibited a decrease in both height and weight in the 150 and 300 mM treatments while quinoa remained constant. *T. halophila* treated with 450 mM and 600 mM NaCl also decreased in weight in later weeks. These findings confirm that quinoa is able to resist stress better than *T. halophila* and also suggest that quinoa may to enter a state of pseudo-dormancy under stress. This state of dormancy was not seen in *T. halophila,* perhaps because a threshold had been crossed during initiation of flowering upon which the plant was committed to growth. Rather, stressed *T. halophila* continually decreased in height and weight over time while its control continually increased in both categories.

Tolerance Mechanisms in Quinoa

The results of this study indicate that there may be several mechanisms responsible for the response of quinoa to salt stress and that its response may be different than that found in *T. halophila*. Soil electroconductivity data (Fig. 2) and sodium accumulation data (Fig. 5) indicated that both plants managed salt in different ways. The steady increase in soil EC suggested that *T. halophila* avoided salt primarily by preventing uptake of NaCl. While other possible explanations for this outcome exist, the data supported the findings of Inan et al. (2004) who observed that *T. halophila* accumulated salt in low amounts in leaf tissue under salt stress compared to its relative, *Arabidopsis*. Vera-Estrella et al. (2005) demonstrated that exclusion occurs in *T. halophila* and it is likely that the low accumulation of salt is managed through this mechanism. Exclusion, or removal of salt from the cytoplasm, is a mechanism primarily employed by glycophytes. While exclusion can play a role in salt tolerance, Glenn et al. (1999) suggested that molecular mechanisms for accumulation within the plant are necessary for a plant to truly function as a halophyte. The plateau in soil EC observed in *C. quinoa* suggested that quinoa has mechanisms for managing salt *within* the plant in addition to limiting NaCl uptake. The mechanistic differences observed in quinoa and *T. halophila* may be due to differences in evolutionary history. 28.3% of the species within the Chenopodiaceae tribe of the Amaranthaceae family are salt tolerant whereas only 0.9% of the species of Brassicaceae are tolerant (Gorham, 1992). This suggests that adaptations contributing salt tolerance in Chenopodiaceae occurred long before adaptations in Brassicaceae and that Chenopodiaceae has had a longer evolutionary period to refine salt tolerance mechanisms than Brassicaceae.

ICP analysis indicated that quinoa was able to effectively manage accumulated salt (Fig. 5). The salt content in both leaves and roots was much higher in salt treated tissue than in control tissue.

This provided a hypothesis that quinoa may have mechanisms other than avoidance of salt uptake since it is unlikely that a halophyte whose primary mechanism of salt tolerance was avoidance would retain salt in a low salt solution. In recovery treated quinoa leaf tissue, salt concentrations remained high in all three cultivars despite having returned to a 50 mM NaCl nutrient solution. Perhaps the mechanism responsible for salt accumulation in quinoa did not allow for the removal of sequestered salt in leaves. A study investigating recovery from salt stress may provide clues to why salt remained in quinoa leaves and if there is a genetic component.

Quinoa also employed compatible solutes as a mechanism for coping with salt stress. Betaine, trehalose, and trigonelline were shown to increase in response to salt. Trigonelline production appeared to be the most directly related to salt stress. It was present in both leaf and root tissue in high quantities prior to stress and even greater quantities during stress. Trigonelline levels also remained elevated in leaves after relief of salt stress. Other studies found trigonelline levels in salt treated tissue to be 218.7 µg g DW^{-1} in G*lycine max* (Cho et al., 1999), 71.89 µmol g FW^{-1} (fresh weight will be diluted compared to dry weight) in *Quercus robur L.* (Oufir et al., 2009), and 1.1 mg g DW^{-1} in *Lycopersicon esculentum* Mill. (Rajasekaran et al., 2001). While absolute betaine accumulation was much lower than trigonelline, it also increased dramatically in response to salt stress and a significant portion of the betaine produced was retained in leaf tissue after salt stress has ceased. Given that trigonelline is a betaine derivative, it is possible that the betaine is found in lower concentrations because it is used directly as a substrate to form trigonelline. The retention of trigonelline and betaine may occur to maintain osmotic equilibrium since there is no apparent mechanism for salt removal in leaf tissue as was demonstrated through the ICP analysis. In roots, half as much betaine was present in all

treatments and the recovery treatment had only slightly more betaine than its control. Perhaps betaine is metabolized and transported to other parts of the plant or it is degraded as it no longer was needed to counteract high levels of salt. Trehalose only increased under high-salt stress in both roots and leaves and was not retained at the end of the recovery treatment. This suggests that trehalose production may be a general response to stress and not salt-specific in quinoa.

It is not uncommon for plants to employ multiple osmoprotectants at varying levels to combat stress. Garcia et al. (1997) reported the accumulation of various compatible solutes including trehalose, sorbitol, and mannitol in rice and Chen et al. (2007) reported the accumulation of betaine and proline in barley. Compatible solutes may also counteract different physiological challenges that arise as stress increases. For example, trehalose has been shown to be involved in increasing membrane fluidity (Crowe et al., 1984) and maintaining enzyme activity in dried conditions (Colaco et al., 1992). Betaine has been shown to protect the oxygen-evolving PSII complex (Murata et al., 1992), enzyme activity, and membrane integrity during salt stress (Sakamoto and Murata, 2002). In quinoa, betaine does not accumulate in concentrations high enough to alter osmotic balance (Sakamoto and Murata, 2002) though it may be involved in other forms of protection (directly or indirectly) while trigonelline likely functions to relieve osmotic stress since it was the only compatible solute that we measured to accumulate in high quantities.

Leaf water content measured in quinoa (Fig. 4) suggested that it was able to effectively retain water under stress. While there was a decrease in the quinoa FW:DW ratio, indicating water loss, the decrease was not as severe as reported in *T. halophila*. After 42 days of treatment, *T. halophila* FW:DW ratios were reported to be 8:1 in the control and 5:1 in the 200 mM NaCl treatment (Inan et al., 2004). *T. halophila* water content decreased from its control by 37.5%

after being treated with 200 mM and quinoa water content decreased from its control by 30% in 450 mM treatment. The greater loss of water at a lower salt concentration suggests that *T. halophila* does not retain water as well as quinoa under salt stress and that quinoa utilizes succulence to minimize the effects of sodium. The decreased stomatal conductance in response to increased salinity in quinoa also likely served to prevent water loss (Table 1).

Real-time PCR also identified SOS1, NHX1, and TIP2 as genes involved in salt stress (Fig. 7). Expression of SOS1 suggested that quinoa was moving sodium out of the cell. Expression of NHX1 suggested that sequestration of sodium from the cytoplasm to the vacuole also occurred in quinoa. TIP 2 expression in quinoa suggested that succulence is occurring by moving water from the vacuole to the cytoplasm. Differences in expression levels of salt regulation genes common to both plants may also contribute to the differences observed between *C. quinoa* and *T. halophila*. Quinoa exhibited constitutive expression of SOS1 and NHX1 in root tissue whereas *T. halophila* has been shown to have induced expression of SOS1, NHX1 (Vera-Estrella et al., 2005) and TIP2 (Yiyue Zhang, 2008) when exposed to saline conditions (Fig. 7). Differences in expression levels may indicate a preventative response rather than initiating expression upon stress. Not only was quinoa better prepared to cope with stress, but two major genes involved in prevention of salt stress were expressed in both roots and leaves which was not the case in *T. halophila*.

Recovery From Salt Stress

Quinoa also showed a remarkable ability to recover from salt stress. The severity of the stress response declined linearly with the reduction of salt concentration as indicated by the increase in stomatal conductance. The positive slope of the linear regression of stomatal conductance on decreasing salt concentration was 0.1524 ± 0.0163 with a correlation of 0.4029. While the slope

and correlation were not as strong as those observed when salt was increased (-0.3316 ± 0.0171 and -0.6333 respectively) it demonstrated that the response to salt was continuous with a gradual response associated with gradual changes in the treatment. The differences in slope and correlation in the recovery treatment could have been due to senescence of the plant as stomatal conductance decreases over time in quinoa. The continuous tolerance to salt stress also suggested that salt stress in quinoa was a complex trait governed by many genes as found in many other organisms including *A. thaliana* (Motoaki Seki, 2002), *T. halophila* (Taji et al., 2004), maize (Ding et al., 2009), and wheat (Kawaura et al., 2006).

Quinoa under recovery treatment increased in leaf water content, as indicated by the FW:DW ratio, but only to levels of the low-salt control harvested 1-2 weeks prior and not to levels of the recovery control that was harvested simultaneously (Fig. 4). This may be due to the remaining salt in the leaves as indicted by ICP analysis. The recovery-control demonstrated that leaf succulence (*i.e.* water content) decreased with senescence, yet recovery treated plants did not exhibit the decreased succulence expected at this point in their life cycle. These results suggested that quinoa entered a dormancy stage in response to salt stress. Retardation of growth under salt stress has been well documented in many organisms including *T. halophila* (Inan et al., 2004), *A. thaliana* (Attia et al., 2008), canola (Chandler and Thorpe, 1987), sugar beet (Ghoulam et al., 2002), and maize (Tas and Basar, 2009). Inhibition of growth, or dormancy, as a mechanism for salt tolerance was described by He et al. (2002). He et al. identified a dormancy-related gene expressed in salt tolerant varieties of rice during salt stress that was homologous to PsDRM1, a gene associated with dormancy in peas (Stafstrom et al., 1998). It is possible that quinoa has similar genes that are expressed during salt stress. The dormancy state in quinoa was also supported by the initiation of flowering that began to develop in recovery

treated quinoa as the salt concentration decreased. The days-to-flower of the untreated quinoa closely resembled the days-to-flower in salt stressed quinoa once the days under salt stress (days in dormancy) were accounted for although it was difficult to determine when salt stress was initiated and terminated. The days-to-flower of the salt stressed quinoa indicated that dormancy was terminated as some point early in the recovery phase of treatment (250-350 mM NaCl) and not at the end (50 mM NaCl). Plants that were allowed more time to recover also developed seed heads similar to those of the control plants and would likely give similar yields; however, these observations are based on preliminary data and further investigation would be required.

The recovery response in quinoa had practical implications in that allowed us to better understand salt stress. Through the recovery response we observed transport of salt to the leaf and retention of compatible solutes in leaves. Salt stress and water stress often lead to the same problems and mechanisms employed tend to overlap (Orcutt and Nilsen, 2000). If mechanisms involved in the recovery from salt stress were also involved in water stress, which is usually temporary, the application of those mechanisms would be beneficial in drought sensitive crops.

Differences Between Cultivars

Varietal comparisons of salt tolerance have been reported in other organisms. Significant differences in growth were observed between varieties of rice (Moons et al., 1995) and barley (Chen et al., 2007) although salt stress impaired growth to a large extent in all of the varieties tested. Betaine accumulation was also reported to vary significantly between varieties of wheat under salt stress and high betaine levels were correlated with high salt tolerance (Zhao et al., 2005). In this study, the physiological components of quinoa valley and altiplano ecotypes were compared in hydroponics. Altiplano ecotypes at times exhibited significant differences from the valley ecotype and the degree of difference between cultivars was modest. Fresh weight harvests

of both ecotypes after treatment in hydroponics showed similar results. CICA 17, the valley ecotype, under 450 mM salt stress showed a 66% decrease in weight relative to its control while Chipaya and Ollague showed a 61% and 62% decrease respectively (Fig. 3). While the difference between the two ecotypes is statistically significant, the difference may be negligible in practical application. CICA 17 exhibited a more pronounced drop in stomatal conductance as the salt concentration increased and was not able to recover from stress as quickly as the altiplano ecotypes (Table 1). There was a large difference in stomatal conductance, yet the fresh weight recovery measurements indicated that even the valley type was recovering from stress at a rate similar to that of the altiplano types. Compatible solute accumulation also exhibited only mild differences between cultivars and ecotypes. No statistically significant differences in accumulation were observed for trigonelline, betaine, or trehalose in salt-treated leaf and root tissue and overall accumulation patterns were consistent across cultivars. The greatest difference in accumulation between cultivars was in recovery-treated tissue. Between altiplano cultivars Chipaya exhibited the tendency to have greater accumulation of all three solutes in leaf tissue while Ollague exhibited greater accumulation in root tissue. Between altiplano and valley ecotypes, decreased accumulation in the valley type was only observed in control tissue. This may have accounted for some variation between ecotypes since the valley type would not anticipate salt stress while high accumulation in altiplano ecotypes indicated preparation for stress.

Conclusions

Our comparison between quinoa and *T. halophila* response to salt suggested interesting mechanisms of salt tolerance in quinoa. Because quinoa is grown as a staple crop and is more salt tolerant than *T. halophila*, applicable advances of salt tolerance in mainstream crops could be

readily identified through studying quinoa. The differences in response to salt that were observed between quinoa and *T. halophila* in this study indicated that each type employed different mechanisms to cope with salt stress. Quinoa was able to accumulate salt using water retention mechanisms such as decreased stomatal conductance, sequestration, and compatible solutes. It was also able to exclude salt in roots and employed SOS1 and SOS-related mechanisms to prevent salt stress. While several mechanisms have been explored in this study, comparisons between cultivars suggest fine-tuning to multiple environments may not have a strong salt component. The complexity of salt tolerance and the potential for novel mechanisms requires a more effective method of exploration. Microarray studies have been identified as an effective method for identifying genes involved in salt stress. Salt stress genes have been identified through the use of a DNA microarray in many organisms including *Arabidopsis* (Gong et al., 2005), *T. halophila* (Taji et al., 2004), maize (Qing et al., 2009), sunflower (Fernandez et al., 2008) and cotton (Hall, 2009, unpublished data). A microarray study comparing the expression profiles of quinoa under high salt, low salt, and recovery conditions would identify many more genes involved in salt stress and perhaps improve our understanding of the physiological mechanisms used by quinoa to tolerate high saline soils.

Tables and Figures

Table 1. Stomatal conductance of Chipaya, Ollague, and CICA 17. Stomatal conductance was taken 24 hours after treatment was administered and was measured twice on each plant for a total of eight measurements per cultivar-treatment combination per day. * is significantly significant different between the control and treatment (95% confidence intervals were used).

Stomatal Conductance ($mmol/m^2s$) in Recovery Treatment					
	Treatment Day				
	1	4	11	16	21
	Salt Concentration (mM)				
Cultivar	**50**	**200**	**450**	**200**	**50**
Chipaya	**217.8**	**123.0**	**93.3**	**99.3**	**153.4**
Control	**193.9**	**184.8**	**191.7**	**146.9**	**128.8**
Difference	**-23.9**	**61.8**	**98.4***	**47.6 ***	**-24.6**
Ollague	**199.7**	**106.4**	**92.6**	**109.3**	**165.9**
Control	**179.4**	**164.1**	**182.4**	**149.6**	**160.0**
Difference	**-20.3**	**57.7***	**89.8***	**40.3**	**-5.9**
CICA17	**222.3**	**146.4**	**79.1**	**108.0**	**167.6**
Control	**218.1**	**229.6**	**203.2**	**186.4**	**213.6**
Difference	**-4.2**	**83.2***	**124.1***	**78.4***	**46.0**

Figure 1. Quinoa and halophila height and weight relative to the control. Plants height and weight were combined over all time points and the average weight relative to the control was calculated. Bars represent standard error and denote significant differences (p=0.05). For each treatment/plant combination n=35.

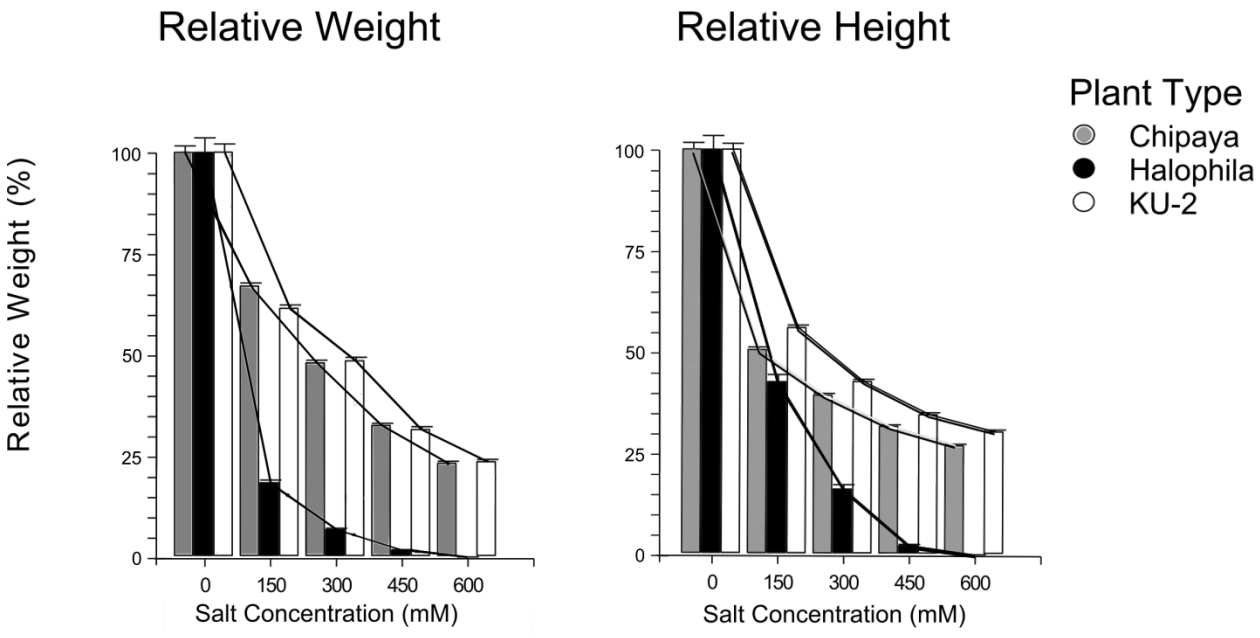


Figure 2. Soil electroconductivity change over time for Chipaya (A), KU-2 (B), and halophila (C). At each time point soil was collected, dried, and electroconductivity measured. N=3 for each time point/treatment combination. Bars represent standard error and denote significant differences (p=0.05).

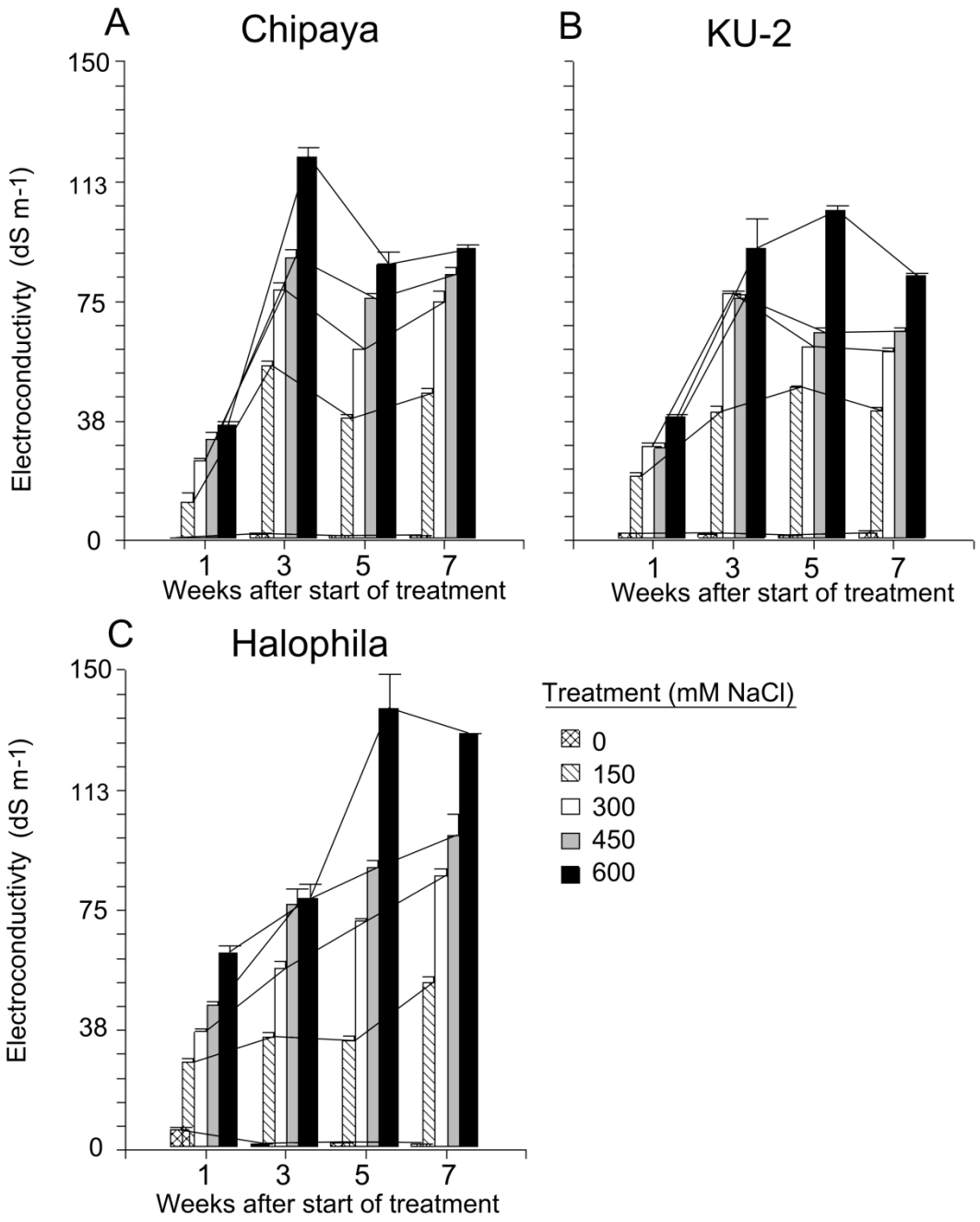


Figure 3. Root and shoot fresh weight of Chipaya, Ollague, and CICA 17 quinoa cultivars grown in a growth chamber in hydroponics under high salt (gradual increase to 450 mM NaCl), low salt (50 mM for duration, harvested with high salt), recovery (gradual increase to 450 mM NaCl followed by gradual decrease to 50 mM NaCl), or recovery control (50 mM for duration harvested with recovery). Measurements were taken at the time of harvest for each treatment. For each cultivar/treatment combination n=16. Bars are mean ± SE.

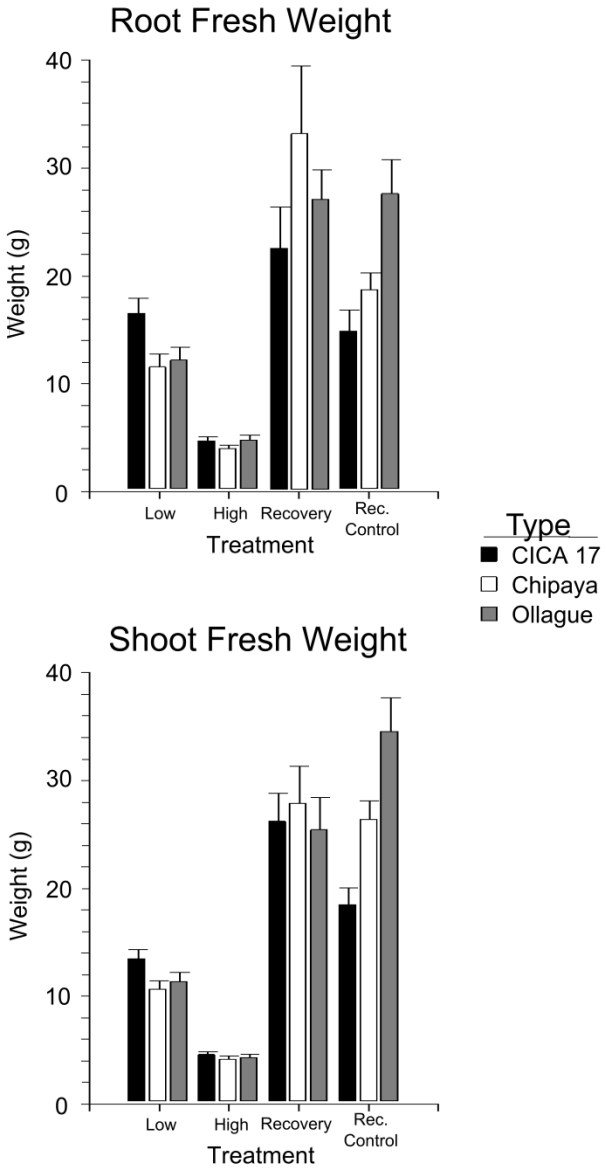


Figure 4. Leaf succulence derived from fresh weight and dry weight measurements of tissue grown under high salt (450 mM), low salt (50 mM harvested with high salt), recovery (450 mM followed by 50 mM), or recovery control (50 mM harvested with recovery) salt treatments. Samples were weighed and combined in equal fresh weight amounts (n=16), and then desiccated. Dried bulk samples were then weighed and leaf succulence calculated. Succulence was then averaged across cultivars (n=3). Bars represent standard error and represent statistically significant similarities or differences (two-sample t-test).

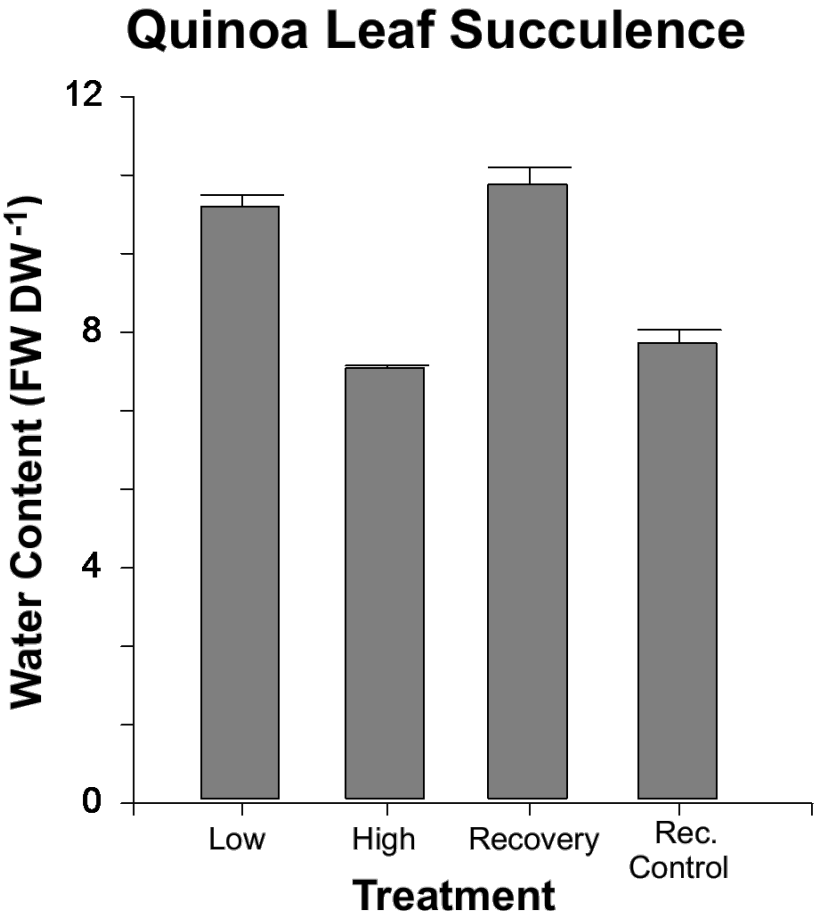
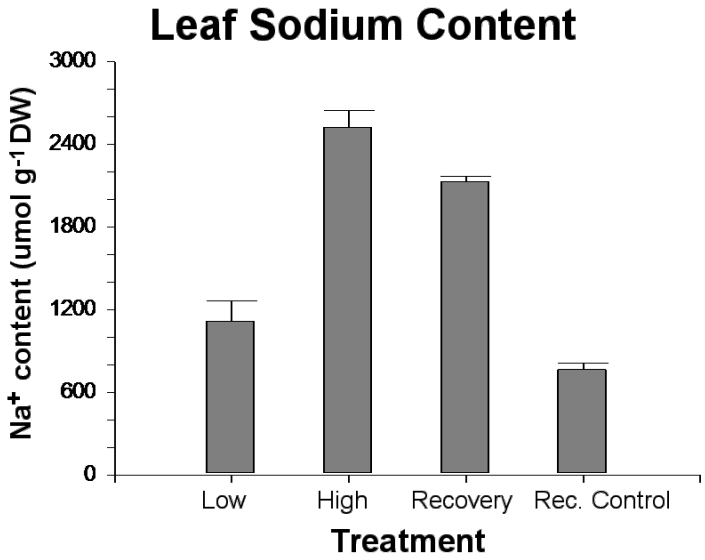


Figure 5. ICP Analysis on quinoa to quantify sodium concentration in leaf and root tissue. Samples were collected from leaf and root tissue under high salt (450 mM), low salt (50 mM harvested with high salt), recovery (450 mM followed by 50 mM), or recovery control (50 mM harvested with recovery) salt treatments. Equal amounts of fresh weight tissue from 16 samples was combined and desiccated (n=16). Following desiccation one ICP analysis was run on each bulked sample. Results were averaged across cultivars (n=3). Bars represent standard error and represent statistically significant similarities or differences (two-sample t-test).



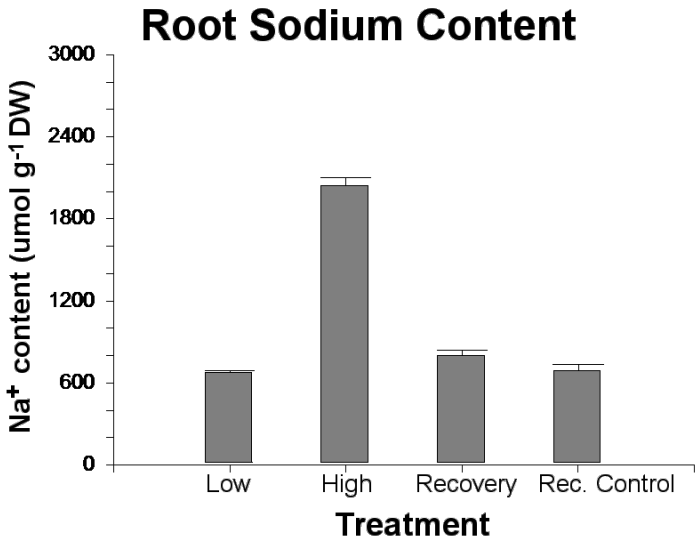


Figure 6. The compatible solutes trigonelline (A,B), betaine (C,D), and trehalose (E,F) were measured in CICA 17 (black), Chipaya (white), and Ollague (gray) leaves (A,C,E) and roots (B,D,F) under high salt (450 mM), low salt (50 mM harvested with high salt), recovery (450 mM followed by 50 mM), or recovery control (50 mM harvested with recovery) salt treatments. For each Chipaya and Ollague, cultivar-treatment combination n=3. For each CICA 17 cultivar-treatment combination, n=1. Bars represent standard error (p=0.05).

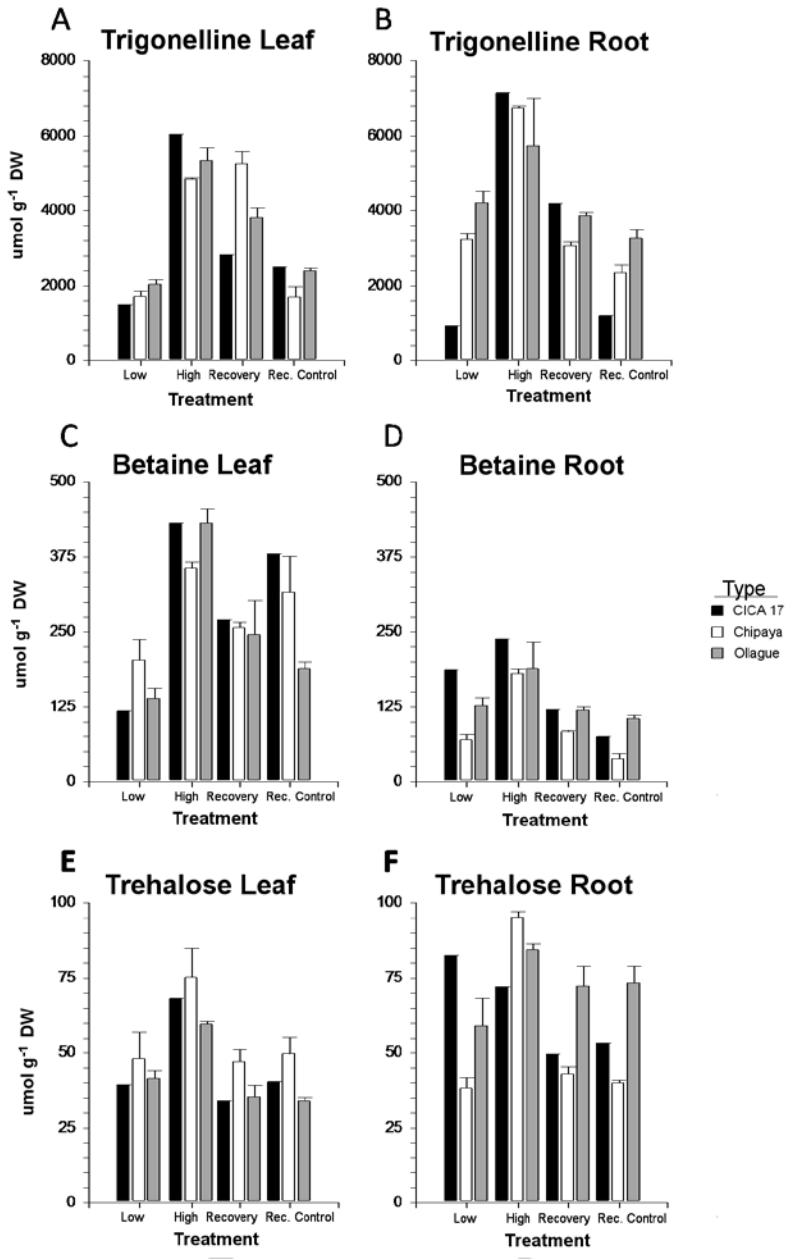
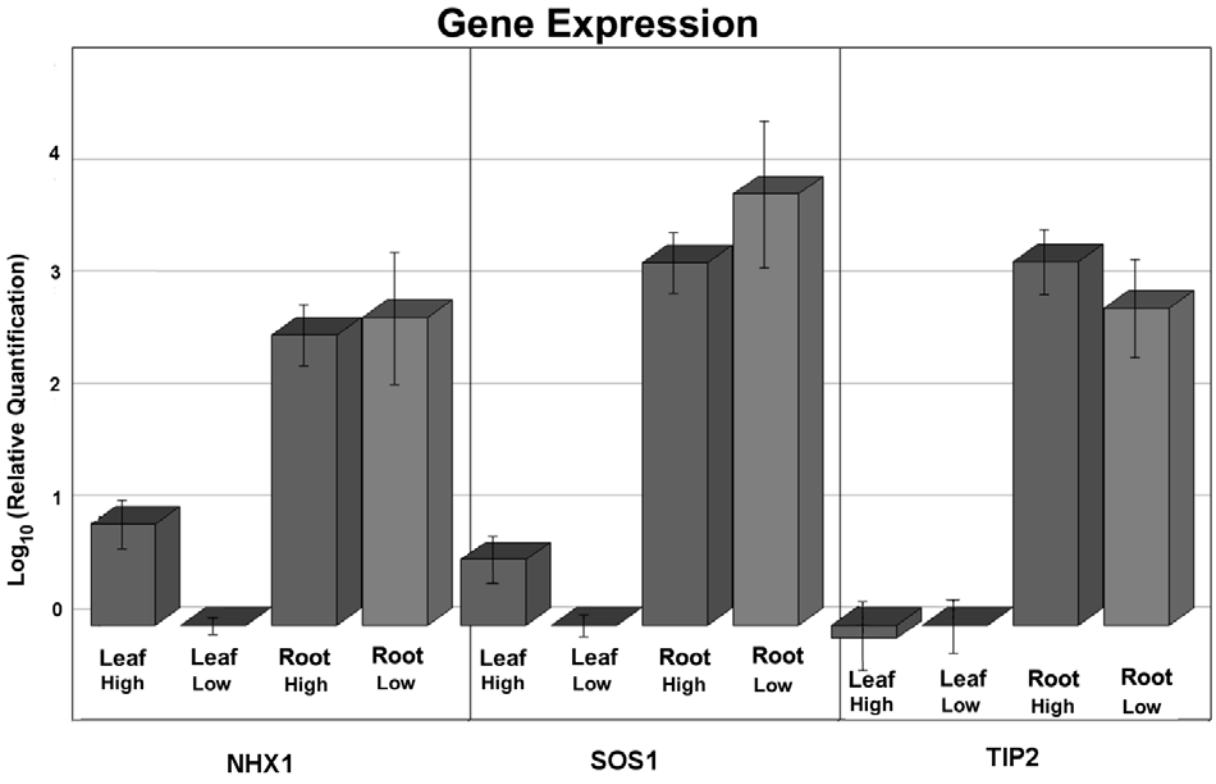


Figure 7. Gene expression of SOS1, NHX1, and TIP2 in quinoa (Ollague) using real-time PCR with GAPDH (not shown) as an endogenous control. High salt leaf (dark red), low salt leaf (green), high salt root (blue), and low salt root (bright red) tissues were compared with low salt leaf serving as a baseline. For each gene-tissue-treatment combination n=6. Bars represent standard error (p=0.05). Differences were also confirmed using a two-sample t-test.



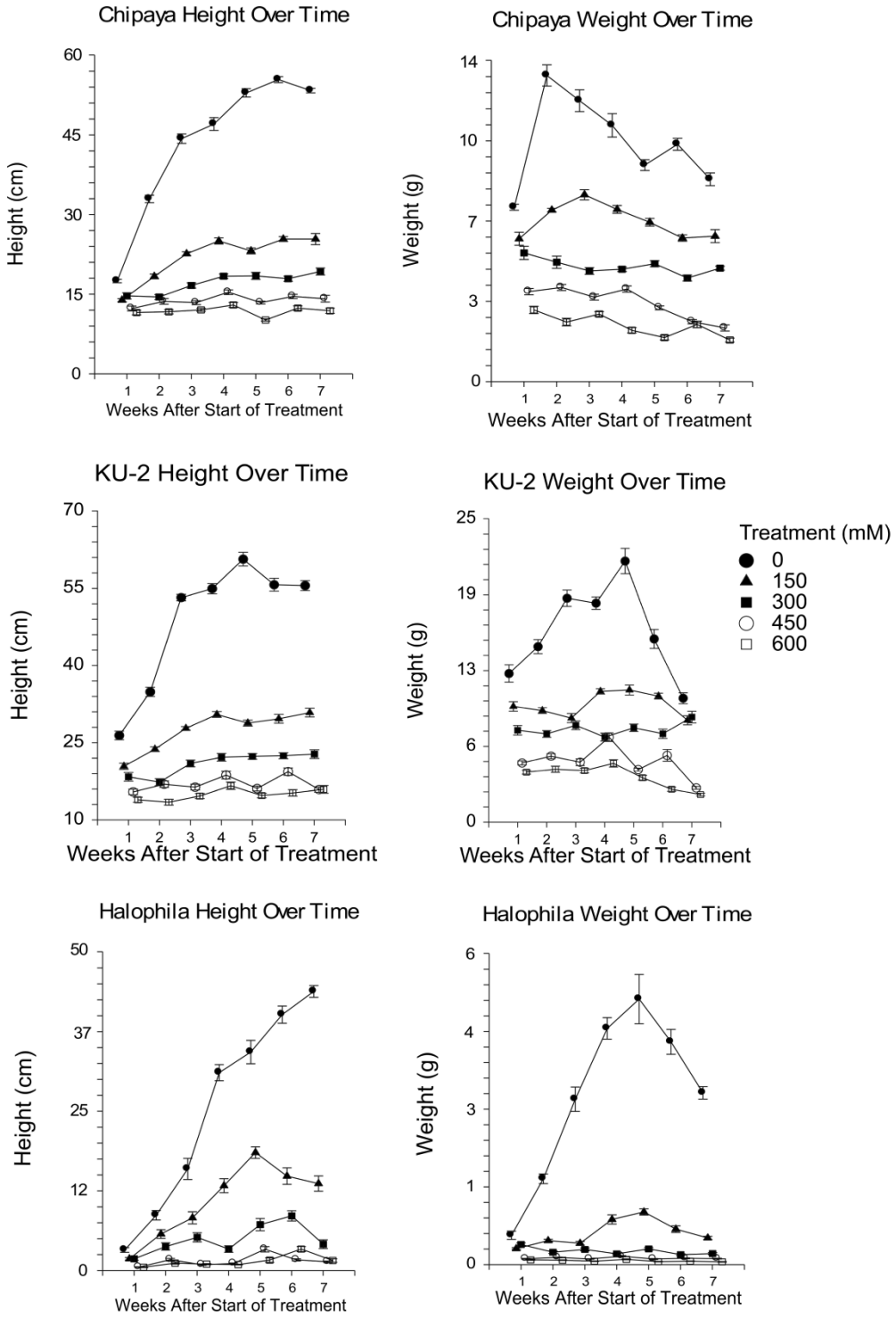
Supplemental Data

Supplemental Table 1. Primer information used for expression analysis using real-time PCR.

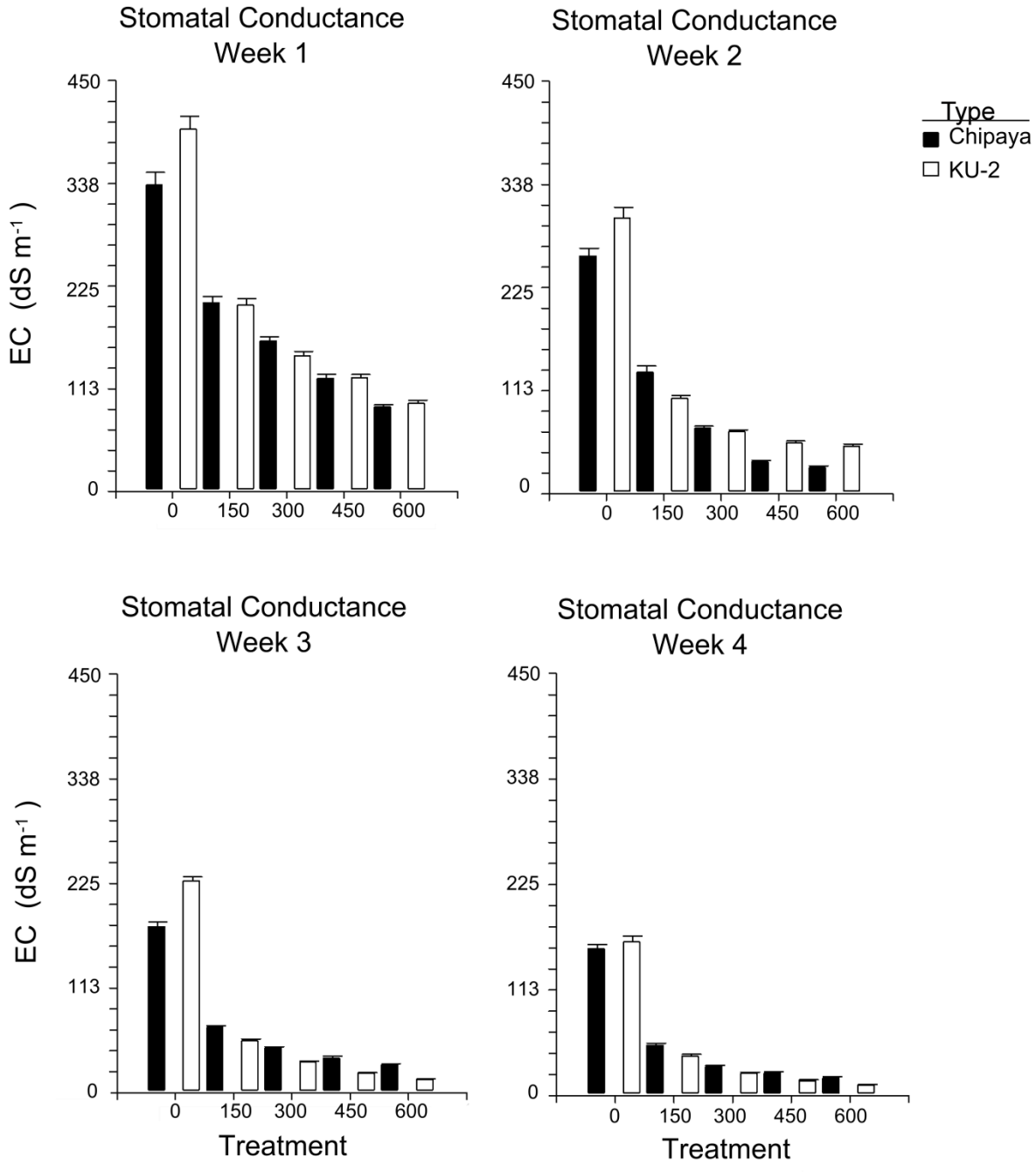
Primer Design			
Gene	**Length**	**Forward/Reverse Sequence**	**Tm**
SOS1	24 mer	5'-TAGCATCAGTGTTFTGGCTCGGAT-3'	60.3 °C
	24 mer	5'-AAAGTCATCACGGTCAGGACACCA-3'	60.2 °C
NHX1	24 mer	5'-ATCAGTTTACGAGGTCAGGGCACA-3'	60.1 °C
	24 mer	5'-GAGGCTTTGTCAGCAACCCAAACA-3'	60.3 °C
TIP2	24 mer	5'-CGCACCAATCGCCATAGGTTTCAT-3'	60 °C
	24 mer	5'-AGTCCACCACCGATAAGAGGACCA-3'	61.3 °C
GAPDH	25 mer	5'-GGTTACAGTCATTCAGACACCATCA-3'	56.7 °C
	21 mer	5'-AACAAAGGGAGCCAAGCAGTT-3'	57.6 °C

Supplemental Figure 1. Halophila (left) and quinoa, cultivar KU-2 (right) two weeks after the start of treatment. Treatments increase from left to right with 0mM (white tag) on the left followed by 150 mM (blue), 300 mM (green), and 450 mM (yellow) with 600 mM (red) on the right.

Supplemental Figure 2. Chipaya, KU-2, and halophila height and weight over time. Plant height and weight were measured weekly after the start of treatment. All treatments are represented and n=5 for each data point. Bars represent standard error.



Supplemental Figure 3. Stomatal conductance in quinoa cultivars measured at one week intervals beginning one week after the start of treatment (week1). Bars represent standard error. Differences were confirmed using a two-sample t-test. For each bar n=10.



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CHAPTER 2: LITERATURE REVIEW

Effects of High Salinity

A major concern in the agricultural industry is the expansion of lands affected by salts and the effects of salinity on crops. There are several reasons that validate this concern. Through irrigation, the soil salinity of agricultural land is continually increasing, giving rise to adverse effects in crop production (Maas, 1987). Increases in soil salinity are causing decreases in yield of as much as 100% in salt sensitive crops such as rice, corn, and peanut (Ayers and Westcot, 1985). Even crops considered to be tolerant do not show the resistance seen in halophytes. Barley, considered the most tolerant mainstream crop, shows a 50% reduction in yield at 200 mM and wheat shows the same reduction in yield at 140 mM (Maas 1987).

Furthermore, the amount of agricultural land affected by salinity is increasing. It is estimated that 900×10^6 hectares of arable land are impacted by high soil salinity (Flowers, 2004). Mühling and Läuchli (2002) estimate that as much as 20% of the earth's arable land is affected by high salinity. As earth's population increases, the need for more agricultural land will force drier areas to become agricultural bases with irrigation as the primary means of watering crops. As saline soils are irrigated, excess water that percolates through the soil and back into rivers and streams carries excess salt back to water sources that are used for irrigation. This in turn will affect any crops irrigated with the water that now carries a greater salt concentration and cause the land being irrigated to become more saline, eventually leading to difficulties associated with salt susceptibility.

Physiological Responses to Salt

A deeper look into the molecular responses to salt stress will help us to better understand potential solutions to the problem. One of the most notable responses to salt is a decrease in enzymatic activity. There is very little variation in the response of various enzymes to salt with the vast majority showing equal sensitivity to salt *in vitro* (Greenway and Munns, 1980). While enzymes react in similar ways, the uptake of ions into the cell varies between plant species. Thus, the impact of salt on enzymatic activity will vary by species and most plant species have some mode of control over ion concentrations within the cell, although some may be more efficient in salt management than others. Salt can also interact with proteins needed for nutrient transport (Orcutt and Nilsen, 2000). One example is the H^+ -ATPase pumps. Many membrane proteins incorporate the H^+ gradient into their mechanisms of nutrient transport into the cell. Yet, Na^+ has an inhibitory effect on H^+ -ATPase which in turn limits the efficiency of the H^+ gradient and decreases nutrient transport into the cell (Kuiper, 1984). Nutrients commonly inhibited include K^+, Ca^{2+}, and Mn^{2+} (Hasegawa et al., 2000). Not only does salt affect proteins but it has been observed by Leopold and Willing (1984) that a linear relationship exists between external salinity and membrane leakage which can be detrimental to the plant.

Other physiological attributes are affected as a response to salt rather than being directly impacted. For example, photosynthesis is decreased as a result of a decrease in stomatal aperture. In an effort to decrease loss of water to the atmosphere during salt stress, stomatal openings are reduced, preventing a higher concentration of salt within the plant (Lovelock and Ball, 2002). Because of this reduction in size, carbon dioxide levels within the plant decrease, causing a reduction in photosynthesis. This has been observed as a decrease in CO_2 fixation associated with low stomatal aperture and water loss (Inan et al., 2004). Soils with high salinity

will also cause a low water potential in the soil which in turn decreases the amount of water available to the plant. Because of this, turgor pressure decreases and inhibition of plant growth is observed (Neumann, 1997).

Molecular Mechanisms for Resistance to Salt Stress

There are several mechanisms implemented by salt tolerant plants when apoplastic Na^+ levels are high. The first is sequestration of sodium ions into the central vacuole of plant cells. This effectively lowers the cytoplasmic Na^+ concentration which allows the plant to continue its metabolic and enzymatic functions until the vacuole reaches saturation. Binzel et al. (1988) exemplified this system with tobacco. They found that when grown in 428 mM NaCl, the plant had a vacuolar Na^+ concentration of 780 mM and a cytosolic concentration less than 100 mM. Furthermore, a gene coding for a vacuolar antiporter, AtNHX1, has been identified and shown to effect salt tolerance (Apse et al., 1999). The only problem with this system is that when saturation of the central vacuole was reached, the cell reached cytoplasmic toxicity and died. Because of this, some plants sequester salt to the vacuoles in older tissue so that the new tissue can continue to develop and function properly (Orcutt and Nilsen, 2000). As long as the plant is able to replace the older tissue with new tissue at an adequate rate, this system will be effective in avoiding the effects of salt tolerance. Along with the growth rate, the saturation level of the vacuole as well as the number of vacuoles within the cytoplasm will impact the level of salt tolerance in the halophyte.

The second mechanism of salt tolerance is salt exclusion in the cytoplasm. During salt stress the cell also activates H^+ -ATPase pumps which drive H^+/Na^+ antiporters, thus transporting Na^+ out of the cell and maintaining a low Na^+ concentration in the cell (Dupont, 1992). The SOS1 gene has been shown to code for an H^+/Na^+ antiporter that controls sodium efflux (Shi et al., 2000).

When used in conjunction with vacuolar sequestration, salinity can be effectively managed. Another mechanism of note is the development of salt glands or epidermal bladder cells which sequester salt away from metabolically active tissue. Such is the case with *Mesembryanthemum crystallinum* which incorporates salt bladders to eliminate salt from tissues (Agarie et al., 2007).

Compatible solutes or osmoprotectants are compounds involved in osmoregulation during salt stress and have been shown to be involved in salt stress in many plants (McNeil et al., 1999; Trinchant et al., 2004; Chen et al., 2007). Osmoprotectants buffer the effects of salt in several ways. When accumulated in high amounts, osmoprotectants can offset the osmotic imbalance caused by a high accumulation of salt in the intercellular space. As salt is excluded from the cell it builds up in the supernatant which causes water to move out of the cell. Compatible solutes are accumulated in the cytoplasm in response to high salt concentrations outside the cell and prevent cellular water loss by balancing the osmotic potential (Yancey, 1994). Osmoprotectants can also provide enzyme protection and maintain membrane integrity under salt stress (Sakamoto and Murata, 2002). One type of osmoprotectants is glycine betaine, also known as and heretofore referred to as betaine. Tobacco exhibited improved salt tolerance when transgenically modified to produce betaine (Holmstrom et al., 2000). Betaine and betaine derivatives such as trigonelline have also been identified, though not quantified, in quinoa seeds (Dini et al., 2006) and they may act as an important component of salt tolerance in chenopodium species. Other osmoprotectants include trigonelline (Cho et al., 1999), pinitol (Adams et al., 1998), sorbitol, trehalose (Rontein et al., 2002), and proline (Ashraf and Foolad, 2007).

Thellungiella halophila: A Known Halophyte

Crops such as rice, corn, and wheat are known as glycophytic crops and can be contrasted with halophytic plants. Orcutt and Nilsen (2000) define halophytes as plants that can cope with saline

environment, typically around 300 mM, without being adversely affected. Investigations into salt stress mechanisms were originally initiated and are now well-characterized in *Arabidopsis* (Ding and Zhu, 1997; Liu and Zhu, 1997; Apse et al., 1999; Zhang et al., 2004; Boursiac et al., 2005). However, while *Arabidopsis* is a model organism and thus easy to characterize, it is not ideal for studying salt tolerance since it is a glycophyte. One model halophyte which has been characterized is *Thellungiella halophila*, also known as salt cress. *T. halophila* is a close relative of *Arabidopsis thaliana* with a genome approximately twice the size and a similar life cycle. *T. halophila* requires a cold treatment of 3-10 days for germination as well as a vernalization period of three weeks (Inan et al., 2004). Because salt cress is a close relative of *Arabidopsis*, Inan et al. (2004) were able to use *Arabidopsis* as a benchmark to characterize salt stress in *T. halophila*. They found that, under salt stress, salt cress root and shoot fresh weight continued to increase in concentrations of up to 500 mM, that weight only slightly decreased over long-term treatment, and that, when returned to normal salt levels, salt cress resumed growth. Zhang et al. (2004) suggested that sequences from *Arabidopsis* could be used to find salt tolerant genes in halophytes which could then be used to genetically engineer salt tolerant crops. This method was used by Vera-Estrella et al. (2005) to identify several genes associated with salt tolerance in salt cress, including SOS1, NHX1, and HKT1, a gene coding for a low affinity Na^+ transporter that regulates Na^+ influx, using an Arabidopsis microarray. Gong et al. (2005) used an Arabidopsis DNA microarray to identify numerous similarities and differences in gene response to salt stress between *Arabidopsis* and *T. halophila*. *T. halophila* was also shown to accumulation statistically significant amounts of proline, an osmoprotectant, under salt stress conditions (Inan et al., 2004).

Quinoa

Chenopodium quinoa (quinoa) is crop grown throughout South America including countries such as Bolivia, Chile, Ecuador, and Peru. Quinoa is an allotetraploid with a basic chromosome number of x=9 and a di-haploid genome size of approximately 967 Mb (Stevens et al., 2006). This dicoteous pseudo-cereal is also high in protein with an excellent balance on amino acids and is gluten-free, making it a crop of interest world-wide (Tapia et al., 1979). Quinoa is also known for its tolerance to high salinity, drought, and cold (Jacobsen et al., 2003). *C. quinoa* belongs to the sub-family Chenopodioideae of the family Amaranthaceae. Within the sub-family Chenopodioidea, approximately 368 species have been classified as tolerant to abiotic stress, or approximately 28% of the family, far more than any other family or sub-family (Orcutt and Nilsen, 2000). Varieties of quinoa from the altiplano of South America grow near salt flats where soil salinity is much higher than soils in the United States. Despite the high salinity as well as cold temperatures and low water supply, altiplano ecotypes thrive in these conditions and even perform better in high salt soil than in low salt soil (Sanchez et al., 2003).

Some varieties of quinoa also show remarkable resistance to salt during germination. For example, Kancolla, an altiplano ecotype, had a germination rate of 75% at a concentration of 57 mS cm^{-1} (Christiansen et al., 1999; Jacobsen et al., 1999). While preliminary data show that quinoa is by definition a halophyte, very little is known about the physiological attributes associated with salt tolerance in quinoa. Jacobsen et al. (2003) reported that salt ion accumulation in the tissues of quinoa occurs in response to salt stress as a means of controlling turgor pressure and transpiration. This is crucial as it will prevent loss of water which could lead to death. They also reported that a reliable indicator of salt stress is stomatal conductance. Turner (2007) characterized the gene sequence and genomic context of a homolog of *A. thaliana*

SOS1 in quinoa suggesting salt exclusion as a possible mechanism, although the function of the quinoa SOS1 homolog has not been demonstrated.

To understand more about the mechanisms of salt tolerance and to confirm whether or not novel mechanisms are employed in quinoa, a physiological comparison to a well-characterized halophyte such as *T. halophila* is needed. A number of obstacles would need to be overcome since these species are not related. Among these are differences in germination, plant size, and morphology. Once these obstacles are overcome, a comparison at varying salt concentrations would determine the degree of tolerance relative to *T. halophila*. Using data previously reported on *T. halophila*, one could then measure compatible solutes and ion accumulation in various tissues and gene expression of genes known to be involved in salt stress to evaluate mechanistic differences between the two species. A DNA microarray would also identify novel mechanisms in quinoa. However, since no quinoa microarray exists, one must first be designed before the analysis can be performed.

Sequencing

Sequencing of cDNA from salt treated quinoa as well as a control is a necessary step for microarray chip design. Traditionally, Sanger sequencing has been used for cDNA sequencing. However, recently several next generation technologies have become much more accessible and efficient. Unlike Sanger sequencing, next generation sequencing (NGS) does not require bacterial cloning. This eliminates bias associated with transcripts that are difficult to clone. NGS also requires much less time. 454 sequencing, a type of NGS, can sequence 400,000 DNA molecules of 250bp each in seven hours. Sanger sequencing can only run 96 DNA molecules of 800bp length, a significant decrease which would require much more time to sequence (von

Bubnoff, 2008). NGS is also much more cost effective than Sanger sequencing when considering the cost per base.

A variety of NGS technologies exist for cDNA sequencing. Three of the most prevalent platforms for sequencing are 454, Illumina (formerly Solexa), and SOLiD. 454 is a pyrosequencing system that uses emulsification PCR and sequence-by-synthesis to detect the sequence of cDNA attached to a bead. Illumina also uses sequencing-by-synthesis but incorporates bridge amplification rather than emulsification PCR. SOLiD incorporates emulsification PCR for amplification as well as sequencing-by-synthesis but uses DNA ligase to attach each nucleotide. The ligation causes a fluorescence which can then be read by the machine. While each of these methods produce a large amount of sequence data, 454 seems to be the best choice for this project. Illumina and SOLiD produce 1300 and 3000Mb per run respectively, but can only produce reads of approximately 35 bases (Mardis, 2008). These technologies are better suited for genome or miRNA sequencing while the longer reads of 454 coupled with the quantity of sequence will best match sequencing of the quinoa transcriptome.

454 sequencing begins with cDNA being ligated with adapters that attach the cDNA to beads. Once attached, the sequence on each bead is amplified so that millions of clonal copies are attached to the bead. Beads are then loaded into a picotiter plate and prepared for sequencing. A primer annealed to the sequence recruits DNA polymerase and nucleotides are added sequentially. Each time a nucleotide is incorporated luciferase activity is read and recorded for each reaction on the plate. This cycle is continued until all strands have been sequenced. Sequence fidelity using the 454 system is 98% accurate as raw data and 99.75% accurate when corrected (Blow, 2007). Once sequencing is completed, the resulting fragments are then assembled into contigs using software provided by 454 Life Sciences.

Microarray Design and Analysis

Since the cDNA used for sequencing originated from mRNA, the contigs resulting from sequencing represent genes transcribed during treatment conditions, in this case under salt stress, and can be used as a source of probes for a microarray. Several methods for microarray chip design are available. One option is a long-oligonucleotide or spotted chip. This was the original design method and can be as accurate as more modern methods although the printing process can cause greater variability (Bammler et al., 2005). The pins used to spot the cDNA on the slide can often unevenly distribute the probes throughout the spot which causes greater variation and difficulty when reading the spot. In order to overcome the problems inherent with spotted microarrays, several companies have developed techniques for printing more uniform and accurate slides.

Because Brigham Young University owns the Agilent platform, this technology will be discussed briefly. First, the desired probes are chosen using eArray software and the sequences sent to Agilent. A slide with one array can contain as many as 244,000 probes. Slides can also be designed with multiple arrays. Agilent offers a 4-plex array with 44,000 probes per array and an 8-plex array with 15,000 probes per array. Using inkjet printing technology short-oligonucleotide probes are constructed one base at a time directly onto the slide. This allows for more efficient use of space as well as a more uniform distribution of probes within the spot. Samples can then be hybridized to the chips and scanned using the Agilent DNA microarray scanner (Agilent, CA). We plan to use sequence data from the 454 sequencing discussed above to create probes that will then be sent to Agilent for chip printing. We will choose 44,000 probes using the eArray software to select for quality probes. This information will then be sent to Agilent to construct a 4-plex array chip.

Once the microarray is designed, RNA can be extracted from salt stressed samples and prepared for hybridization. First, RNA quality needs to be verified using a bio-analyzer. The RNA is then amplified using a TargetAmp RNA amplification kit (Epicenter, WI) and labeled with cy3 or cy5 dyes using a ULS labeling kit (Kreatech, The Netherlands). Two colors are incorporated into the design to compare the transcription levels of different treatments, in this case control and salt stressed tissue. Following labeling, samples are hybridized to the microarrays previously designed and then scanned.

The Agilent system includes software that will be used to analyze the data and isolate probes with differences in expression between the two hybridized samples. These sequences can then be used to identify genes involved in the salt stress reaction. Several of the most prominent candidate genes are verified using quantitative real-time PCR. Significant differences in transcription between the treatments in quinoa could be identified which in turn would identify any novel mechanisms involved in salt stress in quinoa. Naturally, one would expect to detect changes in the expression of SOS1 which has been characterized as a gene involved in salt tolerance in quinoa (Turner, 2007).

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