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Dissecting Salt Tolerance in Soybean by Profiling Differential Physiological Responses under Salt Stress

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Dissecting Salt Tolerance in Soybean by Profiling Differential Physiological Responses under Salt Stress

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Abstract

Saline soils are common worldwide and limit the yield potential of many crops. Plants respond in a variety of ways to the stress imposed by saline soils. Plants under salt stress must first sense their surroundings and transmit a signal alerting the rest of the plant to the saline conditions. Salt tolerance in soybeans is typically defined by exclusion of chloride ions from foliar tissues. Though differences in ion uptake among soybean genotypes is well documented, the key mechanisms employed by tolerant cultivars to cope with salt stress on the whole-plant level are still largely unknown. Objectives of the current research focus on characterization of the differential physiological responses to salt stress between salt-sensitive and salt-tolerant soybean lines and detecting genetic differences which contribute to the ion exclusion mechanisms employed by salt-tolerant lines.

We assessed phytohormone content of two soybean lines following salt stress and found a salt-induced accumulation of abscisic acid suggesting the involvement of this phytohormone in plant abiotic stress responses. The genotype for a newly characterized salt-tolerance gene, *GmCHX1*, was assessed in three salt-sensitive and three salt-tolerant soybean lines. In salt-sensitive soybeans, this cation/H^+ antiporter-encoding gene is reported to contain a copia retrotransposon within its coding sequence. We detected the presence of this transposable element (TE) within three salt-sensitive lines from the U.S. soybean germplasm while this TE was not detected in the three salt-tolerant lines tested

The ability of salt-tolerant soybeans to maintain chlorophyll content, stomatal conductance, and ion exclusion under salt stress demonstrates the wide variety of physiological responses involved in combating this abiotic stress. Determining the key genetic regulators of

each of these responses will enable breeders to enhance the salt tolerance of soybeans and will likely contribute to overall tolerance to abiotic stresses. We show that disruption of the *GmCHX1* coding sequence contributes to the ion inclusion that results in salt-sensitivity in three soybean cultivars from the United States. The functional *GmCHX1* allele is a promising target for selection by breeders looking to protect the yield of future cultivars and elite lines which will probably be cultivated on salt-affected lands.

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Introduction

Causes of Saline Soils

Salt-affected soils are found on every continent and are more common in arid or semiarid regions where annual rainfall is low such as the western United States, north Africa, southeast Asia, and Australia. Data from the Food and Agriculture Organization's World soil database suggests that between 6 and 8% of all land meets the threshold of salinity, equivalent to between 800 million and one billion hectares (FAO, 2008; Tanji, 2002). Saline soils are caused by a high concentration of soluble salt ions in the soil with sodium and chloride being the most soluble and most damaging to plants (Munns and Tester, 2008). Soil salinity is most commonly assessed by measuring soil electrical conductance. Electrical conductance (EC) refers to the ability of a substance to carry an electrical current and increases with ion content of the soil. The SI unit for electrical conductance is Siemens (S) per meter and any soil with an EC level of greater than 4 dS/m is considered saline. This level of soil EC is approximately equivalent to a 40 mM NaCl solution (Tanji, 2002). Deposition of soluble salts onto the soil occurs naturally over time through rainwater, sea spray or in sediment. Certain soil types, especially those high in exchangeable sodium, are prone to release salts via soil degradation. Likewise, poorly drained soils readily accumulate salts. Salts present in precipitation are left behind as water is removed from soils by evapotranspiration.

Although salinization of soils occurs naturally, the process can also be exacerbated by human influences such as irrigation with saline groundwater (Slinger and Tenison, 2007; FAO, 2008). Saline groundwater is present in nearly every state in the United States. However, the depth to saline groundwater is much shallower in some areas compared to others as shown by the map in Figure 1, making these areas more susceptible to salinization (Alley, 2003). Over the past

century, groundwater withdrawals for crop irrigation in Arkansas have continued to increase. Schrader reported in 2001 that groundwater withdrawals for agriculture over the past 40 years have resulted in a 12-meter decline of alluvial aquifer water levels in Arkansas (Schrader, 2001). Recent reports by the USGS of well water-quality from 2003-2007 in Southern Arkansas and Northern Louisiana indicate no major changes in the specific conductance or chloride concentration (Alley, 2003). However, the continued use of saline groundwater in irrigation will result in residual salts that may accumulate to levels inhibitory to crop growth. Major crops grown in Arkansas can be negatively impacted by salt-affected soils making soil salinity a legitimate concern for farmers across the state. For instance, high levels of salt can inhibit germination of rice seeds and soybeans exposed to salt are often stunted. Soils with elevated sodium levels have been identified in several agricultural areas throughout the state of Arkansas including the Stuttgart area where a large percentage of the state's soybeans are produced (Chapman, 1995).

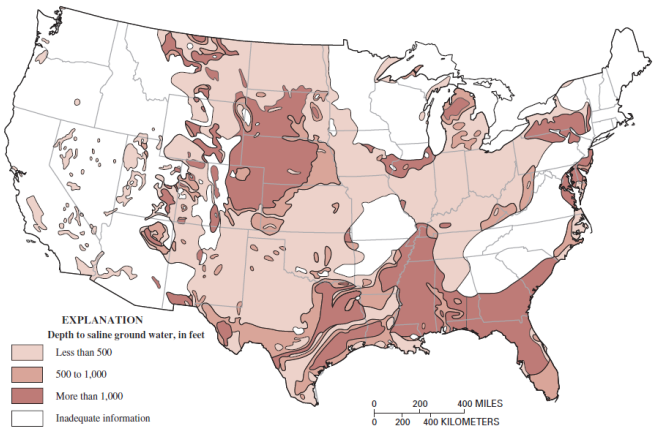


Figure 1. Depth to saline ground water varies across the continental United States (Alley, 2003).

Three types of salt-affected soils exist each with distinct chemical and physical properties which require unique corrective measures. Routine soil testing can be used to establish the type

and extent of salinity. Saline soils are characterized by high levels of soluble salts, which limit available H_2O to plants, and a white or light brown surface crust (Provin and Pitt, 2001). Soils of this type typically contain calcium and magnesium at concentrations which are sufficient to counter the negative effects of the high sodium levels present. Saline-sodic soils are very similar to saline soils with the exception that saline-sodic soils contain a higher ratio of sodium to calcium and magnesium salts (above 15% sodium content) which results in a lower electrical conductance in this soil type compared to saline soils (Chapman, 1995; Provin and Pitt, 2001). Like saline-sodic soils, sodic soils have high concentrations of sodium but are rather low in other soluble salts. Sodic soils also often have a high pH of between 8.5-12.0, which can have a significant negative impact on soil nutrient accessibility and therefore plant growth (Chapman, 1995; Provin and Pitt, 2001). The penetrability of air, rain and irrigation water is often limited in sodic soils with high clay content, making this soil type more susceptible to drying and crusting (Provin and Pitt, 2001).

Unfortunately, few effective options exist for farmers who are faced with reclaiming salt-affected soils for improved agricultural productivity. After determination of soil salinity by a soil test, irrigation water quality should also be assessed and alternative irrigation sources may need to be identified. Improving drainage of soil through deep tillage can increase the movement of water through the soil which may help carry salts past the root zone. Salt leaching is reportedly the most effective method for the removal of salts from the soil's root zone and is particularly effective on soils with good drainage (Qadir et al,2000; Provin and Pitt, 2001). This procedure requires pooling of fresh (salt-free) water on top of the soil and the presence of effective subsurface drains to remove salt-containing water as it infiltrates the soil (FAO, 2008; Provin and Pitt, 2001). The amount of water required to leach a particular soil can be determined by soil

testing. Other methods such as physical scraping of accumulated salts from the soil surface have resulted in limited success (FAO, 2008). Reliable protection of crop yield from the negative effects of soil salinity requires utilization of one or more of these management practices in combination with the use of salt tolerant varieties, if available.

In addition to degradation of farmlands due to agricultural intensification, more land is being urbanized forcing agricultural production to be carried out on marginal lands. Not only do farmers need to produce higher yields than ever due to a growing population, but they must do so under extreme environmental constraints. Increasing salinity tolerance through genetic improvement of crops could provide an economical way for farmers to achieve high yields even when growing on marginal land. Breeding efforts will require a thorough understanding of the tolerance mechanisms utilized by the crop of interest and, more specifically, of the roles of the genes and regulatory elements controlling these mechanisms.

Mechanisms of Salinity Tolerance in Plants

In general, high levels of salts can affect crop growth because they alter microbiological activity in soils and they directly impair the growth and health of plants. Because of the negative impact of saline soils on crop yields, a great deal of research has been done to understand how plants react to this abiotic stress. This body of research has led to the characterization of the salt stress response in plants into two phases: the osmotic phase and the ionic phase. The osmotic phase begins upon root exposure to saline soil with the low osmotic potential of the soil favoring water loss from the plant. These conditions directly inhibit water uptake and also have a major effect on nutrient uptake. From the plant's perspective, this osmotic stress elicits many similar responses as would drought conditions, including a reduction in stomatal conductance.

Reductions in several growth parameters including the rate of new leaf emergence, rate of growth of leaves, and the number of branches is often associated with the osmotic phase of salt stress (Munns and Tester, 2008).

The ionic phase of salt stress is caused by the translocation of ions from the roots to the shoots of the plant leading to accumulation of ions in the foliar tissues after extended exposure to saline conditions. The presence of ions is critical for the functioning of all living cells, but a delicate balance in their concentrations must be maintained. Homeostasis of ions is critical because very high or very low concentrations of ions can inhibit enzyme activities and thus a wide range of cellular processes. Most enzymes can be inhibited by Na^+ concentrations starting at 100 mM (Munns and Tester, 2008; Greenway et al, 1972). As a result of reduced enzyme activity, plants experiencing the ionic phase of salt stress often display an increased rate of senescence in mature leaves along with a decrease in chlorophyll content and photosynthetic activity of these tissues (Munns and Tester, 2008). Likewise, salt ions can also inhibit essential physiological activities of plants. For example, ion accumulation in chloroplasts leads to significant reduction of photosynthesis (Wang et al, 2007).

One way in which plants avoid the negative consequences of growth under saline conditions is by restricting the entry of ions at the root level. Citrus and grapevines, for example, are able to keep Na^+ in the roots and stems and avoid sodium toxicity in their shoots (Flowers, 1988). This method of salinity tolerance is also referred to as ion exclusion and is dependent upon the dynamics of ion exchange at the root-soil interface. Upon crossing the epidermis of the roots, ions may efflux back into the soil or be transported across the endodermis to the xylem (Munns and Tester, 2008). Sodium ions can enter the roots via voltage-independent cation channels or may be exported back into the soil via Na^+/H^+ antiporters (Amtmann and Sanders,

1999; Tester and Davenport, 2003). Once across the endodermal root layer, ions may be loaded into the xylem and carried to the aerial parts of the plant which are often more sensitive to Na^+ and Cl^- toxicity than the roots (Tester and Davenport, 2003).

To regulate xylem Na^+ concentrations, plants have evolved a number of transport proteins including a family of high-affinity potassium transporters (HKT). AtHKT1-1 has been shown to control movement of sodium out of the xylem in *Arabidopsis thaliana* and preliminary work in wheat suggests that HKT1;4-A2 may confer sodium exclusion from leaves (Schachtman and Schroeder, 1994). Overexpression of the plasma membrane Na^+/H^+ antiporter *SOS1*, which is implicated in retrieving Na^+ from the xylem, in *A. thaliana* resulted in improved growth and seed set, reduced Na^+ content and less dramatic decreases in chlorophyll content relative to control plants when under salt stress (Shi et al, 2002). Chloride is able to cross the tonoplast both actively by Cl^- transporters and passively via the apoplast. Which pathway is used is dependent upon the direction of Cl^- movement relative to the Cl^- electrochemical gradient within the plant (White and Broadley, 2001). Voltage-dependent CLCs (passive chloride channels) have been identified in a number of plant species including *Arabidopsis*, rice, corn, and soybean (Zhang et al., 2011). Once ions have entered cells, other membrane transporters are active in regulating localized concentrations. For example, the Na^+ levels in chloroplasts appears to be regulated by a membrane-bound Na^+/H^+ antiporter (Müller et al, 2014).

Plants may close their stomata upon sensing saline or drought conditions by sending an abscisic acid-mediated signal from the roots to the shoots (Munns and Tester, 2008; Davies et al, 2005). Reducing stomatal conductance prevents water loss but also reduces gas exchange and overall transpiration. Via reduced CO_2 uptake, stomatal closure can limit photosynthetic activity and encourage the formation of oxygen radicals ($^1O_2$) which can be converted into other

damaging reactive oxygen species (ROS) and can impose an oxidative stress on plant cells (Sharma et al, 2012). At low concentrations, ROS serve as important signaling molecules. Once the equilibrium between cellular ROS species and their associated scavenging enzymes is disrupted though, ROS can begin to oxidize lipids (Munns and Tester, 2008). For this reason, lipid peroxidation levels have served as a useful gauge of ROS damage in plants grown under stressful abiotic conditions (Tanou et al, 2009; Sharma and Dubey, 2005). In rice, lipid peroxidation levels have been shown to increase with NaCl concentration but salt tolerant rice plants suffer lower levels of lipid peroxidation under salt stress compared to their salt sensitive counterparts (Vaidyanathan et al 2003). ROS-scavenging enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) among others (Gill and Tuteja, 2010). By increasing the production of such enzymes under salt stress, plants can modulate ROS concentrations to a level suitable for signaling purposes. Salt tolerant wheat genotypes, for example, respond to salt stress with a greater increase in SOD, APX, and GR activity compared to salt sensitive wheat genotypes (Sairam et al, 2005). In one study on citrus callus, results suggested that increases in APX activity is key in determining salt tolerance while the activity of other ROS scavenging enzymes is similar between tolerant and sensitive callus (Gueta-Dahan, et al, 1997). The observed variance in the relative importance of each ROS scavenging enzyme among different crop species demonstrates the variety of responses different plants may rely on to adapt to the same stress.

Plants that are able to prevent initial water loss through reduction in stomatal conductance while upregulating ROS scavenging mechanisms to prevent oxidative stress should perform better under salt stress. Transgenic plants overexpressing one or more ROS scavenging enzymes have been created with some success in improving abiotic stress tolerance. For

example, when *SOD* from *Oryza sativa* was overexpressed in *Nicotiana tabacum*, transgenic plants showed improved tolerance to salt, water, and PEG stresses relative to the wild type (Badawi et al, 2004**).** Conversely, *Arabidopsis* mutants with reduced APX levels actually showed greater salt tolerance, demonstrating the flexibility and probable redundancy in plant redox balancing pathways (Miller et al, 2007). Understanding the consequences of manipulating expression of any proposed salt tolerance genes on seed quality and the difficulties of marketability of transgenic crops remain major hurdles in bringing these types of salt-tolerant lines into production.

Tissue tolerance refers to a plant's ability to maintain adequate functions even with high concentrations of ions within plant tissues. A common mechanism of tissue tolerance to salt among plants is the removal of excess ions from the cell cytoplasm into the vacuole by an active process known as vacuolar sequestration. Much like in the roots, tonoplast-located energy-dependent ion transporters are able to move Na^+ and Cl^- across the vacuole as needed to achieve optimal cellular ion concentrations. Several such transporters have been described, including a tonoplast-located Na^+/K^+ antiporter encoded by *GmNHX1* that is induced in soybean leaves by NaCl treatment (Li et al, 2006). In transgenic yeast, cells expressing the protein encoded by *GmNHX1* showed enhanced tolerance to NaCl and increased vacuolar sequestration of Na^+ (Li et al, 2006; Sun et al. 2006). *GmCLC1* encodes a tonoplast-located Cl^- channel in soybean whose expression has been found to be significantly induced in leaves by NaCl treatment. Similar to *GmNHX1*, expression of *GmCLC1* in transgenic yeast resulted in improved salt tolerance which authors attributed to increased sequestration of Cl^- into vacuoles (Li et al. 2006; Phang et al., 2008).

Another mechanism used by plants and other organisms that can combat the damaging effects of high cellular ion concentrations is the stress-induced synthesis of compatible osmolytes including glycine betaine, proline, sorbitol, and mannitol that do not impede normal metabolic reactions within the cell (Munns and Tester, 2008; Hasegawa et al. 2000). Accumulation of these metabolites results in a decrease in the osmotic potential of the cell cytoplasm relative to the vacuole, aiding in the reestablishment of cellular ionic and osmotic homeostasis. Evaluation of plant metabolomes and transcriptomes under salt stress combined with transgenic studies have demonstrated the important role of these osmolytes in combating the negative effects of osmotic stress (Chen and Murata, 2002; Hare and Xu, 1998). Many crop plants increase production of protective osmolytes under a variety of abiotic stresses. For instance, researchers were able to improve the NaCl tolerance of tobacco by introducing a bacterial mannitol synthesis gene (*mt1D*) (Tarczynski et al, 1993). Engineering of the bacterial glycine betaine synthesis gene *codA* allowed glycine betaine synthesis and thereby improved salt tolerance in *Brassica juncea*, a plant species that does not naturally produce glycine betaine (Prasa et al, 2000).

Plants can be categorized according to the ion concentrations of their preferred growth conditions. Halophytes are broadly defined as plants that are able to grow and complete their life cycle under conditions that are considered saline, although a wide range of optimal Na^+ and Cl^- concentrations for different halophytes has been reported (Flowers, 1988; Flowers 1986, Glenn 1999). Glycophytes, on the other hand, have evolved to thrive with comparatively much lower Cl^- concentrations and therefore, rely more heavily on other mechanisms of osmoregulation. Among glycophytes, exclusion of Na^+ or Cl^- does not guarantee physiological tolerance to saline soils (Tester and Davenport, 2003). For instance, a salt-tolerant wild relative of tomato, *Lycopersicon peruvianum*, accumulates higher concentrations of Na^+ than its salt-sensitive

domesticated relative, *L. esculentum* (Tal, 1971; Santa-Cruz et al, 1999). Interestingly, salt-sensitive *Arabidopsis thaliana* mutant *sos1* actually had lower shoot Na^+ content than wild-type plants under salt stress (Ding and Zhu, 1997). Because levels of salt tolerance and mechanisms of tolerance vary greatly among plant species, it is important to remember that a gene or mechanism conferring salinity tolerance in one crop might not necessarily confer the same effects in another crop species.

Most major row crops are considered glycophytes and possess limited salt tolerance leading to a decrease in yield when grown under saline conditions. For example, barley and wheat have soil EC threshold of 6 and 8 dS/m, respectively. With every increase in soil EC unit above the threshold, barley suffers a 5% yield loss and wheat suffers a 7% yield loss on average. Cotton has a threshold and percent yield decrease very similar to both cereals while soybean has a lower threshold at 5 dS/m (Ashraf, 1994). Of the major row crops mentioned, soybean suffers the greatest yield loss with increasing soil salinity (Abel, 1964; Maas and Hoffman 1977).

Importance of Soybean Crop

Soybean [*Glycine max* (L.) Merrill] is a globally important crop that provides protein and oil for a wide array of products. By weight, soybean seed is made up of roughly 40% protein, 20% oil, 35% carbohydrate and 5% ash (Soares et al, 2008). Most soybeans are processed for oil and protein meal with most of the oil destined for use in cooking, biofuels, or manufacturing and most of the protein meal is used as an additive in livestock feed. In fact, only 6% of the world's soybean crop is used directly for human consumption. Traditional soy products may either be fermented or unfermented. Products like natto, soy sauce, miso and tempeh are examples of fermented soy food products. Edamame, tofu, and soy milk represent unfermented soy food

products. Thankfully for soybean farmers, demand for nearly every category of soy foods in the United States is increasing as evidenced by growth of the U.S. retail soy food industry from $1 billion to $4.5 billion over the last 17 years (http://www.soyfoods.org/soy-products/sales-and-trends). Salinity is an ever increasing problem in agriculture, and the ability to maintain or even improve soybean production levels under this constraint will require a better understanding of the genetic components responsible for salt tolerance in the soybean crop.

Effects of Salinity on Soybean Crop and Seed Quality

When grown in 14-15 dS/m soil, twenty soybean cultivars tested gave a yield that was 47.5% of plants grown under non-saline conditions (Chang et al, 1994). Whereas some soybean varieties exhibit higher tolerance than others, there is also variability in the degree of salt tolerance according to the developmental stage of the plant. Saline conditions delay or inhibit germination with these effects being more prominent in salt-sensitive germplasm (Abel, 1969; Phang et al., 2008). The germination stage of soybean is thought to be much more tolerant to salt stress than later stages, although a high degree of tolerance in the germination stage does not necessarily imply the same degree of tolerance in the seedling or adult stage (Phang et al., 2008). Studies of soybean have shown that high salinity may cause reductions in plant height, leaf size, biomass, number of branches, number of pods and weight of seeds (Abel and MacKenzie, 1964; Chang et al, 1994). A major reduction in any one of these categories can severely limit yield potential of the soybean crop and have catastrophic effects on the farmer's financial return.

Not only does salt stress negatively impact germination and growth of soybean plants, but this abiotic stress can also cause a reduction in the agronomic quality of beans harvested from salt-stressed soybean plants. Protein content of soybean seeds is reduced under salt stress

although effects on oil content are inconclusive (Chang et al, 1994). In addition to decreases in the overall productivity of soybean under salt stress, researchers have found that salt stress decreases the number and biomass of root nodules and the efficiency of nitrogen fixation (Singleton and Bohlool, 1984; Delgado et al, 1994). This reduced nodulation of soybeans under salt stress may require farmers who depend on the nitrogen-fixing capabilities of soybean in their crop rotations to find other alternatives for management of soil nitrogen content when growing on salt-affected soils.

Existing Salinity Tolerance in Soybean

Variation in levels of salt tolerance exist in soybean with tolerant and sensitive genotypes being distinguished by ability, or lack thereof, to exclude Cl^- ions from foliar tissues. Sensitivity to Cl^- is greater in cultivated soybean *G. max* compared to its wild relative *G. soja* (Luo et al, 2005a; Zhang et al, 2011). Indeed, it is common for plant species to lose many kinds of biotic and abiotic stress resistance through the process of domestication. Although a negative correlation between leaf chloride content and dry matter production has been reported, a threshold for genotypic classification as sensitive or tolerant has not been officially established (Valencia et al, 2008). Varieties are currently classified as salt sensitive or salt tolerant according to visual ratings of symptoms and by assessment of chloride concentrations in foliar tissues (Valencia et al, 2008; Lee et al, 2008). Much work has been done to determine the genetic basis of salt tolerance in soybean, yet the precise physiological mechanisms controlling this tolerance and the genes controlling those mechanisms are still very poorly understood.

In the late 1960s, experiments in soybean by Abel and colleagues demonstrated a correlation between leaf Cl^- content and leaf chlorosis, suggesting that in soybean chloride may

be more the more toxic component of NaCl stress. (Abel and MacKenzie, 1964). The 3:1 salt-tolerant:salt-sensitive segregation ratio of F_2 progeny from parents with different levels of chloride uptake led Abel to propose that a dominant locus, *Ncl*, was responsible for the leaf chloride exclusion exhibited by soybeans with a tolerant phenotype under salt stress (Abel, 1969). More recent studies have shown that both Na^+ and Cl^- leaf content exhibit a positive correlation with leaf scorch and chlorosis and suggest that the role of both ions in NaCl stress should be explored more fully (Essa 2002, Li et al 2006, Korth lab, unpublished).

Through genetic mapping studies on segregating populations derived from crosses between a salt-sensitive and salt-tolerant parent, a major quantitative trait loci (QTL) has been identified on linkage group N (chromosome 3) in soybean. The alleles associated with markers Sat_091 and Satt237 on chromosome three were found to confer salt tolerance (Valencia et al, 2008). This QTL, often referred to as the S-100 QTL, has been validated through a number of mapping studies and has been found to account for up to 70% of observed variability in salt tolerance in soybean (Valencia et al, 2008; Lee et al, 2004). Recently, a single, dominant gene for salt tolerance was fine-mapped in *G. max* variety Tiefeng 8 to the same region as the previously described S-100 QTL (Guan et al, 2014a). Assessment of allelic variation at this locus within additional Chinese soybean germplasm revealed that in salt-sensitive plants, a retrotransposon insertion was present within the coding sequence of the *Glyma03g32900* locus resulting in a premature stop codon and a truncated transcript in salt-sensitive plants (Guan et al, 2014b). The copia retrotransposon-containing allele was designated *GmSalt3* and the salt tolerant allele *GmSALT3*. The functional *GmSALT3* gene is predicted to encode an endoplasmic reticulum localized cation/H^+ antiporter (Guan et al, 2014b). Presence of the tolerant allele was spatially correlated with geographic regions of salinity within China while the salt-sensitive

allele was more prevalent in non-saline areas. These findings suggest that the allele associated with salinity tolerance has been maintained by positive selection and loss of this allele among the tested Chinese germplasm may be due to associated fitness costs under non-saline conditions.

Resequencing of a recombinant inbred line population led Qi et al to the identification of the same locus associated with salt tolerance (*Glyma03g32900)*, which they named *GmCHX1* (Qi et al, 2014). We will utilize the *GmCHX1* name in reference to the *Glyma03g32900* locus for the remainder of this report. A second group of researchers utilized whole genome resequencing on a diverse group of U.S soybean germplasm including several ancestral lines and were able to identify dependable molecular markers associated with the sensitive and tolerant alleles of the *GmCHX1* gene (Patil et al, 2016). The high levels of accuracy (>90%) afforded by these markers in identifying salt-tolerant and salt-sensitive CHX1 alleles provide a promising tool for the development of soybean lines with improved tolerance to saline conditions.

Salt tolerant soybean rootstock plays a major positive role in ion exclusion and physiological salt tolerance (Ren et al, 2012). However, the mechanisms responsible for stress signaling between the roots and shoots of soybeans under saline conditions is still largely unknown. Unfortunately, physiological adjustments made by tolerant soybean lines both immediately and after extended exposure to saline conditions has not been widely reported. Soybean research has suggested a number of genes whose expression are induced or suppressed in salt tolerant lines under saline conditions and thereby may be involved in the plant's response and adaptation to salt stress (Umezawa et al, 2002; Ren et al, 2012; Hettenhausen et al, 2016; Fan et al, 2013). Validation of these gene expression studies is needed along with further characterization of the genes' roles in salt stress through overexpression and knockout studies of model plants before this information can be utilized in breeding salt-tolerant soybean lines.

Fortunately, likely roles of many putative soybean ion transporter genes, including the previously mentioned *GmNHX1* and *GmCLC1* genes, have been shown through transgenic studies *in vivo* and *in vitro* (Li et al, 2006; Sun et al, 2006; Phang et al, 2008). *GmCAX1*, a plasma membrane localized cation/H^+ antiporter in soybean, was reported to be induced in soybean by treatment with Na^+ and other osmoticum such as PEG. When *GmCAX* 1 was expressed in *A. thaliana*, Na^+ accumulation was reduced and tolerance to Na^+ during germination was improved (Luo et al, 2005b). Several calcium dependent protein kinases (CDPK), which possess both kinase and calcium sensor domains, in soybean were recently reported to be upregulated in response to ABA and drought treatments (Hettenhausen et al, 2016). CDPK knockout studies in other plants have suggested a positive role for CDPKs in ABA-regulated signaling, making this gene family a worthy target for further exploration of its possible role in root-to-shoot stress signaling in soybean (Mori et al, 2006).

There is some evidence to suggest that soybean may increase ROS scavenging activities under salt stress as a means of restoring oxidative balance. When measured in the leaves and roots of salt stressed soybeans, activity levels of the ROS-scavenging enzymes superoxide dismutase (SOD) and ascorbate peroxidase (APX) were increased in tolerant soybean (Yu and Liu, 2003). The increased SOD and APX activity in tolerant soybean was also correlated with a decrease in oxidative damage as indicated by O_2- content. A putative purple acid phosphatase gene in soybean, *GmPAP3*, has also been shown to be induced by salinity, osmotic and oxidative stresses (Liao et al, 2003). Improved growth and reduced lipid peroxidation of transgenic *A. thaliana* expressing *GmPAP3* under saline conditions suggest that this soybean gene could play an important role in redox balancing in soybean.

Objectives

The objectives of the current study are aimed at evaluating differential physiological responses to salt stress between salt-tolerant and salt-sensitive soybean lines and assessing methods of identifying salt tolerant soybean lines for breeder use. By monitoring differences in plant growth, chloride uptake, phytohormone and oxidative stress response between salt-tolerant and salt-sensitive soybean lines, potential biological markers of salt tolerance may be identified. Additionally, the results of this work will contribute to the limited knowledge of the physiological mechanisms that dominate the tolerant response to salt stress in soybean. Infrared thermography will be tested as a new, non-destructive screening method for salt tolerance in soybean. We will also evaluate the *GmCHX1* locus for differences among the soybean lines tested.

Objective 1: Determine differences in physiological responses between salt-sensitive and salt-tolerant soybean lines subjected to salt stress.

Objective 2: Determine the usefulness of infrared thermography as a salt tolerance screening method in soybean.

Objective 3: Evaluate differences in genotype at the *GmCHX1* locus among salt-sensitive and salt-tolerant soybean lines.

Materials and Methods

Plant Growth and Maintenance

Seed from soybean cultivars Clark, Glenn (salt-sensitive), Manokin and Osage (salt-tolerant) were planted into a 10.2- by 10.2- by 8.9-cm square plastic pot containing pasteurized river sand at a density of 3 seeds per pot. Seedlings were germinated and emerged in a greenhouse under 16 hour days with supplemental lights as needed. The average daytime temperature in the greenhouse was between 22-26 °C and average night temperatures between 18-20 °C. Plants were fertilized once prior to the treatment period using 0.5x MiracleGro® All Purpose Fertilizer (24N-8P-16K, with urea as nitrogen source) and every other day throughout the duration of the treatment period.

Salt Treatment

Plants were treated when the first trifoliate was fully emerged, which is defined as the V1 growth stage in the soybean developmental cycle. Treatment consisted of partial flooding with 100mM NaCl of dH_20 for two hours daily. Treatment solutions were supplemented with 0.5x MiracleGro® All Purpose Fertilizer (24N-8P-16K, with urea as nitrogen source) every other day. Each experiment consisted of at least three plants per cultivar per treatment arranged as a completely randomized factorial design and each experiment was repeated at least twice.

Reciprocal Grafting

Reciprocal grafting of soybean seedlings was carried out using the "straw-band" technique reported for use in soybean in 1972 (Bezdicek et al, 1972). Using a razorblade, the upper portion of the rootstock source (two-week old plants) was removed below the cotyledons and a vertical

slice was made into the top of the stem about 1-2cm deep. The hypocotyl of the scion source (one-week old plants) was cut above the cotyledons and the end was cut to form a wedge. Seedlings were spritzed with H_2O to prevent desiccation and the scion was gently inserted into the split rootstock. Grafts were secured with segments of plastic drinking straws and plastic tubing as described in Bezdicek et al, 1972. Each genotype was reciprocally grafted to the other and grafts between scion and rootstock of the same genotype were also made to serve as a control for the grafting procedure. Plastic domes were placed over grafted plants and plants were sprayed with H_2O frequently to prevent desiccation until healing of the graft union.

Phytohormone Analysis

For sample collection, 100 mg of tissue from one leaflet of the first trifoliate was placed into a 2 mL tube (Eppendorf) and immediately frozen in liquid nitrogen. Samples were sent to the Donald Danforth Plant Science Center Proteomics and Mass Spectrometry Facility in St. Louis, Missouri for analysis. Hormone extractions were analyzed at the Danforth Center by LC-MS/MS to detect concentrations of the following phytohormones: abscisic acid (ABA), jasmonic acid (JA), 12-oxo-phytodienoic acid (OPDA), jasmonate isoleucine (JA-Ile) and salicylic acid (SA). The data was normalized based on the internal standards D6ABA, D2JA, and D4SA and hormone concentrations were reported in ng/g fresh weight. Means of each treatment (H_2O and NaCl) within a cultivar were compared by a Student's *t*-test using a p-value of 0.05.

Mineral Analysis

For ion content analysis, a single axial leaflet was collected from each plant from the first-formed trifoliate and placed into a coin envelope. Envelopes containing leaf tissue were

incubated at 31°C for 72 hours to allow complete desiccation of the tissues. Dried tissue was roughly ground using a benchtop coffee grinder and 100 mg of tissue was placed into a 1.5mL Eppendorf microcentrifuge tube. Samples were shipped at room temperature to Arkansas State University for chloride analysis using a Haake Buchler Digital Chloridometer. An additional 10 milligrams of dried tissue from each sample was placed into a labeled ELISA bag for sodium content measurements along with 500 µl of diH_2O. The tissue was macerated by scraping a plastic pestle over the outside of the ELISA bag. The leaf extract was transferred to labeled 1.5 mL Eppendorf tubes and 200 µl of extract was pipetted onto the sensor of a Horiba Na^+ meter (B-722 LAQUAtwin).

Oxidative Stress Assay

The OxiSelect™ TBARS Assay Kit (STA-330, Cell Biolabs, Inc., San Diego, CA) was used to quantify lipid peroxidation via measurement of malondialdehyde (MDA) in plant tissue samples. MDA is a natural by-product of lipid peroxidation and is widely accepted as a marker of oxidative stress in biology. This assay is based on the knowledge that MDA forms a 1:2 product with thiobarbaturic acid (TBA). The following protocol was employed in setting up the assay: an aliquot of 2X TBA diluent was diluted to 1X by addition of diH_2O (250 µl 1X TBA diluent needed per sample). The appropriate volume of TBA powder (1.3 mg per sample) was added to the 1X TBA diluent to create the TBA reagent. Sodium hydroxide was added to the TBA reagent to adjust pH to 3.5 and the solution was mixed to dissolve. Sample buffer was prepared by combining 10 µl butylated hydroxytoluene (BHT) and 990 µl 1X phosphate buffered saline (PBS) per sample. SDS lysis solution was warmed in a 37 °C incubator to thaw and allow resuspension of precipitation.

A standard curve was created by assaying a dilution series of the MDA standard provided in the kit. The standard dilution series was prepared according to the volumes in the table on the following page.

Table 1. Standard curve set up for OxiSelect ™ TBARS assay.

Standard Tubes	**MDA Standard (µL)**	**Water (µL)**	**MDA Standard (µM)**
1	125 µL	875 µL	125
2	250 µL of Tube #1	250 µL	62.5
3	250 µL of Tube #2	250 µL	31.25
4	250 µL of Tube #3	250 µL	15.63
5	250 µL of Tube #4	250 µL	7.81
6	250 µL of Tube #5	250 µL	3.91
7	250 µL of Tube #6	250 µL	1.95
8	250 µL of Tube #7	250 µL	0.98
9	0 µL	250 µL	0.0

Immediately upon collection, one hundred milligrams of fresh tissue was ground in 1 mL PBS + BHT using a plastic pestle. Samples were spun at 10,000 x g for five minutes to pellet cellular debris. One hundred microliters of each sample and each standard was transferred to a new 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany). Standards were assayed in duplicate. One hundred microliters of SDS lysis solution was added to each sample and mixed by pipetting. Samples were incubated for five minutes at room temperature. Two hundred fifty microliters of TBA reagent was added to each sample and standard and tubes were inverted to mix. Samples were incubated at 95 °C for 45-60 minutes followed by cooling in an ice bath for 5 minutes. Samples were then spun at 3000 rpm for 15 minutes. One-hundred fifty microliters of each

standard and sample was assayed in a black 96-well fluorescence microplate with optical bottom (265301; Nalge Nunc International, Rochester, NY). The plate was read using a Biotek Synergy HT fluorescence microplate reader at 540 nm excitation and 590 nm emission and data was collected using the associated KC4 analysis software (BioTek® Instruments, Inc., Winooski, Vermont). Replicates from each biological group were averaged and means of each treatment within a cultivar were compared by Student's *t*-test with a significance level of $p \leq 0.05$.

Infrared Thermography

All plants were planted and treated as previously described. Plants were grown in a Conviron® walk-in growth chamber under a 12-hour light period (light intensity of 4) at 25 °C (Controlled Environments, Ltd., Winnipeg, Canada). Single dH_20-treated plants and NaCl-treated plants were imaged side by side immediately following the two-hour treatment period each day. Plants were imaged inside of a studio light box (Cowboy Studio, Allen, Texas) to diffuse incoming light. Two sheets of amber-colored plexiglass were placed inside the box as a background as mentioned by Sirault et al. (2009). Emissivity of this material is different than that of green plants resulting in an apparent temperature of about two degrees warmer than the air temperature, which provides a homogeneous background and enables rapid separation of seedling images from their background.

All infrared images were captured using the FLIR T420 infrared camera under default imaging settings. Images were analyzed using the FLIR software which allows the average temperature of any given area within an IR image file to be calculated to within $\pm$ 0.1°C. The average temperature was captured for each of the three leaflets of the first (oldest) trifoliate from which the average temperature for each plant was calculated. Seven Clark plants and seven Manokin

plants were imaged and analyzed for both the H_2O and NaCl treatment. The temperature response to salt treatment was calculated by subtracting the average temperature of H_2O-treated plants from the average temperature of NaCl-treated plants of the same cultivar. Temperature response of both cultivars was recorded for six days. Average temperature differences between the cultivars were compared by student's *t*-test at $p \leq 0.05$.

Chlorophyll Content Measurements

Following fourteen days of treatment, ten Clark plants and ten Manokin plants from both H_2O and NaCl treatments were assessed for chlorophyll content using a SPAD-502 Chlorophyll Meter (Konica Minolta; Tokyo, Japan). This instrument detects the absorbance of chlorophyll in both the red and near-infrared regions from which the meter calculates a SPAD value which is proportional to the amount of chlorophyll present in the leaf. One leaf of each plant was assessed for chlorophyll content by placing the leaf inside the measuring head of the meter while avoiding the thick mid-vein. The measuring head was closed and the SPAD value was recorded.

DNA Extraction

The entire axillary leaflet (~500mg) of the first-formed trifoliate was collected in 1.5 mL Safe-Lock Tubes (Eppendorf) and immediately frozen in liquid nitrogen. Samples were stored in -80 °C freezer until time of extraction. Five hundred microliters of CTAB buffer was added to each tube and tissue was pulverized using a plastic pestle and power drill for approximately one minute each or until tissue was completely ground. Samples were incubated in a 65 °C water bath for 45 minutes followed by a brief cooling on ice. Five hundred microliters of chloroform were added to each tube and samples were inverted 6-8 times to ensure adequate mixing.

Samples were spun at 5000 rpm for 15 minutes and the aqueous phase (top, clear phase) was transferred to a new 1.5 mL tube. DNA was precipitated by the addition of 500 µl of 100% isopropanol followed by incubated on ice for 15 minutes. DNA was pelleted by centrifugation at 5000 rpm for 10 minutes. Isopropanol was removed and the pellet was washed with 500 µl of 70% ethanol and spun at 13000 rpm for 10 minutes. The ethanol was removed and the pellets were allowed to air dry for 10-15 minutes or until residual ethanol evaporated. Pellets were dissolved in 50 µl of TE buffer and incubated at 37 °C for 15 minutes followed by vortexing to resuspend the DNA pellet. DNA samples were quantitated using a Bio-Spec Nano spectrophotometer (Shimadzu, Kyoto, Japan) and diluted to 40 ng/µl.

PCR and Gel Electrophoresis

PCR reactions were set up as follows (per reaction): 2.5 µl 10X Genscript Buffer, 0.5 µl dNTPS (10mM each), 0.5 µl Taq polymerase, 1.0 µl forward primer, 1.0 µl reverse primer, 13.5 µl dH2O and 1.0 µl DNA template (40 ng/µl). Reactions were placed in a thermocycler under the following cycling conditions: 1) 94°C for 5 minutes 2) 94°C for 30 seconds 3)60°C for 30 seconds 4)72°C for 30 seconds 5) Repeat steps 2-4 x35 6)72°C for five minutes 7) 4°C for ∞. A combination of 5 µl of PCR product and 2 µl of 6X loading dye were loaded into the wells of a 0.8% TAE agarose gel stained with GelGreen™ nucleic acid gel stain and run at 100V for one hour to visualize PCR results.

Data Analysis

JMP ® Version 13 Basic Analysis developed by SAS was utilized for statistical analyses. The Student's *t*-test was employed for direct comparison of two means. For comparison of multiple

means and for determining the effect of each factor, data were analyzed using analysis of variance (ANOVA) under a full factorial model

Results and Discussion

Physiological Response of Soybean to Salt Stress

Relatively little is known about the specific mechanisms controlling salt tolerance in soybean. Although great strides have been made over the past few decades in the general understanding of ion transport and stress signaling among plants, additional work cataloging the unique responses by different crop plants as well as varieties within a crop are required. We exploited the differential tolerance to salt stress among four soybean lines in order to survey the physiological responses and genetic components associated with tolerance to this abiotic stress. We also evaluated a potential new salt tolerance screening method for use in soybean.

Two soybean varieties were chosen for the initial survey: salt-sensitive Clark and salt-tolerant Manokin. Plants were treated with 100 mM NaCl or dH_2O for 14 days, after which chlorophyll content was measured using a chlorophyll meter (SPAD-502; Konica Minolta; Tokyo, Japan). The mean of chlorophyll measurements from ten dH_2O- and NaCl-treated plants of each cultivar were compared using a One-way ANOVA and a significance levels of $p < 0.05$. NaCl-treated Clark plants showed a significant reduction in chlorophyll content relative to dH_2O-treated Clark plants (Figure 2). The chlorophyll content of the salt-tolerant Manokin plants did not differ significantly between treatments (Figure 2). Under the salt treatment, chlorophyll content of salt-sensitive Clark was significantly reduced compared to chlorophyll content of salt-tolerant Manokin (Figure 2). Similarly, salt-sensitive Union soybeans experienced more severe reductions in chlorophyll content relative to salt-tolerant WF-7 soybeans under salt stress (Ren et

al., 2012). More specifically, NaCl-treated Clark plants suffered a 38.6% reduction in chlorophyll content relative to H_2O-treated Clark plants while NaCl-treated Manokin plants only suffered a 0.35% reduction in chlorophyll content relative to H_2O-treated Manokin plants.

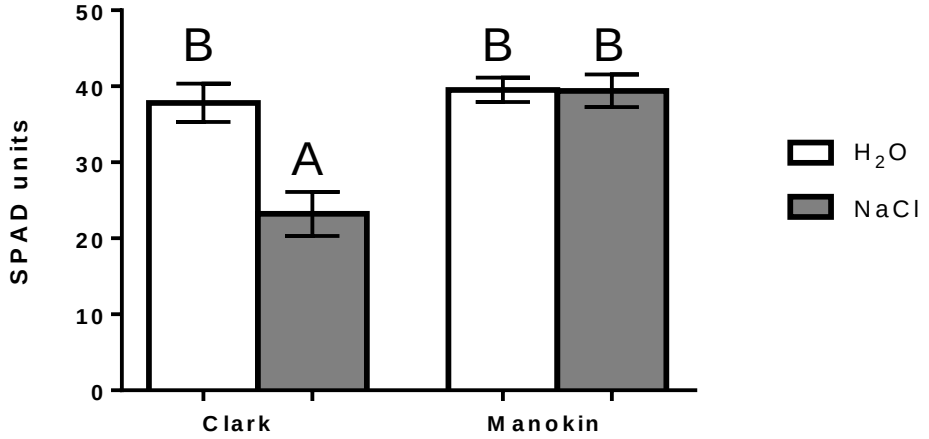


Figure 2. The average chlorophyll content (in SPAD units) was significantly reduced in NaCl-sensitive cv. Clark following 14 days of 100 mM NaCl treatment while chlorophyll content of Manokin was unaffected by the NaCl treatment. Bars that share a letter are not significantly different from on another according to oneway ANOVA; n = 10; $p \leq 0.05$; $\pm$SEM

The fresh weight, plant height, and root dry weight of these plants were also measured to assess any impacts of NaCl treatment on plant growth (Figure 3). Both soybean lines showed significant reductions in fresh weight (Fig. 3A) and plant height (Fig. 3B) due to NaCl treatment, although in both instances the reduction in Manokin plants was less severe. Under NaCl stress, the fresh weight of salt-sensitive Clark plants was significantly less than that of salt-tolerant Manokin plants (Figure 3A). Clark plants showed a 66% reduction in fresh weight due to NaCl treatment while Manokin plants only showed a 34.7% reduction in fresh weight due to NaCl treatment. NaCl treatment caused a 23% reduction in plant height in Clark plants and a 16.4% reduction in plant height in Manokin plants. Both cultivars also showed a small and insignificant decrease in root dry weight under NaCl treatment relative to H_2O treatment (Figure 3C). When

directly comparing NaCl-treated plants, Manokin plants show significantly higher root dry weights than Clark plants. NaCl treatment caused a 49% reduction in root dry weight in Clark plants while Manokin plants only suffered a 22.8% decrease in root dry weight under NaCl treatment.

The SPAD results show that after two weeks of consistent NaCl stress, salt-tolerant Manokin plants are superior at maintaining chlorophyll content relative to salt-sensitive Clark plants. Salt stress has been shown to reduce chlorophyll content and/or photosynthetic rate in a number of plant species including cucumber (*Cucumis sativus* L.), chickpea (*Cicer arietinum* L.), cotton (*Gossypium hirsutum* L.) and wild and cultivated soybeans (Yildirim et al., 2006; Soussi et al., 1998; Brugnoli and Lauteri, 1991; Kao et al., 2003; Lu et al., 2009). Lenis et al. even reported an increase in chlorophyll content under salt stress in some salt-tolerant *Glycine* accessions (2011).

Clear differences in biomass production between the cultivars, as measured by fresh weight and root dry weight, indicate that the salt-tolerant Manokin plants are able to continue active photosynthesis at higher levels than salt-sensitive Clark plants under NaCl stress, which would presumably translate to higher yields. Although we did not directly compare chlorophyll content and photosynthetic rate, these results support previous reports that established a close relationship between chlorophyll levels and photosynthetic rates in soybeans (Buttery and Buzzell, 1977). Farmers dealing with saline soils may take simple chlorophyll measurements to assess the level of salt stress being experienced by their crop in the fields, which could inform irrigation and other management decisions. Breeders could utilize fresh and dry weight data to determine if a correlation between harvest yield and early-season biomass accumulation of

soybeans under salt stress exists. If a strong correlation was found between these factors, breeders could select for higher yields under salt stress much earlier in the breeding process.

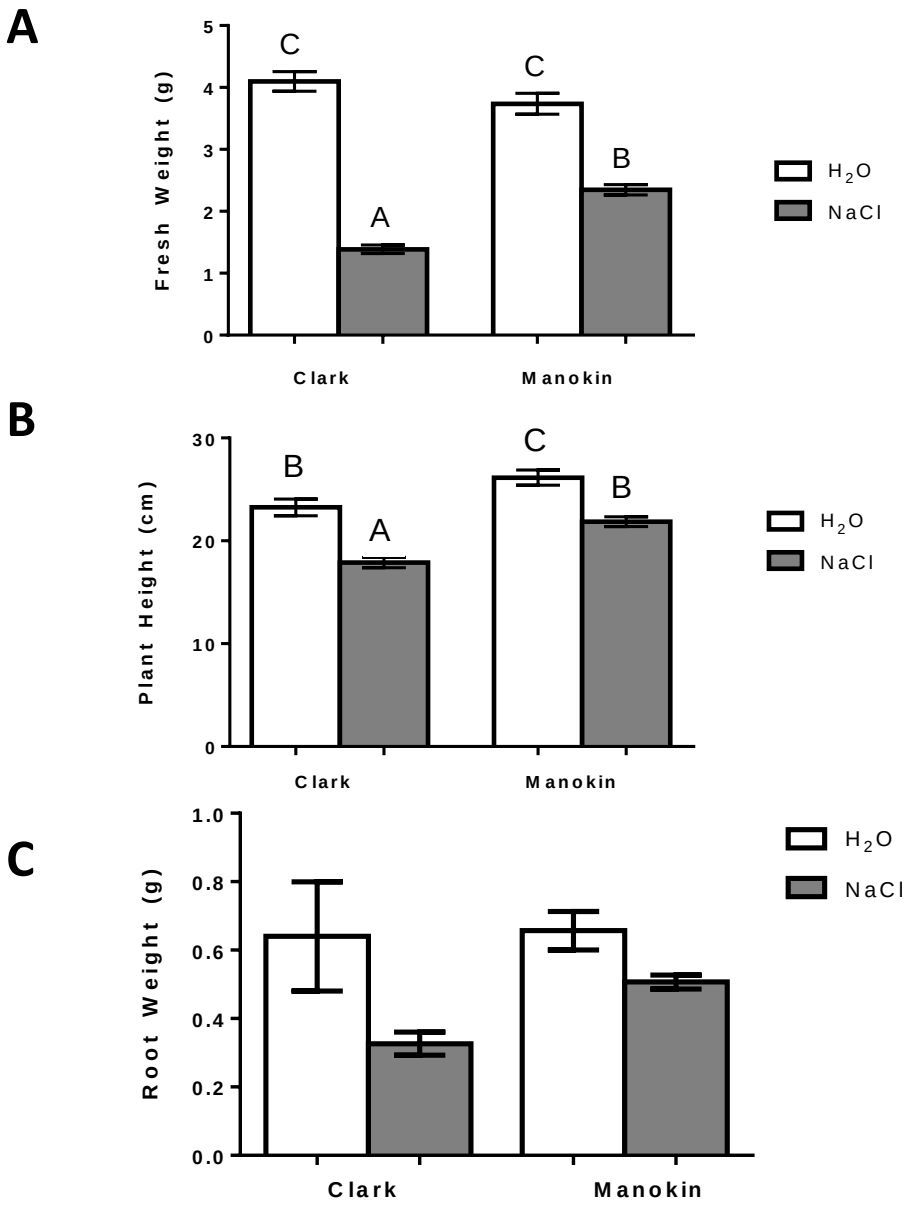


Figure 3. Two soybean cultivars were treated with 100 mM NaCl or dH_2O for 14 days. A) The average fresh weights of both cultivars were significantly reduced in NaCl-treated plants relative to H_2O-treated plants. B) The average height of both cultivars was significantly reduced in NaCl-treated plants relative to H_2O-treated plants. C) Average root dry weight was not significantly impacted by treatment with NaCl. Bars that share a letter are not significantly different from on another according to oneway ANOVA; n = 10; $p \leq 0.05$; $\pm$SEM

Reciprocal Grafting of Soybeans

To determine if the physiological and molecular functions of the roots and/or foliar tissues play a significant role in tolerance and ion exclusion, we performed a reciprocal grafting experiment using two salt-sensitive (Clark and Glenn) and two salt-tolerant (Manokin and Osage) soybean lines. Upon healing of the graft union, grafted plants and ungrafted controls were subjected to 14 days of treatment with 100mM NaCl or dH_2O. Overall, NaCl-treated plants possessing rootstock from salt-sensitive Glenn and Clark lines showed the wilting and chlorotic phenotype associated with chloride uptake and its resulting toxicity (Figure 4A). On the other hand, NaCl-treated plants possessing rootstock derived from salt-tolerant Osage and Manokin lines showed no signs of wilting or leaf scorch following the same treatment (Figure 4B).

A Chloride Sensitive Rootstock

B Chloride Tolerant Rootstock

Figure 4. Soybean plants were reciprocally grafted and treated daily with 100mM NaCl or dH_2O for 14 days. Images are representative of the observed phenotype of each grafting combination and ungrafted controls following treatment; lettering combinations indication scion cultivar (top) and roostock cultivar (bottom). A) Plants with chloride sensitive rootstock (Glenn (G) and Clark (C)) showed wilting, yellowing, and in some cases chlorosis. B) Plants with chloride tolerant rootstock (Osage (O) and Manokin (M)) appeared green and unwilted and looked very similar to water-treated control plants of their respective cultivars

A quantitative assessment of leaf scorch was conducted using a scale based on estimated percent leaf chlorosis (Figure 5, Ledesma et al., 2016). H_2O-treated plants showed no visible symptoms of chlorosis and thus, only NaCl-treated plants were rated. The average leaf scorch score ranged between 5 and 9 among the plants possessing sensitive rootstock (Figure 6). Leaf scorch scores of plants grafted with tolerant rootstock were significantly lower with an average score of 2. These phenotypic data suggest that soybean rootstock plays a major role in the physiological response to salt stress. Furthermore, this experiment demonstrates the potential for salt-sensitive soybean lines to be rescued from this sensitivity via grafting onto tolerant soybean rootstock.

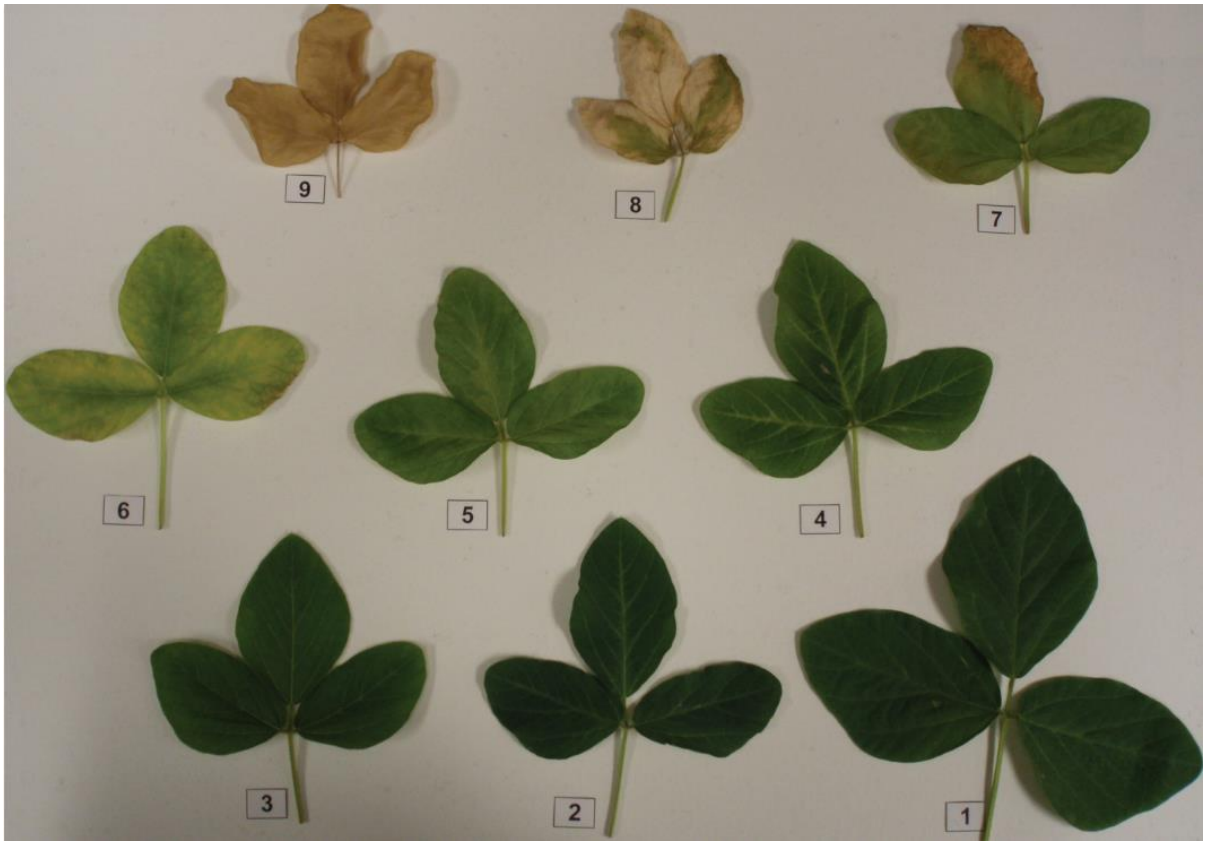


Figure 5. Leaf scorch score rating scale for evaluating soybean salt tolerance (Ledesma et al, 2016). 1 = healthy green leaf from a soybean plant not subjected to salt stress; 2 = dark-green leaf that is stunted due to salt treatment; 3-6 = slight to severe chlorosis; 7 = severe chlorosis and minor necrosis; 8 = minor chlorosis and severe necrosis. 9 = complete necrosis of the soybean leaf due to salt treatment.

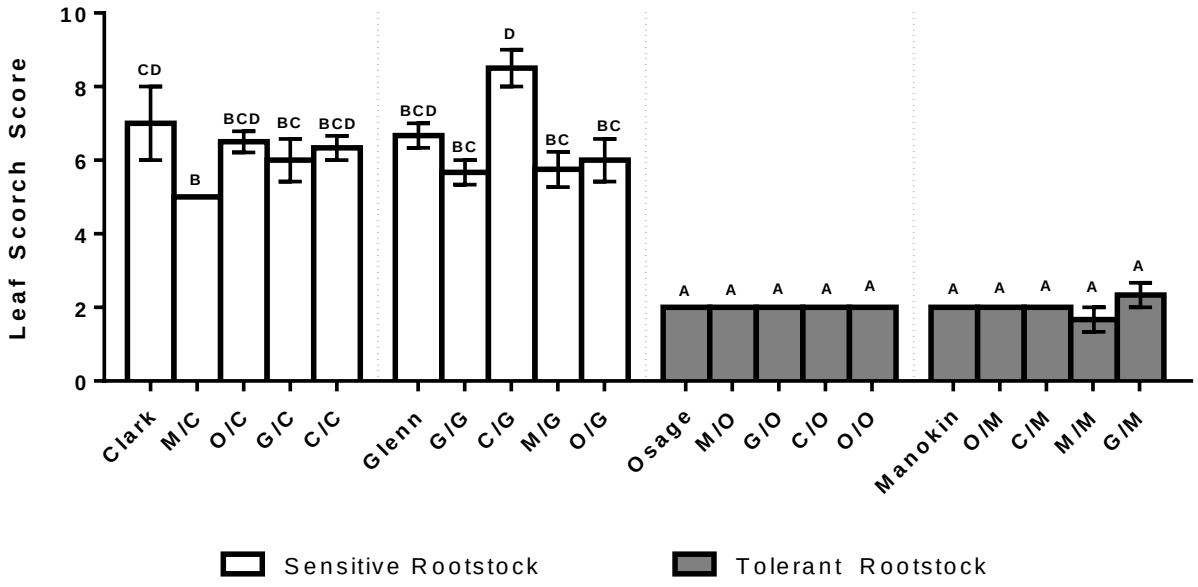


Figure 6. Salt sensitive rootstock led to greater leaf scorch damage following 14 days of 100 mM NaCl treatment. Analyzed by oneway-ANOVA; bars that share a letter are not significantly different from one another; $p \leq 0.05$; $2 \leq n \geq 4$

Decreases in chlorophyll content and greater leaf scorch damage among salt-sensitive soybeans under salt stress suggest that toxic levels of salt ions are able to accumulate in the foliar tissues of these salt-sensitive lines. The differential uptake of chloride by soybeans under salt stress is well-known and has been used as a benchmark for establishing salt sensitivity and tolerance within the soybean crop (Valencia et al, 2008; Lee et al, 2008). It is generally believed that the rootstock plays a dominant role in ion uptake and exclusion given that these tissues are the first exposed to saline soil conditions and also due to the fact that nutrient and water uptake occurs predominantly within the roots whether actively or passively. To test this hypothesis, we analyzed the chloride content of foliar tissue from each grafting combination. Across all combinations, NaCl-treated plants showed increases in mean chloride content relative to H_2O-treated plants, as expected. However, those plants possessing salt sensitive rootstock (Figure 7A) accumulated chloride to nearly three times the level of plants possessing salt tolerant rootstock

(Figure 7B). Chloride exclusion has also been reported as the source of salt tolerance in the soybean cultivar Dare (An et al., 2002).

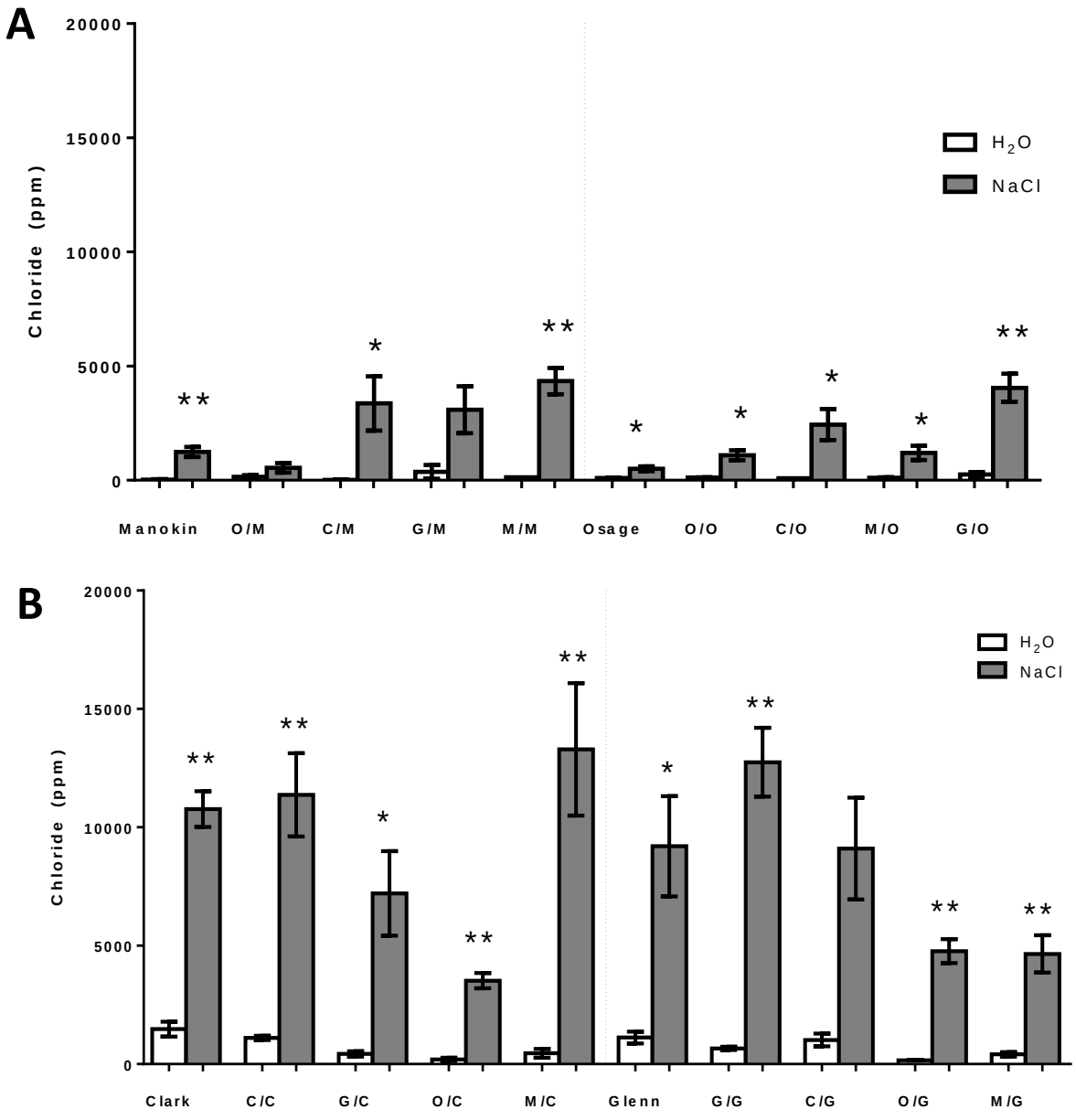


Figure 7. Salt-tolerant rootstock plays a major role in chloride exclusion from the foliar tissues. X-axis labels are written as fractions with genotype of the hypocotyl source represented by the letter in the numerator and genotype of the rootstock source represented by the letter in the denominator: C = Clark, G = Glenn, M = Manokin, O = Osage. A) Plants possessing salt-tolerant rootstock showed uptake of chloride under the salt treatment. B) Plants possessing salt sensitive rootstock also showed uptake of chloride under salt treatment but to a much higher level

Fresh weight of the grafted plants was acquired by cutting plants off at the soil line but no significant differences were observed among the grafting combinations (Figure 8). After measuring fresh weight, the same plants were dried and dry weight was recorded following complete desiccation of the tissues. No significant differences in dry weight were seen among the grafting combinations tested (Figure 9). Although no differences in biomass accumulation were observed in the grafting experiment, the clear differences in chloride content and leaf scorch score demonstrate the importance of root tissues in ion exclusion and physiological salt tolerance.

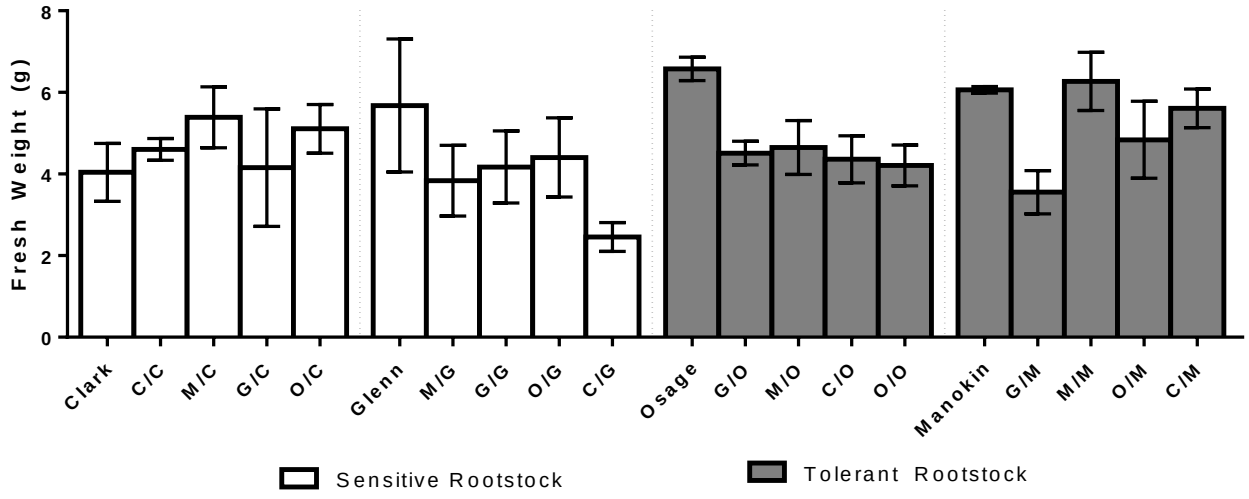


Figure 8. Rootstock source did not have a significant impact on hypocotyl fresh weight following 14 days of NaCl treatment although differences in leaf chloride content were observed at this time. Data represented here reflects the mean fresh weight of NaCl-treated plants only. Analysis by oneway -ANOVA; $p = 0.05$; $2 \leq n \geq 4$

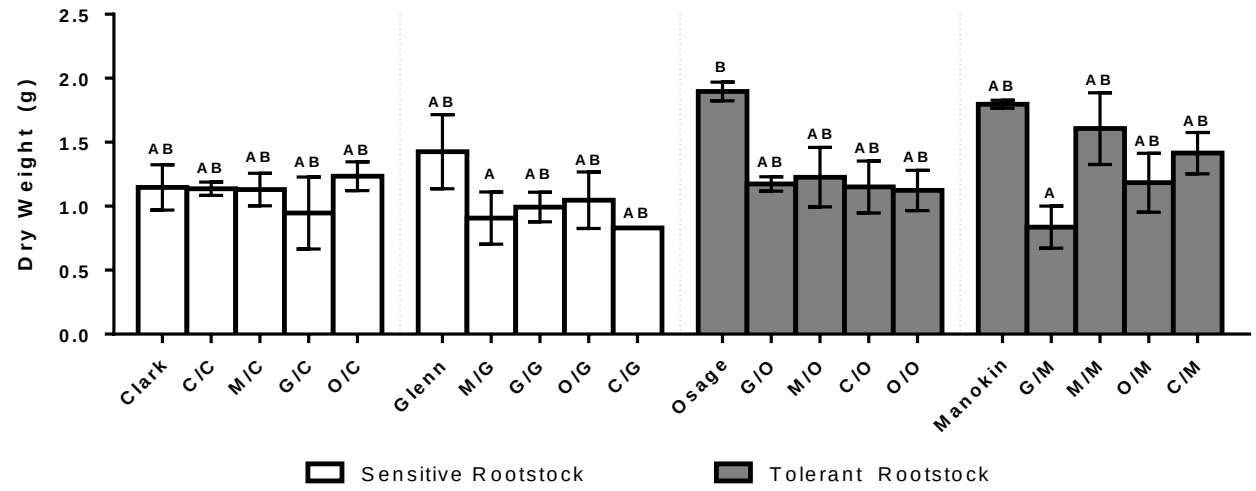


Figure 9. Rootstock source did not have a clear impact on the average dry weight of grafted plants following 14 days of 100 mM NaCl treatment. Data represented here reflects the dry weight of NaCl-treated plants only. Analysis by oneway-ANOVA; Bars that share a letter are not significantly different from one another; $p = 0.05$; $2 \leq n \geq 4$

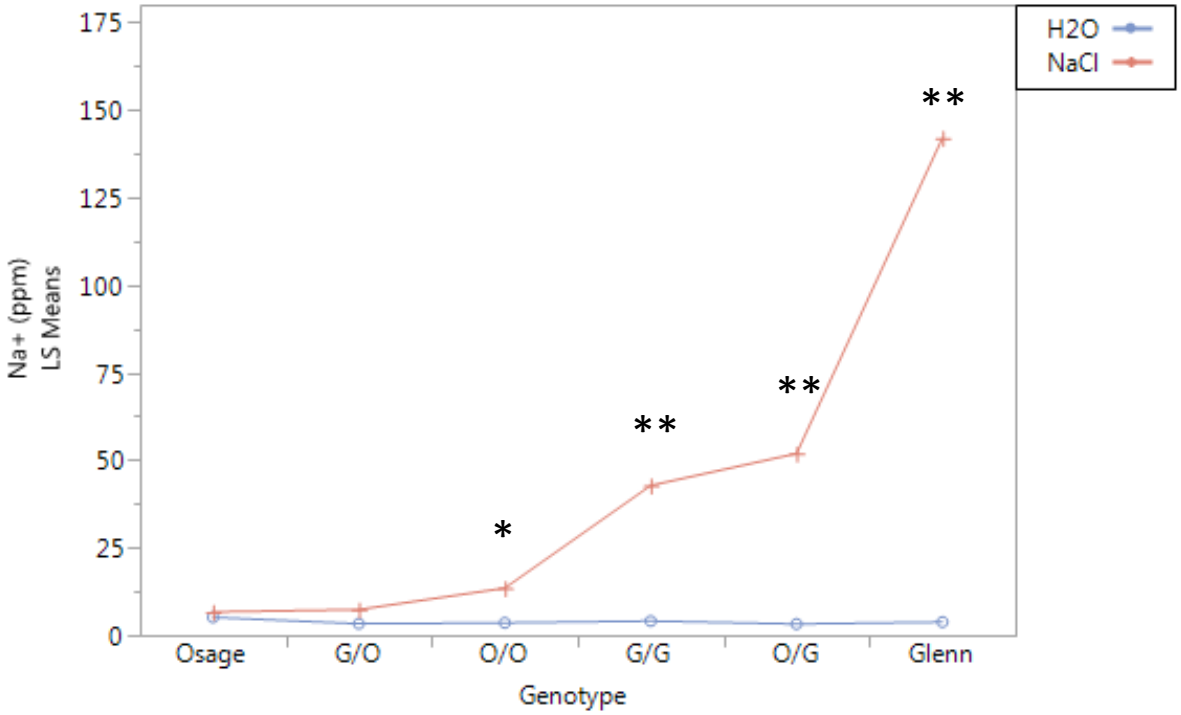


Figure 10. Plants with Glenn rootstock show significantly higher uptake of sodium into foliar tissues following 14 days of 100 mM NaCl treatment. Analysis by one-way-ANOVA; asterisks indicate significant difference between H2O and NaCl treatment within a genotype; $7 \geq n \leq 12$.

To determine if the pattern of sodium uptake follows chloride uptake, the grafting experiment was repeated using salt-sensitive Glenn and salt-tolerant Osage. Plants were analyzed for sodium and chloride content following 14 days of H_2O or 100 mM NaCl treatment. Because sodium and chloride data did not fit a Normal distribution, the data was transformed to a Log Normal distribution. ANOVA and the least-squares means regression analysis was utilized to determine effects of genoptype and treatment on sodium and chloride uptake by grafted plants. According to the least squares means test, the H_2O treatment did not have a significant effect on sodium content among the six genotypes tested ($p = 0.8888$). Conversely, the NaCl treatment showed a significant effect on the level of sodium uptake ($p < 0.0001$) with the extent of the uptake being modulated by genotype. For this reason, visual presentation of sodium data is organized according to genotype (Figure 10). When analyzing the treatment effect within each genotype, all genotypes with Glenn (G/G, O/G, Glenn) as the rootstock source showed a significant increase in sodium content under NaCl treatment ($p < 0.0001$). Additionally, the genotype combination O/O also showed a less significant increase in sodium uptake due to NaCl treatment ($p = 0.0005$). The grafting combinations Osage and G/O did not show a significant change in sodium content due to NaCl treatment. In fact, ungrafted Osage and grafted plants with salt tolerant Osage rootstock showed foliar sodium levels 30-50% of NaCl-treated plants with sensitive rootstock (Figure 13).These results suggest that the cultivar Osage, which is classified as salt tolerant, contributes to sodium exclusion under NaCl treatments to a greater extent than the cultivar Glenn, which is classified as salt sensitive.

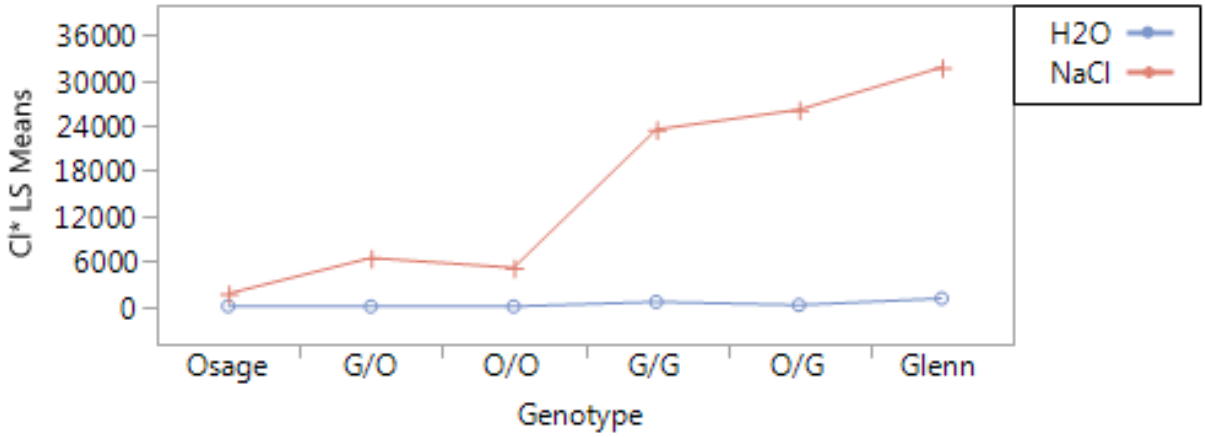


Figure 11. Plants with Glenn rootstock show significantly higher uptake of chloride into foliar tissues following 14 days of 100 mM NaCl treatment. Analysis by oneway-ANOVA; asterisks indicate significant difference between H2O and NaCl treatment within a genotype; $7 \geq n \leq 12$

According to the least-squares model and lognormal transformed chloride data, both the NaCl ($p = 0.0002$) and H_2O ($p < 0.0001$) treatments had a significant effect on chloride uptake in the grafted plants. When analyzing the treatment effect by genotype, all genotype combinations showed a significant increase in chloride content under NaCl treatment compared to H_2O treatment ($p < 0.0001$; Figure 14). However, increases in chloride levels due to NaCl treatment were much larger in magnitude in plants possessing Glenn rootstock (G/G, O/G/ Glenn) compared to plants possessing Osage rootstock (Osage, G/O, O/O) with chloride levels of Glenn rootstock plants around 30% of the chloride levels observed in Osage rootstock plants (Figure 11). As observed with sodium uptake, salt sensitive Glenn soybean appears to be superior at chloride exclusion under NaCl treatment.

Because the same general trend was observed between sodium and chloride uptake among the different genotypes, a direct comparison was made to determine if the uptake of one ion may be correlated to the other. However, no significant correlation was detected between

sodium and chloride uptake when compared by genotype or by treatment. The high degree of variance observed across the datasets as well as a few extreme outliers likely contribute to this lack of correlation. Repeated experiments with additional biological replicates may reveal a closer relationship between the uptake of these two ions under salt-treated conditions.

Previous reports using grafting in soybean have provided conflicting results as to which tissues are responsible for ion exclusion in this plant. Abd-Alla et al. (1998) contributed salt tolerance to improved photosynthetic activity of the foliar tissues from salt-tolerant soybean plants. Conversely, Grattan and Maas reported the control of foliar chloride content by soybean roots and attribute salt tolerance to the root tissues (1985). Taken together, our leaf scorch scores and sodium and chloride data of grafted soybeans demonstrate the significant impact that rootstock has on physiological salt tolerance and ion uptake under salt stress. Interestingly, the final chloride levels of some salt-tolerant scion/salt-sensitive rootstock grafting combinations (i.e. O/C, O/G, and M/G) were similar to chloride levels of a few sensitive scion/tolerant rootstock grafting combinations (i.e. C/M) suggesting that the scion genotype may also play a role in systemic uptake of ions, although the significance of that role has not been clearly demonstrated as a salt-sensitive scion did not always result in increased ion uptake relative to salt-tolerant scions.

As indicated by ion analysis results, even salt-tolerant soybean plants begin to accumulate sodium and chloride under salt stress. However, the level of foliar ions is not enough to cause scorch in these plants. This is an especially important finding for farmers who may not see visible symptoms in their fields yet suffer yield loss due to the stress imposed by saline soil. Routine soil testing can inform farmers if their fields are salt-affected prior to planting, which is essential in choosing the best variety to plant. The results of these experiments could be validated

and given further relevance by expanding the number of cultivars used. It is likely that several salt tolerance mechanisms are at work among the salt tolerant lines. By working with near isogenic lines possessing known differences in genetic salt tolerance (i.e. salt-tolerance associated quantitative trait loci) it may be possible to identify which regions of the soybean genome direct each of these mechanisms within domesticated soybean plants. Furthermore, this type of research may also shed light on if and how regions of the genome interact to make a soybean plant more or less susceptible to growth in saline conditions.

Phytohormone Signaling of Soybean under Salt Stress

Phytohormones are thought to be important components of both biotic and abiotic stress signaling in plants. Recent evidence suggests that complex crosstalk, mediated by transcription factors and MAP kinases, may occur between the jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) mediated pathways (Fujita et al, 2006). The role of these phytohormones in the soybean response to salt stress was explored by measuring their content in H_2O-treated and NaCl-treated soybeans following 144 hours (6 days) of treatment. At 144 hours, both salt-sensitive Clark and salt-tolerant Manokin plants showed a significant increase in ABA content under the NaCl treatment (Figure 12), indicating that ABA is salt-induced but this induction does not differ significantly between the two soybean lines tested. Induction of ABA by salt or drought stress has been documented in many plant species and ABA is thought to be the primary component of abiotic stress signaling in plants, although the specific downstream effects of ABA induction differ between species (Zhu, 2002; Hoad, 1975; Finkelstein and Rock, 2002; Zeevaart and Creelman, 1988). *Arabidopsis* mutants with reduced ABA content germinated better under salt stress than wild-type plants (Koornneef et al., 1984). In *Brassica*

napus, higher salt salt tolerance was associated with lower ABA concentration under salt stress (He and Cramer, 1996).

ABA is induced under drought conditions in *Arabidopsis thaliana* and has been shown to inhibit lateral root growth in this plant species under stress conditions (De Smet et al., 2006). Conversely, exogenous applications of ABA stimulated lateral root formation in rice (*Oryza sativa)* (Chen et al., 2006). These discordant results between different crops and the many secondary messengers of ABA signaling that have been identified suggest the plasticity of this signaling pathway and the broad range of environmental responses in which this phytohormone may play a role (Cutler et al., 2010).

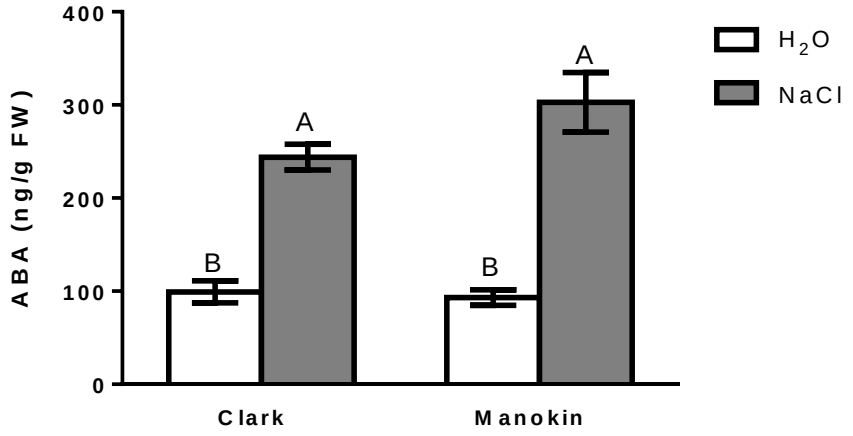


Figure 12. The average abscisic acid content of both soybean cultivars Clark and Manokin was significantly increased following 6 days of 100 mM NaCl treatment. Tissue was analyzed by LC/MS-MS. Data was analyzed by one-way ANOVA; error bars indicate SEM; N= 5; $p < 0.05$. Bars that share a letter are not significantly different from one another.

In addition to JA, content of the JA precursor oxophytodienoic acid (12-OPDA) and of the downstream product jasmonyl isoleucine (JA-Ile) were also measured. Neither 12-OPDA content (Figure 13A) nor JA content (Figure 13B) were significantly affected by NaCl treatment in either of the lines tested. JA-Ile content was not significantly different between treatments for

Clark or Manokin (Figure 13C). Salicylic acid content of Clark plants was significantly reduced under NaCl treatment while SA content was unchanged in Manokin plants (Figure 14).

With very few significant differences between Clark and Manokin plants under NaCl stress, no clear trends emerged to allow association of phytohormone content and salt tolerance. Field and lab studies have demonstrated a close correlation between ABA content and stomatal aperture in a number of plants (Khalil and Grace, 1993; Tardieu et al, 1992, Wartinger et al, 1990; Zhang and Davies, 1990). The induction of ABA in both lines under salt stress is therefore not surprising given the essential role this hormone plays in regulating stomatal conductance and thus water loss from the plant. The JA-Ile content of the two lines did differ significantly from each other under the H_2O treatment suggesting that these lines may maintain different basal levels of this phytohormone which could impact the speed with which soybean plants from each line are able to respond to stress. The reduction of SA in Clark plants under NaCl stress may suggest that the induction of ABA observed from the same plants has a negative effect on the production of SA. Evaluation of phytohormone content over an extended timeline using additional soybean varieties might reveal a clearer pattern of induction and suppression of these hormones in soybeans under salt stress.

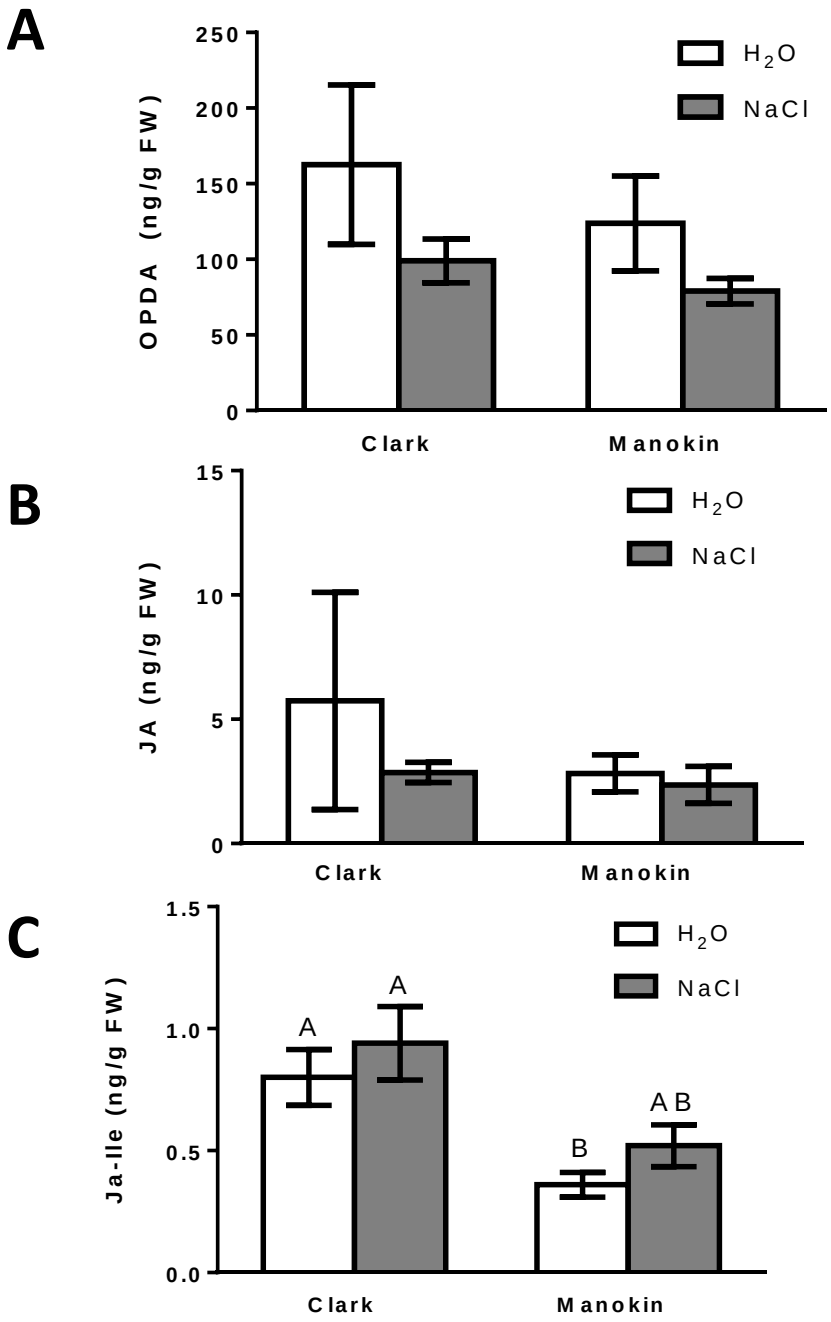


Figure 13. Foliar levels of OPDA, JA, and JA-Ile were not significantly affected following 6 days of 100 mM NaCl treatment. A) The average OPDA content of both Clark and Manokin plants was unaffected by NaCl treatment. B) The average JA content of both Clark and Manokin plants was unaffected by NaCl treatment. C) Ja-Ile content of Clark and Manokin plants was not significantly affected by NaCl treatment. Analyzed by oneway ANOVA; error bars indicate SEM; N= 5; p = 0.0; bars that share a letter are not significantly different from one another.

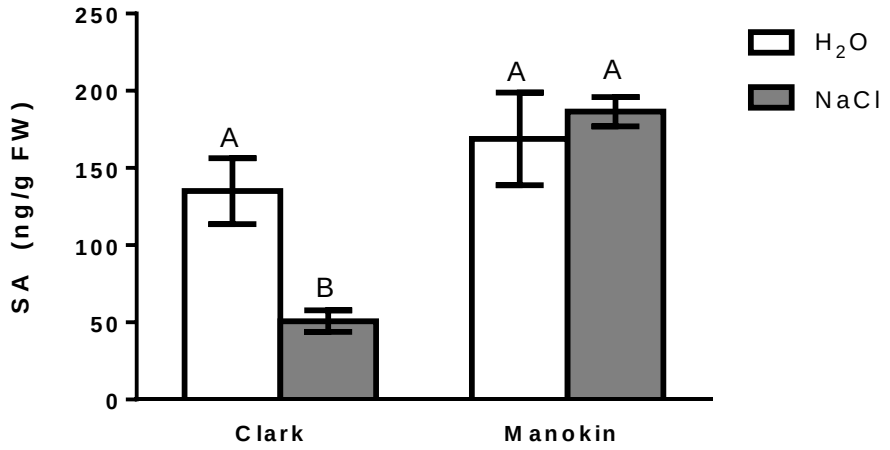


Figure 14. Salicylic acid content is significantly reduced in salt sensitive cv. Clark following 6 days of 100 mM NaCl treatment. SA content is unaffected in salt tolerant cv. Manokin following the same NaCl treatment.

Oxidative Stress Signaling of Soybean under Salt Stress

In addition to signaling mediated by phytohormones, signals associated with reactive oxygen species are also thought to play an important role in abiotic stress response in plants. Depending on their cellular concentrations, reactive oxygen species may serve as a signaling molecule or as a catalyst of lipid peroxidation, thus measurements of lipid peroxidation have proven to be useful indicators of oxidative stress in some plants (Tanou et al, 2009; Sharma and Dubey, 2005). Malondialdehyde (MDA), a by-product of lipid peroxidation, has been used to measure lipid peroxidation levels of plants under salt stress and the change in MDA content under salt stress has been shown to be a helpful marker for salt tolerance in some plants (Vaidyanathan et al 2003, Sharma et al., 2012). We assessed MDA content of four soybean lines following 48 hours and 144 hours of NaCl or H_2O treatment. After 48 hours of treatment, only salt-tolerant Osage plants showed a significantly higher MDA content in NaCl-treated plants relative to H_2O-treated plants (Figure 15A). NaCl-treated Manokin plants showed a statistically

insignificant increase relative to H_2O-treated plants at this time point. After 144 hours of NaCl treatment, both salt-tolerant varieties showed significantly higher MDA under NaCl treatment (Figure 15B). Salt-sensitive varieties showed no significant differences due to treatment at either time point.

Interestingly, these data show that the two salt-tolerant varieties suffered greater lipid peroxidation under salt stress than the two salt-sensitive varieties tested. These results are in contrast to previous reports in wheat and soybean that suggest a correlation between salt sensitivity and lipid peroxidation under salt stress (Sairam et al., 2005; Khan et al, 2009). For example, the salt-tolerant soybean BB52 showed decreasing levels of MDA with increasing levels of NaCl whereas salt-sensitive Lee68 and N23232 showed increases in MDA levels under increasing salt concentrations (Phang et al, 2008). Salt-sensitive rice seedlings accumulated higher levels of MDA under salt stress compared to salt-tolerant seedlings (Mishra et al., 2013). ROS are known to be very damaging to chloroplasts, however, a reduction in photosynthesis was not observed in salt-tolerant Manokin plants in previous experiments where chlorophyll content was assessed (Figure 2). These experiments should be repeated with additional soybean lines at additional time points, however results suggest that MDA content under salt stress may not be a dependable marker of salt tolerance across all soybean varieties. Lipid peroxidation indicates an overwhelming of the ROS scavenging capacity of a plant. Thus, monitoring of ROS scavenging enzyme levels in these same soybean varieties may reveal variable induction of the enzymes primarily responsible for redox balance in these plants. Furthermore, knock-outs and overexpression of the genes encoding these enzymes in soybean could reveal necessity and sufficiency of any one of these enzymes to improve salinity tolerance.

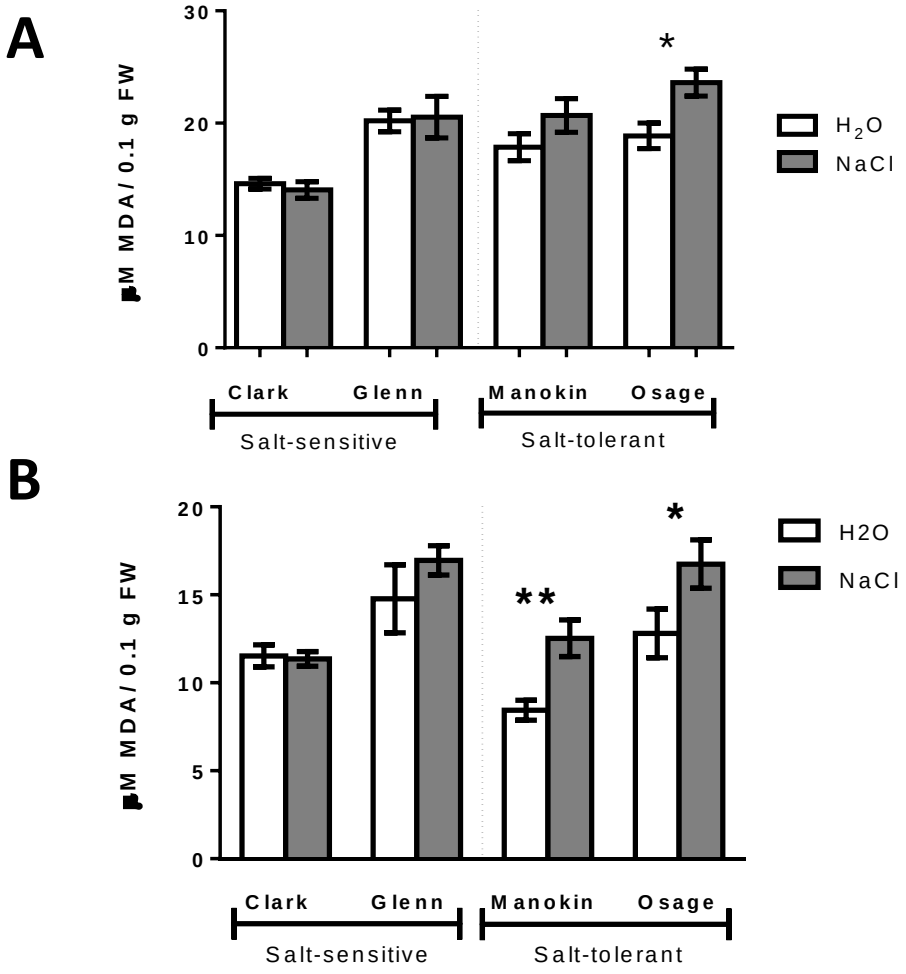


Figure 15. Salt-tolerant plants showed increases in MDA content following NaCl treatment. A) Following 48 hours of treatment with 100 mM NaCl, MDA content of salt-tolerant Osage plants was significantly increased. B) Following 144 hours of treatment with 100 mM NaCl, MDA content of both salt-tolerant varieties was significantly increased. * $p < 0.05$ ** $p < 0.1$

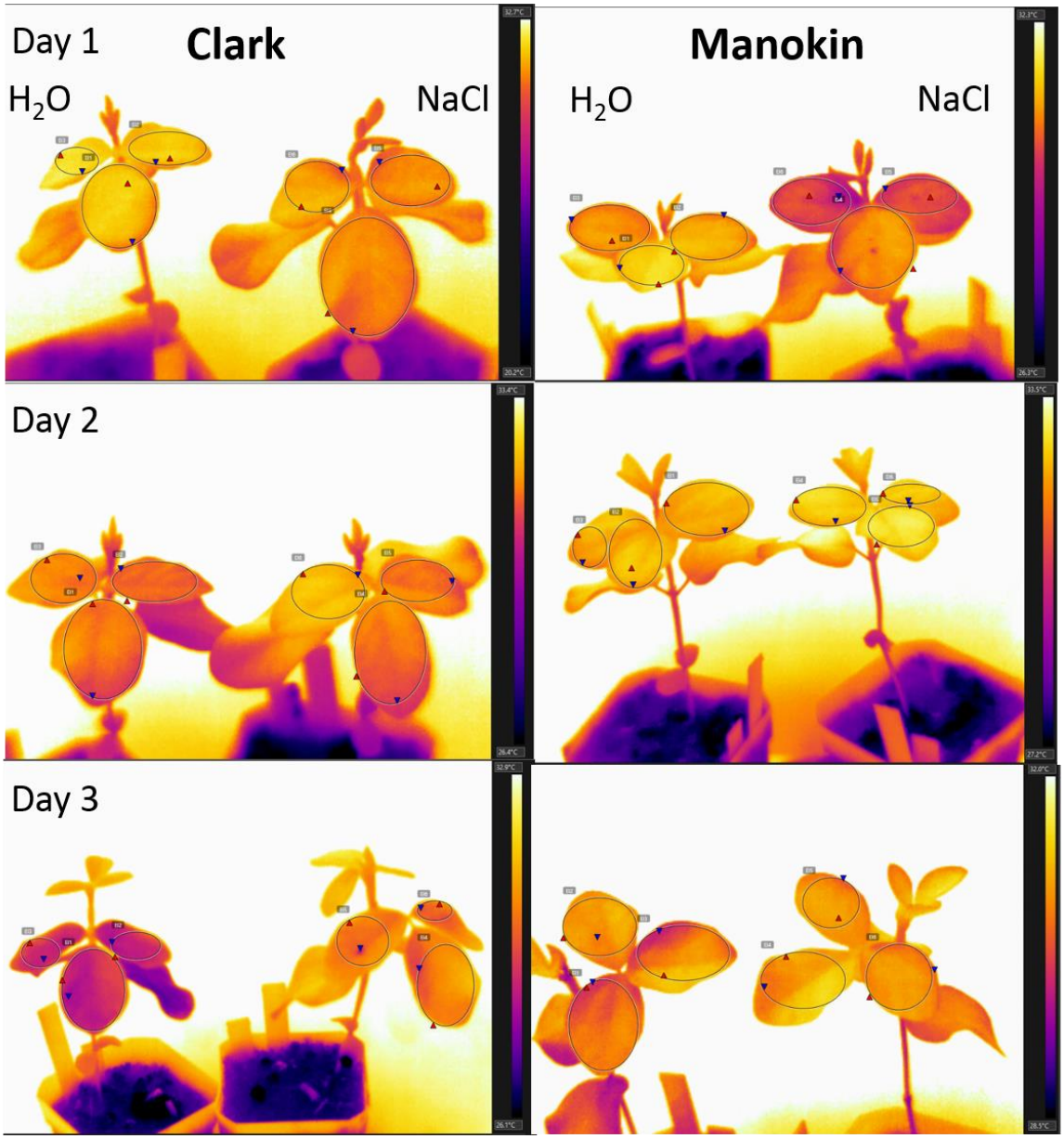
Screening Salt Tolerance in Soybean by Infrared Thermography

As previously mentioned, screening for salt tolerance in soybean is typically accomplished through the analysis of leaf chloride content and/or the assignment of leaf scorch scores. Analysis of foliar chloride is a destructive method that requires removal of the tissues to be tested. Both chloride content and leaf scorch methods require that plants be treated with salt for an extended period to allow adequate uptake of salts for accurate detection of differences between lines. Infrared thermography is a commonly used method of assessing crop and plant health and relies on the relationship between plant transpiration and leaf temperature (Sirault et al., 2008. By acquiring infrared thermographs of salt-treated soybeans that differ in their salt tolerance, we hoped to establish a relationship between the level of salt tolerance and leaf temperature under salt stress. This method would allow for fast, affordable, and non-destructive salt tolerance screening of soybean germplasm. IR thermography was previously used to establish a correlation stomatal closure and high plant temperature (Jones, 1999). This screening method has been used to identify stomatal control mutants in *Arabidopsis* and barley and has been reported as a screening method for salt tolerance in wheat (Sirault et al., 2009).

Following each day of treatment, infrared thermographs were captured of seven plants of each cultivar from each treatment. The average leaf surface temperature of each cultivar from each treatment was calculated and the average difference between water- and NaCl-treated plants was used to determine the effect of salt treatment on leaf temperature. Throughout the treatment period, NaCl-treated Manokin plants showed a temperature difference of 0.5 °C or less compared to water-treated plants, indicating that salt tolerant Manokin plants responded to NaCl treatment with a small increase in temperature (Figure 16). The temperature difference for Clark plants ranged from about 0.2 to 1.2°C between treatments, and was significantly higher than the

temperature difference for Manokin plants on day 3 of the treatment (Figure 16). This data also shows that the salt-sensitive line suffered from larger leaf temperature increases earlier in the treatment period. Notably, the average temperature of water-treated plants was significantly different between the two cultivars with higher basal temperatures in the salt-tolerant Manokin plants. The insignificant and delayed increase in leaf temperature seen in salt-tolerant Manokin plants indicates that these plants are able to maintain relatively normal transpiration levels under stress. Comparatively, salt-sensitive Clark plants experienced larger increases in temperature under salt stress, which could reflect a decrease in transpiration rate although transpiration rate was not measured directly.

A



B

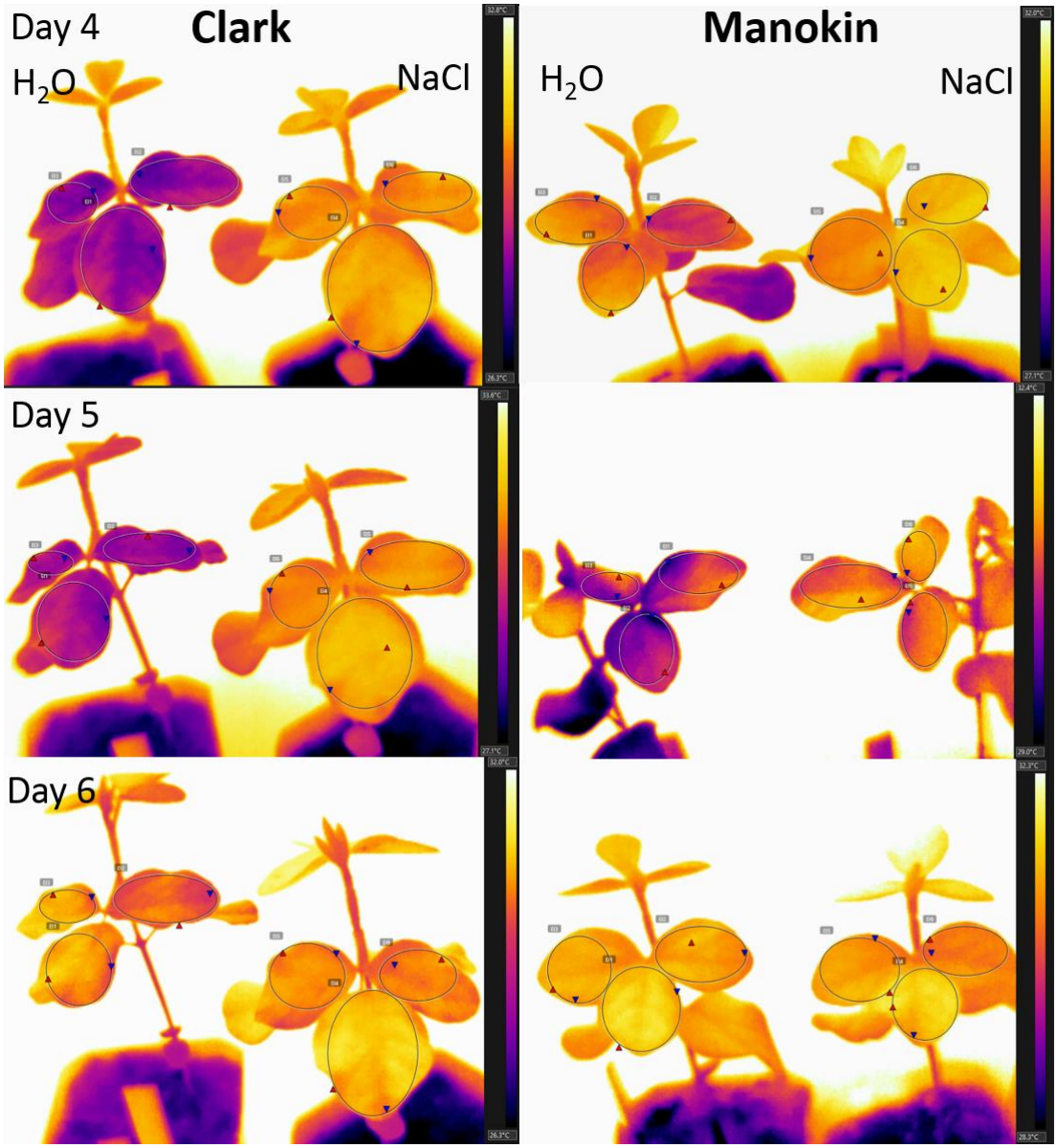


Figure 16. Infrared thermographs of soybean plants over the course of 6 days indicate that NaCl-treated plants are generally warmer than H2O-treated plants of the same variety. A) IR thermographs of Clark and Manokin plants following the first three days of treatment. On day 3, NaCl-treated Clark plants appear much warmer than H2O-treated Clark plants. B) IR thermographs of Clark and Manokin plants following days 4-6 of treatment. Both lines show an increase in temperature for NaCl-treated plants. Circles within each image represent the area of pixels for which temperature data was acquired.

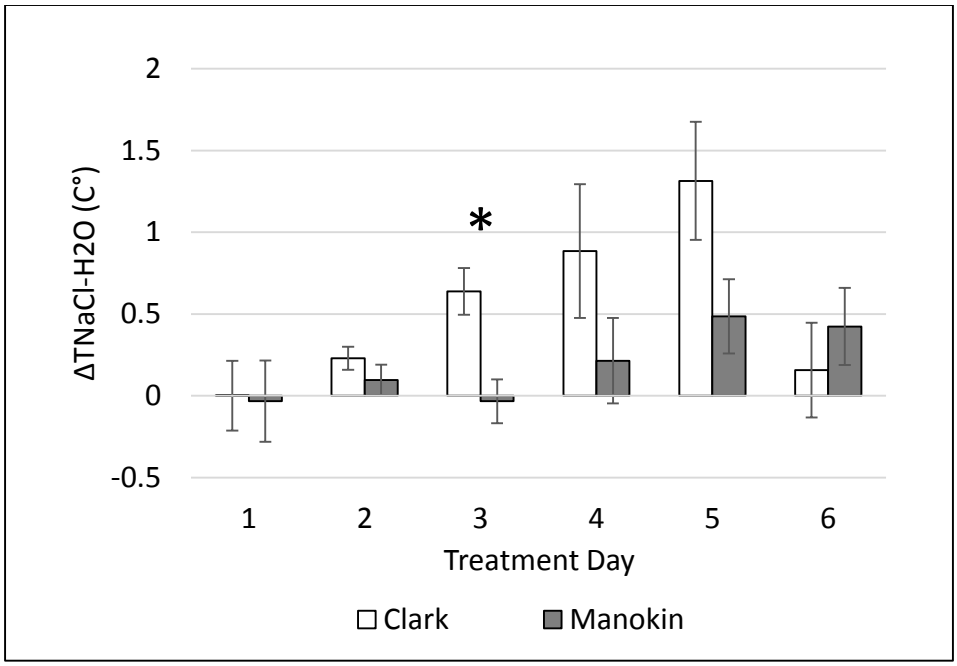


Figure 17. The temperature difference between NaCl- and H_2O-treated plants is significantly different between salt-sensitive Clark and salt-tolerant Manokin following 3 days of 100 mM NaCl treatment. Over the course of six days of treatment, Clark plants show larger differences in temperature between treatments for the first five days. This increase in temperature due to NaCl treatment is only significantly different from Manokin on day 3 of the treatment according to student's *t*-test; n = 7; error bars indicate $\pm$ SEM; p = 0.05.

Stomatal conductance is one of the major sources of resistance to transpiration and measurements of stomatal conductance can serve as an indicator of transpiration potential. To determine whether the observed temperature differences could be due to a difference in stomatal conductance, and hence transpiration, we measured stomatal conductance of the same plants used in the IR experiment. For fourteen days, stomatal conductance values were recorded using a leaf porometer following NaCl or H_2O treatment each day. The ratio of the average stomatal conductance of NaCl-treated to H_2O-treated plants was used as a metric to assess the effect of NaCl treatment on transpiration rate in Clark and Manokin soybeans. The conductance ratios for both soybean lines showed a general downward trend throughout the treatment period (Figure 17). The average conductance ratio of Clark plants was consistently lower than that of Manokin

plants, which could help explain the observed temperature differences of these plants. Epidermal leaf peels were made from Clark, Glenn, Manokin and Osage plants to determine whether differences in stomatal density could explain observed differences in stomatal conductance. However, no clear differences in stomatal density between salt sensitive and salt tolerant lines were detected (Appendix, Figure 21).

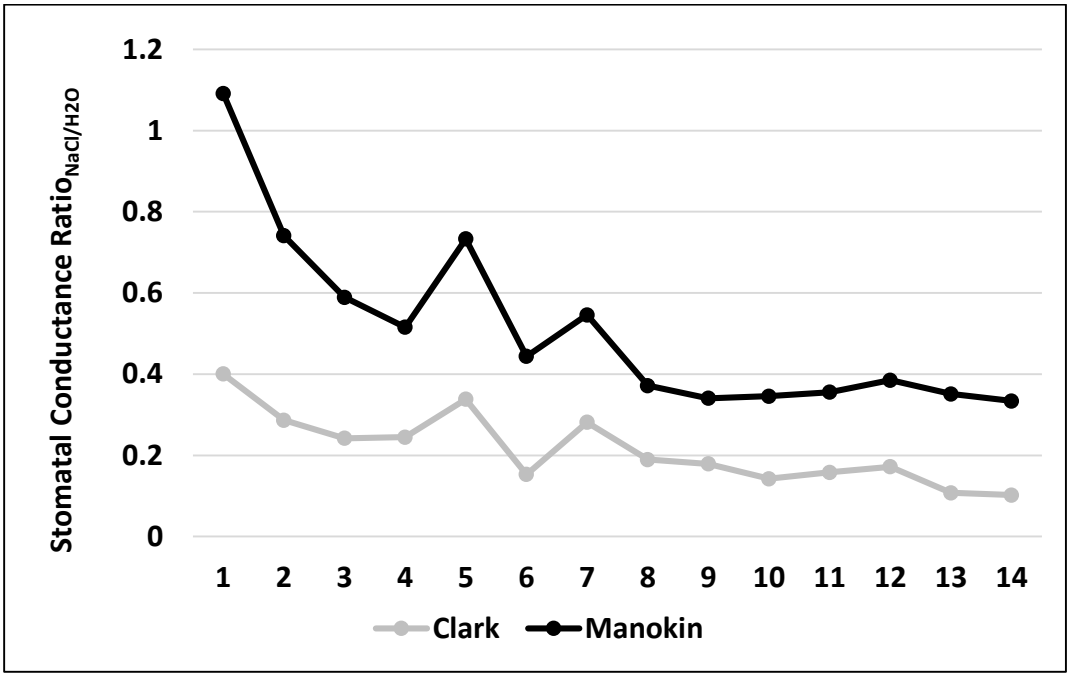


Figure 18. The ratio of stomatal conductance between NaCl- and H_2O-treated Clark plants was consistently lower than in Manokin plants over the course of the treatment. A general decline in stomatal conductance ratios of both soybean lines was observed throughout the 14 days of treatment.

Because significant temperature differences were observed on only one day of treatment throughout the IR thermography experiment, the usefulness of IR thermography as a salt tolerance screening method cannot yet be concluded. To further assess the suitability of IR thermography for use by soybean breeders as a salt tolerance screening method, this experiment would need to be repeated with more replications and additional soybean lines. Testing a number

of salt concentrations would also be useful in optimizing this screening method. Practical considerations and plant-to-plant variation in leaf temperature may make this method more useful for comparing genetic materials in a field setting where temperature of whole canopies can be compared.

Assessment of *GmCHX1* Genotype in Soybean

GmCHX1 is a locus associated with salt tolerance in soybean located within a previously reported salt tolerance quantitative trait loci. Guan et al. assessed allelic variation of this locus within Chinese soybean germplasm and reported an allele containing a 3.78 kb retrotransposon insertion which disrupts the production of functional transcripts from this locus (Guan et al., 2014b). The transponson-containing allele was strongly associated with salt sensitivity in the lines they tested. To assess whether this same transposon was present at the *GmCHX1* locus in our salt sensitive soybean lines, we designed two PCR primer sets to amplify the genomic sequence within the *GmCHX1* locus. The first set of primers was designed to amplify within exon 3 of the locus (Figure 19, orange arrows) and served as a positive control which should produce a PCR product of 322 bp from both alleles at the *GmCHX1* locus. The second set of primers was designed to amplify within the transposon and should only produce a product from DNA samples containing this transposon (Figure 19, red arrows). DNA from six soybean lines (three salt-sensitive and three salt-tolerant) was tested with both primer sets to determine the genotype of each line at the *GmCHX1* locus.

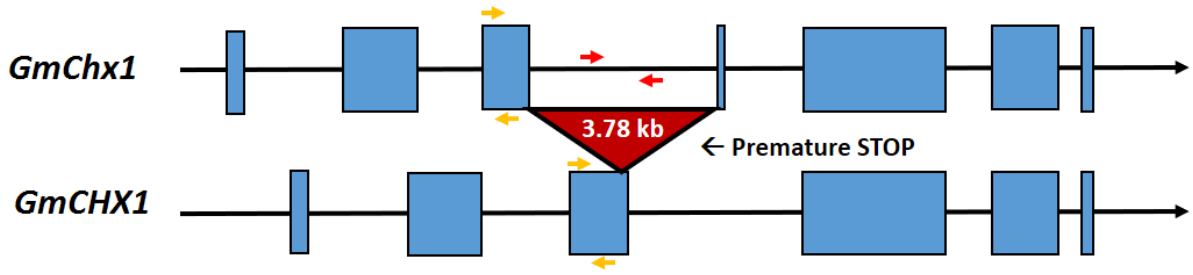


Figure 19. PCR primers were designed to amplify the genomic sequence within exon 3 of the *GmCHX1* locus. The orange primer set should produce a product of 322 bp while the red primer set should produce a product of 565 bp, allowing side by side analysis of the presence of both fragments.

All of the samples produced a 322 bp band corresponding to the 5' region of exon 3 (Figure 20). The salt-sensitive lines (Clark, Glenn, Williams 82) also produced a 565 bp band corresponding to the retrotransposon insert sequence (Figure 20 B, C, D, respectively). This product was absent from the three salt-tolerant samples tested (Osage, Lee68, Manokin), indicating absence of the retrotransposon (Figure 20, E, F, G, respectively). These results suggest that the *GmCHX1* locus may be a helpful DNA marker for predicting phenotype under salt stress. At least ten individuals from each cultivar were tested for both PCR products. Surprisingly, four out of the total 18 individuals from cv. Glenn tested negative for the insert sequence (data not shown) suggesting that there may be ongoing segregation of this locus in this population of Glenn plants. As salinization of soils and degradation of land quality continues, use of molecular breeding tools will prove essential in meeting grower demands for high-yielding varieties with tolerance to a variety of stresses. Incorporation of markers specific to the salt-tolerant *GmCHX1* allele into routine molecular marker analysis of soybean breeding programs could greatly improve the efficiency with which salt tolerant soybean lines are selected and bred and could eliminate the need for costly phenotypic screening.

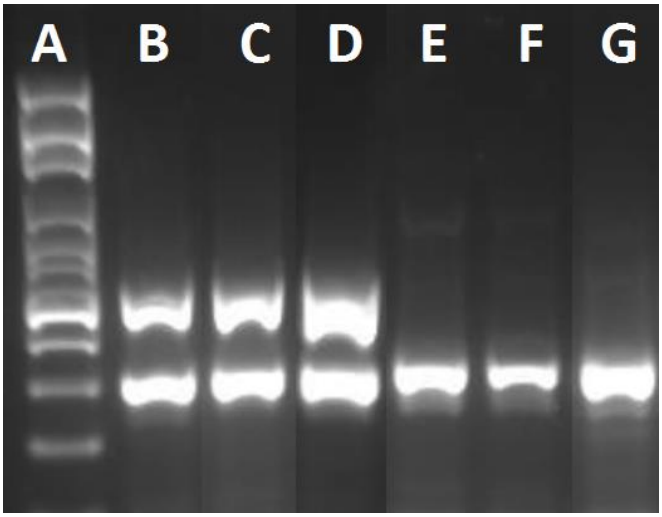


Figure 20. All salt sensitive soybean lines tested possess DNA sequences of a retrotransposon insert within the GmCHX1 coding sequence. A) 1kb molecular marker. The 565 bp PCR product derived from genomic DNA from salt sensitive cv. Clark (lane B), cv. Glenn (lane C), and cv. Williams 82 (lane D) indicates that the retrotransposon insert is present. Absence of the 565 bp PCR product from the salt tolerant cultivars Osage (lane E), Lee68 (lane F), and Manokin (lane G) indicates that the transposon insertion is absent in the plant tested. Amplification of the 322 bp fragment in each sample served as a positive control to demonstrate quality and amplication of DNA template.

Conclusion

The physiological and molecular mechanisms responsible for salinity tolerance in plants has been well studied over the past three to four decades and several informative reviews have been published that summarize the key mechanisms employed by plants under salt stress and, when known, the genetic components that control and modulate these mechanisms (Hasegawa et al., 2000; Zhu et al., 2001; Munns and Tester, 2008; Blumwald, 2000; Roy et al., 2014). A great deal is known about the general response of plants to salt stress, however, the relative importance of each of these mechanisms differs from one crop species to the next. Our results indicate that ion exclusion is the primary determinant of salt tolerance in soybean and that this exclusion ability is largely dependent upon the root tissues. Furthermore, we confirmed that a functional *GmCHX1* gene corresponds to salt tolerance in several U.S. soybean varieties and, in agreement with previous reports, is likely the genetic source of ion exclusion in these lines (Guan et al, 2014a, 2014b, Qi et al, 2014). Through a survey of physiological responses to salt stress, we determined that salt-tolerant soybeans are able to perform very similarly under both water and salt treatments. Salt-sensitive soybeans, on the other hand, suffered in chlorophyll levels, fresh weights and root dry weights, and stomatal conductance under salt stress. Additionally, we established a salt-induced increase in abscisic acid content among all soybean lines tested, which suggests that phytohormone signaling may play a prominent role in the salt stress response of soybean. We propose that ion exclusion is the primary mechanism determining salt sensitivity of soybean but that additional mechanisms are responsible for modulating the degree of sensitivity or tolerance observed among different soybean lines.

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Appendix

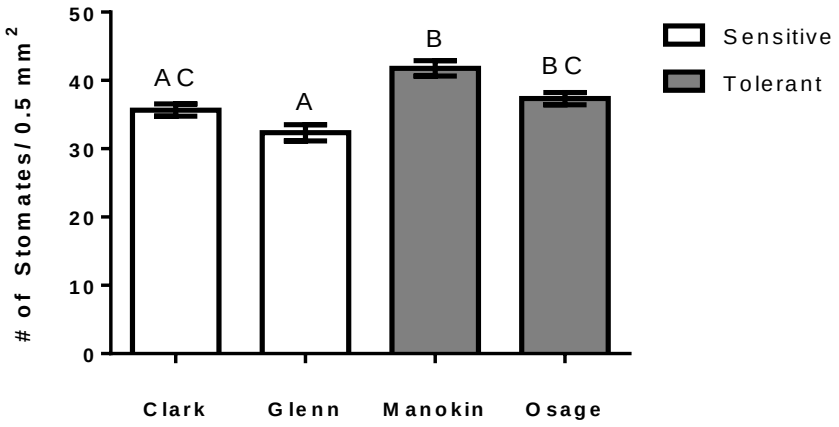

Figure 21. Stomatal density of four soybean cultivars. Analyzed by ANOVA, ±SEM; n=3; $p < 0.05$

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