8-2013

Genome-Wide Association Mapping Identifies QTLs and Candidate Genes for Salt Tolerance in Soybean

Lei Huang
University of Arkansas, Fayetteville

GENOME-WIDE ASSOCIATION MAPPING IDENTIFIES QTLS AND CANDIDATE GENES FOR SALT TOLERANCE IN SOYBEAN

GENOME-WIDE ASSOCIATION MAPPING IDENTIFIES QTLS AND CANDIDATE GENES FOR SALT TOLERANCE IN SOYBEAN

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Crop, Soil, and Environmental Sciences

By

Lei Huang
Northeast Normal University
Bachelor of Science in Biotechnology, 2010

August 2013
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

_____
Dr. Pengyin Chen
Thesis Director

_____
Dr. Andy Pereira
Committee Member

_____
Dr. Kristofor R. Brye
Committee Member

_____
Dr. Kenneth L. Korth
Committee Member

_____
Dr. Vibha Srivastava
Committee Member

ABSTRACT

Salinity is a common abiotic stress causing soybean yield loss worldwide. Use of tolerant cultivars is an effective and economic approach to coping with this stress. Toward this, research is needed to identify salt-tolerant germplasm and better understand the genetic and molecular basis of salt tolerance in soybean. The objectives of this study were to identify salt-tolerant genotypes, to search for SNPs and QTLs associated with salt tolerance, and to identify candidate salt tolerance genes. A total of 192 diverse soybean lines and cultivars were screened for salt tolerance in the greenhouse based on visual leaf scorch scores after 15 - 18 days of 120 ml NaCl stress, among which 94 were tolerant while 87 were sensitive. These genotypes were further genotyped using the SoySNP50K iSelect BeadChip with 52,041 single nucleotide polymorphism (SNP) markers, among which 37,281 SNPs were polymorphic with minor allele frequency (MAF) > 5% and present in 75% of all genotypes. Genome-wide association mapping showed that 62 SNP markers representing 6 genomic regions on Chromosomes 2, 3, 5, 6, 8, and 18, respectively were significantly associated with salt tolerance ($P < 0.001$). 52 SNP markers on Chromosome 3 are mapped at or near the major salt tolerance QTL previously identified in S-100 (Lee *et al.*, 2004). Three SNPs on Chromosome 18 map near the salt tolerance QTL previously identified in Nannong1138-2 (Chen *et al.*, 2008). The other significant SNPs represent four putative minor QTLs for salt tolerance newly identified in this study. Ten genes, which are mapped at or near (< 35 kb) the significant SNPs, appear to be potential candidates involved in ion metabolisms and salt stress responses in soybean. Gene expression analysis indicated that GmUBC2, an ubiquitin-conjugating enzyme, and GmNHX1, a vacuolar Na^+/H^+ antiporter, are both up-regulated in salt-tolerant (Lee 68 and S-100) and salt-sensitive genotypes (Dare and Glenn). However, GmUBC2 expression is higher in salt-tolerant genotypes than in

salt-sensitive genotypes. As for GmNHX1, Dare exhibited a higher level of expression than the other three genotypes. These results imply potential roles of GmUBC2 and GmNHX1 in conferring salt tolerance in soybean.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to Dr. Stewards for bringing me to the University of Arkansas for my master's study.

I would like to thank my academic advisor Dr. Pengyin Chen for giving the opportunity to join the soybean breeding program and for his patience, guidance, support, commitment, and encouragement. Not only has he always been patient and supportive for my research as an academic advisor, but also he's been kindly concerned and ready to help with my daily life. He gives my opportunities to meet great academic and industrial colleagues and scientists, and helps me develop my career goals as well as social skills; He encourages and guides me to perform actively in academic and daily living communities. Thank you Dr. Chen for all you've done for me.

I would like to thank my committee members Dr. Andy Pereira, Dr. Ken Korth, Dr. Vibha Srivastava, and Dr. Kristofor Brye for serving on my committee and for their patience, kindness, understanding, support, and advice.

I would like to thank Dr. Chengjun Wu and Dr. A. Shi for instructions and advice on molecular experiments and data analysis.

I sincerely feel grateful for all the current and past members of the soybean breeding program at the University of Arkansas. Best wishes and special thanks to Ailan Zhang, Baishuang Yu, Chunyan Yang, Cindy Lopez, Chengjun Wu, David Moseley, Innan Cervantes, Jane Mokua, Jiao Wang, Jody Hedge, Luciano Jaureguy, Lindsay Mack, Liliana Florez, Mariola Klepadlo, Moldir Orazaly, Qibin Ma, Sarah Paulson, Shi Sun, Tina Hart, Victor Hugo Nakase, Yoko Yoshikawa, and Xujun Fu.

I am very thankful to the staff of the Department of Crop, Soil, and Environmental Sciences at the University of Arkansas for their generous help throughout my master's program.

I am also very grateful to John Guerber for his help with all the greenhouse management and research.

I would also like to thank my friends Annie Guo, Cheng Li, Fan Wang, Jack Xu, Jingjing Tong, Jing Li, Jin Zhang, Hongxiang Yan, Hu Wang, Shu Yang, Ting Liu, Xin Xiao, Yaohua Chen, and Yiwei Qian, who have been supporting and helping me all the time during my master's program.

Most importantly, I would like to express my deepest gratitude to my family who show endless love and support to me wherever I am.

DEDICATION

The thesis is dedicated to my family and friends, who have been giving me endless love and support during my master's program.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER IV

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

Figure 1. Relative expression of GmUBC2 in the first trifoliate leaves of the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**) and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. CK represents the untreated control. Error bars represent standard deviation (n=3).

Figure 2. Relative expression of GmNHX1 in the first trifoliate leaves of the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**) and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. CK represents the untreated control. Error bars represent standard deviation (n=3).

Figure 3. Comparative expression of GmUBC2 in the first trifoliate leaves between the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**), and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. Error bars represent standard deviation (n=3).

Figure 4. Comparative expression of GmNHX1 in the first trifoliate leaves between the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**), and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. Error bars represent standard deviation (n=3).

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Salinity Problems

Soil salinity is the dissolved salt content in soil, often measured in electrical conductivity (EC) of the water extracted from a saturated soil (called saturation extract) and expressed in dS/m (deci Siemens per meter). Soil is classified as salt-affected when the ECe is 2 dS/m or more (FAO, 1988), which can sacrifice crop production (Table 1).

Salt-affected soils are classified into three main groups: saline soils (excessive neutral soluble salts consisting of chlorides and sulphates of sodium, calcium and magnesium.), sodic soils (excessive exchangeable sodium and salts capable of alkaline hydrolysis, e.g. Na_2CO_3) and saline-sodic soils (excessive both neutral soluble salts and exchangeable sodium) (FAO, 1988). Sodium chloride is the predominant salt in salt-affected soils (Munns and Tester, 2008). High sodium concentration in soil causes aggregate swelling and soil dispersion (Warrence *et al.*, 2002). Soil dispersion causes clay particles to plug soil pores, reducing soil permeability, which adversely affect plant up-take of water.

The primary formation of salt-affected soils occurs naturally. Climate weathering of soil parental materials rich in salts releases various types of salts mainly consisting of chlorides of sodium, calcium, and magnesium. Rainwater and wind carrying oceanic salts are other natural causes of increasing salts. In arid and semiarid areas, salt accumulates over a long period of time as the rate of evaporation far exceeds that of precipitation. Agricultural activities such as improper irrigation and drainage practices can also result in the increase of soil salinity. Heavy irrigation concentrates salts year-by-year when the irrigation water delivering salts losses through a combination of evaporation and transpiration (known as evapotranspiration) and there is no proper drainage for leaching and removal of salts. These agricultural practices have led to

huge losses in arable lands. Today, nearly 20% of the cultivated land and nearly half of the irrigated land worldwide are salt-affected (Zhu, 2001).

Soybean Production and Uses

Soybean [*Glycine max* (L.) Merr.] is the cultivated species under the genus *Glycine* Willd. The genus *Glycine* Willd. includes two subgenera, *Glycine* and *Sojo*. The subgenus *Soja* (Moench) F.J. Herm. consists of two annual species, the cultivated soybean, *Glycine max* (L.) Merr., and the wild soybean, *Glycine sojo* Sieb. & Zucc., while the subgenus *Glycine* includes at least 16 wild perennial species.

Soybean originated from Southeast Asia with first domestication recorded in 11th century BC in China, and was introduced to the US in 1765. It is now one of the most important crop species in the world. In 2011, a total of 251.5 million metric tons of soybean were produced worldwide (Soy Stats, 2012). Among the major soybean producers, the US accounted for 33% of the total production, followed by Brazil (29%), Argenina (19%), and China (5%). In the US, Iowa and Illinois were the major two producers with a production of 12.69 and 11.33 million metric tons, respectively. Arkansas ranked the ninth with a production of 3.38 million metric tons (Soy Stats, 2012).

With a high content of protein and oil, soybean is widely consumed for edible and industrial uses. Traditional soybean foods for human consumption include nonfermented products such as soy milk, soybean sprouts, edamame, tofu, tofu skin, etc., and fermented products such as miso, soy sauce, fermented bean paste, natto, tempeh, etc. (Soy Stats, 2012). Other soybean foods include full fat soy flour, roasted soybean, soy nuts, etc. Soybean is also an important source for dietary supplements such as soy isoflavones, vitamin E, and phytosterols (Soy Stats, 2012). Soybean meal is used as a filler and source of protein in animal diets,

including swine, poultry, cattle, horse, sheep, and fish feed. In 2011, the US produced 35.6 million metric tons of soybean meal (Soy Stats, 2012). Soybean is also a major source of edible fats and oils. In 2011, soybean accounted for 66% of edible fats and oils consumption in the US (Soy Stats, 2012). Beside edible uses, soybean oil is widely used in a variety of industrial applications. Soybean oil is currently a major feedstock for biodiesel production. It can also be used as a carrier for agricultural pesticides. In 2011, industrial products accounted for 20% of soybean oil consumption in the US (Soy Stats, 2012).

Effects of Salt Stress on Soybean

Salt-affected soil has become a common abiotic stress causing agricultural loss. Crops under salt stress are primarily subjected to ion damage and osmotic stress, which often cause secondary stresses such as oxidative imbalance (Zhu, 2001).

The physiological responses of soybean under salt stress have been extensively studied. Abel and Mackenzie (1964) reported delayed seed germination in low salt concentrations and significantly reduced germination rate in high salt concentration. Salt stress inhibits seedling growth, decreases nodulation, and results in considerable reduction in agronomic traits including height, seed weight, leaf size, biomass, pods number, and yield (Abel and MacKenzie, 1964; Singleton and Bohlool, 1984; Chang *et al.*, 1994, Essa, 2002; Katerji *et al.*, 1998; Serraj *et al.*, 1998; Wang and Shannon, 1999; Katerji *et al.*, 2003). Salt stress also causes severe leaf chlorosis, leaf scorch, and even plant death (Abel, 1969; Parker *et al.*, 1983, 1987). However, Soybean species and genotypes vary greatly in salt stress response and tolerance (Abel and MacKenzie, 1964; Nukaya *et al.*, 1982; Parker *et al.*, 1983; Grattan and Maas, 1988a, 1988b;

Pantalone *et al.*, 1997). It is therefore likely to minimize yield loss by use of salt-tolerant cultivars on salinity-affected fields, as is widely proposed.

A number of studies have indicated that salt-induced injury mainly results from excessive Na^+ accumulation, which adversely affects nutrition absorption, cytosolic enzyme activities, photosynthesis, and metabolism (for review, Niu *et al.*, 1995; Zhu, 2003; Mahajan and Tuteja, 2005). However, chloride toxicity in several crops has also been revealed (Wilson, 1967; Lessani and Marschner, 1978; Shannon, 1997; Pantalone *et al.*, 1997; Ping *et al.*, 2002). In soybean, most studies implicate that salt injury is mainly caused by Cl^- toxicity while few others emphasize the toxic effects of Na^+ or both over Cl^- (Abel and MacKenzie, 1964; Parker *et al.*, 1983; Yang and Blanchar, 1993; Wang and Shannon, 1999; Essa, 2002; An *et al.*, 2002; Luo *et al.*, 2005b; Lenis *et al.*, 2011; Zhang *et al.*, 2011). Soybean genotypes are classified as either Cl^- includers, in which Cl^- is mainly translocated to the foliage in the early stage of salt stress, or Cl^- excluders, in which Cl^- is mostly stored in roots during the early period of salt stress. On one hand, excessive Cl^- inclusion is commonly believed to cause leaf scorch in soybean based on the well paralleled correlation between leaf scorch symptoms and leaf chloride content. On the other hand, Cl^- exclusion is thought as the main salt tolerance mechanism in soybean due to the negative correlation between leaf chloride content and salt tolerance in some soybean and woody perennial species (Philip and Broadley, 2001). However, this correlation needs more verification as Pantalone *et al.* (1997) also found several salt-tolerant *Glycine* accessions with high leaf chloride content. Despite the role of Cl^- in salt injury of soybean, Li *et al.* (2006c) reported a less Na^+ accumulation in salt-tolerant soybean cultivars than in salt-sensitive cultivars. Another study indicated that Na^+ exclusion rather than Cl^- exclusion primarily accounts for the salt tolerance of wild soybean species (Luo *et al.*, 2005b). The findings above may be due to the genetic

variability among *Glycine* species and genotypes. However, whether sodium or chloride is more crucial for salt-induced damage in soybean is still unclear.

Salt Tolerance Screening

Effective methods for evaluating soybean salt tolerance are critical for the development of salt-tolerant soybean cultivars. Several methods have been developed (Parker *et al.*, 1983; Yang and Blanchar, 1993; Pantalone *et al.*, 1997; An *et al.*, 2002; Lee *et al.*, 2004; Valencia *et al.*, 2008; Lee *et al.*, 2008). Parker *et al* (1983) as well as Yang and Blanchar (1993) evaluated salt tolerance of soybean plants grown in fields with high salt content. However, the variability of salt across the fields retained as a problem, subjecting soybean plants to uneven salt stress. The field screening method also involved other variable factors such as soil uniformity and fertility, temperature and light intensity, which were hardly controllable but associated with plant injury (Pathan *et al.*, 2007). Hydroponics method appears as a more reliable screening method, in which soybean seedlings are grown in a nutrient solution added with certain amount of NaCl under controlled greenhouse conditions (Pantalone *et al.*, 1997; An *et al.*, 2001; Lee *et al.*, 2004; Valencia *et al.*, 2008). Soybean genotypes are evaluated for salt tolerance based on the visual leaf scorch ratings. However, this method is costly and labor intensive as the nutrients need changing before the evaluation (Lee *et al.*, 2008). Lee *et al.* (2008) improved the hydroponics method by substitution of a nutrient solution for a sandy soil as the growth medium, and termed this new method as PC (plastic cone-tainer) method since soybean plants were grown in sandy soil-filled plastic cone-tainers immersed in a NaCl solution. In this method, both leaf scorch rating and leaf chloride content measurement were carried out to evaluate the salt tolerance in

soybean. The PC method appeared be highly correlated with the hydroponics method (Lee *et al.*, 2008).

Using the methods mentioned above, a number of salt-tolerant soybean cultivars have been identified. Parker *et al.* (1983), Shao *et al.* (1995), and Yang and Blanchar (1993) reported 33, 10, and 19 Cl^- tolerant U.S. cultivars, respectively. Xu *et al.* (1999) classified 8 Chinese soybean cultivars as highly salt-tolerant. Variation in Cl^- tolerance was also found among 12 perennial *Glycine* accessions, among which *G. argyrea* 1626 and *G. clandestine* 1389 were more tolerant than the sensitive cultivar Jackson and the tolerant cultivar Lee, broadening the potential utility for the enhancement of salt tolerance in soybean (Pantalone *et al.*, 1997).

Genetics of Salt Tolerance in Soybean

Abel (1969) reported the first study on inheritance of the capacity for Cl^- inclusion and exclusion in soybean. The Cl^- excluders (Lee, N53-509) and Cl^- includers (Jackson, N53-505, B54-842) were used to make crosses. The parents and their progeny were evaluated for saline tolerance and leaf chloride content. Crosses between parents with similar Cl^- accumulation produced F_2 and F_3 offspring similar to the parents in chloride content. In eight crosses between parents different in chloride accumulation, F_2 plants segregated into an excluder : includer ratio of 3 : 1. F_3 progeny of F_2 excluder plants further segregated into an excluder : segregating ratio of 1 : 2 while F_3 progeny of F_2 includer plants showed no segregation. Backcrossing the F_1 plants from a cross between an includer and an excluder led to an includer : segregating ratio of 1 : 1 when using the includer as the recurrent parent, and an excluder : segregating ratio of 1 : 1 when using the excluder as the recurrent parent. Therefore, Abel (1969) proposed that a single dominant gene controls Cl^- tolerance in soybean. Shao *et al.* (1994) also studied the inheritance

of salt tolerance in soybean by crossing salt-tolerant varieties and salt-sensitive varieties, and evaluating the F_1, F_2 and F_3 progeny for salt tolerance in a saline field irrigated with a mixture of seawater and freshwater in a ratio of 1:1. Shao *et al.* (1994) found similar results, and concluded that a single gene confers salt tolerance, which is dominant over salt sensitivity. In contrast to the results of Abel (1969) and Shao *et al.* (1994), Luo *et al.* (2004) reported that minor genes control salt tolerance in soybean. Xu and Tuyen (2012) attributed the different results to the differences in genetic background of the parents and evaluation methods for salt tolerance. Lee *et al.* (2009) then studied the inheritance of salt tolerance in a wild soybean accession PI 483463. The results showed that F_2 plants from the cross between the tolerant PI 483463 and the sensitive cultivar Hutcheson segregated into a tolerant : sensitive ratio of 3 : 1. The $F_{2:3}$ plants segregated into a tolerant : segregating : sensitive ratio of 1 : 2 : 1. F_2 plants from the cross between PI 483463 and the tolerant cultivar S-100 segregated into a tolerant : sensitive ratio of 15 : 1. Lee *et al.* (2009) thus concluded that the salt tolerance of PI 483463 is controlled by a single dominant gene, which is different from the one in S-100.

Linkage and Association Mapping

Molecular markers such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphic DNA (AFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP) have been widely used for identifying quantitative trait loci (QTLs) for a variety of traits of economical and agronomical importance in crops. The markers/QTLs can be further applied for map-based cloning and marker-assisted selection in development of crop cultivars with traits of interest.

DNA marker-assisted selection (MAS) can significantly improve the efficiency and broaden the scope of crop breeding (Xu and Crouch, 2008).

Linkage mapping for salt tolerance and the related traits has been reported in various crops such as *Lycopersicum* (Breto *et al.*, 1994), barley (Mano and Takeda, 1997), tomato (Foolad and Chen, 1999), wheat (Lindsay *et al.*, 2004), and rice (Gong *et al.*, 1999; Koyama *et al.*, 2001; Lin *et al.*, 2004). Lin *et al* (2004) detected eight QTLs for salt tolerance-related physiological traits in rice. Of these QTLs, two major QTLs, qSKC-7 and qSKC-1, were associated with shoot Na^+ content and K^+ content, respectively (Lin *et al.*, 2004). SKC-1 was further isolated by map-based cloning and demonstrated to encode a Na^+ transporter, which was involved in regulation of Na^+/K^+ homeostasis under salt stress (Ren *et al.*, 2005). Lindsay *et al.* (2004) reported a locus, named as *Nax1* (Na^+ exclusion), on chromosome 2AL of durum wheat by using AFLP, RFLP and microsatellite markers. *Nax1* explained 38% phenotypic variation of the Na^+ exclusion trait, which was defined as a low Na^+ concentration in the leaf blade and related to wheat salt tolerance (Lindsay *et al.*, 2004).

In soybean, a few studies have addressed the QTLs conditioning salt tolerance in wild and cultivated soybean cultivars (Zhong *et al.*, 1997; Lee *et al.*, 2004; Hamwieh and Xu, 2008; Chen *et al.*, 2008; Tuyen *et al.*, 2010; Hamwieh *et al.*, 2011). Lee *et al.* (2004) identified a major QTL for salt tolerance on molecular linkage group (LG) N (Chromosome 3) in the salt-tolerant cultivar S-100 by use of RFLP and SSR markers. Screening 27 U.S soybean cultivars descended from S-100 or Tokyo for salt tolerance using both the phenotypic method and the SSR markers, Sat_091 and Satt237, which flank the salt tolerance QTL, showed that the presence of Sat_091 and Satt237 alleles from S-100 was always correlated with the salt tolerance of S-100 descendants (Lee *et al.*, 2004). The salt tolerance alleles in the cultivar Lee classified as chloride

excluder by Abel (1969) also derive from S-100 (Lee *et al.*, 2004). It is reported that that S-100 is the source of salt tolerance shown in nearly 20% of soybean cultivars in the southern U.S. (Shannon and Carter, 2003). Lee *et al.* (2004) thus suggested the use of the two markers, Sat_091 and Satt237, in commercial marker-assisted breeding programs in the U.S. Hamwieh and Xu (2008) as well as Hamwieh *et al.* (2011) further identified the same salt tolerance QTL in a wild soybean accession JWS156-1, and soybean cultivars FT-abyara and Jin dou No.6, and confirmed the correlation between the associated SSR markers and salt tolerance in these genotypes. However, different alleles of these SSR markers exist between JWS156-1/FT-abyar/Jin dou No.6 and S-100, suggesting that specific salt tolerance alleles from S-100 were not always present in other salt-tolerant genotypes (Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011). More verification is thus needed for using these markers for salt tolerance screening. In addition, Chen *et al.* (2008) detected eight QTLs for percentage of plant survival (PPS), plant survival days (PSD), and visual salt tolerance ratings (TR), which are traits conditioning salt tolerance, and confirmed the major QTL identified by Lee *et al.* (2004). A major QTL was found between markers Sat_164 and Sat_358 on linkage group G (chromosome 18) (Chen *et al.*, 2008). Tuyen *et al* (2010) revealed another QTL for alkaline salt tolerance on linkage group D2 from the wild soybean (*Glycine soja* Sieb & Zucc.) accession JWS156-1.

Association mapping (AM), based on the variation and extent of linkage disequilibrium (LD) across the genome within a population, has recently become an alternative method to detection of molecular markers and QTLs associated with specific traits in plants. LD refers to the nonrandom association of alleles at different loci (Cardon and Bell, 2001). The decay of LD over physical distance in a population determines marker density needed to perform an effective AM (Yu and Buckler, 2006). A more rapidly decayed LD requires higher marker coverage. If

LD exists between a marker and a locus associated with a trait, then specific marker alleles or haplotypes (i.e., genotype combinations at groups of linked markers) can be significantly associated with phenotypic values (Cardon and Bell, 2001).

AM has at least three advantages over linkage mapping. Linkage mapping deals with limited recombination events in a bi-parental population while AM exploits historical and evolutionary recombination events in a collection of individuals with unobserved ancestry through many generations, and thus has a higher mapping resolution (Zhu *et al.*, 2008). AM also saves research time compared to linkage mapping, which needs time to develop a mapping population. Greater allele number is another advantage over linkage mapping.

AM generally falls into two categories: candidate-gene association mapping and genome-wide association mapping (Zhu *et al.*, 2008). Candidate-gene association mapping depends on genetic polymorphisms in selected genes that potentially control specific traits. It requires information on sequence and function of candidate genes. Nowadays, annotated genome sequences of several model species and application of genomic technologies (i.e., sequencing, SNP genotyping, gene expression, comparative genomics, bioinformatics, linkage mapping, etc.) have advanced and facilitated candidate-gene association mapping (Zhu *et al.*, 2008). Genome-wide association mapping exploits genetic variation across the whole genome to detect marker-trait associations using a large number of genetic markers (Zhu *et al.*, 2008). As high throughput sequencing has made it affordable to identify and genotype hundreds of thousands of SNP markers across a large set of samples, genome-wide association mapping is gaining more favorability in genetic study among researchers (Zhu *et al.*, 2008). For example, the recently developed SoySNP50K iSelect Beadchip provides a powerful tool for characterizing soybean

genetic diversity and linkage disequilibrium, and for constructing high resolution linkage maps with over 50,000 SNP markers (Song *et al.*, 2013).

Assembled genotypes used in an AM often contain groups of subpopulations and within-group familial relatedness (Yu and Buckler, 2006). LD resulting from admixture of subpopulations can cause spurious marker-trait associations if no correction applied in statistical analysis. To deal with population structure issue and familial relatedness in population-based samples, several statistical methods have been developed: genomic control (GC) (Devlin and Roeder, 1999), structured association (SA) (Falush *et al.*, 2003; Pritchard and Rosenberg, 1999; Prichard *et al.*, 2000a), mixed model approach (Yu *et al.*, 2006), and principal component approach (Price *et al.*, 2006). On one hand, GC estimates the degree of statistical inflation caused by population structure using a set of random markers, with an assumption of a similar effect of such structure on all loci in a population (Zhu *et al.*, 2008). On the other, SA, mixed model, and principal component approach explicitly account for genetic relatedness using genotypic information, and incorporate population structure (Q), relative kinships (K), and/or principal component analysis for further statistical analysis. These methods have been utilized to reduce false positives generated by population structure accordingly (Zhu *et al.*, 2008).

Mechanisms of Salt Tolerance

In an effort to develop crop cultivars with enhanced salt tolerance, understanding the mechanisms of salt stress response and tolerance has remained as one of the key challenges for plant biologists and crop breeders. Extensive studies have been carried out to reveal salt tolerance mechanisms in model plants and various crops (For review, Flowers, Troke & Yeo, 1977; Zhu, 2001, 2002, 2003; Tester and Davenport, 2003; Chinnusamy *et al.*, 2005; Parida and

Das, 2005; Moller *et al.*, 2007; Munns and Tester, 2008). Phang *et al.* (2008) summarized and classified the salt tolerance mechanisms of soybean mainly into four categories: maintenance of ion homeostasis, accumulation of osmoprotectants, restoration of oxidative balance, and other metabolic and structural adaptations.

Ion homeostasis

Regulation of Na^+ homeostasis in cytoplasm under salt stress involves three cooperative mechanisms: restriction of Na^+ influx, active Na^+ efflux, and Na^+ compartmentation (for review, Niu *et al.*, 1995; Zhu *et al.*, 2003). The Salt Overly Sensitive (SOS) pathway has been well defined and recognized as a key mechanism for Na^+ exclusion and ion homeostasis under salt stress (Ji *et al.*, 2013). In *Arabidopsis*, Ca^{2+} signal elicited by salt stress activates SOS3 (a calcineurin B-like Ca^{2+} sensor protein)-SOS2 (a protein kinase) kinase complex. The activated complex then activates SOS1, a plasma membrane Na^+/H^+ antiporter, which mediates Na^+ efflux, pumping toxic Na^+ out of cytosol and inhibiting the transport of Na^+ from roots to shoots under salinity, and AtNHX1, a tonoplast Na^+/H^+ antiporter, which regulates Na^+ compartmentation, pumping excessive Na^+ into the vacuole (for review, Zhu *et al.*, 2003; Chinnusamy *et al.*, 2005). Overexpression of SOS1 in transgenic *Arabidopsis* can result in less Na^+ in the shoots and xylem transpirational stream, and enhance salt tolerance (Shi *et al.*, 2003). The induction of AtNHX1 in *Arabidopsis* under both salt- and ABA-stress was also reported (Shi and Zhu, 2002). Overexpression of AtNHX1 in genetically engineered *Arabidopsis*, tomato, and canola has been shown to enhance salt tolerance (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001). Cation channels or transporters regulate Na^+ influx into root cell cytosol under salinity (Chinnusamy *et al.*, 2005). The high-affinity K^+ transporters (HKT) also mediate Na^+ uptake and

transport in plants (Wang and Wu, 2013). Members of HKT transporters confer salt tolerance in plants (Horie *et al.*, 2009). Further studies have shown that most plant HKT transporters actually function as Na^+ transporters, and only few are K^+/Na^+ symporters (Wang and Wu, 2013).

In soybean, SOS1 homologue (GmSOS1) is also induced by salt stress (Phang *et al.*, 2008), but its role in salt tolerance remains unclear. Li *et al.* (2006b) isolated a tonoplast NHX homolog (GmNHX1) from soybean. GmNHX1 is up-regulated by salt and drought stress (Sun *et al.*, 2006; Phang *et al.*, 2008). Overexpression of GmNHX1 was found to enhance the salt tolerance in transgenic tobacco and *Lotus corniculatus* (Li *et al.*, 2006b; Sun *et al.*, 2006). A HKT homolog (GmHKT1) modulating Na^+ and K^+ transport in soybean was recently isolated (Chen *et al.*, 2011). Overexpression of GmHKT1 enhanced the salt tolerance in genetically engineered tobacco (Chen *et al.*, 2011).

Plasma membrane and vacuolar H^+-ATPase and/or H^+-PPase produce proton force for the activity of Na^+/H^+ antiporters (Chinnusamy *et al.*, 2005). *Arabidopsis* overexpressing AVP1, a vacuolar H^+-PPase, had increased Na^+ sequestration into vacuole, higher leaf relative water content as well as enhanced salt and drought tolerance (Gaxiola *et al.*, 2001). In soybean, homologs of H^+-PPase and subunit C of vacuolor H^+-ATPase were induced by salt stress (Phang, 2008).

Cl^- homeostasis is crucial for salt tolerance in soybean. However, little is known about genes conferring Cl^- homeostasis (Li *et al.*, 2006b). A soybean vacuolar chloride channel (GmCLC1) was first isolated, and induced under salt stress (Li *et al.*, 2006b). It was implicated to function in the sequestration of Cl^- into vacuoles, and thus increase the salt tolerance in transgenic tobacco BY-2 cells (Li *et al.*, 2006b). A further study confirmed the role of GmCLC1 in Cl^- transport and suggested that GmCLC1 is regulated by pH (Wong *et al.*, 2012). Zhou *et al.*

(2010) isolated another CLC-type chloride channel (GmCLCnt) from soybean, which was up-regulated by NaCl. Overexpression of GmCLCnt increased salt tolerance in Arabidopsis, implying the important role of GmCLCnt in salt tolerance (Zhou *et al.*, 2010).

Osmotic adjustment and osmoprotection

Osmotic adjustment is an essential attribute of salt tolerance in plants (Shabala and Cuin, 2007). On one hand, plant absorb inorganic ions such as Na^+ and Cl^-, which, though toxic, can be used as cheap osmotica for maintenance of normal cell turgor under saline conditions, assuming that they are efficiently sequestered into vacuoles (Shabala and Cuin, 2007). On the other, plants accumulate organic compatible solutes such as proline, betaine, polyols, sugar alcohols, and soluble sugars for osmotic adjustment (Zhu, 2003; Phang *et al.*, 2008). These compatible solutes are also called osmoprotectants due to their additional protective functions such as scavenging ROS (reactive oxygen species) as well as stabilizing proteins and cell membranes (Le Rudulier *et al.*, 1984; Papageorgiou and Murata, 1995; Bohnert and Jensen, 1996; Chen and Murata, 2000; Phang *et al.*, 2008). Salt stress induces genes involved in synthesis of osmoprotectants, which accumulate in a positive correlation with osmotic stress tolerance (Zhu, 2002). In addition to osmotic adjustment, protective effects of the osmoprotectants have been shown to contribute to enhanced stress tolerance as a few transgenic studies have demonstrated (Shen *et al.*, 1997; Nanjo *et al.*, 1999; Hong *et al.*, 2000). For example, reduced free radicals and enhanced salt tolerance were found in transgenic tobacco expressing a mutated P5CS (delta1-pyrroline-5-carboxylate synthetase) gene in lack of proline feedback inhibition, suggesting the function of proline in ROS detoxification (Hong *et al.*, 2000). Nanjo *et al.* (1999) found enhanced salt tolerance and constitutive freezing tolerance in genetically engineered *Arabidopsis* with

suppressed proline degradation. Other transgenic studies have also indicated the significant role of proline in plant response to salt and osmotic stress (Kishor *et al.*, 1995; Zhu *et al.*, 1998). In soybean, osmoprotectants such as *Glycine* betaine, trigonelline, and pinitol accumulate under salinity, and significantly enhance the salt tolerance. However, proline as an osmoprotectant in soybean retains controversial due to several conflicting findings (Krackhard and Guerrier, 1995; Guo and Weng, 2004). Phang *et al.* (2008) presumably attributed the incongruity to the different experimental conditions and genetic variability of the germplasms used in those studies. However, more verification is needed.

Restoration of oxidative balance

Abiotic stresses including salt stress cause oxidative damage by induction of enhanced cellular ROS (Reactive Oxygen Species), which normally maintain low and balanced, and function as signaling components regulating various cellular processes such as programmed cell death, abiotic stress responses, pathogen defense and systemic signaling (reviewed by Mittler, 2002). Redox sensitive receptors-like kinases and two component histidine kinases are thought to sense ROS (Chinnusamy *et al.*, 2005). A mitogen activated protein kinase (MAPK) signaling cascade is then activated, which regulates several transcription factors and ultimately leads to plant defense against oxidative stress (Chinnusamy *et al.*, 2005). In addition, changes in Ca^{2+} and calmodulin levels under the stress of hydrogen peroxide (H_2O_2, an important ROS) suggest the involvement of a Ca^{2+}-mediated pathway in plant defense against oxidative stress (Mittler, 2002). A MAPK cascade includes MAPKKKs (mitogen activated protein kinase kinase kinase), MAPKKs (mitogen activated protein kinase kinase), and MAPKs (mitogen activated protein kinase). In *Arabidopsis*, ROS stress imposed by H_2O_2 induces a MAPKKK (ANP1), which

activates two MAPKs, AtMPK3 and AtMPK6, through MAPK cascade (Moon *et al.*, 2003). AtNDPK2 (*Arabidopsis* nucleoside diphosphate kinase 2), which can be also induced by H_2O_2 and positively interact with AtMPK3 and AtMPK6, is thus implicated in mediating a MAPK cascade transducing H_2O_2 signaling (Moon *et al.*, 2003). Transgenic tobacco overexpressing NPK1 (an ortholog of ANP1), rice overexpressing OsMAPK5 (a rice MAPK), and *Arabidopsis* overexpressing NDPK2 showed enhanced tolerance to multiple abiotic stresses including salinity (Kovtun *et al.*, 2000; Xiong and Yang, 2003; Moon *et al.*, 2003). In addition to the MAPK cascade transducing ROS signaling, studied suggested that another salt-stress activated MAPK cascade in *Arabidopsis* exists, including AtMEKK1 (a MAPKKK), AtMEK1/AtMKK2 (two MAPKKs), and AtMPK4 (a MAPK), and is negatively regulated by MKP1 (*Arabidopsis* MAPK phosphatase 1). However, the role of MAPKKKs, MAPKKs and MAPKs in salt stress and salt stress-induced ROS signaling transduction in soybean remains unknown.

Plants accumulate various antioxidants including non-enzymatic (e.g. ascorbate, glutathione and carotenoids) and enzymatic (e.g. superoxide dismutase, catalase and peroxidase) ROS scavengers to restore oxidative balance and alleviate the oxidative damage. It was also suggested that ROS production avoidance mechanisms play an important role in plant defense against oxidative stress (Mittler, 2002). Expression of genes involved in antioxidant synthesis is broadly believed to be enhanced under oxidative stress induced by salinity. For instance, Takemura *et al.* (2002) reported an increased transcript level of SOD (superoxide dismutase), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, in *Bruguiera gymnorrhiza* under salt stress. Salt-tolerant plants accumulate more antioxidants than salt-sensitive plants (Gossett *et al.*, 1994; Dionisio-Sese and Tobita, 1998; Tsugane *et al.*, 1999; Mittova *et al.*, 2000; Moradi and Ismail, 2007). A number of transgenic studies further indicated

that overexpressing ROS scavenging enzymes in transgenic plants enhances the tolerance to various abiotic stresses (Wang *et al.*, 1999; Roxas *et al.*, 1997, 2000). A positive correlation between salt tolerance and antioxidant activities in soybean has also been documented (Phang *et al.*, 2008). Several soybean genes such a putative purple acid phosphatase (GmPAP3) and an antiquitin-like protein (GmTP55) have been shown to be involved in plant response to salinity and oxidative stress as well as defense against ROS (Phang *et al.*, 2008).

Objectives

To improve salt tolerance in soybean through plant breeding, identification of salt-tolerant genotypes and a deeper understanding of both genetic and molecular bases of salt tolerance are required. In this study, a set of selected elite soybean breeding lines and cultivars were evaluated for salt tolerance. Of these genotypes, the salt-tolerant and salt-sensitive genotypes grouped as an admixture population, were genome-wide scanned through the SoySNP50K iSelect BeadChip with 52,041 single nucleotide polymorphism (SNP) markers to explore the genetic diversity and linkage disequilibrium within this population. Associating the phenotypic and genotypic data, genome-wide association mapping was conducted to detect marker-trait associations for salt tolerance. Expression analysis of salt-responsive genes in salt-tolerant and salt-sensitive genotypes under NaCl-induced salt stress was also examined to identify candidate salt tolerance genes in soybean. The overall objectives of this study were to identify salt-tolerant genotypes, to search for SNP markers and QTLs associated with salt tolerance, and to identify candidate salt tolerance genes in soybean.

REFERENCES

Abel, G.H. 1969. Inheritance of the capacity for chloride inclusion and chloride exclusion in soybeans. *Crop Sci.* 9, 697–698.

Abel, G.H. and A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. *Crop Sci.* 4, 157–161.

An P., S. Inanaga, Y. Cohen, U. Kafkafi, and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. *J. Plant Nutr.* 25, 407–423.

Apse, M.P., G.S. Aharon, W.S. Snedden and E. Blumwald. 1999. Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis. Science* 285, 1256–1258.

Bohnert, H.J. and R.G. Jensen. 1996. Strategies for engineering water stress tolerance in plants. *Trends Biotechnol*. 14, 89-97.

Breto, M.P., M.J. Asins, and E.A. Carbonell. 1994. Salt tolerance in *Lycopersicum* species. III. Detection of quantitative traits loci by means of molecular markers. *Theor. Appl. Genet.* 88, 395–401.

Cardon, L., and J. Bell. 2001. Association study designs for complex diseases. *Nat. Rev. Gen.* 2, 91–99.

Chang, R.Z., Y.W. Chen, G.H. Shao and C.W. Wan. 1994. Effect of salt stress on agronomic characters and chemical quality of seeds in soybean. *Soybean Sci.* 13, 101–105.

Chen, H., S. Cui, S. Fu, J. Gai and D. Yu. 2008. Identification of quantitative trait loci associated with salt tolerance during seedling growth in soybean (*Glycine max* L.). *Aust. J. Agric. Res.* 59, 1086–1091.

Chen, H.T., H. He and D.Y. Yua. 2011. Overexpression of a novel soybean gene modulating Na^+ and K^+ transport enhances salt tolerance in transgenic tobacco plants. *Physiol. Plantarum* 141, 11–18.

Chen, T.H. and N. Murata. 2000. Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr. Opin. Plant Biol.* 5, 250–257.

Chinnusamy, V., A. Jagendorf and J.K. Zhu. 2005. Understanding and Improving Salt Tolerance in Plants. *Crop Sci.* 45, 437–448.

Devlin, B., and K. Roeder. 1999. Genomic control for association studies. *Biometrics* 55, 997–-1004.

Dionisio-Sese, M.L., S. Tobita. 2000. Effects of salinity on sodium content and photosynthetic responses of rice seedlings differing in salt tolerance. *J. Plant Physiol.* 157, 54-58.

Diwan, N. and P.B. Cregan. 1997. Automated sizing of fluorescent labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean. *Theor. Appl. Genet.* 95, 723–733.

Essa, T.A. 2002. Effect of salinity stress on growth and nutrient composition of three soybean [*Glycine max* (L.) Merr.] cultivars. *J. Agron. Crop Sci.* 188, 86–93.

Falush, D., M. Stephens, and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* 164, 1567–1587.

FAO. 1988. Salt-affected soils and their management. Publications Division, Food and Agriculture Organization of the United Nations, Rome, Italy.

Flowers, T.J., M.L. Koyama, S.A. Flowers, C. Sudhakar, K.P. Singh, and A.R. Yeo. 2000. QTL: their place in engineering tolerance of rice to salinity. *J. Exp. Bot.* 51, 99–106.

Flowers, T.J., P.F. Troke and A.R. Yeo. 1977. The mechanism of salt tolerance in halophytes. *Rev. PL Physiol.* 28, 89-121.

Foolad, M.R. and F.Q. Chen. 1999. RFLP mapping of QTLs conferring salt tolerance during the vegetative stage in tomato. *Theor. Appl. Genet.* 99, 235-243.

Gaxiola, R.A., J. Li, S. Undurraga, V. Dang, G.J. Allen, S.L. Alper and G.R. Fink. 2001. Drought- and salt-tolerant plants result from overexpression of the *AVP1* H-pump. *Proc. Natl. Acad. Sci. USA* 98, 11444–11449.

Gong, J.M., P. He, Q. Qian, L.S. Shen, L.H. Zhu, and S.Y. Chen. 1999. Identification of salt-tolerance QTL in rice (*Oryza sativa* L.). *Chin. Sci. Bull.* 44, 68-71.

Gorham, J., J. Bridges, J. Dubcovsky, J. Dvorak, P.A. Hollington, M.C. Luo and J.A. Khan. 1997. Genetic analysis and physiology of a trait for enhanced K^+/Na^+ discrimination in wheat. *New Phytol.* 137, 109–116.

Gossett, D.R., E.P. Millhollon and M.C. Lucas. 1994. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop Sci.* 34, 706-14.

Grattan, S.R. and E.V. Maas. 1988a. Effect of salinity on phosphate accumulation and injury in soybean: I. Influence of $CaCl_2$/NaCl ratios. *Plant Soil* 105, 25–32.

Grattan, S.R. and E.V. Maas. 1988b. Effect of salinity on phosphate accumulation and injury in soybean. II. Role of substrate Cl^- and Na^+. *Plant Soil* 109, 65–71.

Guo, B. and Y. Weng. 2004. Salt tolerance mechanism and molecular markers of genes associated with salt tolerance in soybean. *Chin. Bull. Bot.* 21, 113–120.

Hamwieh, A. and D.H. Xu. 2008. Conserved salt tolerance quantitative trait locus (QTL) in wild and cultivated soybeans. *Breed Sci.* 58, 355–359.

Hamwieh, A., D.D. Tuyen, H. Cong, E.R. Benitez, R. Takahashi, and D.H. Xu. 2011. Identification and validation of a major QTL for salt tolerance in soybean. *Euphytica* 179, 451–459.

Hong, Z., K. Lakkineni, Z. Zhang and D.P.S. Verma. 2000. Removal of feedback inhibition of delta1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiol.* 122, 1129–1136.

Horie, T., F. Hauser, and J.I. Schroeder. 2009. HKT transporter-mediated salinity resistance mechanisms in *Arabidopsis* and monocot crop plants. *TRENDS Plant Sci*. 14, 660–668.

Katerji, N., J.W. van Hoorn, A. Hamdy and M. Mastrorilli. 2003. Salinity effect on crop development and yield, analysis of salt tolerance according to several classification methods. *Agric. Water Manage.* 62, 37-66.

Katerji, N., J.W. van Hoorn, A. Hamdy, M. Mastrorilli and F. Karam. 1998. Salinity and drought, a comparison of their effects on the relationship between yield and evapotranspiration. *Agric. Water Manage.* **36**, 45–54.

Kishor, P.B.K., Z. Hong, G.H. Miao, C.A.A. Hu and D.P.S. Verma. 1995. Overexpression of [delta]-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* 108, 1387–1394.

Kovtun, Y., W.L. Chiu, G. Tena and J. Sheen. 2000. Functional analysis of oxidative stressactivated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. USA* 97, 2940-2945.

Koyama, M.L., A. Levesley, R.M.D. Koebner, T.J. Flowers, A.R. Yeo. 2001. Quantitative trait loci for component physiological traits determining salt tolerance in rice. *Plant Physiol.* 125, 406–422.

Krackhard, M. and G. Guerrier. 1995. Effect of osmotic and ionic stresses on proline and organic acid contents during inhibition and germination of soybean seeds. *J. Plant Physiol.* 1146, 725–730.

Le Rudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith and R.C. Valentine. 1984. Molecular biology of osmoregulation. *Science* 224, 1064-1068.

Lee J.D, G. Shannon, T.D. Vuong, and H.T. Nguyen. 2009. Inheritance of salt tolerance in wild soybean (*Glycine soja* Sieb. & Zucc.) accession PI483463. *J. Hered.* 100, 798–801.

Lee, G.J., H.R. Boerma, M.R. Villagarcia, X. Zhou, T.E. Carter Jr, Z. Li, and M.O. Gibbs. 2004. A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. *Theor. Appl. Genet.* 109, 1610–1619.

Lee, J.D., L. Scotty, Smothers, D. Dunn, M. Villagarcia, C.R. Shumway, T.E. Carter, Jr., and J.G. Shannon. 2008. Evaluation of a Simple Method to Screen Soybean Genotypes for Salt Tolerance. *Crop Sci.* 48, 2194–2200.

Lenis, J.M., M. Ellersieck, D.G. Blevins, D.A. Sleper, H.T. Nguyen, D. Dunn, J.D. Lee, and J.G. Shannon. 2011. Differences in ion accumulation and salt tolerance among *Glycine* accessions. *J. Agron. Crop Sci.*197, 302–310.

Lessani, H., and H. Marschner. 1978. Relation between salt tolerance and long-distance transport of sodium and chloride in various crop species. *Aust. J. Plant Physiol.* 5, 27–37.

Li, W.Y., F.L. Wong, S.N. Tsai, T.H. Phang TH, G. Shao and H.M. Lam. 2006b. Tonoplast-located *GmCLC1* and *GmNHX1* from soybean enhance NaCl tolerance in transgenic bright yellow (BY)-2 cells. *Plant Cell Environ.* 29, 1122–1137.

Li, X., P. An, S. Inanaga, E. Eneji and K. Tanabe. 2006c. Salinity and defoliation effects on soybean growth. *J. Plant Nutr*. 29, 1499–1508.

Lin, H.X., M.Z. Zhu, M. Yano, J.P. Gao, Z.W. Liang, W.A. Su, X.H. Hu, Z.H. Ren and D.Y. Chao. 2004. QTLs for Na^+ and K^+ uptake of the shoots and roots controlling rice salt tolerance. *Theor. Appl. Genet.* 108, 253-260.

Lindsay, M.P., E.S. Lagudah, R.A. Hare, and R. Munns. 2004. A locus for sodium exclusion (*Nax1*), a trait for salt tolerance, mapped in durum wheat. *Functional Plant Biology* 31, 1105–1114.

Luo, Q., B. Yu and Y. Liu. 2005b. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *J. Plant Physiol.* 162, 1003–1012.

Mahajan, S. and N. Tuteja. 2005. Cold, salinity and drought stresses: an overview. *Archives of Biochemistry and Biophysics* 444, 139-158.

Mano, Y. and K. Takeda. 1997. Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare* L.). *Euphytica* 94, 263–272.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405–410.

Mittova, V., M. Volokita, M. Guy and M. Tal. 2000. Activities of SOD and the ascorbate–glutathione cycle enzymes in subcellular compartments in leaves and roots of the

cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. *Physiol. Plantarum* 110, 42–51.

Moller, I.M., P.E. Jensen and A. Hansson. 2007. Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* 58, 459-481.

Moon, H., B. Lee, G. Choi, D. Shin, D.T. Prasad, O. Lee, S.S. Kwak, D.H. Kim, J. Nam, J. Bahk, J.C. Hong, S.Y. Lee, M.J. Cho, C.O. Lim and D.J. Yun. 2003. NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants *Proc. Natl. Acad. Sci. USA* 100, 358-363.

Moradi, F. and A. Ismail. 2007. Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. *Ann. Bot.* 99, 1161–1173.

Munns, R. and M. Tester. 2008. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681.

Nanjo, T., M. Kobayashi, Y. Yoshiba, K. Wada, H. Tsukaya, K. Yamaguchi-Shinozaki and K. Shinozaki. 1999. Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J.* 18, 185-193.

Niu, X., Bressan, R.A., Hasegawa, P.M., and Pardo, J.M. 1995. Ion homeostasis in NaCl stress environments. *Plant Physiol.*109, 735–742.

Nukaya, A., M. Masui, and A. Ishida. 1982. Salt tolerance of green soybeans as affected by various salinities in sand culture. *J. Jpn. Soc. Hort. Sci.* 50, 487-496.

Pantalone, V.R., W.J. Kenworthy, L.H. Slaughter, and B.R. James. 1997. Chloride tolerance in soybean and perennial *Glycine* accessions. *Euphytica* 97, 235–239.

Papageorgiou, G. and N. Murata. 1995. The unusually strong stabilizing effects of *Glycine* betaine on the structure and function of the oxygen-evolving photosystem I1 complex. *Photosynth Res.*44, 243-252.

Parida, A.K. and A.B. Das. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotox. Environ. Safe.* 60, 324–349.

Parker, M.B., G.J. Gascho, T.P. Gains. 1983. Chloride toxicity of soybeans grown on Atlantic Coast Flatwoods soils. *Agron. J.* 75, 439–443.

Parker, M.B., T.P. Gaines, and G.J. Gascho. 1986. Sensitivity of Soybean cultivars to soil chloride. *University of Georgia: Tifton, GAUGA Res. Bull.* 347.

Parker, M.B., T.P. Gains, J.E. Hook, G.J. Gascho, and B.W. Maw. 1987. Chloride and water stress effects on soybean in pot culture. *J. Plant Nutr.*10, 517–538.

Pathan, M.S., J.D. Lee, J.G. Shannon, H.T. Nguyen. Recent advances in breeding for drought and salt stress tolerance in soybean. In: Jenks MA, Hasegawa PM, Jain SM, editors. Advances in molecular-breeding toward drought and salt-tolerant crops. Dordrecht (the Netherlands): Springer; 2007. p. 739-773.

Phang, T.H., G.H. Shao and H.M. Lam. 2008. Salt tolerance in soybean. *J. Integr. Plant Biol.* 50, 1196–1212.

Philip, J., and R. Broadley. 2001. Chloride in soils and its uptake and movement with the plant: A review. *Ann. Bot.* 88, 967–988.

Ping, A., S. Inanaga, Y. Cohen, U. Kafkafi and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. *J. Plant Nutr.* 25, 407–423.

Price, A.L., N.J Patterson, R.M. Plenge, M.E. Weinblatt, N.A. Shadick, and D. Reich. 2006. Principal component analysis corrects for stratification in genome-wide association studies. *Nat. Genet*. 38, 904–909.

Pritchard, J.K., and N.A. Rosenberg. 1999. Use of unlinked genetic markers to detect population stratification in association studies. *Am. J. Hum. Genet*. 65, 220–228.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000a. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Ren, Z.H., J.P. Gao, L.G. Li, X.L. Cai, W. Huang, D.Y. Chao, M.Z. Zhu, Z.Y. Wang, S. Luan and H.X. Lin. 2005. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat. Genet.* 37, 1141-1146.

Roxas, V.P., Jr., R.K. Smith, E.R. Allen, and R.D. Allen. 1997. Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.* 15, 988–991.

Roxas, V.P., S.A. Lodhi, D.K. Garrett, J.R. Mahan, and R.D. Allen. 2000. Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. *Plant Cell Physiol.* 41, 1229–1234.

Rubio, F., W. Gassmann and J.I. Schroeder. 1995. Sodium driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science* 270, 1660–1663.

Rus, A., S. Yokoi, A. Sharkhuu, M. Reddy, B.H. Lee, T.K. Matsumoto, H. Koiwa, J.K. Zhu, R.A. Bressan and P.M. Hasegawa. 2001a. AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. *Proc. Natl. Acad. Sci. USA* 98, 14150–14155.

Sairam, R.K. and A. Tyagi. 2004. Physiology and molecular biology of salinity stress tolerance in plants, *Curr. Sci.* 86, 407–421.

Serraj, R., H. Vasquez-Diaz and J.J. Drevon JJ. 1998. Effects of salt stress on nitrogen fixation, oxygen diffusion, and ion distribution in soybean, common bean, and alfalfa. *J. Plant Nutr.* 21, 475–488.

Shabala, S., and T.A. Cuin. 2007. Potassium transport and plant salt tolerance. *Physiol. Plantarum* 133, 651–669.

Shannon, J.G., and T.E. Carter, Jr. 2003. Breeding soybeans for tolerance to abiotic stress. p. 139–150. *In* Proc. Congr. AAPRESID, XI, Rosario, Argentina. 26–29 Aug. 2003. Asociación Argentina de Productores en SiembraDirecta, Rosario, Argentina.

Shannon, M.C. 1997. Adaptation of plants to salinity. *Adv. Agron.* 60, 75–120.

Shao G.H., R.Z. Chang, Y.W. Chen, and S.R. Yan. 1994. Study on inheritance of salt tolerance in soybean. *Acta Agronomica Sinica* 20, 721–726.

Shao, G.H., R.H. Chang, Y.W. Chen. 1995. Screening for salt tolerance to soybean cultivars of the United States. *Soybean Genet. Newslett.* 22, 32–42.

Shen, B., R.G. Jensen and H.J. Bohnert. 1997. lncreased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. *Plant Physiol.* 113, 1177-1183.

Shi, H., and J. K. Zhu. 2002. Regulation of expression of the vacuolar Na^+/H^+ antiporter gene AtNHX1 by salt stress and ABA. *Plant Mol.* 50, 543–550.

Shi, H., B.H. Lee, S.J. Wu and J.K. Zhu. 2003. Overexpression of a plasma membrane Na^+/H^+ antiporter improves salt tolerance in*Arabidopsis. Nature Biotechnol.* 21, 81–85.

Shi, H., F.J. Quintero, J.M. Pardo and J.K. Zhu. 2002. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na transport in plants. *Plant Cell* 14, 465–477.

Shi, H., M. Ishitani, C.S. Kim and J.K. Zhu. 2000. The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na/H antiporter. *Proc. Natl. Acad. Sci. USA* 97, 6896–6901.

Singleton, P.W. and B.B. Bohlool. 1984. Effect of salinity on nodule formation by soybean. *Plant Physiol.* 74, 72-76.

Song Q, D.L. Hyten, G. Jia, C.V. Quigley, E.W. Fickus, RL. Nelson, and P.B. Cregan. 2013. Development and evaluation of SoySNP50K, a high-density genotyping array for soybean. *PLoS ONE* 8, e54985.

Song, Q.J., L.F. Marek, R.C. Shoemaker, K.G. Lark, V.C. Concibido, X. Delannay, J.E. Specht, and P.B. Cregan. 2004. A new integrated genetic linkage map of the soybean. *Theor. Appl. Genet.* 109, 122-128.

Soy Stats. 2012. A reference guide to important soybean facts and figures. http://www.soystats.com/2012/default.htm (accessed 18 June. 2012).

Sun, Y.X., D. Wang, Y.L. Bai, N.N. Wang and Y. Wang. 2006. Studies on the overexpression of the soybean *GmNHX1* in *Lotus corniculatus*: the reduced Na^+ level is the basis of the increased salt tolerance. *Chin. Sci. Bull.* 51, 1306–1315.

Takemura, N. Hanagata, Z. Dubinsky and I. Karube. 2002. Molecular characterization and response to salt stress of mRNAs encoding cytosolic Cu^{2+}/Zn^{2+} superoxide dismutase and catalase from *Bruguiera gymnorrhiza*, *Trees* 16, 94–99.

Tester, M. and R. Davenport. 2003. Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.* 91, 503-527.

Tuyen, D.D., S.K. Lal and D.H. Xu. 2010. Identification of a major QTL allele from wild soybean (*Glycine soja* Sieb. & Zucc.) for increasing alkaline salt tolerance in soybean. *Theor. Appl. Genet.* 121, 229–236.

Valencia, R., P.Y. Chen, T. Ishibashi, and M. Conatser. 2008. A rapid and effective method for screening salt tolerance in soybean. *Crop Sci.* 48, 1773–1779.

Wang, D. and M.C. Shannon. 1999. Emergence and seedling growth of soybean cultivars and maturity groups under salinity. *Plant and Soil* 214, 117–124.

Wang, J., H. Zhang, and R.D. Allen. 1999. Overexpression of an *Arabidopsis* peroxisomal ascorbate peroxidase gene in tobacco increases protection against oxidative stress. *Plant Cell Physiol.* 40, 725–732.

Wilson, J.R. 1967. Response to salinity in *Glycine*: 3. Dry matter accumulation in three Australian species and *G. javanica*. *Aust. J. Exp. Agric. Anim. Husb.* 7, 50–56.

Wong, T.H., M.W. Li, X.Q. Yao, and H.M. Lam. 2012. The GmCLC1 protein from soybean functions as chloride ion transporter. *J. Plant Physiol*. 170, 101–104.

Xiong, L., and Y. Yang. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid inducible mitogen-activated protein kinase. *Plant Cell* 15, 745–759.

Xu, Y. and J.H. Crouch. 2008. Marker-assisted selection in plant breeding: from publications to practice. *Crop Sci.* 48, 391–407.

Xu, Z., R. Chang, L. Qiu, J. Sun, X. Li. 1999. Evaluation of soybean germplasm in China. In: Proceedings of the world soybean research conference VI. , Chicago, 4–7 August 1999. Superior Printing, Champagne, pp 156–165.

Yang J. and R.W. Blanchar. 1993. Differentiation chloride susceptibility in soybean cultivars. *Agron. J.* 85, 880–885.

Yu, J., and E.S. Buckler. 2006. Genetic association mapping and genome organization of maize. *Curr. Opin. Biotechnol*. 17, 155–160.

Yu, J., G. Pressoir, W.H. Briggs, I. Vroh Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B Holland, S. Kresovich, and E.S. Buckler. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nat. Genet.* 38, 203–208.

Zhang X. K., Q.H., J.H. Cao, and B.J. 2011. Differential Cl^-/salt tolerance and NaCl-induced alternations of tissue and cellular ion fluxes in *Glycine max*, *Glycine soja* and their hybrid seedlings. *J. Agronomy and Crop Sci.* 197, 329-339.

Zhang, H.X. and E. Blumwald. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnol.* 19, 765–768.

Zhang, H.X., J.N. Hodson, J.P. Williams and E. Blumwald. 2001. Engineering salt-tolerant Brassica plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. USA* 98, 12832–12836.

Zhong, M., Z.A. Hu and P.M. Gresshoff. 1997. Search for molecular markers of salt tolerance of soybean by DNA amplification fingerprinting. *Soybean Genet. Newslett.* 24, 81–82.

Zhou, G.A., and L.J. Qiu. 2010. Identification and functional analysis on Abiotic stress response of soybean Cl^- channel gene *GmCLCnt*. *Agr. Sci. China* 9, 199–206.

Zhu, B., J. Su, M.C. Chang, D.P.S. Verma, Y.L. Fan and R. Wu. 1998. Overexpression of a pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water and salt stress in transgenic rice. *Plant Sci.* 139, 41–48.

Zhu, C., M. Core, E. Buckler, and J. Yu. 2008. Status and prospects of association mapping in plants. *The Plant Genome* 1, 5-20.

Zhu, J.K. 2001. Plant salt tolerance. *TRENDS Plant Sci.* 6, 66-71.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53, 247–273.

Zhu, J.K. 2003. Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol.* 6, 441–445.

Table 1. Soil salinity classes and crop growth (FAO, 1988).

Soil Salinity Class	Conductivity of the Saturation Extract (dS/m)	Effect on Crop Plants
Non saline	0 - 2	Salinity effects negligible
Slightly saline	2 - 4	Yields of sensitive crops may be restricted
Moderately saline	4 - 8	Yields of many crops are restricted
Strongly saline	8 - 16	Only tolerant crops yield satisfactorily
Very strongly saline	> 16	Only a few very tolerant crops yield satisfactorily

CHAPTER II

SALT TOLERANCE SCREENING OF ELITE SOYBEAN BREEDING LINES AND CULTIVARS

ABSTRACT

Salinity has been recognized as a significant yield-limiting problem for soybean production worldwide. Fortunately, genetic variation in salt tolerance among soybean genotypes is available, giving the potential of developing salt-tolerant cultivars through genetic improvement. The objective of this study was to evaluate the salt tolerance of 192 elite soybean breeding lines and cultivars based on leaf scorch symptoms. Among them, S-100 and Lee 68 were used as salt-tolerant checks while Glenn and Dare were used as salt-sensitive checks. Soybean seedlings were subjected to salt stress using 120 mM NaCl solution, and subsequently scored using a scale from 1 (plants with healthy leaves) to 5 (plants completely dead). The screening was conducted twice with four replications in 2012 and 2013, respectively. ANOVA indicated significant variation in salt response among genotypes. There was also a significant year effect as well as a genotype (G) by year (Y) effect. The broad sense heritability on an entry mean basis was 0.95, demonstrating a rather small environmental effect on salt response and high probability of selection for salt tolerance among genotypes. A total of 94 genotypes were classified as salt-tolerant (< 3.13) while 87 were salt-sensitive (> 3.62) and 6 salt-intermediate (3.13 – 3.62). The classifications of the remaining five genotypes were inconsistent between the experiments in two years, largely contributing to the G x Y effect in ANOVA. The salt-tolerant genotypes identified in this study can be used in soybean production and/or parental materials for development of salt-tolerant cultivars.

INTRODUCTION

Salt stress is a major abiotic stress adversely affecting the overall plant health and yield of soybean worldwide. It was reported that yields of salt-stressed soybean are reduced 12% at 4.2 dS/m and 46% at 7.0 dS/m in comparison with yields of soybean at 0.8 dS/m (Katerji *et al.*, 2003). Salinity primarily imposes two stresses on plants, osmotic stress and salt toxicity. Osmotic stress reduces the rate and amount of water plants take up from soils while excessive accumulation of salts such as Na^+ and Cl^- , two predominant ions in saline soils, are toxic to plants (Lenis *et al.*, 2011).

In soybean, salt tolerance often negatively correlates with the leaf chloride content under salt stress (Abel and MacKenzie, 1964; Parker *et al.*, 1983; Yang and Blanchar, 1993; Wang and Shannon, 1999; Essa, 2002; An *et al.*, 2002). According to the chloride accumulation capacity in stems and leaves, soybean genotypes are classified into excluders with relatively low leaf chloride content, and includers with relatively high leaf chloride content. Excluders show a higher level of salt tolerance than includers based on the severity of the leaf scorch symptom (Abel and MacKenzie, 1964; Parker *et al.*, 1983; Yang and Blanchar, 1993; Wang and Shannon, 1999; Essa, 2002; An *et al.*, 2002).

Most researchers proposed that chloride exclusion is the main salt tolerance mechanism in soybean. However, the mechanism seems to be different among some salt-tolerant perennial *Glycine* accessions (Pantalone *et al.*, 1997). These accessions also accumulated relatively high level of chloride in leaves. Pantalone *et al.* (1997) thus implicated that some perennial accessions may even utilize Cl^- for osmotic adjustment, and that mechanisms other than chloride exclusion confer salt tolerance in these accessions. Luo *et al.* (2005b) further reported that Na^+ is more toxic to wild soybean (*Glycine soja* Sieb. & Zucc.) than Cl^-. Salt tolerance in wild soybean (*Glycine soja*) is primarily regulated by withholding Na^+ instead of Cl^- in roots and decreasing

Na^+ content in leaves (Luo *et al.*, 2005b). Läuchli and Wieneke (1979) reported that the up-take of both Na^+ and Cl^- increased in both salt-tolerant and salt-sensitive cultivars along with the increase of NaCl concentrations in nutrient solution. Recently, Lenis *et al.* (2011) examined Na^+ and Cl^- accumulation and salt tolerance of four *Glycine* species, *G. max, G. soja, G. tomentella,* and *G. argyrea*. In agreement with the study of Läuchli and Wieneke (1979), they found that all accessions had an increase in both Na^+ and Cl^- content in leaves with increased NaCl concentrations. Salt-tolerant accessions accumulated less Na^+ and Cl^- in leaves than sensitive accessions. However, salt injury and tolerance level appeared to be more associated with Na^+ than with Cl^- regardless of *Glycine* species. These controversial findings indicate that the genetic variability and different salt tolerance mechanisms exist among *Glycine* species and accessions within species.

Different screening methods have been applied to exploiting genetic variability for salt tolerance in cultivated soybean and wild accessions: field screening (Parker *et al.*, 1983; Yang and Blanchar, 1993), greenhouse hydroponics screening (Pantalone *et al.*, 1997; An *et al.*, 2002; Lee *et al.*, 2004; Valencia *et al.*, 2008), and greenhouse PC (plastic cone-tainer) screening (Lee *et al.*, 2008). Field-based screening involves hardly controllable factors such as soil uniformity and fertility, temperature, light intensity, etc., which affect plant responses to salt stress, while hydroponics method tends to be more costly and labor intensive. PC method is considered as an inexpensive, reliable, and manageable technique for salt tolerance screening, and it uses sandy soil instead of nutrient solution as the growth medium (Lee *et al.*, 2008). In this study, a greenhouse screening method similar to the PC method was used for evaluating salt tolerance in soybean. Previous studies imply that several salt-tolerant ancestors of U.S. cultivars exist (Lee *et al.*, 2004). In addition to recently detected genetic variability for salt tolerance, determination

and introduction of new sources of salt tolerance within cultivated soybean is important for improving salt tolerance in U.S breeding programs. Given that, the objective of this study was to evaluate salt tolerance of elite soybean breeding lines and cultivars, in hopes that screening results may provide breeders with new resources to breed for salt-tolerant cultivars.

MATERIALS AND METHODS

Greenhouse Screening

A total of 192 diverse elite soybean breeding lines and cultivars (Table 1) were tested for salt tolerance in a greenhouse at the Rosen Center at the University of Arkansas. Salt-tolerant cultivars Lee68 and S-100 (Lee *et al.,* 2008; Valencia *et al.,* 2008) and salt-sensitive cultivars Dare (Valencia *et al.,* 2008) and Glenn (unpublished data) were used as checks. For each genotype, 10 ~ 12 seeds were sowed in a 3.5 inch plastic pot (Plasticflowerpots.net, Lake Worth, FI) containing approximately 300g loamy sand (Kibler, Arkansas) as the growth medium Soil particle analysis based on a 2-hour hydrometer method described by Arshad *et al.* (1996) showed that the loamy sand consists of 83.5% sand, 11.0% clay and 5.5% silt. To prevent the loamy sand from leaking out through drainage holes, a fold piece of paper towel (approximately 10 cm by 10 cm) was placed at the bottom of each pot. Pots were placed in trays (17 3/4" x 25 1/2" x 1", U.S. Plastic Corp., Lima, Ohio) for the purposes of watering and salt treatment. Plants were grown at 25 ± 2 °C with 14h photoperiod, and fertilized once per week using the Miracle-Gro® All Purpose Plant Food (The Scotts Miracle-Gro Company, Marysville, Ohio) according to the manufacturer's instruction. The plant fertilizer contains 24% N, 8% P_2O_5, 16% K_2O, 0.02% B, 0.07% Cu, 0.15% Fe, 0.05% Mn, 0.06% Zn, and 0.0005% Mo (The Scotts Miracle-Gro Company, Marysville, Ohio). At VC stage, each pot was tined to five plants. At V1 stage when

the first trifoliate leaf was about to expand, 3.5 L of 120 mM NaCl solution was added to each tray of treated plants at 1:00 p.m. for 2 hours per day, and reached one-third depth (about 1 inch) of pots, while 3.5 L of tap water was used for control plants. The electrical conductivity (EC) of NaCl solution and tap water was 12.6 dS/m and 0.17 dS/m, respectively. The severity of leaf scorch symptom was scored when salt-sensitive checks were completely dead (approximately 15 ~ 18 days after the initiation of salt stress). The scoring scale was from 1 to 5: 1, plants with normal healthy leaves; 2, one-third or less leaves showing scorch symptoms; 3, half or less leaves showing scorch symptoms; 4, two-third or more leaves showing scorch symptoms or only upmost leaves surviving; 5, plants completely dead (Valencia *et al.*, 2008). Plants in each pot were rated individually, and then averaged to get a final score for each pot. Each pot was a replication. The test used a randomized complete block design with four replications, and was conducted twice in 2012 and 2013 to confirm the results.

Statistical Analysis of Phenotypic Data

The Shapiro-Wilk (*w*) test was performed using JMP 9.0 (SAS Institute, Cary, NC) to test the normality of the leaf scorch scores for the evaluated genotypes. The PROC GLM procedure in SAS 9.3 (SAS Institute Inc., 2011) was performed to determine overall differences of leaf scorch scores among genotypes, blocks (replications), and years. Broad-sense heritability (H^2) of leaf scorch score on an entry basis was computed using the following equation (Wang *et al.*, 2008):

$$H^2 = MS_g/\left[MS_g + \left(MS_{gy}/y\right) + \left(MS_e/ry\right)\right],$$

where MS_g is the genotype mean square error, MS_{gy} is genotype by year mean square, MS_e is the error mean square, r is the number of replications/blocks, and y is the number of environments (year).

The MEANS/LSD procedure in SAS 9.3 (SAS Institute Inc., 2011) was used to test the average of leaf scorch score for each genotype, and to determine the differences between genotypes. Salt response type of genotypes was determined using the means of two tolerant ($Mean_T$) or sensitive ($Mean_S$) checks ± two standard deviations (2SD) (Table 2). Genotypes with a score falling into the scale of ($Mean_T$ – 2SD, $Mean_T$ + 2SD) were classified as tolerant; genotypes with a score falling into the scale of ($Mean_S$ – 2SD, $Mean_S$ + 2SD) were classified as sensitive; and genotypes with a score falling into the scale of ($Mean_S$ + 2SD, $Mean_T$ – 2SD) were classified as intermediate.

RESULTS

A total of 192 diverse soybean genotypes including cultivars, breeding lines, and germplasm accessions were selected for salt tolerance screening in this research (Table 1). The leaf scorch symptoms of each genotype were scored in two separate experiments in 2012 and 2013. The scores of all genotypes in 2012 ranged from 1.00 to 5.00 with an average of 3.07, while the scores in 2013 ranged from 1.25 to 5.00 with an average of 3.19 (Table 2). For the tolerant checks, Lee 68 and S-100 had mean scores of 1.86 and 1.00 in 2012 and 2.30 and 1.75 in 2013, respectively. In contrast, the sensitive checks Dare and Clenn had scores of 4.73 and 4.65 in 2012 and 4.40 and 4.45 in 2013, respectively.

The leaf scorch score distributions for 2012, 2013, and two-year combined data were tested for normality using the Shapiro-Wilk (*w*) test at a significant level of $P < 0.05$ (Figure 1).

None of the data sets was normally distributed ($P_{2012} > 0.05$; $P_{2013} > 0.05$; $P_{combined} > 0.05$), but all obviously exhibited a bimodal distribution, suggesting that salt tolerance in soybean is most likely a qualitative trait controlled by single genes/QTLs with large effects, and that the genotypes screened in this study are fixed for the salt tolerance alleles.

The analysis of variance (ANOVA) (Table 3) had a R^2 value of 0.86, indicating that this model captured the most variations and had a reasonably good petition of variations. ANOVA showed a significant variation among the genotypes. There was also a significant year effect as well as a genotype (G) by year (Y) effect. Genotypic effect was the largest in proportion followed by the year effect. Replication effect was not significant, and G x Y effect was almost negligent relative to the error term, indicating leaf scorch ratings were consistent and reliable in classifying the genotypes under testing.

Genotypes were classified as tolerant, sensitive, or intermediate using the score means of tolerant or sensitive checks ± two standard deviations. A genotype was classified into a response category only if it had similar leaf scorch scores and fell into the same category in both experiments. Consequently, 94 genotypes were classified as tolerant, while 87 were sensitive and 6 intermediate (Tables 4 and 5). The remaining five genotypes were inconsistent between the two screening experiments, and therefore their responses to salt stress need to be confirmed. The tolerant genotypes exhibited significantly higher leaf scorch scores than the sensitive genotypes with an average difference of about 2.5 in leaf scorch score (Table 4). The six intermediate genotypes had an average of 3.36. As compared to the tolerant or sensitive checks, 60 and 8 genotypes were more tolerant than the tolerant S-100 and Lee 68, respectively while 30 and 32 genotypes were more sensitive than the sensitive Dare and Glenn, respectively (Table 5).

DISCUSSION

Effective methods for salt tolerance evaluation in soybean are crucial for development of salt-tolerant cultivars (Lee *et al.*, 2008). A simple greenhouse screening method was used in this study. Leaf scorch was rated as a measure for salt tolerance as suggested by Valencia *et al.* (2008). In contrast to a hydroponics method using nutrient solution as the growth medium, our method used loamy sand in regular plastic pots, which reduced the labor and cost for salt tolerance screening. Lee *et al.* (2008) used a similar screening method, in which sandy soil was used as the growth medium in plastic cone-tainers (PC). However, only one plant in each cone-tainer can be screened in the PC method while in our study, five plants in each pot were screened, allowing for better assessment of true response of a given genotype to salt stress. Our method, as demonstrated by the consistence across replications and years in current research, appears to be as effective as, if not more than, the hydroponics method and the PC method in determining salt-tolerant and salt-sensitive genotypes.

The concentration of salt solution used for salt stress can affect the final results (Lee *et al.*, 2008). A highly positive correlation between the salt concentration and leaf scorch symptom was observed in all annual soybean genotypes (Lenis *et al.*, 2011). Low concentration can lengthen the duration of salt stress needed for significantly differential symptoms to appear between salt-tolerant and salt-sensitive genotypes (Lee *et al.*, 2008) while high concentration may reduce the differences in symptoms between them. Lee *et al.* (2008) proposed that a 50 to 100 mM NaCl solution was effective while Valencia *et al.* (2008) suggested using a 120 mM NaCl solution. In our study, a 120 mM NaCl solution was used and appeared to be effective in differentiating the phenotypic responses of soybean genotypes to salt stress.

The duration from salt stress initiation to leaf scorch symptom expression was slightly different between two experiments conducted in 2012 and 2013. Sensitive checks died three days earlier in 2013 (15 d) than in 2012 (18 d) possibly due to differences in temperature. The 2012 experiment was conducted in early winter while the 2013 experiment in early spring. Although the greenhouse parameters were set up the same for the two experiments, the actual environmental conditions may have varied in terms of temperature, light, or interior airflow. Lee *et al.* (2008) assumed that higher temperature likely accounts for the earlier appearance of leaf scorch symptoms. On one hand, higher temperature causes plant to transpire more, and therefore take up more water as well as ions including Cl^- (Lee *et al.*, 2008). On the other, it facilitates the evaporation from soil, which thus absorbs more NaCl solution to stress the plants. Indeed, the temperature during the 2012 experiment was noticeably cooler compared to the temperature during the 2013 experiment. Despite possible temperature effect on timing of leaf scorch symptoms, leaf scorch scores for most genotypes were consistent between the two experiments, as indicated by a rather small G x Y effect in ANOVA (Table 3). Therefore, screening results should be reliable as long as genotypes under question are screened in the same batch at the same time. The high broad sense heritability (0.95) also suggests that screening based on leaf scorch symptoms is efficient in differentiating responses of soybean genotypes to salt stress.

In this study, 94 genotypes were classified as tolerant while 87 were sensitive and 6 were intermediate. The results for nearly all the genotypes were consistent with the published and unpublished data (Table 1). Only five genotypes were inconsistent for salt responses between two experiments, namely R95-1705, R05-1772, R09-4571, Chu chou, and Georgian. This was most likely due to insufficient plants per pot, replications or inconsistent environmental effects in the greenhouse. These genotype need to be rescreened to confirm their responses to salt stress.

The tolerant and sensitive genotypes in this study can be used for breeding salt tolerance in soybean and constructing genetic populations for gene discovery. It is worth pointing out that the tolerance and sensitivity to salt stress based on leaf scorch symptoms need to be further verified through tissue analysis of leaf chloride content, although classifications of genotypes in this study may not change even with additional data on leaf chloride content.

REFERENCES

Abel, G.H. and A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. *Crop Sci.* 4, 157–161.

An, P., S. Inanaga, U. Kafkafi , A. Lux, and J. Sugimoto. 2001.Different effect of humidity on growth and salt tolerance of two soybean cultivars. *Biol. Plant.* 44:405–410.

An P., S. Inanaga, Y. Cohen, U. Kafkafi, and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. *J. Plant Nutr.* 25, 407–423.

Arshad, M.A., B. Lowery, and B. Grossman. 1996. Physical tests for monitoring soil quality. p.123–142. In: J.W. Doran and A.J. Jones (eds.) Methods for assessing soil quality. Soil Sci. Soc. Am. Spec. Publ. 49. SSSA, Madison, WI.

Essa, T.A. 2002. Effect of salinity stress on growth and nutrient composition of three soybean [*Glycine max* (L.) Merr.] cultivars. *J. Agron. Crop Sci.* 188, 86–93.

Hamwieh, A., D.D. Tuyen, H. Cong, E.R. Benitez, R. Takahashi, and D.H. Xu. 2011. Identification and validation of a major QTL for salt tolerance in soybean. *Euphytica* 179, 451–459.

Katerji, N., J.W. van Hoorn, A. Hamdy and M. Mastrorilli. 2003. Salinity effect on crop development and yield, analysis of salt tolerance according to several classification methods. *Agric. Water Manage.* 62, 37-66.

Läuchli, A., and J. Wieneke. 1979. Studies on growth and distribution of Na^+, K^+ and Cl^- in soybean varieties differing in salt tolerance. *Zeitschrift für Pflanzenernährung und Bodenkunde*, 146, 799–814.

Lee, G.J., H.R. Boerma, M.R. Villagarcia, X. Zhou, T.E. Carter Jr, Z. Li, and M.O. Gibbs. 2004. A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. *Theor. Appl. Genet.* 109, 1610–1619.

Lee, J.D., L. Scotty, Smothers, D. Dunn, M. Villagarcia, C.R. Shumway, T.E. Carter, Jr., and J.G. Shannon. 2008. Evaluation of a Simple Method to Screen Soybean Genotypes for Salt Tolerance. *Crop Sci.* 48, 2194–2200.

Lenis, J.M., M. Ellersieck, D.G. Blevins, D.A. Sleper, H.T. Nguyen, D. Dunn, J.D. Lee, and J.G. Shannon. 2011. Differences in ion accumulation and salt tolerance among *Glycine* accessions. *J. Agron. Crop Sci.*197, 302–310.

Luo, Q., B. Yu and Y. Liu. 2005b. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *J. Plant Physiol.* 162, 1003–1012.

Pantalone, V.R., W.J. Kenworthy, L.H. Slaughter, and B.R. James. 1997. Chloride tolerance in soybean and perennial *Glycine* accessions. *Euphytica* 97, 235–239.

Parker, M.B., G.J. Gascho, T.P. Gains. 1983. Chloride toxicity of soybeans grown on Atlantic Coast Flatwoods soils. *Agron. J.* 75, 439–443.

SAS Institute Inc. 2011. SAS/STAT® 9.3 User's Guide. Cary, NC: SAS Institute Inc.

Valencia, R., P.Y. Chen, T. Ishibashi, and M. Conatser. 2008. A rapid and effective method for screening salt tolerance in soybean. *Crop Sci.* 48, 1773–1779.

Wang, D. and M.C. Shannon. 1999. Emergence and seedling growth of soybean cultivars and maturity groups under salinity. *Plant and Soil* 214, 117–124.

Wang, J., P.E. McClean, R. Lee., R.J. Goos, and T. Helms. 2008. Association mapping of iron deWciency chlorosis loci in soybean (*Glycine max* L. Merr.) advanced breeding lines. *Theor. Appl. Genet.* 116, 777–787.

Yang J. and R.W. Blanchar. 1993. Differentiation chloride susceptibility in soybean cultivars. *Agron. J.* 85, 880–885.

Table 1. Soybean genotypes screened for salt tolerance using a potting soil method in greenhouse experiments in 2012 and 2013.

Entry	Genotype	PI number/Pedigree[†]	Origins[‡]	References[§]
1	Caviness	Asgrow A5403 x Hutcheson	Arkansas	P. Chen, unpublished raw data, 2011
2	Davis	PI 553039	Arkansas	Lee *et al.*, 2004; Yang *et al.*, 1993
3	Desha	PI 633610	Arkansas	P. Chen, unpublished raw data, 2011
4	Hood 75	PI 559371	Arkansas	A. Shi, personal communication, 2011
5	Lee 68	PI 559369	Arkansas	Valencia *et al.*, 2008; Luo *et al.*, 2005
6	Lonoke	PI 633609	Arkansas	P. Chen, unpublished raw data, 2011
7	Narow	PI 553052	Arkansas	P. Chen, unpublished raw data, 2011
8	Osage	PI 648270	Arkansas	P. Chen, unpublished raw data, 2011
9	Ozark	PI 633970	Arkansas	P. Chen, unpublished raw data, 2011
10	R01-416F	Jackson x KS4895	Arkansas	P. Chen, unpublished raw data, 2011
11	R02-3065	HBK 5990 x Anand	Arkansas	P. Chen, unpublished raw data, 2011
12	R03-1250	PIO 9592 x KS4895	Arkansas	P. Chen, unpublished raw data, 2011
13	R04-1250RR	Ozark x 98601	Arkansas	P. Chen, unpublished raw data, 2011
14	R04-1274RR	R96-3427 x 98601	Arkansas	P. Chen, unpublished raw data, 2011
15	R04-342	R97-1650 x 98601	Arkansas	P. Chen, unpublished raw data, 2011
16	R04-572	MD 4900 x Ozark	Arkansas	P. Chen, unpublished raw data, 2011
17	R05-1415	MFS-591 x V96-4486	Arkansas	P. Chen, unpublished raw data, 2011
18	R05-1772	R95-1705 x V96-4181	Arkansas	P. Chen, unpublished raw data, 2011
19	R05-235	P9594 x Ozark	Arkansas	P. Chen, unpublished raw data, 2011
20	R05-269	P9594 x Ozark	Arkansas	P. Chen, unpublished raw data, 2011
21	R05-374	Lonoke x DP4748	Arkansas	P. Chen, unpublished raw data, 2011
22	R05-3817	R96-3427 x 605	Arkansas	P. Chen, unpublished raw data, 2011
23	R05-4114	R98-1523 x 98601	Arkansas	P. Chen, unpublished raw data, 2011
24	R06-4433	Lonoke x P9594	Arkansas	P. Chen, unpublished raw data, 2011
25	R07-10244	S99-2281 x UA 4805	Arkansas	P. Chen, unpublished raw data, 2011
26	R07-10322	R97-1634 x V00-3824	Arkansas	P. Chen, unpublished raw data, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
27	R07-129	R01-332 x IA3017	Arkansas	P. Chen, unpublished raw data, 2011
28	R07-1685	5002T x Ozark	Arkansas	P. Chen, unpublished raw data, 2011
29	R07-1738	R00-1076 x R01-2373	Arkansas	P. Chen, unpublished raw data, 2011
30	R07-1769	R00-1551 x R00-684	Arkansas	P. Chen, unpublished raw data, 2011
31	R07-1826	R00-1551 x R01-315	Arkansas	P. Chen, unpublished raw data, 2011
32	R07-1857	R01-2373 x R01-315	Arkansas	P. Chen, unpublished raw data, 2011
33	R07-5351	LS96-1631 x R96-3427	Arkansas	P. Chen, unpublished raw data, 2011
34	R07-6654	Lonoke x R00-33	Arkansas	P. Chen, unpublished raw data, 2011
35	R07-6669	Lonoke x R00-33	Arkansas	P. Chen, unpublished raw data, 2011
36	R07-7775	R95-1705 x Satelite	Arkansas	P. Chen, unpublished raw data, 2011
37	R08-1178	S98-1375 x R97-1634	Arkansas	P. Chen, unpublished raw data, 2011
38	R08-141	R00-1076 x R00-1940	Arkansas	P. Chen, unpublished raw data, 2011
39	R08-265	R00-1551 x R00-1940	Arkansas	P. Chen, unpublished raw data, 2011
40	R08-2797	DP4748S x K 1599	Arkansas	P. Chen, unpublished raw data, 2011
41	R08-3206	R00-2267 x S00-9980-22	Arkansas	P. Chen, unpublished raw data, 2011
42	R08-3211	R00-2267 x S00-9980-22	Arkansas	P. Chen, unpublished raw data, 2011
43	R08-991	S97-1688 x Caviness-RR	Arkansas	P. Chen, unpublished raw data, 2011
44	R09-1237	R01-4910 x IA2064	Arkansas	P. Chen, unpublished raw data, 2011
45	R09-1831RR	R99-2512 x R01-4787	Arkansas	P. Chen, unpublished raw data, 2011
46	R09-2567	TN01-235 x UA 4805	Arkansas	P. Chen, unpublished raw data, 2011
47	R09-3742	R95-1705 x Osage	Arkansas	P. Chen, unpublished raw data, 2011
48	R09-400	R00-1076 x BA 743303	Arkansas	P. Chen, unpublished raw data, 2011
49	R09-4010	R00-1076 x IA2065	Arkansas	P. Chen, unpublished raw data, 2011
50	R09-430	BA 743303 x R00-684	Arkansas	P. Chen, unpublished raw data, 2011
51	R09-4571	DP 4748 x S01-9794	Arkansas	P. Chen, unpublished raw data, 2011
52	R09-886	R01-315 x JTN-01	Arkansas	P. Chen, unpublished raw data, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
53	R95-1705	Hutcheson x Barc-7	Arkansas	P. Chen, unpublished raw data, 2011
54	R97-1634	P9592 x Holladay	Arkansas	P. Chen, unpublished raw data, 2011
55	R98-209	Asgrow A6297 x Clifford	Arkansas	P. Chen, unpublished raw data, 2011
56	UA 4805	Hartz 5545 x KS4895	Arkansas	P. Chen, unpublished raw data, 2011
57	UARK 5798	Hutcheson x Walters	Arkansas	P. Chen, unpublished raw data, 2011
58	UARK 5896	PI 619232	Arkansas	P. Chen, unpublished raw data, 2011
59	Walters	PI 544354	Arkansas	P. Chen, unpublished raw data, 2011
60	Wu-Kung 509	PI 201423	Australia	A. Shi, personal communication, 2011
61	FT-Abyara	PI 628838	Brazil	Hamwieh *et al.*, 2011
62	Anwei	FC 31572	China	A. Shi, personal communication, 2011
63	S-100	PI 548488	China-Heilongjiang	Valencia *et al.*, 2008; Lee *et al.*, 2008
64	Changteh	PI 179823	China-Henan	A. Shi, personal communication, 2011
65	Shang tsai	PI 103079	China-Henan	A. Shi, personal communication, 2011
66	Paoting	PI 179825	China-Hubei	A. Shi, personal communication, 2011
67	Charlee	PI 548446	China-Jiangsu	A. Shi, personal communication, 2011
68	Clemson	PI 548448	China-Jiangsu	A. Shi, personal communication, 2011
69	Georgian	PI 548455	China-Jiangsu	A. Shi, personal communication, 2011
70	Great White	PI 165671	China-Jiangsu	A. Shi, personal communication, 2011
71	Nanking 332	PI 165675	China-Jiangsu	A. Shi, personal communication, 2011
72	Perfume	PI 165676	China-Jiangsu	A. Shi, personal communication, 2011
73	7902	PI 92707	China-Jilin	A. Shi, personal communication, 2011
74	Morse	PI 548390	China-Liaoning	Shao *et al.*, 1995
75	Laredo	PI 548463	China-Shaanxi	A. Shi, personal communication, 2011
76	Jin dou No. 6	PI 574484	China-Shanxi	Hamwieh et al 2011
77	Pan-San	PI 171437	China-Sichuan	A. Shi, personal communication, 2011
78	Barchet	PI 548443	China-Zhejiang	A. Shi, personal communication, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
79	Biloxi	PI 548444	China-Zhejiang	A. Shi, personal communication, 2011
80	Cherokee	PI 548447	China-Zhejiang	A. Shi, personal communication, 2011
81	Sixth Moon	PI 60273	China-Zhejiang	A. Shi, personal communication, 2011
82	Delmar	PI 548548	Delaware	Shao *et al.*, 1995
83	Cibao	PI 153681	El Salvador	A. Shi, personal communication, 2011
84	Bragg	PI 548660	Florida	Parker *et al.*, 1983; Lee *et al.*, 2004
85	Foster	PI 548970	Florida	Parker *et al.*, 1983
86	Branca do Rio Grande	PI 203400	France	A. Shi, personal communication, 2011
87	Gasoy 17	PI 553046	Georgia	Parker *et al.*, 1983
88	Gordon	PI 553047	Georgia	Lee *et al.*, 2004
89	Wright	PI 553042	Georgia	Parker *et al.*, 1983; Lee *et al.*, 2004
90	Clark	PI 548533	Illinois	Valencia *et al.*, 2008
91	Clark 63	PI 548532	Illinois	Shao *et al.*, 1995
92	Flanklin	PI 548563	Illinois	Shao *et al.*, 1995
93	Will	PI 518672	Illinois	Shao *et al.*, 1995
94	Williams	PI 548631	Illinois	Valencia *et al.*, 2008
95	Williams 79	PI 518670	Illinois	Shao *et al.*, 1995
96	Williams 82	PI 518671	Illinois	Shao *et al.*, 1995
97	Punjab-1	PI 198078	India	A. Shi, personal communication, 2011
98	Bonus	PI 548517	Indiana	Shao *et al.*, 1995
99	C1943	PI 599811	Indiana	Lee *et al.*, 2008
100	Giant Speckled	FC 31592	Indonesia	A. Shi, personal communication, 2011
101	Java 29	PI 148259	Indonesia	A. Shi, personal communication, 2011
102	Ringgit	PI 192867	Indonesia-Java	A. Shi, personal communication, 2011
103	Aka Saya	PI 200446	Japan	A. Shi, personal communication, 2011
104	Aokimame	PI 200454	Japan	A. Shi, personal communication, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
105	Chu chou	PI 157413	Japan	A. Shi, personal communication, 2011
106	Gaku Bun	PI 200466	Japan	A. Shi, personal communication, 2011
107	Hanashirazu	PI 200469	Japan	A. Shi, personal communication, 2011
108	Houjaku Kuwazu	PI 416937	Japan	A. Shi, personal communication, 2011
109	Kaifuu seitou	PI 506820	Japan	Lee *et al.*, 2008
110	Komata	PI 200492	Japan	A. Shi, personal communication, 2011
111	Shimo Baba	PI 200524	Japan	A. Shi, personal communication, 2011
112	Tamana	PI 200542	Japan	A. Shi, personal communication, 2011
113	Tamanishiki	PI 200543	Japan	A. Shi, personal communication, 2011
114	Tokyo	PI 418080	Japan	Lee *et al.*, 2004
115	Yashiro Zairai No.2	PI 200550	Japan	A. Shi, personal communication, 2011
116	Yonekadake	PI 200551	Japan	A. Shi, personal communication, 2011
117	Kurakake Daizu	PI 81037	Japan-Hokkaido	A. Shi, personal communication, 2011
118	Chasengoku	PI 224269	Japan-Hyogo	A. Shi, personal communication, 2011
119	Shariin	PI 84967	Japan-Hyogo	A. Shi, personal communication, 2011
120	Tookichi	PI 208788	Japan-Hyogo	A. Shi, personal communication, 2011
121	Zyuninyoshi	PI 208789	Japan-Hyogo	A. Shi, personal communication, 2011
122	Kiizaya	PI 94159	Japan-Kagoshima	A. Shi, personal communication, 2011
123	Koshoku Akidaizu	PI 85897	Japan-Shizuoka	A. Shi, personal communication, 2011
124	Douglas	PI 548555	Kansas	Shao *et al.*, 1995
125	Raub 16.1422	PI 197182	Malaysia	A. Shi, personal communication, 2011
126	Manokin	PI 559932	Maryland	Lee *et al.*, 2004
127	Miles	PI 548598	Maryland	Shao *et al.*, 1995
128	Wye	PI 548633	Maryland	Shao *et al.*, 1995
129	Centennial	PI 548975	Mississippi	parker et al., 1983; Lee et al., 2004
130	Deltapine 726	PI 556907	Mississippi	A. Shi, personal communication, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
131	Dorman	PI 548653	Mississippi	A. Shi, personal communication, 2011
132	Hill	PI 548654	Mississippi	A. Shi, personal communication, 2011
133	Lee	PI 548656	Mississippi	Pantalone *et al.*, 1997
134	Pickett 71	PI 548982	Mississippi	P. Chen, unpublished raw data, 2011
135	Avery	PI 518663	Missouri	Yang *et al.*, 1993
136	Custer	PI 548546	Missouri	Shao *et al.*, 1995
137	Hartwig	PI 543795	Missouri	Lee *et al.*, 2008
138	Jake	PI 643912	Missouri	P. Chen, unpublished raw data, 2011
139	Oksoy	PI 548602	Missouri	Shao *et al.*, 1995
140	Pershing	PI 548604	Missouri	Shao *et al.*, 1995
141	0197	PI 471938	Nepal	Hamwieh et al 2011
142	Dare	PI 548987	North Carolina	Valencia *et al.*, 2008
143	Jackson	PI 548657	North Carolina	Pantalone *et al.*, 1997; Tuyen *et al.*, 2010
144	Johnston	PI 508267	North Carolina	Lee *et al.*, 2004
145	N98-4445A	PI 636691	North Carolina	Lee *et al.*, 2008
146	NC-ROY	PI 617045	North Carolina	P. Chen, unpublished raw data, 2011
147	Ransom	PI 548989	North Carolina	Parker *et al.*, 1983
148	Arksoy	PI 548438	North Korea	A. Shi, personal communication, 2011
149	Chinuikon	PI 83874	North Korea	A. Shi, personal communication, 2011
150	Haberlandt	PI 548456	North Korea	Lee *et al.*, 2004
151	Heihokuta	PI 88820	North Korea	A. Shi, personal communication, 2011
152	Koshu	PI 90243	North Korea	A. Shi, personal communication, 2011
153	Kulat	PI 219698	Pakistan	A. Shi, personal communication, 2011
154	Soya Otootan	PI 215755	Peru-Huanuco	A. Shi, personal communication, 2011
155	Amarilla	PI 159922	Peru-Lima	A. Shi, personal communication, 2011
156	Glycine H	PI 159925	Peru-Lima	A. Shi, personal communication, 2011

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
157	Honduras	PI 159924	Peru-Lima	A. Shi, personal communication, 2011
158	Tumbes	PI 159927	Peru-Lima	A. Shi, personal communication, 2011
159	Coker 237	PI 556536	South Carolina	Parker *et al.*, 1983
160	Coker 317	PI 556623	South Carolina	Parker *et al.*, 1983
161	Coker 488	PI 556537	South Carolina	Parker *et al.*, 1983
162	Dillon	PI 592756	South Carolina	Lee *et al.*, 2004
163	Alki ball	PI 157394	South Korea	A. Shi, personal communication, 2011
164	Kahei	PI 82588	South Korea	A. Shi, personal communication, 2011
165	Maganolia	PI 548467	South Korea	A. Shi, personal communication, 2011
166	So ran du	PI 157493	South Korea	A. Shi, personal communication, 2011
167	Ringgit	PI 204335	Suriname	A. Shi, personal communication, 2011
168	Fiskeby III	PI 438471	Sweden	Lenis *et al.*, 2011
169	Avoyelles	PI 548442	Taiwan	A. Shi, personal communication, 2011
170	Otootan	PI 548479	Taiwan	A. Shi, personal communication, 2011
171	CNS	PI 341246	Tanzania	A. Shi, personal communication, 2011
172	5002T	PI 634193	Tennessee	P. Chen, unpublished raw data, 2011
173	Bedford	PI 548974	Tennessee	Yang *et al.*, 1993
174	DP 5634	N/A	Tennessee	A. Shi, personal communication, 2011
175	Forrest	Dyer x Bragg	Tennessee	Lee *et al.*, 2004; Lee *et al.*, 2008
176	Ogden	PI 548477	Tennessee	Lee *et al.*, 2004
177	Coker 425	PI 556744	United States	Yang *et al.*, 1993
178	Coker 485	PI 556743	United States	Yang *et al.*, 1993
179	Hartz 5164	PI 556834	United States	Yang *et al.*, 1993
180	Hartz 5171	PI 556721	United States	Yang *et al.*, 1993
181	Hartz 5252	PI 556718	United States	Yang *et al.*, 1993
182	Hartz 6130	PI 556803	United States	Yang et al., 1993

Table 1. *continued.*

Entry	Genotype	PI number/Pedigree	Origins	References
183	Hartz 6200	PI 556917	United States	Yang *et al.*, 1993
184	HBK R4924	N/A	United States	Valencia *et al.*, 2008
185	HBK R5226	N/A	United States	P. Chen, unpublished raw data, 2011
186	Pioneer 9531	PI 556775	United States	Yang *et al.*, 1993
187	Pioneer 9581	PI 556800	United States	Yang *et al.*, 1994
188	Santa Maria	FC 31919	Venezuela	A. Shi, personal communication, 2011
189	Essex	PI 548667	Virginia	Lee *et al.*, 2004; Yang *et al.*, 1993
190	Hutcheson	PI 518664	Virginia	Lee *et al.*, 2004; Lee *et al.*, 2008
191	Glenn	N/A	Virginia	P. Chen, unpublished raw data, 2011
192	Smith Super	FC 32176	Unknown	A. Shi, personal communication, 2011

† The PI numbers derived from the USDA Germplasm Resources Information Network (available at http://www.ars-grin.gov/npgs). The pedigree information derived from the public and private soybean breeding programs.

‡ The origins refer to where the genotypes were developed or collected from.

§ References, the published or unpublished information on responses of genotypes to salt stress.

Table 2. Descriptive statistics for leaf scorch scores, on a 1-5 scale, of 192 soybean genotypes evaluated in two greenhouse experiments conducted in 2012 and 2013.

Year[†]	No. Genotypes	Mean	Range	Tolerant checks		Sensitive checks	
				Lee 68	S-100	Dare	Glenn
2012	192	3.07	1.00 - 5.00	1.86 ± 0.45[‡]	1.00 ± 0.00	4.73 ± 0.22	4.65 ± 0.41
2013	192	3.19	1.25 - 5.00	2.30 ± 0.38	1.75 ± 0.96	4.40 ± 0.71	4.45 ± 0.55
Combined[§]	192	3.13	1.13 - 4.92	2.08 ± 0.45	1.37 ± 0.74	4.56 ± 0.52	4.55 ± 0.46
Mean ± SD[¶]				1.73 ± 0.70		4.56 ± 0.47	
Mean ± 2SD[#]				1.73 ± 1.40		4.56 ± 0.94	

† The same experiment was conducted twice in 2012 and 2013, respectively.

‡ Mean ± standard deviation.

§ Combined, the pooled data averaged from both experiments in 2012 and 2013.

¶ Mean ± SD, the overall mean of scores of two tolerant ($Mean_T$) or sensitive ($Mean_S$) checks ± standard deviation.

Mean ± 2SD, the overall mean of scores of two tolerant ($Mean_T$) or sensitive ($Mean_S$) checks ± two standard deviations.

Table 3. Analysis of variance for leaf scorch scores of 192 soybean genotypes evaluated in two greenhouse experiments conducted in 2012 and 2013.

Source of variation	DF†	Sum of Squares	Mean Square	F-value	P-value	R^2
Model	386	2594.18	6.72	17.59	<.0001	0.86
Replication	3	0.34	0.11	0.29	0.829	
Year	1	9.48	9.48	24.80	<.0001	
Genotype	191	2353.00	12.32	32.24	<.0001	
Genotype × Year	191	231.37	1.21	3.17	<.0001	
Error	1149	439.11	0.38			
Corrected Total	1535	3033.29				

† DF, degree of freedom.

Table 4. Descriptive statistics for leaf scorch scores of salt-tolerant, salt-intermediate, and salt-sensitive genotypes on a 1-5 scale used in two greenhouse experiments conducted in 2012 and 2013.

Salt stress response†	No. genotypes	2012		2013		Combined†	
		Mean ± SD‡	Range	Mean ± SD	Range	Mean ± SD	Range
Tolerant	94	1.85 ± 0.59	1.00 - 3.10	2.04 ± 0.52	1.25 - 3.12	1.95 ± 0.44	1.12 - 3.11
Intermediate	6	3.30 ± 0.17	3.14 - 3.58	3.41 ± 0.11	3.25 - 3.60	3.36 ± 0.12	3.26 - 3.59
Sensitive	87	4.49 ± 0.40	3.64 - 5.00	4.37 ± 0.41	3.65 - 5.00	4.39 ± 0.32	3.77 - 4.92

† Salt response type was determined using the means of two tolerant or sensitive checks ± two standard deviations (Table 2). Genotypes with a score < 3.13 (1.73 – 1.40, 1.73 + 1.40) were classified as tolerant; genotypes with a score > 3.62 (4.56 – 0.94, 4.56 + 0.94) were classified as sensitive; and genotypes with a score falling into the scale of 3.13 - 3.62 (1.73 + 1.40, 4.56 – 0.94) were classified as intermediate.

‡ Combined, the pooled data averaged from the 2012 and 2013 experiments.

§ SD, standard deviation.

Table 5. Leaf scorch scores of salt-tolerant, salt-intermediate, and salt-sensitive genotypes on a 1-5 scale used in two greenhouse experiments conducted in 2012 and 2013.

Genotype				Response[§]
	2012	2013	Mean[‡]	
Hartz 5252	1.0	1.3	1.1	Tolerant
Johnston	1.0	1.3	1.1	Tolerant
NC-ROY	1.1	1.4	1.2	Tolerant
FT-Abyara	1.0	1.5	1.3	Tolerant
Coker 237	1.2	1.5	1.3	Tolerant
R07-10244	1.1	1.6	1.3	Tolerant
Kiizaya	1.5	1.3	1.4	Tolerant
R07-1826	1.2	1.5	1.4	Tolerant
S-100	1.0	1.8	1.4	Tolerant
Morse	1.6	1.3	1.4	Tolerant
Gordon	1.1	1.8	1.4	Tolerant
Wright	1.2	1.8	1.5	Tolerant
Hartwig	1.0	2.0	1.5	Tolerant
Kaifuu seitou	1.5	1.5	1.5	Tolerant
Avoyelles	1.8	1.3	1.5	Tolerant
Miles	1.3	1.8	1.5	Tolerant
R08-3206	1.6	1.5	1.5	Tolerant
Paoting	1.6	1.5	1.5	Tolerant
R08-2797	1.2	1.9	1.5	Tolerant
7902	1.6	1.5	1.6	Tolerant
R05-3817	1.7	1.5	1.6	Tolerant
R07-10322	1.9	1.4	1.6	Tolerant
R09-430	1.2	2.0	1.6	Tolerant
Barchet	1.0	2.3	1.6	Tolerant
Coker 488	1.3	2.0	1.6	Tolerant
R07-129	1.6	1.7	1.6	Tolerant
R09-2567	1.1	2.2	1.6	Tolerant
Hartz 5164	1.6	1.8	1.7	Tolerant
Pan-San	2.1	1.3	1.7	Tolerant
R07-1769	1.1	2.3	1.7	Tolerant
Biloxi	1.6	1.8	1.7	Tolerant
UARK 5896	1.3	2.2	1.7	Tolerant
Jin dou No. 6	1.2	2.3	1.7	Tolerant
Honduras	2.0	1.5	1.8	Tolerant
Osage	1.6	2.0	1.8	Tolerant

Table 5. *continued.*

Genotype				Response[§]
	2012	2013	Mean[‡]	
Will	1.5	2.0	1.8	Tolerant
R97-1634	1.4	2.2	1.8	Tolerant
R09-886	1.7	1.9	1.8	Tolerant
Hartz 6130	1.9	1.8	1.8	Tolerant
Yonekadake	1.9	1.8	1.8	Tolerant
R07-1738	1.4	2.3	1.8	Tolerant
Tamana	2.4	1.3	1.8	Tolerant
R05-4114	1.8	2.0	1.9	Tolerant
Deltapine 726	1.1	2.7	1.9	Tolerant
Glycine H	2.3	1.5	1.9	Tolerant
Jake	1.9	1.9	1.9	Tolerant
R05-235	1.6	2.3	1.9	Tolerant
Fiskeby III	2.1	1.8	1.9	Tolerant
Otootan	1.5	2.4	1.9	Tolerant
R08-991	1.7	2.2	2.0	Tolerant
R07-6654	1.8	2.2	2.0	Tolerant
R04-1274RR	1.7	2.3	2.0	Tolerant
HBK R5226	1.7	2.3	2.0	Tolerant
Kurakake Daizu	1.7	2.3	2.0	Tolerant
Tumbes	2.8	1.3	2.0	Tolerant
Lee	1.7	2.4	2.0	Tolerant
Hartz 6200	1.2	2.8	2.0	Tolerant
Kulat	2.1	2.0	2.0	Tolerant
Giant Speckled	2.9	1.3	2.1	Tolerant
Lee 68	1.9	2.3	2.1	Tolerant
R08-265	1.7	2.4	2.1	Tolerant
Santa Maria	3.0	1.3	2.1	Tolerant
Dillon	2.1	2.2	2.1	Tolerant
Forrest	1.8	2.5	2.1	Tolerant
Avery	1.7	2.6	2.2	Tolerant
R05-269	1.4	3.0	2.2	Tolerant
Smith Super	2.9	1.4	2.2	Tolerant
Ringgit (Indonesia)	2.1	2.3	2.2	Tolerant
Centennial	2.1	2.3	2.2	Tolerant
Changteh	2.2	2.3	2.2	Tolerant

Table 5. *continued.*

Genotype				Response[§]
	2012	2013	Mean[‡]	
R03-1250	2.2	2.3	2.2	Tolerant
Ringgit (Suriname)	2.2	2.3	2.2	Tolerant
Bedford	2.4	2.1	2.2	Tolerant
Ransom	2.3	2.2	2.2	Tolerant
Charlee	2.2	2.3	2.2	Tolerant
Pershing	2.3	2.3	2.3	Tolerant
Yashiro Zairai No.2	2.8	1.8	2.3	Tolerant
Soya Otootan	2.2	2.4	2.3	Tolerant
5002T	2.4	2.2	2.3	Tolerant
Manokin	2.3	2.5	2.4	Tolerant
R07-5351	2.4	2.3	2.4	Tolerant
R08-1178	1.8	2.9	2.4	Tolerant
Zyuninyoshi	2.9	2.0	2.4	Tolerant
R04-342	2.0	3.0	2.5	Tolerant
R02-3065	2.3	2.7	2.5	Tolerant
Anwei	2.4	2.8	2.6	Tolerant
Hill	2.5	2.9	2.7	Tolerant
R09-4010	3.0	2.4	2.7	Tolerant
Oksoy	2.7	2.8	2.7	Tolerant
Cherokee	2.9	2.9	2.9	Tolerant
Hartz 5171	3.0	3.0	3.0	Tolerant
R06-4433	3.0	3.0	3.0	Tolerant
Coker 425	3.1	3.1	3.1	Tolerant
R09-1831RR	3.1	3.1	3.1	Tolerant
Coker 317	3.3	3.3	3.3	Intermediate
R09-400	3.1	3.5	3.3	Intermediate
Amarilla	3.2	3.5	3.3	Intermediate
R05-374	3.3	3.4	3.3	Intermediate
R08-141	3.2	3.4	3.3	Intermediate
Tookichi	3.5	3.4	3.4	Intermediate
Hutcheson	3.7	3.6	3.6	Sensitive
Koshu	3.7	3.9	3.8	Sensitive
R08-3211	3.6	3.9	3.8	Sensitive
Nanking 332	3.8	3.8	3.8	Sensitive
Cibao	4.0	3.7	3.8	Sensitive

Table 5. *continued.*

Genotype				Response[§]
	2012	2013	Mean[‡]	
CNS	3.9	3.8	3.8	Sensitive
R07-1685	3.8	4.0	3.9	Sensitive
Davis	4.0	3.8	3.9	Sensitive
CAVINESS	4.1	3.7	3.9	Sensitive
R05-1415	3.8	4.0	3.9	Sensitive
Gaku Bun	4.1	3.8	3.9	Sensitive
N98-4445A	3.7	4.3	4.0	Sensitive
Williams 79	4.2	3.7	4.0	Sensitive
Perfume	4.3	3.7	4.0	Sensitive
Pioneer 9581	4.0	4.0	4.0	Sensitive
Heihokuta	4.1	4.0	4.0	Sensitive
Great White	4.4	3.8	4.1	Sensitive
Shimo Baba	4.4	3.8	4.1	Sensitive
Chinuikon	3.7	4.5	4.1	Sensitive
Wye	3.9	4.3	4.1	Sensitive
Pickett 71	4.2	4.0	4.1	Sensitive
Raub 16.1422	4.0	4.3	4.1	Sensitive
Koshoku Akidaizu	4.3	4.0	4.1	Sensitive
R09-3742	3.9	4.4	4.1	Sensitive
HBK R4924	4.6	3.7	4.1	Sensitive
Williams 82	4.4	3.9	4.1	Sensitive
Shang tsai	4.3	4.0	4.2	Sensitive
Komata	4.4	4.0	4.2	Sensitive
Williams	4.1	4.3	4.2	Sensitive
DP 5634	4.1	4.4	4.2	Sensitive
Gasoy 17	4.5	4.0	4.2	Sensitive
Tamanishiki	4.7	3.8	4.2	Sensitive
Punjab-1	4.0	4.5	4.2	Sensitive
Coker 485	4.0	4.5	4.3	Sensitive
0197 (Nepal)	4.3	4.3	4.3	Sensitive
Essex	4.5	4.2	4.3	Sensitive
Haberlandt	3.9	4.8	4.3	Sensitive
Clemson	3.7	5.0	4.4	Sensitive
Sixth Moon	4.7	4.0	4.4	Sensitive
Hanashirazu	5.0	3.8	4.4	Sensitive

Table 5. *continued.*

Genotype				Response[§]
	2012	2013	Mean[‡]	
Hood 75	4.4	4.4	4.4	Sensitive
Aokimame	4.3	4.5	4.4	Sensitive
Cutler 71	3.8	5.0	4.4	Sensitive
Wu-Kung 509	4.6	4.3	4.4	Sensitive
So ran du	4.3	4.7	4.5	Sensitive
Chasengoku	4.9	4.0	4.5	Sensitive
Pioneer 9531	3.9	5.0	4.5	Sensitive
C1943	4.1	4.9	4.5	Sensitive
Aka Saya	5.0	4.0	4.5	Sensitive
Arksoy	4.5	4.5	4.5	Sensitive
Ozark	4.2	4.9	4.5	Sensitive
Delmar	4.6	4.5	4.5	Sensitive
Branca do Rio Grande	4.6	4.5	4.5	Sensitive
Glenn	4.7	4.5	4.6	Sensitive
Ogden	4.6	4.5	4.6	Sensitive
Dare	4.7	4.4	4.6	Sensitive
Laredo	4.9	4.3	4.6	Sensitive
Java 29	4.8	4.4	4.6	Sensitive
R07-1857	4.6	4.6	4.6	Sensitive
Custer	4.4	4.8	4.6	Sensitive
R04-1250RR	4.3	4.9	4.6	Sensitive
Douglas	5.0	4.3	4.6	Sensitive
Missoy	4.5	4.8	4.6	Sensitive
Foster	4.9	4.4	4.6	Sensitive
Bragg	4.5	4.8	4.6	Sensitive
Lonoke	4.7	4.6	4.7	Sensitive
NAROW	4.7	4.7	4.7	Sensitive
R07-6669	4.7	4.6	4.7	Sensitive
UA 4805	4.9	4.4	4.7	Sensitive
R01-416F	4.6	4.7	4.7	Sensitive
Bonus	4.7	4.8	4.7	Sensitive
Clark	4.7	4.8	4.7	Sensitive
R98-209	4.8	4.7	4.7	Sensitive
Kahei	4.8	4.8	4.8	Sensitive
R09-1237	4.9	4.7	4.8	Sensitive

Table 5. *continued.*

Genotype				Response[§]
	2012	2013	Mean[‡]	
Tokyo	5.0	4.5	4.8	Sensitive
UARK 5798	4.7	4.8	4.8	Sensitive
Alki ball	4.8	4.8	4.8	Sensitive
Desha	4.8	4.8	4.8	Sensitive
Flanklin	4.8	4.8	4.8	Sensitive
R07-7775	4.7	5.0	4.8	Sensitive
Houjaku Kuwazu	5.0	4.8	4.9	Sensitive
Dorman	4.9	5.0	4.9	Sensitive
R04-572	5.0	4.8	4.9	Sensitive
Shariin	4.8	5.0	4.9	Sensitive
Walters	4.9	5.0	4.9	Sensitive
Jackson	4.8	5.0	4.9	Sensitive

† Leaf scorch scores were based on a 1-5 rating scale with 1 representing the most tolerant and 5 representing the most sensitive. Scores for each genotype were averages over four replications.

‡ Mean, the average over the two experiments in 2012 and 2013.

§ Salt response type was determined using the means of two tolerant or sensitive checks ± two standard deviations (Table 2). Genotypes with a score < 3.13 (1.73 – 1.40, 1.73 + 1.40) were classified as tolerant; genotypes with a score > 3.62 (4.56 – 0.94, 4.56 + 0.94) were classified as sensitive; and genotypes with a score falling into the scale of 3.13 - 3.62 (1.73 + 1.40, 4.56 – 0.94) were classified as intermediate.

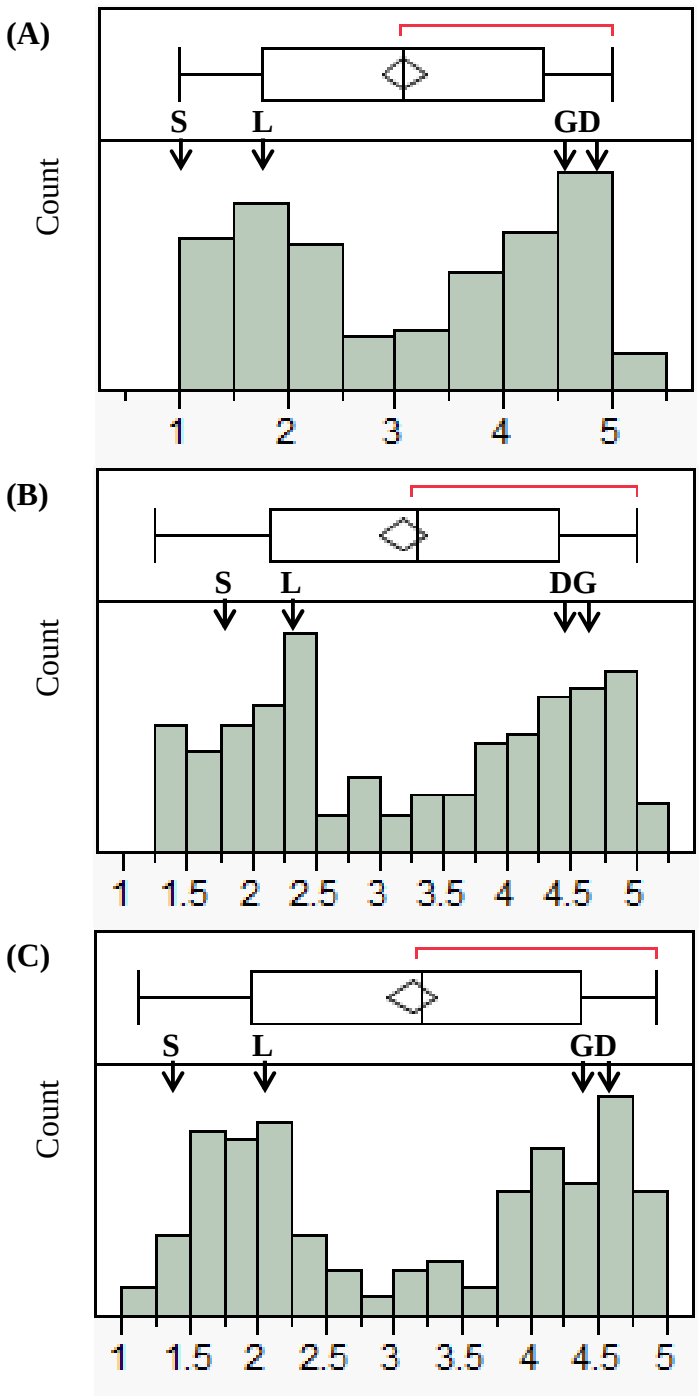

Figure 1. Distributions of leaf scorch scores of 192 genotypes evaluated in greenhouse experiments conducted in 2012 (**A**) and 2013 (**B**). The distribution of two-year combined data (**C**) was also showed using averages of the 2012 and 2013 experiments. The arrows represent the salt-tolerant or salt-sensitive checks: **L**: Lee 68; **S**: S-100; **D**: Dare; and **G**: Glenn.

CHAPTER III

GENOME-WIDE ASSOCIATION MAPPING OF SALT TOLERANCE IN SOYBEAN

ABSTRACT

Association mapping is an alternative to linkage mapping for detection of marker-trait associations. The main objective of this study was to employ single nucleotide polymorphism (SNP)-based genome-wide association mapping to uncover the SNPs and QTLs associated with salt tolerance in soybean. A total of 181 diverse soybean breeding lines and cultivars, 94 salt-tolerant and 87 salt-sensitive, were genotyped using 52,041 evenly spaced SNPs, from which 37,281 SNPs with stringent quality were selected for further analysis. These genotypes were divided into five subgroups using STRUCTURE and UPGMA cluster analysis. Linkage disequilibrium (LD) analysis revealed that the average LD across the genome extended up to 1.4 Mb for $r^2 > 0.1$ and 0.5 Mb for $r^2 > 0.2$. After controlling the population structure and individual relatedness, and selecting the statistical models that minimized spurious marker-trait associations, a total of 62 SNPs representing six salt tolerance QTLs on Chromosomes 2, 3, 5, 6, 8, and 18 were identified ($P < 0.001$). Among them, 52 SNPs on Chromosome 3 map within or near a major salt tolerance quantitative trait locus (QTL) previously identified in S-100 (Lee *et al.*, 2004) while three SNPs on Chromosome 18 map near a salt tolerance QTL previously identified in Nannong1138-2 (Chen *et al.*, 2008). The other SNPs represent four putative minor QTLs newly identified in this study. Ten candidate genes were identified, which are mapped at or near (< 35 kb) the significant SNPs, and potentially involved in ion metabolisms and salt stress responses. These results suggest that the genome-wide association mapping with high marker density is an effective method for uncovering QTLs and candidate genes for salt tolerance in soybean.

INTRODUCTION

To improve salt tolerance of soybean cultivars, one of the challenges is to understand the genetic background for the trait in soybean. A few studies have been focused on the inheritance of salt tolerance in soybean (Abel, 1969; Shao *et al.*, 1994; Luo *et al.*, 2004). Abel (1969) and Shao *et al.* (1994) proposed that a single dominant gene symbolized as *Ncl* controls the soybean salt tolerance while Luo *et al.* (2004) concluded that minor genes confer this trait. Xu and Tuyen (2012) attributed the inconsistent results to the genetic variability of parental cultivars and the different methods evaluating salt tolerance. QTL analysis has repeatedly detected a major salt tolerance QTL on linkage group N (Chromosome 3) in cultivated S-100, FT-abyara, Jin dou No.6, and a wild soybean accession JWS156-1 (Lee *et al.*, 2004; Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011). Lee *et al.* (2004) suggested that this QTL is most likely the gene *Ncl* locus because the tolerance allele in the cultivar Lee used in Abel's (1969) study derives from S-100. In contrast, Chen *et al.* (2008) reported a major QTL on linkage group G (Chromosome 18) and seven minor QTLs on other linkage groups, which are associated with salt tolerance or the related traits. Later, Lee *et al.* (2009) studied the inheritance of salt tolerance in a wild soybean accession PI483463, and concluded that a single dominant gene different from the *Ncl* controls the salt tolerance in PI483463. However, more genetic studies are needed to validate the gene/QTLs newly identified.

Association mapping (AM) is an alternative method for uncovering molecular markers and QTLs associated with traits in plants by exploiting the genetic variability and linkage disequilibrium of a diverse population (Zhu *et al.*, 2008). A series of AM studies have been reported, focusing on various traits in crop species such as maize (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.), barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), tomato

(*Lycopersicon esculentum* Mill.), sorghum (*Sorghum bicolor* (L.) Moench), and potato (*Solanum tuberosum* L.) (Zhu *et al.*, 2008).

In soybean, several AM studies have discovered or verified a series of markers and QTLs associated with quality and agronomic traits in soybean such as seed sucrose content, free amino acid content, 100-pod fresh weight, 100-seed fresh weight, seed protein content, seed oil content, and iron deficiency chlorosis (Jun *et al.*, 2008; Wang *et al.*, 2008; Shi *et al.*, 2010; Hou *et al.*, 2011; Mamidi *et al.*, 2011). Most of them used a limited number of SSR markers, and thus restricted the mapping resolution. Mamidi *et al.* (2011) conducted a genome-wide association analysis using 1,536 SNP markers, and identified multiple genomic regions associated with iron deficiency chlorosis in soybean.

So far, no AM study on salt tolerance in soybean has ever been reported. Given the inconsistent results of previous linkage mapping studies on salt tolerance in soybean, an AM approach seems to be an appropriate way to uncover markers and QTLs associated with this trait. The goal of this study was to conduct a genome-wide discovery scan using 52,041 SNPs with a high mapping resolution to detect SNPs and QTLs associated with salt tolerance in soybean. Candidate genes at or near the significant SNPs were also discussed. The population used in this study consisted of advanced soybean breeding lines and cultivars collected from private and public breeding programs as well as USDA germplasm collection. Statistical procedures accounting for the existing population structure and familial relatedness within this diverse population were applied to minimize possible false marker-trait associations.

MATERIALS AND METHODS

Plant Materials and Phenotypic data

A total of 181 diverse soybean genotypes, 94 salt-tolerant and 87 salt-sensitive (Table 5, Chapter II), were selected based on the phenotypic data. The leaf scorch scores averaged over the two experiments in 2012 and 2013 were used for association analysis.

DNA Extraction and SNP Genotyping

Fifteen seeds of each genotype were planted in a plastic tray (10 1/2" x 21" x 2 1/2", Plasticflowerpots.net, Lake Worth, FI) filled with Sunshine Redi-earth Professional Growing Mixes (Sun Gro Horticulture Distribution Inc, Bellebue, WA) at 25 ± 2 ℃ with a 14h photoperiod in the Greenhouse 3.4 at the Rosen Center at the University of Arkansas. At V1 stage, the first trifoliate leaves were collected from ten plants of each genotype, and stored at -80 ℃ for subsequent DNA extraction. Genomic DNA was extracted using the hexadecyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). Briefly, leaf samples were grounded in liquid nitrogen with a mortar and a pestle. The extraction buffer was then added to the grounded sample followed by chloroform:isoamyl alcohol (24:1) to remove the protein. After centrifugation, the supernatant was transferred into a 1.5 ml tube, and added with 95% cold ethanol for DNA precipitation. 75% ethanol was then used to wash DNA pellets. The next day, the DNA was dissolved by 0.1 × TE buffer, and the concentration was measured using Bio-Tek PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). The DNA solution was stored at -80°C. Each sample was further genotyped using the SoySNP50K iSelect BeadChip with 52,041 single nucleotide polymorphism (SNP) markers

spanning the whole soybean genome (Song *et al.*, 2013). SNP markers with a MAF < 5% and absent in more than 25% of all genotypes were excluded from subsequent analyses.

Gene Diversity and Linkage Disequilibrium

Gene diversity, heterozygosity, and polymorphic information content (PIC) for SNPs on each chromosome and across the genome were calculated using PowerMarker 3.25 (Liu and Muse, 2005). Linkage disequilibrium (LD) was estimated as the squared allele frequency correlation (r^2) among loci using TASSEL 3.0 (Bradbury *et al.*, 2007). Loci were considered to be in significant LD if $P < 0.001$. The LD value (r^2) for each pair of loci was plotted against physical distance (Mb) using the SGPLOT procedure in SAS 9.3 (SAS Institute Inc., 2011). The LD decay scatter plots were generated for the whole genome and individual chromosomes. The LD decay distance was estimated for $r^2 > 0.1$ and 0.2 using the LOESS procedure in SAS 9.3 (SAS Institute Inc., 2011).

Population Structure and Kinship

Distance-based analysis for the 181 soybean genotypes using Euclidean-inferred ancestry for each genotype was carried out by TASSEL 3.0 (Bradbury *et al.*, 2007). A phylogenetic tree was generated by the UPGMA (unweighed pair group method using arithmetic averages) cluster analysis. A total of 829 and 2,486 informative SNPs were selected for estimation of population structure and kinship, respectively. Population structure was first inferred by the model-based software STRUCTURE 2.3.4 to estimate the number of subpopulations (Pritchard *et al.*, 2000a). Each genotype was given a percentage value estimating its subpopulation membership within the whole population. The admixture model with correlated allele frequencies was used. K value (the number of subpopulations) was set from 1 to 10, for each of which, ten runs were performed. For

each run, a burn-in of 10,000 cycles was conducted, followed by 50,000 iterations. To determine the optimum K value, the posterior probabilities for successive adjacent K (K2 vs. K3, K3 vs. K4, etc.) were compared by Wilcoxson two sample t-test (Rosenberg *et al.*, 2001) using PROC NPAR1WAY procedure in SAS 9.3 (SAS Institute Inc., 2011). The smaller K value in the first non-significant Wilcoxson test ($P < 0.001$) was chosen as the optimum K value. The subpopulation membership percentage values of the first run for the smaller K were used to generate the Q-matrix. Principal component analysis (PCA) was also performed by TASSEL 3.0 to infer the population structure. As suggested by Mamidi *et al.* (2011), the number of principal components collectively explaining more than 25% of the variation was chosen for subsequent analyses. Pairwise kinship coefficients (K-matrix) described by Loiselle *et al.* (1995) and Ritland (1996) were then calculated using SPAGeDi 1.4 (Hardy and Vekemans, 2002), which estimated the probability of recent co-ancestry between genotypes. All negative kinship values were set to zero as suggested by Yu *et al.* (2006). The Q-matrix, PCA, and K-matrix were used for association analyses.

Marker-Trait Association Analyses

Nine different linear regression models (Table 1), similar to the ones described by Mamidi *et al.* (2011), were implemented using TASSEL 3.0 to identify marker-trait associations. Three general linear models (GLMs) considered only fixed effects while the remaining six mixed linear models (MLMs) considered both random and fixed effects. In these models, y is a vector for the phenotypic observation, α is a vector for the fixed effects of the SNPs, β is a vector for the fixed effects of the population structure, ν is a vector for the random effects of the individual relatedness, and ε is a vector for the residual effects (Mamidi *et al.*, 2011). X is the SNP

genotypes, P is the matrix of the principal components (PCs), Q is the Q-matrix inferring subpopulation memberships of genotypes, K^L is the Loiselle *et al.* (1995) matrix of kinship coefficients, and K^R is the Ritland (1996) matrix of kinship coefficients.

An ideal model is expected to have the observed P-values for all marker loci uniformly distributed (Yu *et al.*, 2006). For each model, the observed P-values for all marker loci were ranked from the smallest to the largest. The expected P-values were calculated as (i/n), where i is the rank of the observed P-value, and n is the total number of markers used in the model (Stich *et al.*, 2008). To determine the deviation of the observed P-values from the uniform distribution, the mean of the squared difference (MSD) between the observed and expected P-values of all marker loci was calculated using the equation described by Mamidi *et al.* (2011):

$$MSD = [\sum_{i=1}^{n}(p_i - i/n)^2]/n,$$

where i is the rank number, p_i is the ith ranked P-value, and n is the number of markers. Low MSD values indicate a less degree of deviation of the observed P-values from the distribution (Stich *et al.*, 2008). Significant SNPs were selected only from the models that have low MSD values.

RESULTS

Single Nucleotide Polymorphism Marker Analysis

A total of 181 diverse soybean genotypes were genotyped using the SoySNP50K iSelect BeadChip with 52,041 SNPs spanning the whole genome (Song *et al.*, 2013). These SNPs showed a wide distribution in minor allele frequencies (MAFs), ranging from monomorphic (MAF = 0) to equal allele frequency (MAF ≈ 50%) with an average MAF of 20% across the 181 (Figure 1). Of the 52,041 SNPs, the SNPs with a MAF < 5% and absent in more than 25% of all

genotypes were excluded from subsequent analyses, leaving 37,281 informative SNPs. The remaining SNPs were distributed on 20 chromosomes with an average of 1,862 SNPs on each chromosome (Table 2). The average physical distance between SNPs was 25.5 kb. Table 3 shows the gene diversity, polymorphic information content (PIC), and heterozygosity for individual chromosomes and the whole genome assessed by the 37,281 SNPs. Across the genome, the gene diversity ranged from 0.05 to 0.50 with an average of 0.349. The polymorphic information content (PIC) values ranged from 0.049 to 0.375 with an average of 0.280. The heterozygosity ranged from 0.00 to 0.947 with an average of 0.091. The low average of heterozygosity indicated that the majority of the genotypes were homozygous for most SNP loci because of the biallelic nature of SNPs and the selfing nature of *Glycine max* (Mamidi *et al.*, 2011).

Linkage Disequilibrium Decay

All 37,281 SNPs were used to determine the LD decay distance in the population of 181 soybean genotypes tolerant or sensitive to salt stress. As shown in Table 4, the LD decays in terms of physical distance (Mb) for $r^2 > 0.1$ ranged from 0.5 Mb to 3.2 Mb with an average of 1.4 Mb across all chromosomes while the LD decays for $r^2 > 0.2$ ranged from 0.2 Mb to 1.3 Mb with an average of 0.5 Mb.

Population Structure and Kinship Analysis

Of the 37,281 SNPs, 829 and 2,468 evenly distributed across the genome according to the physical positions were used to estimate population structure and relative kinship, respectively. Population structure was first estimated using the software STRUCTURE 2.3.4 (Pritchard *et al.*,

2000a). This analysis determined that the population consisted of five subpopulations (Figure 2). Membership probabilities for each subpopulation were assigned to each genotype. With the maximum membership probability, 35, 11, 37, 70, and 27 genotypes were assigned to Subpopulations 1 to 5, respectively (Figure 2). UPGMA cluster analysis also divided this population into five clusters. In contrast to the subpopulations derived from STRUCTURE, 3, 27, 28, 16, and 104 genotypes were assigned to Clusters 1 to 5 (Figure 3). Principal component analysis was also implemented to evaluate the population structure using TASSEL 3.0. Five principal components explained a total of 28.6% variance, among which the first to fifth components explained 10.1, 7.9, 4.6, 3.3, and 2.7% of the variance, respectively.

The two procedures described by Loiselle *et al.* (1995) and Ritland (1996) respectively were implemented in SPAGeDi 1.4 to calculate pairwise kinship coefficients. Both procedures gave similar kinship coefficients. Figure 4 shows the distributions of kinship coefficients using Loiselle *et al.* (1995) and Ritland (1996) methods. For both methods, about 70% of the values were less than 0.05, whereas about 25% of the values ranged from 0.05 to 0.25. These results indicate that the 181 soybean genotypes have a low to a moderate level of genetic relatedness. Two K-matrices, K^L and K^R, were developed for subsequent association analyses using Loiselle *et al.* (1995) and Ritland (1996) methods, respectively.

Marker-Trait Associations

The leaf scorch scores averaged over two experiments in 2012 and 2013 were used for statistical model analyses (Tables 4 and 5, Chapter II). The genotypic and phenotypic data were implemented into nine different models described in Table 1 to detect marker-trait associations. Table 5 shows the number of SNPs associated with leaf scorch scores at different significant

levels ($P < 0.05$, 0.01, or 0.001). The general linear model (GLM) SFA that did not consider population structure and kinship gave the most significant SNPs at all levels. The other two GLMs, Q and PCA, which considered population structure and principal components respectively, gave similar numbers of significant SNPs at all levels (2589 vs. 2671 at $P < 0.05$; 867 vs. 764 at $P < 0.01$; 182 vs. 166 at $P < 0.001$). In contrast to the GLMs, six mixed linear models (MLMs), which considered kinship or both kinship and population structure, all gave a smaller number of significant SNPs at all levels. Among the MLMs, all had similar numbers of significant SNPs except for K^R, which had a relatively larger number of significant SNPs at all levels. In association analysis, an ideal model is expected to exhibit a uniform distribution of the observed P-values (Mamidi *et al.*, 2011). To determine which models were the best fits for detecting marker-trait associations in this study, the mean of squared difference (MSD) between the observed P-values and the expected P-values for all marker loci was calculated for each model (Table 5). Consequently, the models K^R, $Q+K^R$, $PCA+K^R$, and $PCA+K^L$ had much lower MSD values compared to the others, and thus were chosen to select significant SNPs from.

A total of 62 SNPs were significantly associated with salt tolerance ($-\log10 (P) > 3$), among which 3, 52, 1, 1, 2, and 3 SNPs map on Chromosomes 2, 3, 5, 6, 8, and 18, respectively (Table 6). Among these SNPs, 50 had a MAF > 0.2, 35 had a MAF > 0.3, and 19 had a MAF > 0.4. Most of the significant SNPs with a high MAF (> 0.3) were located on Chromosome 3 and 18. The leaf scorch score means of the genotypes with major alleles ranged from 1.91 to 3.77 while the means of those with minor alleles ranged from 1.68 to 3.96. The differences between major allele and minor allele means ranged from 0.69 to 2.06. Overall, these SNPs explained 7.0 – 54.1% of the phenotypic variation. For each chromosome, the significant SNPs on Chromosomes 2, 3, 5, 6, 8, and 18 accounted for 8.4 – 8.7%, 9.5 – 54.1%, 7.9%, 8.9%, 7.6%,

and 7.0 – 7.4% of the phenotypic variation, respectively. Of the 52 significant SNPs on Chromosome 3, 41, 19, 10, and 8 SNPs explained more than 10%, 20%, 30%, and 40% of the phenotypic variation, respectively. Within an interval of 0.51 Mb spanned by 25 consecutive significant SNPs from the position 40153205 to 40663609 on Chromosome 3, 17 SNPs explained more than 25% of the variation, indicating a strong salt tolerance QTL within this interval. The significant SNPs on Chromosomes 2, 5, 6, 8, and 18 explained 7.0 – 8.9% of the variation, indicating five putative salt tolerance QTLs with minor effects, which require further research validation.

The soybean reference genome (SoyBase, available at https://www.soybase.org) provides a useful tool to identify candidate genes that are potentially involved in ion metabolisms and salt stress responses. Ten genes at or near (< 35 kb) the significant SNPs according to the physical positions were selected (Table 7), among which seven were on Chromosome 3 whereas three were on Chromosomes 5, 8, and 18, respectively. The potential functions of these genes were discussed subsequently, and appeared to be related to plant responses to salt stress.

DISCUSSION

Instead of traditional linkage mapping which uses a population derived from a bi-parental cross, the genome-wide association mapping (AM) was conducted using a diverse population of soybean breeding lines and cultivars in this study. When conducting association analysis, it is important to estimate the pairwise linkage disequilibrium (LD) between markers and LD decay distances within an association mapping panel (Chen *et al.*, 2012). For detection of a marker-trait association, a marker needs to be in significant LD with a locus associated with the trait (Cardon and Bell, 2001). The LD decay distance thus determines the mapping resolution and marker

density required for an effective association analysis (Zhu *et al.*, 2008). A short LD decay distance leads to a high mapping resolution with a large number of markers while a longer LD decay distance lowers the mapping resolution, but only requires a relatively small number of markers (Flint-Garcia *et al.*, 2003; Zhu *et al.*, 2008). Generally, a higher LD decay distance is expected in self-pollinated crops such as wheat, than in cross-pollinated crops such as maize (Zhu *et al.*, 2008). Previous studies have addressed the LD decay distances in soybean (Jun *et al.*, 2008; Mamidi *et al.*, 2011). The LD extended up to 50 cM for $r^2 > 0.1$ and 10 cM for $r^2 > 0.2$ in 96 soybean accessions from Korea, China, and Japan (2008) using 150 SSR markers (Jun *et al.*, 2008). In another study, the LD decay distances assessed by about 850 SNP markers for $r^2 > 0.1$ were 7.0 Mb (19.3 cM) and 5.9 Mb (19.7 cM) for two independent populations collected from multiple soybean breeding programs in US (Mamidi *et al.*, 2011). The LD decay distances vary greatly from one study to another, and depend on various factors, especially the size and genetic variation of the population as well as the number of markers (Chen *et al.*, 2012). In our study, the average LD decay distances across the genome were 1.4 Mb for $r^2 > 0.1$ and 0.5 Mb for $r^2 > 0.2$ (Table 4), much lower than those previously reported. The shorter LD decay distance may result from the larger size (181 genotypes) and higher diversity (given the diverse origins, where 181 genotypes were collected from) population as well as the much larger number of markers used in our study. The 37,281 SNPs gave an average marker distance of 25.5 kb, much lower than the LD decay distance, which therefore gave a sufficient power for the association analysis.

Despite the advantages of AM such as reduced research time and higher mapping resolution, one of the key challenges for AM is to ensure that any marker-trait associations are genetically significant and not spurious associations due to population structure, familial relatedness, and/or co-ancestry (Mamidi *et al.*, 2011). Several statistical models have been

developed to account for each or combination of these factors with potential confounding effects (Zhu *et al.*, 2008). The assembled genotypes in this study can be structured, given the different geographic origins and multiple breeding programs, where they were collected. Here, nine models (Tables 1 and 5) were applied. The best models were determined by comparing the means of the squared differences (MSD) between observed P-values and expected P-values for these models (Stich *et al.*, 2008). Results indicated that the models K^R, $Q+K^R$, $PCA+K^R$, and $PCA+K^L$ had much lower MSD values than the others. In contrast, the SFA model without considering either population structure or relatedness had the highest MSD value, and gave six to eight times more significant ($P < 0.001$) marker-trait associations, the majority of which could be spurious. These results imply that testing multiple models is necessary before marker-trait associations are determined (Mamidi *et al.*, 2011).

As an alternative to linkage mapping, AM is expected to uncover previously reported QTLs. Of the 62 significant SNPs identified in this study, 52 on Chromosome 3 are located within or near the major salt tolerance QTL region previously identified in the cultivars S-100, FT-abyara, and Jin dou No.6 and the wild accession JWS156-1 (Lee *et al.*, 2004; Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011). Among the 52 SNPs, 25 consecutive SNPs spanning an interval of 0.51 Mb explained much higher phenotypic variation (30% in average) as compared to the others, suggesting that this major QTL most likely resides within this interval (Table 6). It was also supported by the greater mean differences between tolerance allele and sensitive allele genotypes at those SNP loci (Table 6). Three SNPs on Chromosome 18 are mapped near a salt tolerance QTL previously identified in Nannong1138-2 (Chen *et al.*, 2008). The other SNPs on Chromosomes 2, 5, 6, and 8 represent four putative salt tolerance QTLs newly identified in this study. These markers had minor effects, and explained 7.0 – 8.4% of the phenotypic variation.

Overall, two salt tolerance QTLs, including the repeatedly reported major salt tolerance QTL on Chromosome 3, have been confirmed in this study while four new putative minor QTLs have been identified. However, these putative QTLs need further validation with different genetic background.

Previous studies have proposed that salt tolerance in soybean is dominated by a single gene *Ncl* (Abel, 1969; Shao *et al.*, 1994), especially given that a major salt tolerance QTL (implied as the *Ncl* locus) on Chromosome 3 has been repeatedly identified in soybean cultivars and a wild soybean accession (Lee *et al.*, 2004; Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011). However, other studies suggest that multiple minor genes or QTLs control salt tolerance in soybean (Luo *et al.*, 2004; Chen *et al.*, 2008). The conflicting results may be mainly due to the differences in genetic background of genotypes and phenotypic screening methods implemented in these studies. In contrast, our study appears to indicate the presence of both a major QTL and minor QTLs for salt tolerance. The majority of the significant SNPs on Chromosome 3 had large effects, and indicated a major salt tolerance QTL while the others had minor effects, and indicated several minor QTLs on other chromosomes. The diverse population used in our study may include genotypes with different salt tolerance mechanisms, and thus resulted in discovery of multiple QTLs with major or minor effects while previous studies only used bi-parental populations with limited genetic resources for salt tolerance, and therefore was unable to detect as many salt tolerance QTLs at one time.

Another potential goal of AM is to use marker-trait associations as departure points to uncover candidate genes associated with the trait (Mamidi *et al.*, 2011). A series of AM studies have identified SNPs at or near the genes known to control the traits studied (Atwell *et al.*, 2010; Mamidi *et al.*, 2011). Given the high marker density in this study, it was expected to identify

some candidate genes potentially involved in ion homeostasis and salt stress responses in soybean. As the average LD decay distance for $r^2 > 0.2$ was 0.5 Mb, within which loci are considered to be in LD, it was therefore reasonable to search for candidate genes within an interval of 0.5 Mb from the significant SNPs. As suggested by Mamidi *et al.* (2011), a gene was preliminarily selected if this gene was linked to a significant SNP within the interval distance and there was no insignificant SNP between them. The functions of the gene were then explored according to the public references and gene annotations derived from the SoyBase (http://www.soybase.org). Given these criteria, a total of ten genes were selected (Table 7).

Two genes, Glyma03g32890 and Glyma03g32900, were identified within the major salt tolerance QTL region on Chromosome 3 (Table 7). Both, annotated as K^+/H^+ antiporters (SoyBase, available at http://www.soybase.org), are most likely to be involved in salt tolerance. In plants, K^+ plays crucial roles in many fundamental processes including enzyme activation, membrane transport, anion neutralization, and osmoregulation (Clarkson and Hanson, 1980; Wang and Wu, 2013). Under high salinity (NaCl), Na^+ competes with K^+ for uptake, disrupting K^+ homeostasis in plants (Parida *et al.*, 2005). Evidence has demonstrated that K^+ acquisition and homeostasis is crucial for plants under salt stress (Loupassaki *et al.*, 2002; Chen *et al.*, 2005). Other studies have repeatedly named the cytosolic K^+/Na^+ ratio as the key determinant for plant salt tolerance despite the high quantity of toxic Na^+ in cytosol (Shabala and Cuin, 2007). Therefore, K^+ protects plants from Na^+ toxicity. In soybean, however, studies have been mainly focused on the accumulation and transport of Na^+ and Cl^-. Some propose that Cl^- exclusion is the most crucial mechanism for salt tolerance in soybean while others emphasize the importance of Na^+ over Cl^- or both. In spite, a few studies indeed show a positive correlation between leaf K^+ content and salt tolerance in soybean cultivars and wild *Glycine* species (Abdel-Samad *et al.*,

1997; Shereen *et al.*, 2001; Essa, 2002; Kao *et al.*, 2006). This correlation does not necessarily rule out the important roles of Na^+ and Cl^- in responses of soybean to salt stress. Most likely, the homeostasis of Na^+, K^+, and Cl^- all contributes to salt tolerance in soybean while few contrasting findings may be mainly due to the genetic variation among those studied soybean genotypes and *Glycine* species. In plants, K^+ uptake and transport is mediated by K^+ transporters and channels, which mainly derive from the gene families KUP/HAK/KT, HKT, NHX, and CHX (Wang and Wu, 2013). Some have been showed to be up-regulated by salt stress and play an important role in salt tolerance (Su *et al.*, 2002; Cellier *et al.*, 2004; Obata *et al.*, 2007; Bassil *et al.*, 2011; Barragan *et al.*, 2012). Chen *et al.* (2011) recently isolated a novel HKT-like transporter in soybean, which was involved in K^+ and Na^+ transport, and enhanced salt tolerance. Given the above evidence, it will be highly interesting to investigate the functions of the genes, Glyma03g32890 and Glyma03g32900, and their potential roles in K^+ and Na^+ homeostasis and salt tolerance in soybean.

These two genes, although promising, do not preclude other candidates within the same or other QTL regions from affecting salt tolerance. It is worth noting that the SNP closest to or within the two genes did not show a relatively high R^2 value while the nearby SNPs showed much higher R^2 values (Table 7). It is likely that specific genes or motifs within the region spanned by the nearby SNPs are the actual determinants, which may regulate the predicted K^+/H^+ antiporters.

Fortunately, a few genes seemly involved in regulatory processes were uncovered. The gene Glyma03g32410 is mapped at or near three consecutive SNPs explaining 25.6 – 28.2% of the variation within the major QTL region on Chromosome 3 (Table 7). It is annotated as a pre-mRNA processing factor (SoyBase, available at http://www.soybase.org; Table 7). In plants, pre-

mRNA processing factors are involved in post-transcriptional regulations of genes. Accumulating studies suggest that pre-mRNA processing is a new target of salt toxicity (Yan *et al.*, 2005). Forment *et al.* (2002) reported that expression of *Arabidopsis* SR-like splicing factors, which are involved in pre-mRNA processing, conferred salt tolerance in yeast and transgenic plants, suggesting the importance of maintaining efficient pre-mRNA processing in plants under salt stress. A strong induction of an mRNA splicing factor in rice under salt stress provided further supportive evidence (Yan *et al.*, 2005). Thus, it will be interesting to study the functions of the gene Glyma03g32410 in soybean under salt stress.

Two genes likely involved in ubiquitin 26S proteasome system (UPS) were identified. The gene Glyma03g32800 (26S proteasome regulatory subunit) is mapped at the SNP explaining about 40.5% of the variation within the major QTL region on Chromosome 3 while Glyma18g46940 (ubiquitin-conjugating enzyme E2) is mapped near the SNP explaining 7.1% of the variation and representing a salt tolerance QTL on Chromosome 18 (Table 7). Both ubiquitin-conjugating enzyme and 26S proteasome are essential components in UPS, which conducts proteolysis, and thereby regulates most intracellular processes in plants (Smalle and Vierstra *et al.*, 2004). Accumulating studies suggest that UPS also plays important roles in plant tolerance to abiotic stresses. For instance, overexpression of a soybean ubiquitin-conjugating enzyme E2 gene, GmUBC2, was demonstrated to confer salt tolerance through modulating abiotic stress-responsive gene expression in *Arabidopsis* (Zhou *et al.*, 2010).

Three genes potentially involved in salt stress signaling transduction were also identified: Glyma03g33240 (Ca^{2+}-transporting ATPase), which is mapped near the SNP explaining 10.7 % of the variation within the major QTL region on Chromosome 3; Glyma05g09310 (pyruvate kinase), which is mapped near the SNP explaining 7.9 % of the variation and representing a

putative salt tolerance QTL on Chromosome 5; and Glyma08g24580 (Ca^{2+}-transporting ATPase), which is mapped near the SNP explaining 7.6% of the variation and representing a putative salt tolerance QTL on Chromosome 8 (Table 7). In plants, Ca^{2+}-transporting ATPase mediates the efflux of cytoplasmic Ca^{2+}, which transduces salt stress signals. Chung *et al.* (2000) identified a plasma membrane Ca^{2+}-transporting ATPase (GmSCA1) in soybean, which was strongly induced under salt stress. As for pyruvate kinase, it requires K^+ to be activated (Wang and Wu, 2013). Pyruvate kinase is highly sensitive to cytoplasmic K^+, and its activity can be directly inhibited by low cytoplasmic K^+ in root cells (Wang and Wu, 2013). Therefore, it is thought as a potential K^+ sensor (Wang and Wu, 2013). Considering the importance of K^+ homeostasis for salt tolerance as aforementioned, the gene Glyma05g09310 annotated as a pyruvate kinase appears to be a candidate.

The genes Glyma03g32850 and Glyma03g32940 may also be involved in ion homeostasis and salt stress responses in soybean, both of which are mapped at the SNPs with high R^2 values within the major QTL region (Table 7). The former is annotated as a heat shock protein 70 kda (Hsp70) while the latter encodes a UNC93-like protein (SoyBase, available at http://www.soybase.org). It is well known that Hsp70 responds to environmental stresses such as heat, cold, drought, salt, and other stresses (Wang *et al.*, 2004). Overexpression of Hsp70 genes is positively correlated with thermotolerance, and results in increased tolerance to salt, water, and high-temperature stress in plants (Wang *et al.*, 2004). As for UNC93, it was selected because it is potentially an ion channel regulatory protein in mammals (de la Cruz *et al.*, 2003), although no study has addressed its functions in plants.

It is worth nothing that none of the candidate genes identified and discussed in the present study seems to be involved in chloride metabolisms, considering the previously proposed

notion that salt tolerance in soybean is mainly regulated by chloride exclusion (Abel and MacKenzie, 1964; Parker *et al.*, 1983; Yang and Blanchar, 1993; Wang and Shannon, 1999; An *et al.*, 2002; Zhang *et al.*, 2011). In spite, several uncharacterized genes also exist, which are mapped at or near highly significant SNPs within the major QTL region. These unknown genes may have potential roles in responses of soybean to salt stress.

In summary, a genome-wide association analysis was conducted using phenotypic (leaf scorch) and genotypic (SNP) data to study the genetic basis of salt tolerance in soybean. A diverse population consisting of 94 salt-tolerant and 87 salt-sensitive genotypes was used. With statistical models correcting the population structure and relatedness, multiple SNP loci were uncovered, representing the major salt tolerance QTL on Chromosome 3 and five putative minor QTLs on Chromosomes 2, 5, 6, 8, and 18, respectively. However, the minor QTLs need further validation. The soybean reference genome and extensive knowledge and studies on plant stress responses in public enabled the identification of ten candidate genes, which are mapped at or near the significant SNPs and are potentially involved in salt tolerance in soybean. The SNPs and QTLs for salt tolerance identified in this study may be potentially used for marker-assisted selection in U.S. soybean breeding programs. The candidate genes may provide new research targets to investigate the salt tolerance mechanisms in soybean.

REFERENCES

Abdel-Samad, H.M., and M.A.K. Shaddad. 1997. Salt tolerance of soybean cultivars. *Biol. Plantarum* 39, 263–269.

Abel, G.H. 1969. Inheritance of the capacity for chloride inclusion and chloride exclusion in soybeans. *Crop Sci.* 9, 697–698.

Abel, G.H. and A.J. MacKenzie. 1964. Salt tolerance of soybean varieties (*Glycine max* L. Merrill) during germination and later growth. *Crop Sci.* 4, 157–161.

An P., S. Inanaga, Y. Cohen, U. Kafkafi, and Y. Sugimoto. 2002. Salt tolerance in two soybean cultivars. *J. Plant Nutr.* 25, 407–423.

Atwell, S., Y.S. Huang, B.J. Vilhjalmsson, G. Willems, M. Horton, Y. Li, D. Meng, A.A. Platt, M.A. Tarone, T.T. Hu, R. Jiang, N.W. Muliyati, X. Zhang, M.A. Amer, I. Baxter, B. Brachi, J. Chory, C. Dean, M. Debieu, J. de Meaux, J.R. Ecker, N. Faure, J.M. Kniskern, J.D. Jones, T. Michael, A. Nemri, F. Roux, D.E. Salt, C. Tang, M. Todesco, M.B. Traw, D. Weigel, P. Marjoram, J.O. Borevitz, J. Bergelson, and M. Nordborg. 2010. Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. *Nature* 465, 627–631.

Barragan, V., E.O. Leidi, Z. Andres, L. Rubio, A. De Luca *et al.* 2012. Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis*. *Plant Cell* 24, 1127–1142.

Bassil, E., H. Tajima, Y.C. Liang, M.A. Ohto, K. Ushijima *et al.* 2011. The *Arabidopsis* Na^+/H^+ antiporters NHX1 and NHX2 control vacuolar pH and K^+ homeostasis to regulate growth, flower development, and reproduction. *Plant Cell* 23, 3482–3497.

Bradbury, P.J., Z. Zhang, D.E. Kroon, T.M. Casstevens, Y. Ramdoss, and E.S. Buckler. 2007. Software for association mapping of complex traits in diverse samples. *Bioinformatics* 23, 2633–2635.

Cardon, R.L., and J.I. Bell. 2001. Association study designs for complex disease. *Nat. Rev. Genet.* 2, 91–99.

Cellier, F., G. Conejero, L. Ricaud, D.T. Luu, M. Lepetit, *et al.* 2004. Characterization of *AtCHX17*, a member of the cation/H^+ exchangers, CHX family, from *Arabidopsis thaliana* suggests a role in K^+ homeostasis. *Plant J.* 39, 834–46.

Chen, H., S. Cui, S. Fu, J. Gai and D. Yu. 2008. Identification of quantitative trait loci associated with salt tolerance during seedling growth in soybean (*Glycine max* L.). *Aust. J. Agric. Res.* 59, 1086–1091.

Chen, H.T., H. He and D.Y. Yua. 2011. Overexpression of a novel soybean gene modulating Na^+ and K^+ transport enhances salt tolerance in transgenic tobacco plants. *Physiol. Plantarum* 141, 11–18.

Chen, X.J., D.H. Min, T.A. Yasir, and Y.G. Hu. 2012. Genetic diversity, population structure and linkage disequilibrium in elite Chinese winter wheat investigated with SSR markers. *Plos ONE* 7, e44510.

Chen, Z., I. Newman, M. Zhou, N. Mendham, G. Zhang, and S. Shabala. 2005. Screening plants for salt tolerance by measuring K^+ flux: a case study for barley. *Plant, Cell and Environ.* 28, 1230–1246.

Chung, W.S., S.H. Lee, J.C. Kim, W.D. Heo, M.C. Kim, C.Y. Park *et al.* 2000. Identification of a calmodulin-regulated soybean Ca^{2+}-ATPase (SCA1) that is located in the plasma membrane. *Plant Cell* 12, 1393–1407.

Clarkson, D.T., and J.B. Hanson. 1980. The mineral nutrition of higher plants. *Annu. Rev. Plant Physiol.*31, 239–98.

De la Cruz, I.P., Levin, J.Z., Cummins, C., Anderson, P., and Horvitz, H.R. 2003. *Sup-9, sup-10* and *unc-93* may encode components of a two-pore K^+ channel that coordinates muscle contraction in *Caenorhabditis elegans*. *J. Neurosci*. *23*, 9133–9145.

Doyle J.J. and J.L. Dolyle. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12, 13–15.

Essa, T.A. 2002. Effect of salinity stress on growth and nutrient composition of three soybean [*Glycine max* (L.) Merr.] cultivars. *J. Agron. Crop Sci.* 188, 86–93.

Flint-Garcia, S.A., J.M. Thornsberry, E.S. Buckler. 2003. Structure of linkage disequilibrium in plants. *Annu. Rev. Plant. Biol.* 54, 357–374.

Forment, J., M. Á. Naranjo, M. Roldán, R. Serrano, and O. Vicente. 2002. Expression of *Arabidopsis* SR-like splicing proteins confers salt tolerance to yeast and transgenic plants. *Plant J.* 30, 511–519.

Hamwieh, A. and D.H. Xu. 2008. Conserved salt tolerance quantitative trait locus (QTL) in wild and cultivated soybeans. *Breed Sci.* 58, 355–359.

Hamwieh, A., D.D. Tuyen, H. Cong, E.R. Benitez, R. Takahashi, and D.H. Xu. 2011. Identification and validation of a major QTL for salt tolerance in soybean. *Euphytica* 179, 451–459.

Hardy, Q.J., and X. Vekemans. 2002. SPAGeDi: A versatile computer program to analyze spatial genetic structure at the individual or population levels. *Mol. Ecol. Res*. 2, 618–620.

Hou, J.F., C.L. Wang, X.J. Hong, J.M. Zhao, C.C. Xue, N. Guo, J.Y. Cai, and H. Xing. 2011. Association analysis of vegetable soybean quality traits with SSR markers. *Plant Breeding* 130, 444–449.

Jun, T.H., K. Van, M.Y. Kim, S.H. Lee, and D.R. Walker. 2008. Association analysis using SSR markers to find QTL for seed protein content in soybean. *Euphytica* 162, 179–191.

Kao, W.Y., T.T. Tsai, H.C. Tsai, and C.N. Shi. 2006. Response of three *Glycine* species to salt stress. *Environ. Exper. Bot*. 56, 120–125.

Läuchli, A., and J. Wieneke. 1979. Studies on growth and distribution of Na^+, K^+ and Cl^- in soybean varieties differing in salt tolerance. *Zeitschrift für Pflanzenernährung und Bodenkunde*, 146, 799–814.

Lee J.D, G. Shannon, T.D. Vuong, and H.T. Nguyen. 2009. Inheritance of salt tolerance in wild soybean (*Glycine soja* Sieb. & Zucc.) accession PI483463. *J. Hered.* 100, 798–801.

Lee, G.J., H.R. Boerma, M.R. Villagarcia, X. Zhou, T.E. Carter Jr, Z. Li, and M.O. Gibbs. 2004. A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. *Theor. Appl. Genet.* 109, 1610–1619.

Lenis, J.M., M. Ellersieck, D.G. Blevins, D.A. Sleper, H.T. Nguyen, D. Dunn, J.D. Lee, and J.G. Shannon. 2011. Differences in ion accumulation and salt tolerance among *Glycine* accessions. *J. Agron. Crop Sci.*197, 302–310.

Liu, K., and S.V. Muse. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* 21, 2128–2129.

Loiselle, B.A., V.L. Sork, J. Nason, and C. Graham. 1995. Spatial genetic structure of a tropical understory shrub, Psychotriaoffi cinalis (Rubiaceae). *Am. J. Bot.* 82, 1420–1425.

Loupassaki, M.H., K. S. Chartzoulakis, N.B. Digalaki, and I.I. Androulakis. 2002. Effect of salt stress on concentration of nitrogen, phosphorous, potassium, calcium, magnesium, an sodium in leaves, shoots, and roots of six olive cultivars. *J. Plant Nutr.* 25, 2457–2482.

Luo, Q., B. Yu and Y. Liu. 2005b. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *J. Plant Physiol.* 162, 1003–1012.

Luo, Q.Y., B.J. Yu, Y.L. Liu, Y.M. Zhang, Y.L. Xue and Y. Zhang. 2004. The mixed inheritance analysis of salt tolerance in cultivars of *Glycine max*. *Soybean Sci.* 23, 239–244.

Mamidi, S., S. Chikara, R.J. Goos, D.L. Hyten, S.M. Moghaddam, P.B. Cregan, and P.E. McClean. 2011. Genome-wide association analysis identifies candidate genes associated with iron deficiency chlorosis in soybean. *The Plant Genome* 4, 154–164.

Obata, T., H.K. Kitamoto, A. Nakamura, A. Fukuda, and Y. Tanaka. 2007. Rice shaker potassium channel OsKAT1 confers tolerance to salinity stress on yeast and rice cells. *Plant Physiol.* 144, 1978–1985.

Parida, A.K., and A.B. Das. 2005. Salt tolerance and salinity effects on plants: a review. *Ecotox. Environ. Safe.* 60, 324–349.

Parker, M.B., G.J. Gascho, T.P. Gains. 1983. Chloride toxicity of soybeans grown on Atlantic Coast Flatwoods soils. *Agron. J.* 75, 439–443.

Pritchard, J.K., M. Stephens, and P. Donnelly. 2000a. Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.

Ritland, K. 1996. Estimators for pairwise relatedness and individual inbreeding coefficients. *Genet. Res.* 67, 175–186.

Rosenberg, N.A., T. Burke, K. Elo, M.W. Feldman, P.J. Freidlin, M.A.M. Groenen, J. Hillel, A. Mäki-Tanila, M. Tixier-Boichard, A. Vignal, K. Wimmers, and S. Weigend. 2001. Empirical evaluation of genetic clustering methods using multilocus genotypes from 20 chicken breeds. *Genetics* 159, 699–713.

SAS Institute Inc. 2011. SAS/STAT® 9.3 User's Guide. Cary, NC: SAS Institute Inc.

Shabala, S., and T.A. Cuin. 2007. Potassium transport and plant salt tolerance. *Physiol. Plantarum* 133, 651–669.

Shao G.H., R.Z. Chang, Y.W. Chen, and S.R. Yan. 1994. Study on inheritance of salt tolerance in soybean. *Acta Agronomica Sinica* 20, 721–726.

Shereen, A., R. Ansari and A.Q. Soomro. 2001. Salt tolerance in soybean (*Glycine max* L.): effect on growth and ion relations. *Pak. J. Bot.* 33, 393–402.

Shi, A., P. Chen, B. Zhang, and A. Hou. 2010. Genetic diversity and association analysis of protein and oil content in food-grade soybeans from Asia and the United States. *Plant Breeding* 129, 250—256.

Smalle, J., and R.D. Vierstra. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* 55, 555–590

Song Q, D.L. Hyten, G. Jia, C.V. Quigley, E.W. Fickus, RL. Nelson, and P.B. Cregan. 2013. Development and evaluation of SoySNP50K, a high-density genotyping array for soybean. *PLoS ONE* 8, e54985.

Stitch, B., J. Mohring, H.P. Piepho, M. Heckenberger, E.S. Buckler, and A.E. Melchinger. 2008. Comparison of mixed-model approaches for association mapping. *Genetics* 178, 1745–1754.

Su, H., D. Golldack, M. Katsuhara, C. Zhao, and H.J. Bohnert. 2001. Expression and stress-dependent induction of potassium channel transcripts in the common ice plant. *Plant Physiol.* 125, 604–614.

Wang, D. and M.C. Shannon. 1999. Emergence and seedling growth of soybean cultivars and maturity groups under salinity. *Plant and Soil* 214, 117–124.

Wang, J., P.E. McClean, R. Lee., R.J. Goos, and T. Helms. 2008. Association mapping of iron deWciency chlorosis loci in soybean (*Glycine max* L. Merr.) advanced breeding lines. *Theor. Appl. Genet.* 116, 777–787.

Wang, W., B. Vinocur, O. Shoseyov, and A. Altman. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *TRENDS Plant Sci.* 9, 244–252.

Wang, Y., and W.H. Wu. 2013. Potassium transport and signaling in higher plants. *Annu. Rev. Plant Biol.* 2013. 64, 451–76.

Warrence, N.J., J.W. Bauder, and K.E. Pearson. 2002. Basics of salinity and sodicity effects on soil physical properties. *Departement of Land Resources and Environmental Sciences, Montana State University-Bozeman, MT.*

Xu, D.H., and D. D. Tuyen. 2012. Genetic studies on saline and sodic tolerances in soybean. *Breeding Sci.* 61, 559–565.

Yan, S.P., Z.C. Tang, W.A. Su, and W.N. Sun. 2005. Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics 5,* 235–244

Yang J. and R.W. Blanchar. 1993. Differentiation chloride susceptibility in soybean cultivars. *Agron. J.* 85, 880–885.

Yu, J., G. Pressoir, W.H. Briggs, I. Vroh Bi, M. Yamasaki, J.F. Doebley, M.D. McMullen, B.S. Gaut, D.M. Nielsen, J.B Holland, S. Kresovich, and E.S. Buckler. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nat. Genet.* 38, 203–208.

Zhang X. K., Q.H., J.H. Cao, and B.J. 2011. Differential Cl^-/salt tolerance and NaCl-induced alternations of tissue and cellular ion fluxes in *Glycine max, Glycine soja* and their hybrid seedlings. *J. Agronomy and Crop Sci.* 197, 329–339.

Zhou, G.A., R.Z. Chang, and L.J. Qiu. 2010. Overexpression of soybean ubiquitin-conjugating enzyme gene GmUBC2 confers enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in *Arabidopsis*. *Plant Mol. Biol.* 72, 357–367.

Zhu, C., M. Core, E. Buckler, and J. Yu. 2008. Status and prospects of association mapping in plants. *The Plant Genome* 1, 5–20.

Table 1. The statistical models used for testing marker-trait associations in the population of 181 diverse soybean genotypes tolerant or sensitive to salt stress[†].

Model	Statistical models	Information related to the models
SFA[‡]	$y = X\alpha + \varepsilon$	y is related to *X*, without any correction
Q[§]	$y = X\alpha + Q\beta + \varepsilon$	y is related to *X*, with correction for Q
PCA[¶]	$y = X\alpha + P\beta + \varepsilon$	y is related to *X*, with correction for PCA[#]
K^L	$y = X\alpha + K^L v + \varepsilon$	y is related to *X*, with correction for K^L
K^R	$y = X\alpha + K^R v + \varepsilon$	y is related to *X*, with correction for K^R
$Q+K^L$	$y = X\alpha + Q\beta + K^L v + \varepsilon$	y is related to *X*, with correction for Q and K^L
$Q+K^R$	$y = X\alpha + Q\beta + K^R v + \varepsilon$	y is related to *X*, with correction for Q and K^R
$PCA+K^L$	$y = X\alpha + P\beta + K^L v + \varepsilon$	y is related to *X*, with correction for PCA and K^L
$PCA+K^R$	$y = X\alpha + P\beta + K^R v + \varepsilon$	y is related to *X*, with correction for PCA and K^R

† This table is modified from Mamidi *et al.* (2011).

‡ SFA, a single factor analysis of variance without considering population structure and kinship.

§ Q, population structure; five subpopulations were determined and implemented in the analysis.

¶ PCA, principal component analysis.

Five principal components (PCs) explaining 28.6% variance were used for analysis.

Table 2. Summary of 37,281 SNPs used for association analysis in the population of 181 diverse soybean genotypes tolerant or sensitive to salt stress. Only SNPs with a MAF higher than 5% and present in more than 75% of all genotypes were selected.

Chromosome	Linkage group	No. polymorphic SNPs	Physical distance (Mb)	Average distance (kb)
1	D1a	1511	55.9	37.0
2	D1b	2194	51.7	23.5
3	N	1583	47.8	30.2
4	C1	1665	49.2	29.6
5	A1	1640	41.9	25.6
6	C2	1835	50.7	27.6
7	M	1952	44.7	22.9
8	A2	2276	47.0	20.7
9	K	1783	46.8	26.3
10	O	1829	51.0	27.9
11	B1	1464	39.2	26.8
12	H	1279	40.1	31.4
13	F	2416	44.4	18.4
14	B2	1769	49.7	28.1
15	E	2152	50.9	23.7
16	J	1647	37.4	22.7
17	D2	1749	41.9	24.0
18	G	3260	62.3	19.1
19	L	1990	50.6	25.4
20	I	1237	46.8	37.8
unknown†	unknown	50		
Mean		1862	47.5	25.5
Total		37281		

† Unknown, the exact location of SNPs cannot be determined.

Table 3. Gene diversity, heterozygosity, and polymorphic information content (PIC) assessed by 37,281 informative SNPs in the population of 181 soybean genotypes tolerant or sensitive to salt stress.

Chromosome	No. polymorphic SNPs	Gene diversity			Heterozygosity			PIC		
		Mean	Min.†	Max.‡	Mean	Min.	Max.	Mean	Min.	Max.
1	1511	0.351	0.051	0.500	0.095	0.000	0.942	0.282	0.049	0.375
2	2194	0.359	0.050	0.500	0.086	0.000	0.936	0.286	0.049	0.375
3	1583	0.356	0.050	0.500	0.097	0.000	0.879	0.285	0.049	0.375
4	1665	0.359	0.050	0.500	0.085	0.000	0.914	0.286	0.049	0.375
5	1640	0.361	0.050	0.500	0.103	0.000	0.902	0.288	0.049	0.375
6	1835	0.344	0.050	0.500	0.092	0.000	0.926	0.276	0.049	0.375
7	1952	0.350	0.050	0.500	0.104	0.000	0.931	0.281	0.049	0.375
8	2276	0.338	0.050	0.500	0.087	0.000	0.929	0.272	0.049	0.375
9	1783	0.343	0.050	0.500	0.076	0.000	0.897	0.277	0.049	0.375
10	1829	0.316	0.056	0.500	0.082	0.000	0.947	0.258	0.054	0.375
11	1464	0.347	0.050	0.500	0.094	0.000	0.914	0.279	0.049	0.375
12	1279	0.349	0.050	0.500	0.082	0.000	0.868	0.279	0.049	0.375
13	2416	0.340	0.050	0.500	0.088	0.000	0.931	0.274	0.049	0.375
14	1769	0.370	0.050	0.500	0.102	0.000	0.923	0.294	0.049	0.375
15	2152	0.361	0.050	0.500	0.084	0.000	0.874	0.288	0.049	0.375
16	1647	0.344	0.050	0.500	0.088	0.000	0.919	0.277	0.049	0.375
17	1749	0.349	0.050	0.500	0.083	0.000	0.879	0.280	0.049	0.375
18	3260	0.359	0.050	0.500	0.102	0.000	0.887	0.287	0.049	0.375
19	1990	0.341	0.050	0.500	0.098	0.000	0.873	0.276	0.049	0.375
20	1237	0.345	0.050	0.500	0.088	0.000	0.863	0.277	0.049	0.375
unknown§	50	0.312	0.073	0.500	0.080	0.000	0.617	0.255	0.070	0.375
Overall mean	1862	0.349	0.050	0.500	0.091	0.000	0.947	0.280	0.049	0.375

† Min., minimum.

‡ Max., maximum.

§ Unknown, the exact location of SNPs cannot be determined.

Table 4. Average linkage disequilibrium (LD) decay distance (Mb) of 20 chromosomes for r^2 > 0.1 and 0.2 in the population of 181 diverse soybean genotypes tolerant or sensitive to salt stress.

Chromosome	LD decay distance (Mb)	
	$r^{2\dagger}$ > 0.1	r^2 > 0.2
1	1.2	0.5
2	1.1	0.4
3	0.5	0.2
4	1.1	0.6
5	1.1	0.5
6	0.9	0.3
7	1.5	0.5
8	2.9	0.8
9	1.2	0.4
10	0.9	0.3
11	0.6	0.2
12	1.1	0.4
13	1.3	0.3
14	2.6	0.3
15	1.9	1.0
16	0.7	0.3
17	0.9	0.3
18	3.2	1.0
19	2.9	1.3
20	0.9	0.3
Average	1.4	0.5

† r^2, the estimated value of LD as the squared allele frequency correlation among loci.

Table 5. Test statistics for the nine models for association analysis in the population of 181 soybean genotypes tolerant or sensitive to salt stress.

Model	No. of P-values <0.05	No. of P-values <0.01	No. of P-values <0.001	MSD†
SFA	5975	2225	535	1.61E-02
Q‡	2589	867	182	3.65E-04
PCA§	2671	764	166	2.08E-04
K^R	2366	622	120	5.70E-05
K^L	1856	385	79	2.06E-04
$Q+K^R$	2115	530	86	2.61E-05
$Q+K^L$	1902	433	70	1.22E-04
$PCA+K^R$	1868	466	80	7.29E-05
$PCA+K^L$	1818	431	62	9.64E-05

† MSD, mean square deviation.

‡ Q, population structure inferred by STRUCTURE software.

§ PCA, principal component analysis.

Table 6. Statistical summary of single nucleotide polymorphisms (SNPs) significantly associated with salt tolerance in the population of 181 soybean genotypes tolerant or sensitive to salt stress.

SNP	Chr.[†]	Position (bp)	-log10 (P)	R^2 (%)	Minor allele freq.	Tol.[‡] allele	Sen.[§] allele	Tol. allele mean	Sen. allele mean	Diff.[¶]
Gm02_11494870_T_C	2	11494870	3.70	8.4	0.13	C	T	3.0	4.0	1.0
Gm02_11495939_C_T	2	11495939	3.70	8.4	0.13	T	C	3.0	4.0	1.0
Gm02_11698384_C_A	2	11698384	3.81	8.7	0.12	C	A	3.0	3.9	0.9
Gm03_38172118_A_G	3	38172118	4.25	11.0	0.37	A	G	2.5	3.4	1.0
Gm03_39868800_T_C	3	39868800	3.73	9.0	0.31	C	T	2.4	3.4	1.1
Gm03_39912307_T_G	3	39912307	3.73	9.0	0.31	G	T	2.4	3.4	1.1
Gm03_39915523_C_T	3	39915523	3.97	9.8	0.30	T	C	2.4	3.4	1.1
Gm03_40018337_C_T	3	40018337	4.12	10.1	0.31	T	C	2.4	3.5	1.1
Gm03_40019280_A_G	3	40019280	4.11	10.2	0.32	G	A	2.4	3.5	1.1
Gm03_40052612_T_C	3	40052612	3.98	9.7	0.37	C	T	2.4	3.5	1.1
Gm03_40060562_C_T	3	40060562	3.90	9.7	0.34	T	C	2.4	3.5	1.1
Gm03_40062964_C_T	3	40062964	3.88	9.6	0.36	T	C	2.4	3.5	1.1
Gm03_40153205_T_C	3	40153205	8.74	25.6	0.45	C	T	2.3	3.8	1.5
Gm03_40154304_A_G	3	40154304	8.74	25.6	0.45	G	A	2.3	3.8	1.5
Gm03_40155554_T_G	3	40155554	8.67	28.2	0.41	G	T	2.2	3.8	1.6
Gm03_40197155_A_C	3	40197155	7.29	20.9	0.40	C	A	2.5	4.0	1.5
Gm03_40278033_G_A	3	40278033	3.81	9.3	0.36	A	G	2.4	3.5	1.1
Gm03_40421296_A_G	3	40421296	4.15	10.0	0.16	G	A	2.9	4.2	1.3
Gm03_40452899_C_A	3	40452899	7.19	20.2	0.35	A	C	2.6	4.1	1.5
Gm03_40466433_C_T	3	40466433	8.68	27.2	0.46	T	C	2.3	4.0	1.7
Gm03_40467180_G_A	3	40467180	9.08	27.5	0.45	A	G	2.3	4.0	1.7

Table 6. *continued.*

SNP	Chr.	Position (bp)	-log10 (P)	R^2 (%)	Minor allele freq.	Tol. allele	Sen. allele	Tol. allele mean	Sen. allele mean	Diff.
Gm03_40494556_A_G	3	40494556	13.35	43.5	0.44	G	A	2.1	4.0	1.9
Gm03_40516071_A_G	3	40516071	11.25	35.2	0.42	G	A	2.3	4.1	1.8
Gm03_40546463_C_A	3	40546463	12.87	41.5	0.49	A	C	2.3	4.1	1.8
Gm03_40557941_A_G	3	40557941	12.79	40.5	0.48	G	A	2.3	4.1	1.8
Gm03_40565405_G_A	3	40565405	15.34	50.9	0.47	A	G	2.1	4.1	2.0
Gm03_40584028_A_G	3	40584028	13.37	43.5	0.48	G	A	2.2	4.1	1.9
Gm03_40585266_G_A	3	40585266	13.14	41.6	0.49	A	G	2.3	4.3	1.8
Gm03_40593882_G_A	3	40593882	15.82	54.1	0.47	A	G	2.0	4.1	2.1
Gm03_40597392_C_T	3	40597392	5.44	14.6	0.25	T	C	2.8	4.0	1.2
Gm03_40600088_A_G	3	40600088	5.03	13.5	0.24	G	A	2.8	4.0	1.2
Gm03_40602759_G_A	3	40602759	4.06	10.0	0.15	A	G	2.9	4.2	1.3
Gm03_40606894_T_C	3	40606894	15.82	54.1	0.47	C	T	2.0	4.1	2.1
Gm03_40613405_T_C	3	40613405	6.70	18.3	0.29	C	T	2.6	4.1	1.5
Gm03_40656449_A_G	3	40656449	9.45	29.5	0.50	G	A	2.3	4.0	1.7
Gm03_40661459_A_G	3	40661459	11.30	35.9	0.49	G	A	2.2	3.9	1.7
Gm03_40663609_G_A	3	40663609	9.23	28.4	0.49	A	G	2.3	4.0	1.7
Gm03_40677040_G_A	3	40677040	4.58	11.6	0.16	A	G	2.9	4.2	1.3
Gm03_40699200_G_T	3	40699200	4.36	11.0	0.32	T	G	2.7	3.9	1.2
Gm03_40700660_T_C	3	40700660	4.48	11.5	0.33	C	T	2.7	3.9	1.2
Gm03_40740328_T_G	3	40740328	6.32	16.8	0.19	G	T	2.8	4.3	1.5
Gm03_40768058_C_A	3	40768058	6.58	18.3	0.21	A	C	2.8	4.3	1.5
Gm03_40800808_A_G	3	40800808	4.36	10.6	0.13	G	A	2.9	4.3	1.4
Gm03_40895159_T_C	3	40895159	4.18	10.7	0.30	T	C	2.7	3.9	1.2

Table 6. *continued.*

SNP	Chr.	Position (bp)	-log10 (P)	R^2 (%)	Minor allele freq.	Tol. allele	Sen. allele	Tol. allele mean	Sen. allele mean	Diff.
Gm03_41100337_C_T	3	41100337	4.35	10.4	0.26	T	C	2.3	3.4	1.1
Gm03_41194560_G_A	3	41194560	4.33	10.8	0.24	A	G	2.2	3.4	1.2
Gm03_41205784_A_G	3	41205784	4.22	10.1	0.24	G	A	2.2	3.4	1.2
Gm03_41206640_A_G	3	41206640	4.37	10.8	0.24	G	A	2.2	3.4	1.2
Gm03_41212195_C_T	3	41212195	4.10	9.8	0.22	T	C	2.2	3.4	1.2
Gm03_41234338_T_C	3	41234338	4.32	10.8	0.24	C	T	2.2	3.4	1.2
Gm03_41236752_T_C	3	41236752	4.25	10.1	0.23	C	T	2.2	3.4	1.2
Gm03_41245330_C_T	3	41245330	4.02	9.5	0.23	T	C	2.2	3.4	1.2
Gm03_41258466_T_C	3	41258466	6.27	17.8	0.30	C	T	2.7	4.1	1.4
Gm03_41301876_A_C	3	41301876	5.69	17.1	0.33	C	A	2.3	3.6	1.3
Gm03_44583239_A_C	3	44583239	4.04	10.3	0.30	C	A	2.8	4.0	1.2
Gm05_9081936_A_G	5	9081936	3.51	7.9	0.15	G	A	2.4	3.2	0.8
Gm06_8039368_C_T	6	8039368	3.64	8.9	0.29	T	C	2.6	3.4	0.8
Gm08_18707057_T_C	8	18707057	3.45	7.6	0.09	C	T	2.0	3.2	1.2
Gm08_18772502_C_T	8	18772502	3.45	7.6	0.09	T	C	2.0	3.2	1.2
Gm18_56568068_C_T	18	56568068	3.08	7.0	0.46	T	C	2.8	3.5	0.7
Gm18_56571714_C_T	18	56571714	3.10	7.1	0.45	T	C	2.8	3.5	0.7
Gm18_58234790_A_G	18	58234790	3.25	7.4	0.11	G	A	3.0	4.1	1.1

† Chr., chromosome.

‡ Tol., tolerance.

§ Sen., sensitive.

¶ Diff., the difference between major allele mean and minor allele mean.

Table 7. Genes mapped at or near the significant SNPs and potentially involved in ion homeostasis and salt stress responses.

SNP	Chr.†	Position (bp)	R^2 (%)	Gm gene model‡	Start of model (bp)	End of model (bp)	Distance from SNP (bp)	Gene annotations§
Gm03_40154304_A_G	3	40154304	25.6	Glyma03g32410	40153390	40163904	0¶	Pre-mRNA processing factor 39-like
Gm03_40557941_A_G	3	40557941	40.5	Glyma03g32800	40556875	40561932	0	26S proteasome regulatory subunit
Gm03_40585266_G_A	3	40585266	41.6	Glyma03g32850	40584885	40588047	0	Heat shock protein 70 kda
Gm03_40613405_T_C	3	40613405	18.3	Glyma03g32890	40613121	40618246	0	K^+/H^+ antiporter
Gm03_40613405_T_C	3	40613405	18.3	Glyma03g32900	40623066	40634673	9661	K^+/H^+ antiporter
Gm03_40661459_A_G	3	40661459	35.9	Glyma03g32940	40656951	40662253	0	UNC93-like protein
Gm03_40895159_T_C	3	40895159	10.7	Glyma03g33240	40885110	40891987	3172	Ca^{2+}-transporting ATPase
Gm05_9081936_A_G	5	9081936	7.9	Glyma05g09310	9044232	9047185	34751	Pyruvate kinase
Gm08_18772502_C_T	8	18772502	7.6	Glyma08g24580	18739243	18745823	26679	Ca^{2+}-transporting ATPase
Gm18_56571714_C_T	18	56571714	7.1	Glyma18g46940	56589214	56592077	17500	Ubiquitin-conjugating enzyme E2 5-like

† Chr., chromosome.

‡ The Gm models refer to the Glyma1.1 gene models derived from the SoyBase (http://www.soybase.org).

§ The annotations derive from the SoyBase (http://www.soybase.org).

¶ The distance for the SNP marker within the model is set as zero.

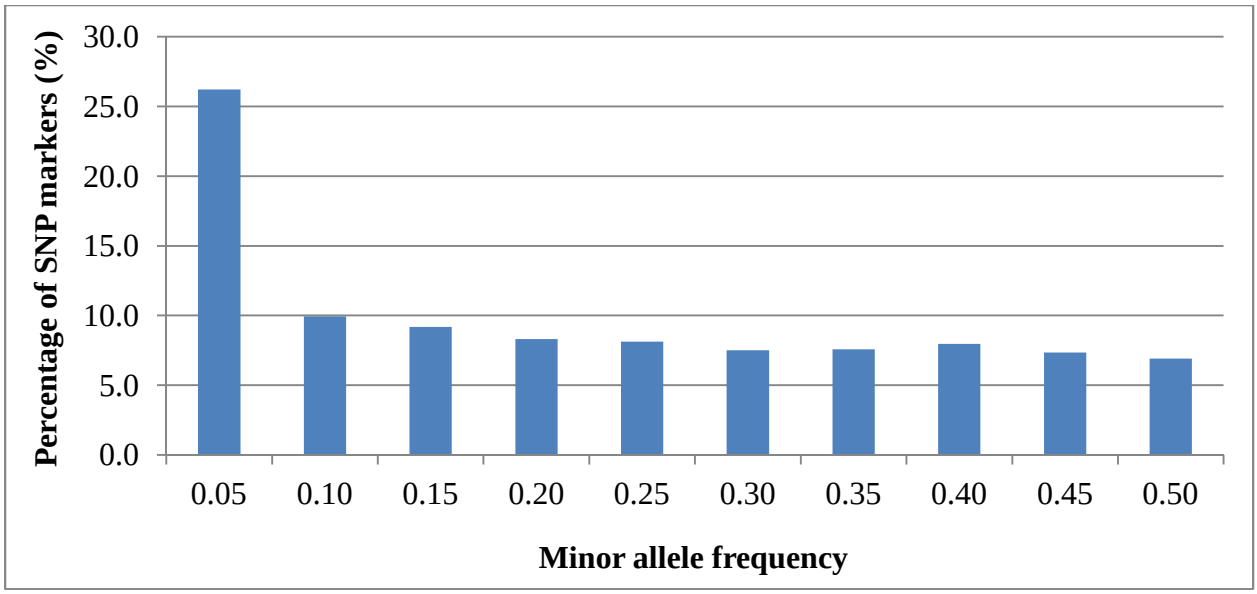

Figure 1. Distribution of minor allele frequencies (MAF) of 52,041 SNPs in the population of 181 diverse soybean genotypes tolerant or sensitive to salt stress.

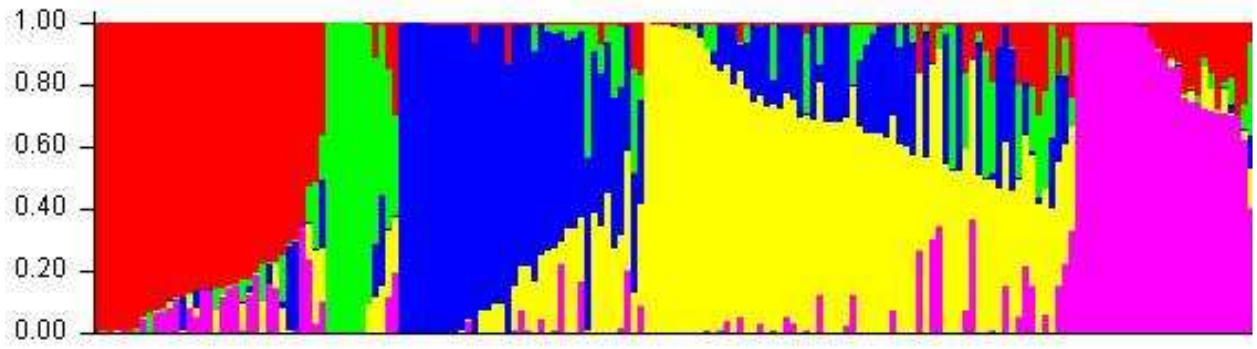

Figure 2. Bar plots by STRUCTURE analysis showing five subpopulations of 181 diverse soybean genotypes tolerant or sensitive to salt stress. Each vertical bar represents a specific genotype. The vertical coordinate of each subpopulation indicates the membership coefficients for each genotype. Colors represent the assigned subpopulations: red zone = Subpopulation 1; green zone = Subpopulation 2; blue zone = Subpopulation 3; yellow zone = Subpopulation 4; and pink zone = Subpopulation 5.

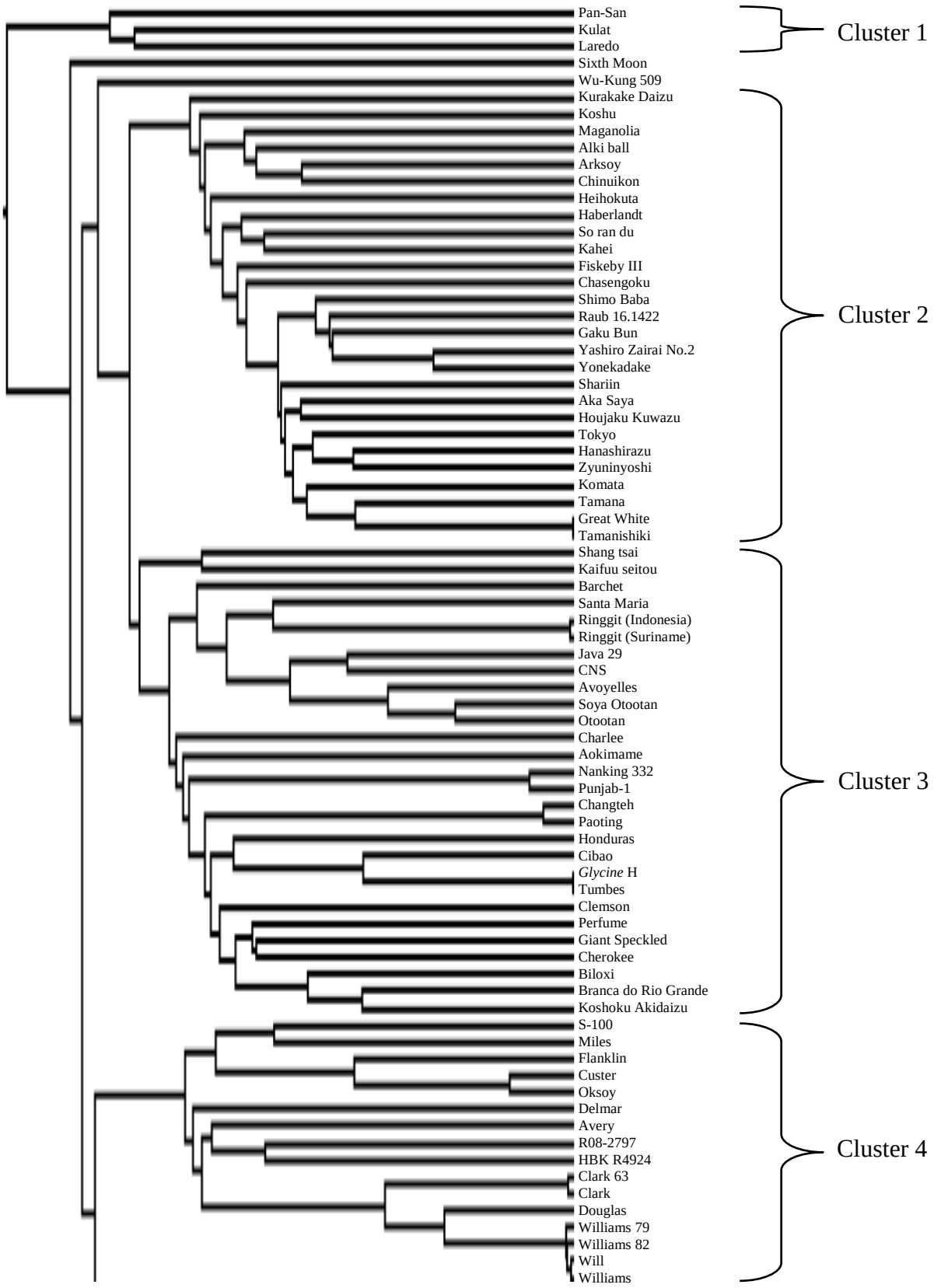

Figure 3. Dendrogram grouping of 181 diverse soybean genotypes tolerant or sensitive to salt stress into five clusters by distance-based UPGMA cluster analysis (*continued on next page*).

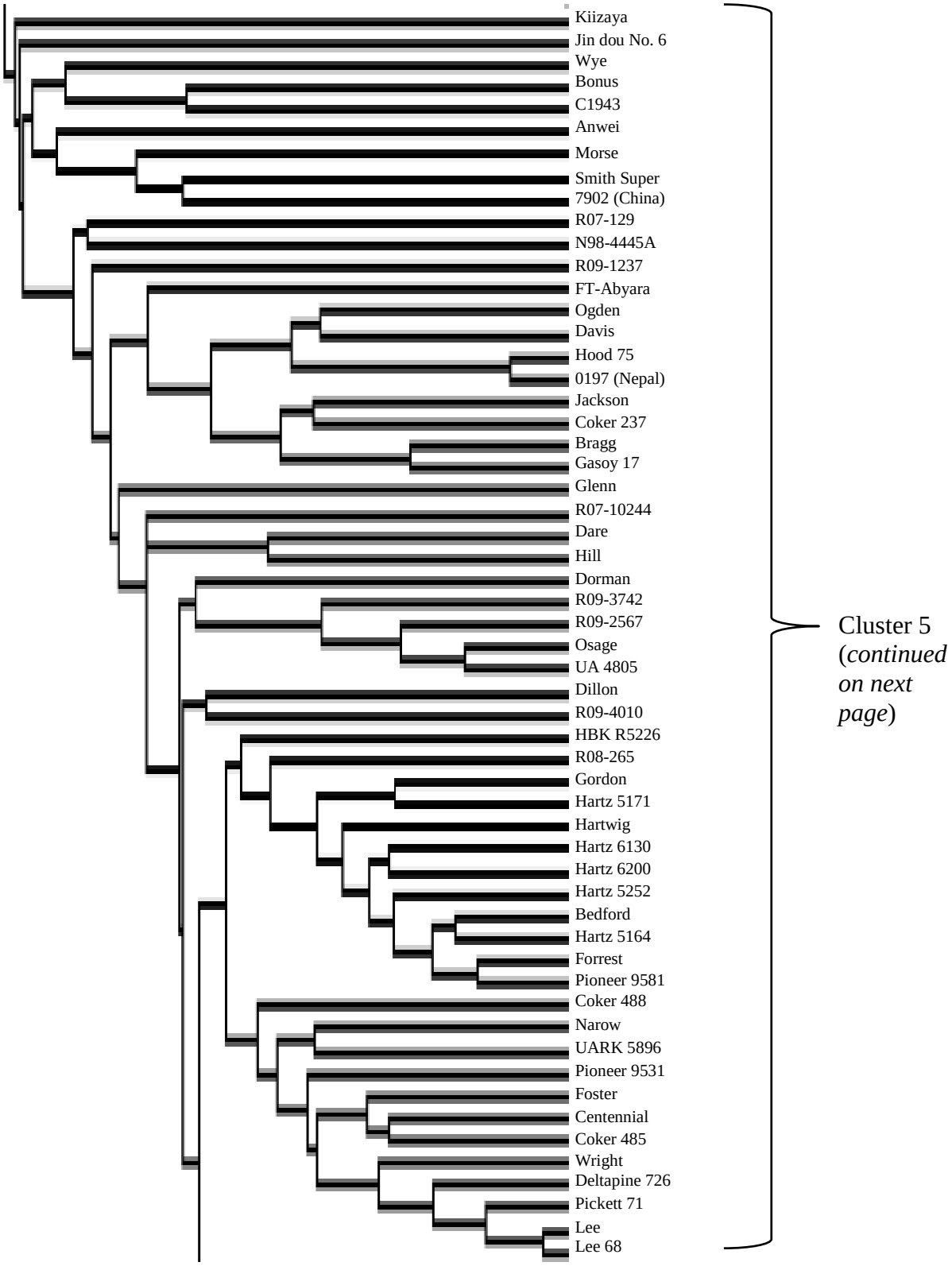

Figure 3. Dendrogram grouping of 181 diverse soybean genotypes tolerant or sensitive to salt stress into five clusters by distance-based UPGMA cluster analysis (*continued on next page*).

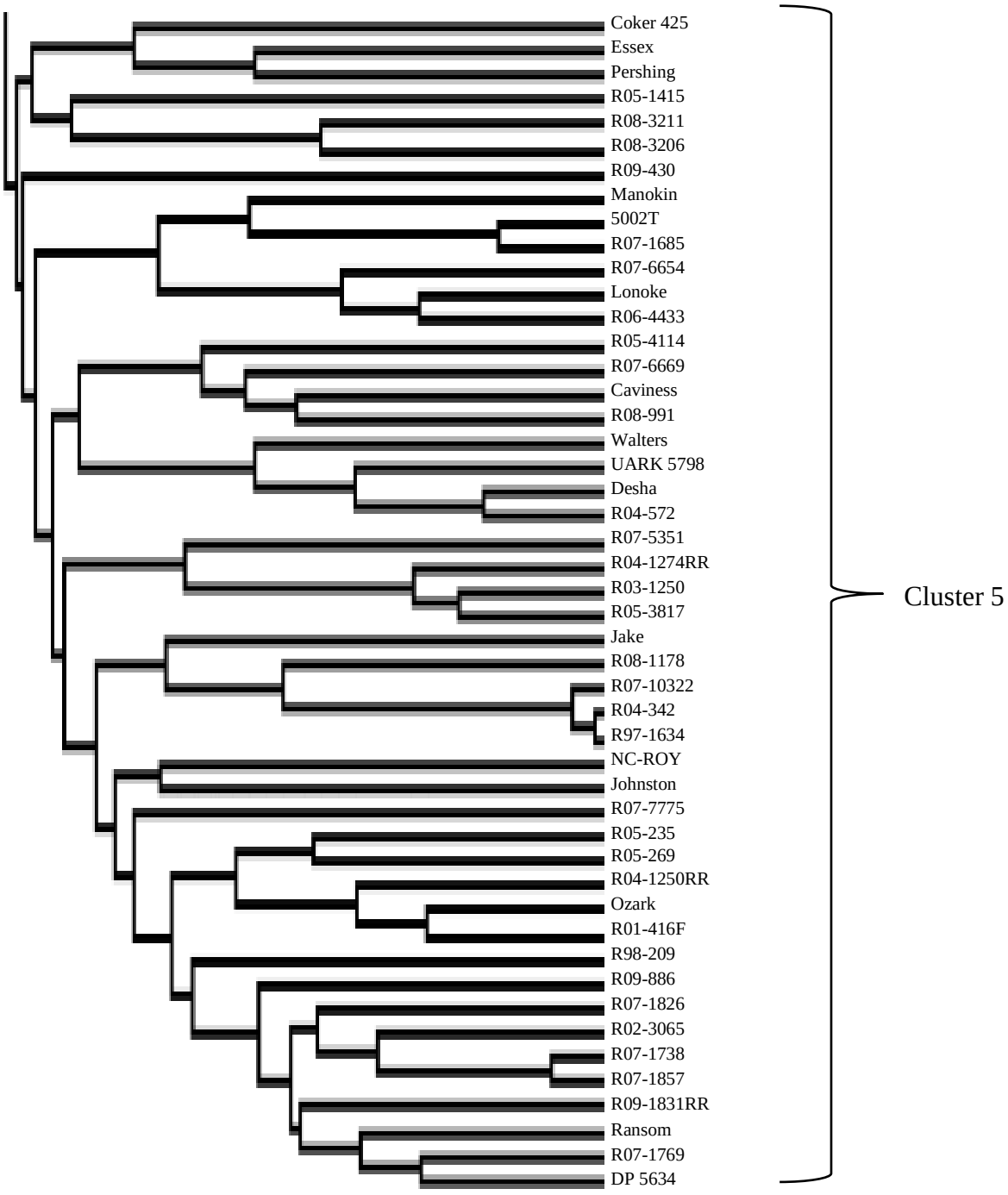

Figure 3. Dendrogram grouping of 181 diverse soybean genotypes tolerant or sensitive to salt stress into five clusters by distance-based UPGMA cluster analysis.

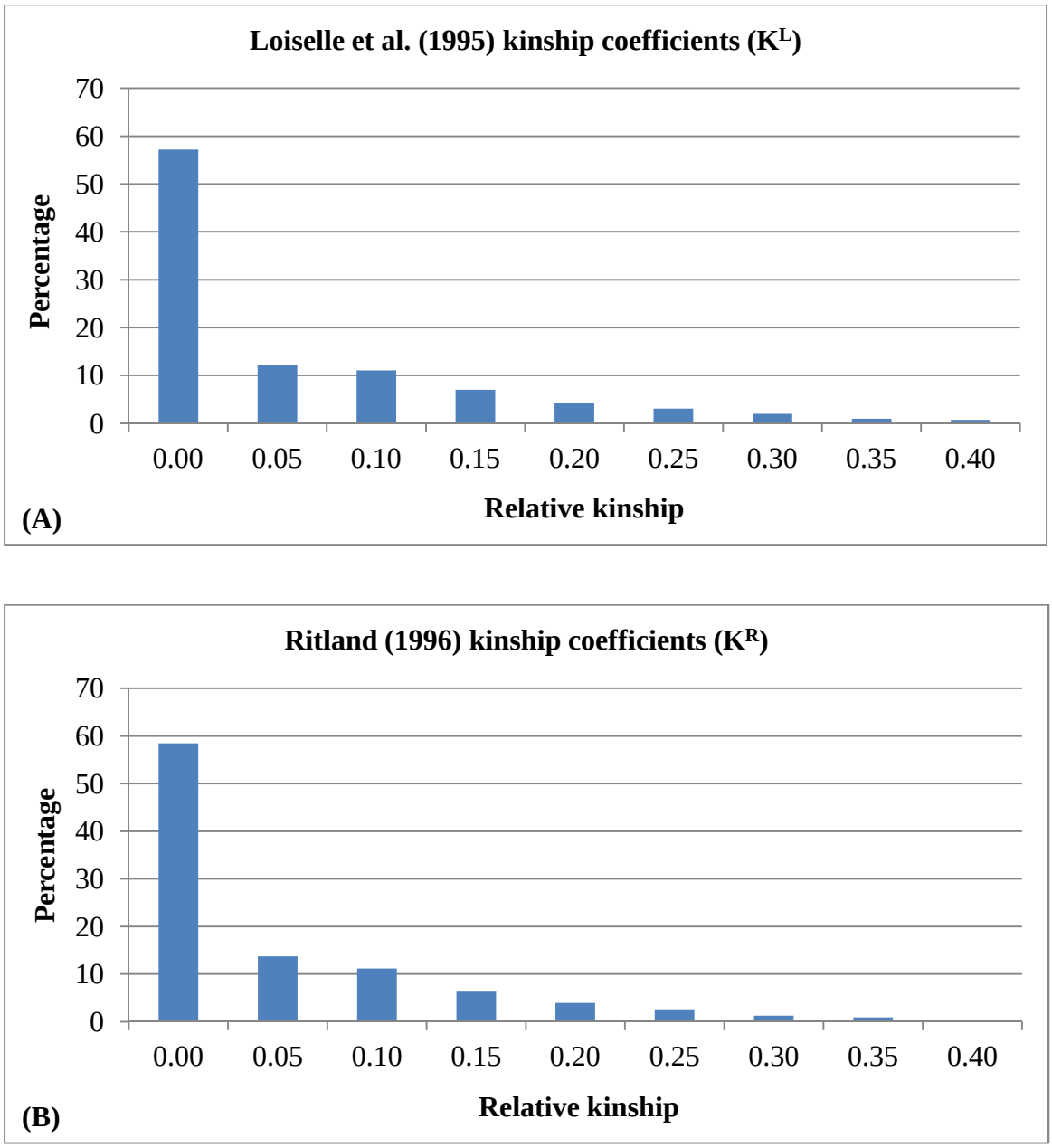

Figure 4. The distributions of pairwise kinship coefficients of 181 soybean genotypes with tolerant or sensitive responses to salt stress, K^L and K^R, described by Loiselle *et al.* (1995) (**A**) and Ritland (1996) (**B**), respectively. Values greater than 0.5 are not shown, and account for only 1.5% and 1.4% of the (**A**) and (**B**) distributions, respectively.

CHAPTER IV

EXPRESSION ANALYSIS OF SALT-RESPONSIVE GENES IN SOYBEAN UNDER SALT STRESS

ABSTRACT

Understanding the salt tolerance mechanisms in soybean has remained as one of the key challenges to facilitate the development of salt-tolerant soybean cultivars. The objective of this study was to identify candidate salt tolerance genes in soybean through gene expression analysis. Quantitative real-time (RT) PCR was performed to comparatively examine the expression of two salt-responsive genes in the first trifoliate leaves of salt-tolerant and salt-sensitive cultivars under salt stress. The GmUBC2, an ubiquitin-conjugating enzyme gene, and GmNHX1, a vacuolar Na^+/H^+ antiporter gene, were both up-regulated in salt-tolerant (Lee 68 and S-100) and sensitive cultivars (Dare and Glenn). However, the GmUBC2 expression was higher in salt-tolerant cultivars than in salt-sensitive cultivars. Dare exhibited a higher level of GmNHX1 expression in leaves than the other three genotypes. The up-regulation and differential expression of GmUBC2 and GmNHX1 among salt-tolerant and salt-sensitive genotypes suggested their potential roles in conferring salt tolerance in soybean.

INTRODUCTION

Extensive studies have been focused on the mechanisms of salt tolerance in plants, which involves many genes, given its complexity. One of the key mechanisms is to maintain ion homeostasis by restricting the accumulation of toxic Na^+, which affects the dynamic equilibrium of other essential ions like Ca^{2+} and K^+ (Tester and Davenport, 2003). Na^+/H^+ antiporters mediate Na^+ exclusion and ion homeostasis through two major processes: Na^+ extrusion from cells and Na^+ compartmentation into vacuoles (Ji *et al.*, 2013).

The plasma membrane Na^+/H^+ antiporters extrude Na^+, and restrict the vascular long-distance Na^+ transfer from roots to shoots, protecting photo-synthetically active tissues from excessive Na^+ (Shi *et al.*, 2002). The vacuolar Na^+/H^+ antiporters function in compartmentation of cytosolic Na^+ into vacuoles. Na^+ compartmentation not only reduces the Na^+ toxicity in cytoplasm, but also allows plants to use vacuolar Na^+ as an osmoticum for maintenance of osmotic potential in cells. Overexpression of plasma membrane/vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*, Brassica, tomato, rice, and wheat all showed improved salt tolerance (Sun *et al.*, 2006). In soybean, the ability of Na^+ exclusion was also found to contribute to salt tolerance (Luo *et al.*, 2005b). A tonoplast Na^+/H^+ antiporter (GmNHX1) in soybean was induced by NaCl (Li *et al.*, 2006; Sun *et al.*, 2006). The salt-tolerant soybean cultivars showed a higher expression of GmNHX1 in roots and however, a lower expression in leaves than the salt-sensitive cultivars. A further study demonstrated a higher concentration of Na^+ in vacuoles of cells ectopically expressing GmNHX1, suggesting that GmNHX1 functions in compartmentation of toxic Na^+ into vacuoles (Li *et al.*, 2006).

Ubiquitin 26S system (UPS), which functions in intracellular protein degradation, also plays an important role in plant abiotic stress tolerance (Lyzenga and Stone, 2012). Ubiquitin

(Ub) is first activated by ubiquitin-activating enzyme (E1) and transferred to an ubiquitin-conjugating enzyme (Ubc or E2) to form an E2-Ub thiolester (Smalle and Vierstra *et al.*, 2004). The Ub is then delivered to the target protein either alone or in conjunction with an Ub ligase (Ubl or E3), and modifies the protein. This modified protein is subsequently degraded by the 26S proteasome (Smalle and Vierstra *et al.*, 2004). A soybean E2 gene GmUBC2 was recently identified (Zhou *et al.*, 2010). The GmUBC2 was induced by salt and drought stress, and involved in the regulation of ion homeostasis, osmolyte synthesis, oxidative stress, and abiotic stress responses. Overexpression of GmUBC in *Arabidopsis* led to improved salt and drought tolerance (Zhou *et al.*, 2010).

In this study, the expression of GmNHX1 and GmUBC2 were examined in both salt-tolerant and salt-sensitive cultivars under salt stress. The roles of these genes in salt tolerance in soybean were discussed.

MATERIALS AND METHODS

Plant Materials and Salt Treatment

Two salt-tolerant cultivars S-100 and Lee68 and two salt-sensitive cultivars Dare and Glenn were used in this study. 8 ~ 10 seeds were planted in a 3.5 inch pot (Plasticflowerpots.net, Lake Worth, FI) filled with loamy sand (Kibler, Arkansas) with a proportion of paper towel (8 cm × 8 cm) underneath. Soil particle analysis based on a 2-hour hydrometer method described by Arshad *et al.* (1996) showed that the loamy sand consists of 83.5% sand, 11.0% clay and 5.5% silt. Pots were placed in trays (10 1/2" x 21" x 2 1/2", Plasticflowerpots.net, Lake Worth, FI) for the purposes of watering and salt treatment. The temperature was set at 25 ± 2 °C with a 14h photoperiod in the greenhouse, and fertilized once per week by Miracle-Gro® All Purpose Plant

Food (The Scotts Miracle-Gro Company, Marysville, Ohio) according to the manufacturer's instruction. The plant fertilizer contains 24% N, 8% P_2O_5, 16% K_2O, 0.02% B, 0.07% Cu, 0.15% Fe, 0.05% Mn, 0.06% Zn, and 0.0005% Mo (The Scotts Miracle-Gro Company, Marysville, Ohio). At VC stage, each pot was tined to three plants. When the first trifoliate leaf expanded, the treatment plants were salt stressed by adding 10L of 250 mM NaCl solution to the trays. The solution covered one-second of pots in height, and reached the plants through the drainage holes. The control plants were watered using tap water in the same manner for the same period of time. The electrical conductivity (EC) of the NaCl solution and tap water was 26.5 dS/m and 0.17 dS/m, respectively. Three trifoliate leaves were collected individually as three replications at each of the four time points (0, 1, 6, and 24h) after initiation of salt stress. All leaf samples were immediately frozen in liquid nitrogen, and stored at -80 °C for subsequent RNA isolation.

RNA Isolation

Total RNA were extracted from all leaf samples using *TRIzol*® Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the optimized manufacturer's protocol. RNA was then treated by the Ambion® DNA-*free*™ DNase Treatment & Removal Reagents (Invitrogen Life Technologies, Carlsbad, CA) to remove the contaminating DNA. The concentration of resulting DNA-free RNA was determined by the measured absorbance at 260 nm through a Nanodrop spectrophotometer. RNA was then stored at -80 ℃.

Quantitative Real-Time (RT) PCR

Two genes, GmNHX1 (a vacuolar Na^+/H^+ antiporter) and GmUBC2 (an ubiquitin-conjugating enzyme gene), were selected for expression analysis (Table 1). Primers derived from the references shown in Table 1. The quantitative real time RT-PCR (qPCR) reactions were conducted to examine the expression level of the candidate genes using the SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR green (Invitrogen, Valencia, CA) according to the manufacturer's protocols. The soybean actin gene ACT11 was used as internal control to normalize the expression value of each reaction (Table 1). Melting curve and gel electrophoresis analysis of amplification products were conducted to examine the specificity of the reactions. The relative expression of genes was calculated according to the method described by Livak and Schmittgen (2001).

RESULTS AND DISCUSSION

Quantitative real-time (RT) PCR was performed to measure GmUBC2 mRNA levels in the first trifoliate leaves of salt-tolerant and-sensitive soybean cultivars under salt stress. GmUBC2 transcripts were detected in all cultivars for both control and treatment groups. At 1h, the expression level for all cultivars did not differ significantly between control and treatment groups (Figure 1). At 6h, the expression levels under treatment increased significantly compared to those of control groups in all cultivars. The salt-tolerant Lee 68 and salt-sensitive Glenn and Dare had an about 2-fold increase while the salt-tolerant S-100 had a 3-fold increase (Figure 1). At 24h, the expression levels in S-100, Glenn, and Dare under treatment did not change significantly in comparison to those at 6h while the level in Lee 68 increased by 3-fold compared to that at 6h (Figure 1). These results indicated that the expression of GmUBC2 is up-regulated

by salt stress in both salt-tolerant and salt-sensitive cultivars. When comparing the expression among cultivars, the levels did not differ significantly at 1h (Figure 3). However, at 6h, the expression level was significantly higher in the salt-tolerant S-100 than those in the others. The expression levels in the salt-tolerant S-100 and Lee 68 were significantly higher than those in the salt-sensitive Dare and Glenn at 24h (Figure 3). These results indicated that GmUBC2 is differentially expressed in leaves among different cultivars under salt stress, and generally salt-tolerant cultivars have a higher expression than salt-sensitive cultivars.

The expression of GmNHX1, a vacuolar Na^+/H^+ antiporter, were also examined by qRT-PCR. At 1h and 6h, the expression levels did not show significant differences between the control and treatment groups for all cultivars (Figure 2). However, at 24h, the expression of the treatment groups increased significantly compared to that of the control groups for all cultivars, among which S-100, Glenn, and Lee 68 had a 2.5- to 3-fold increase while Dare had a 5.5-fold increase (Figure 2). These results indicated that GmNHX1 is up-regulated after 24h by salt stress in both salt-tolerant and salt-sensitive cultivars. When comparing the expression among cultivars, the levels did not differ significantly at 1h and 6h (Figure 4). However, at 24h, the expression level in the salt-sensitive Dare was significantly higher than those in the others. These results indicated that GmNHX1 is differentially expressed among different cultivars under salt stress.

Ubiquitin 26S proteasome system (UPS), which involves a conjugation cascade consisting of the ubiquitin-activating enzyme (UBA; E1), ubiquitin-conjugating enzyme (UBC; E2), and ubiquitin ligase (E3), modulates the level of regulatory proteins and removes disorder or unfolded proteins that may accumulate due to abiotic stresses (Lyzenga and Stone, 2012). Extensive studies on the UPS have been mainly focused on the E3 enzymes while considerably

less is known about the E1 and E2 enzymes (Lyzenga and Stone, 2012). Recent studies on the E2 enzymes indicated the importance of these enzymes for plants under abiotic stresses (Wan *et al.*, 2010; Zhou *et al.*, 2010; Li and Schmidt, 2010; Lyzenga and Stone, 2012). A soybean E2 enzyme, GmUBC2, is up-regulated under salt and drought conditions (Zhou *et al.*, 2010). Overexpression of GmUBC2 enhanced the salt tolerance in *Arabidopsis* (Zhou *et al.*, 2010). In the present study, the expression level of GmUBC2 was compared between the salt-tolerant and salt-sensitive cultivars. In agreement with the results by Zhou *et al.* (2010), the GmUBC2 was up-regulated in all cultivars under salt stress. The salt-tolerant cultivars exhibited a higher expression, providing new evidence for the importance of the GmUBC2 in salt tolerance in soybean. Furthermore, another E2 enzyme gene identified and discussed in the association mapping study suggests that multiple E2 enzymes are involved in salt tolerance in soybean.

Under salt stress, soybean cultivars show different capacities of Na^+ exclusion from the shoots, despite of the prevailing notion that soybean possesses salt tolerance mainly by the mechanism of Cl^- exclusion. Luo *et al.* (2005b) and Lenis *et al.* (2011) reported less Na^+ accumulation in the shoots of salt-tolerant cultivars. Indeed, the salt-tolerant S-100 and Lee 68 accumulate a higher level of Na^+ in leaves than the salt-sensitive Dare under salt stress (Valencia *et al.*, 2008). Therefore, S-100 and Lee 68 may only require a lower ability of sequestering excessive Na^+ into vacuoles in leaves than Dare. In the present study, the lower expression of GmNHX1, which was implied to function in sequestration of Na^+ into vacuoles (Sun *et al.*, 2006), in S-100 and Lee 68 than in Dare, appears to explain the former findings. However, the other salt-sensitive Glenn did not show significant difference in GmNHX1 expression, compared to S-100 and Lee 68, suggesting that other mechanisms other than the sequestration of Na^+ also exist, and affect salt tolerance in soybean.

In summary, this study compared the expression levels of two candidate genes GmUBC2 and GmNHX1 between salt-tolerant and salt-sensitive cultivars under salt stress. As expected, both were up-regulated in all cultivars. However, the expression of GmUBC2 was higher in salt-tolerant genotypes than in salt-sensitive genotypes while the expression of GmNHX1 was higher in salt-sensitive Dare than in the others. The differential expression between salt-tolerant and salt-sensitive cultivars further suggested their important roles in salt tolerance in soybean.

REFERENCES

Hu, R., C. Fan, H. Li, Q. Zhang, and Y.F. Fu. 2009. Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *BMC Mol. Biol.* 10, 93.

Ji, H., J.M. Pardo, G. Batelli, M.J. Van Oosten, R.A. Bressan, and X. Li. 2013. The Salt Overly Sensitive (SOS) pathway: established and emerging roles. *Mol. Plant.* 6, 275–286.

Lenis, J.M., M. Ellersieck, D.G. Blevins, D.A. Sleper, H.T. Nguyen, D. Dunn, J.D. Lee, and J.G. Shannon. 2011. Differences in ion accumulation and salt tolerance among *Glycine* accessions. *J. Agron. Crop Sci.*197, 302–310.

Li, W.Y., F.L. Wong, S.N. Tsai, T.H. Phang TH, G. Shao and H.M. Lam. 2006b. Tonoplast-located *GmCLC1* and *GmNHX1* from soybean enhance NaCl tolerance in transgenic bright yellow (BY)-2 cells. *Plant Cell Environ.* 29, 1122–1137.

Livak, K. and T. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta delta CT method. *Methods* 25, 402–408.

Luo, Q., B. Yu and Y. Liu. 2005b. Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *J. Plant Physiol.* 162, 1003–1012.

Lyzenga, W.J., and S.L. Stone. 2012. Abiotic stress tolerance mediated by protein ubiquitination. *J. Exp. Bot.* 63, 599–616.

Shi, H., F.J. Quintero, J.M. Pardo and J.K. Zhu. 2002. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *Plant Cell* 14, 465–477.

Smalle, J., and R.D. Vierstra. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* 55, 555–590.

Sun, Y.X., D. Wang, Y.L. Bai, N.N. Wang and Y. Wang. 2006. Studies on the overexpression of the soybean *GmNHX1* in *Lotus corniculatus*: the reduced Na^+ level is the basis of the increased salt tolerance. *Chin. Sci. Bull.* 51, 1306–1315.

Table 1. Genes used in quantitative real-time (RT) PCR.

Genes	Description	Accessions	Primers	Reference
GmUBC2	An ubiquitin-conjugating enzyme gene	BT089210	Forward: ctcacatctatccagtcattgcttt Reverse: actaaacattcgagctgcttca	Sun *et al.* (2006)
GmNHX1	A vacuolar Na^+/H^+ antiporter gene	AY392759	Forward: tttggacctttgattcgttgcg Reverse: cgccatcaaacagaatcacagaag	Zhou *et al.* (2010)
ACT11†	Actin; A housekeeping cytoskeletal and structural gene	BW652479	Forward: cagagaaagtgcccaaatcatgt Reverse: ttgcatacaaggagagaacagctt	Hu *et al.* (2009)

† ACT11 was used as the internal control in qRT-PCR.

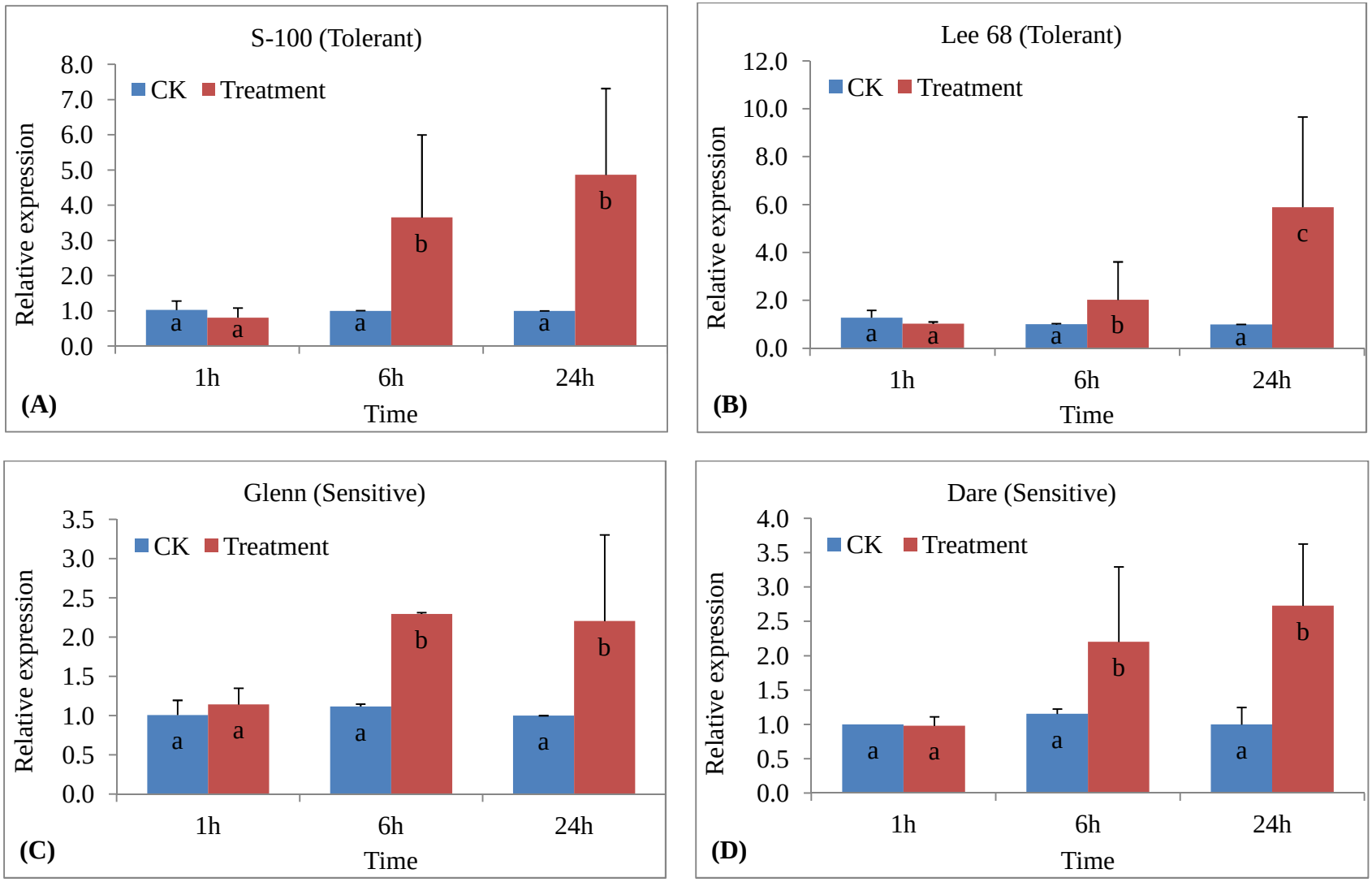

Figure 1. Relative expression of GmUBC2 in the first trifoliate leaves of the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**) and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. CK represents the untreated control. Error bars represent standard deviation (n=3).

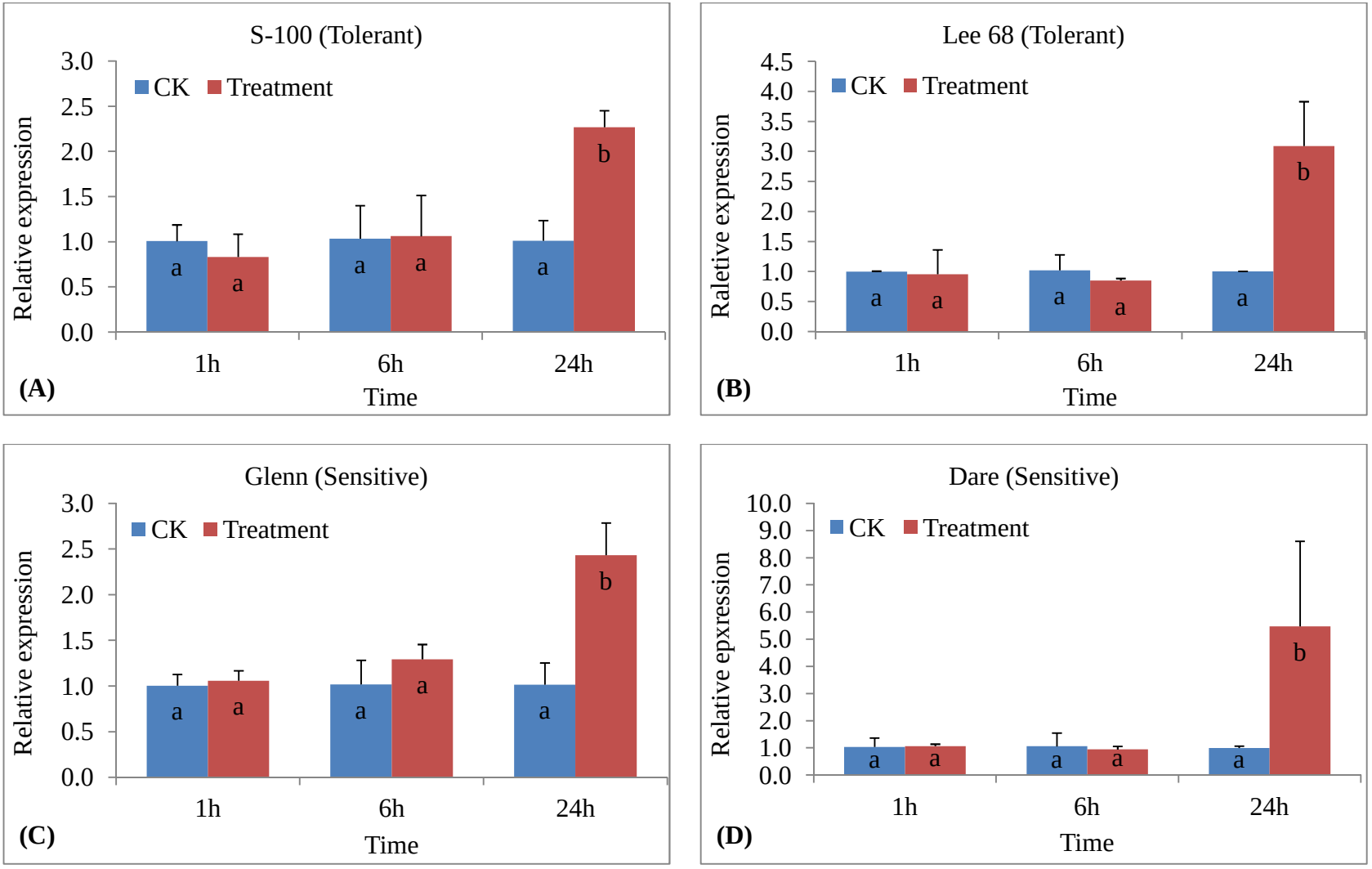

Figure 2. Relative expression of GmNHX1 in the first trifoliate leaves of the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**) and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. CK represents the untreated control. Error bars represent standard deviation (n=3).

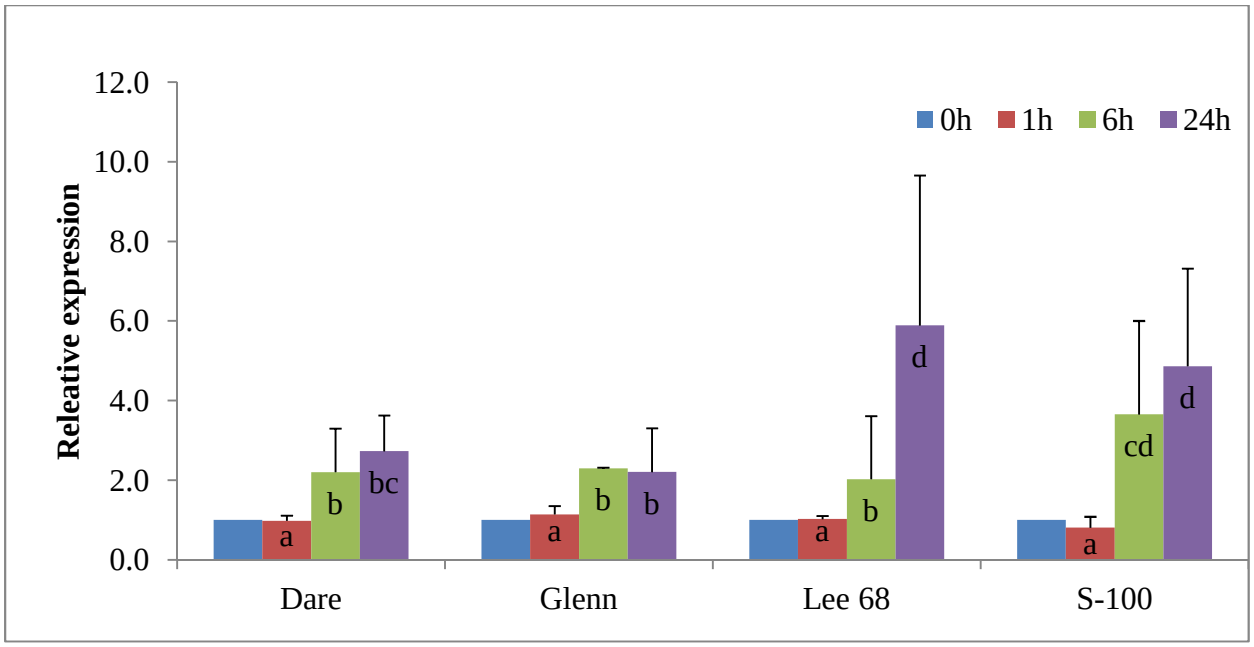

Figure 3. Comparative expression of GmUBC2 in the first trifoliate leaves between the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**), and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. Error bars represent standard deviation (n=3).

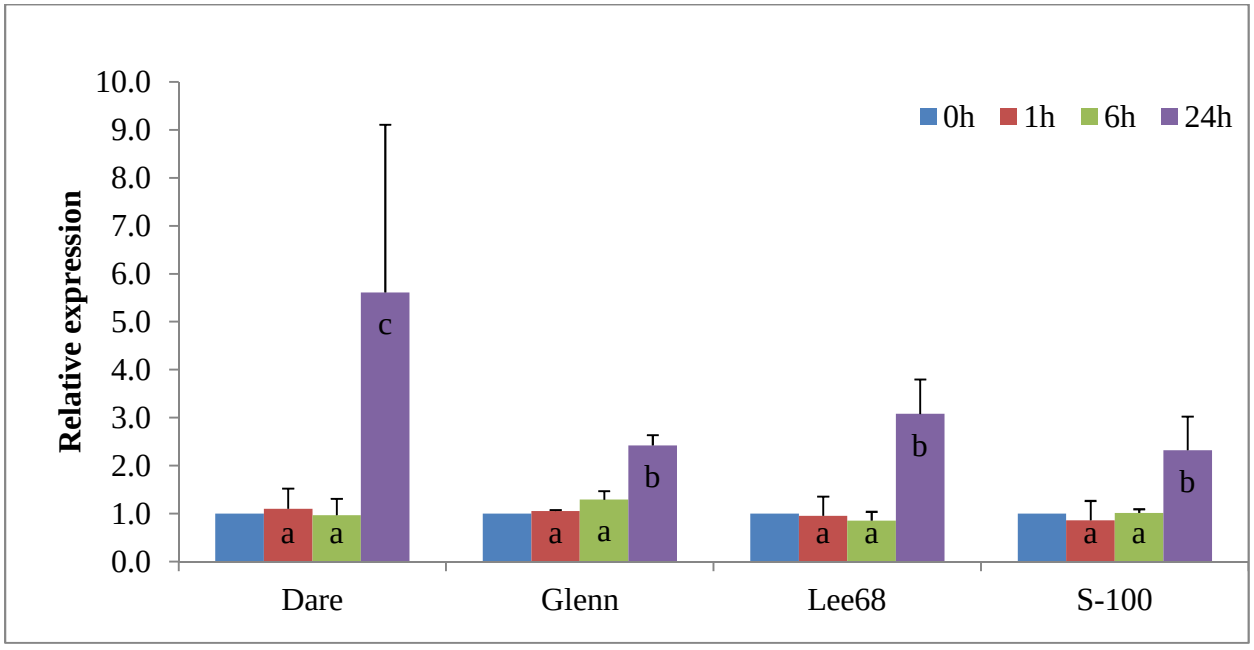

Figure 4. Comparative expression of GmNHX1 in the first trifoliate leaves between the salt-tolerant S-100 (**A**) and Lee 68 (**B**), and the salt-sensitive Glenn (**C**), and Dare (**D**) at 1, 6, and 24h under salt stress. The soybean ACT11 gene was used as internal control. Error bars represent standard deviation (n=3).

CHAPTER V

OVERALL CONCLUSIONS

Salt stress causes yield loss in soybean production. Development of salt-tolerant cultivars is highly desirable to minimize the adverse effect of salt stress. In this study, a total of 192 diverse soybean lines and cultivars were screened for salt tolerance in the greenhouse 94 genotypes were classified as salt-tolerant while 87 were salt-sensitive.

These genotypes, 181 in total, were further genotyped using the SoySNP50K iSelect BeadChip with 52,041 SNPs, from which 37,281 informative SNPs were selected for association analysis. After controlling the population structure and familial relatedness, and selecting the statistical models that minimized spurious marker-trait associations, the genome-wide association mapping revealed 62 significant SNPs representing six salt tolerance quantitative trait loci (QTLs) on Chromosomes 2, 3, 5, 6, 8, and 18 ($P < 0.001$). Among them, 25 SNPs spanning an interval of 0.51 Mb on Chromosome 3 explained the most variation, and indicated a major QTL previously identified in S-100 (Lee *et al.*, 2004) while three SNPs on Chromosome 18 are mapped near a salt tolerance QTL previously identified in Nannong1138-2 (Chen *et al.*, 2008). The other SNPs represent four putative minor QTLs newly identified in this study, which need further validations with different genetic background. According to the soybean reference genome and extensive knowledge and studies on plant stress responses in public, ten genes at or near (< 35 kb) the significant SNPs appear to be candidates involved in ion metabolisms and salt stress responses.

Gene expression analysis indicated that GmUBC2, an ubiquitin-conjugating enzyme, and GmNHX1, a vacuolar Na^+/H^+ antiporter, are both up-regulated in leaves of salt-tolerant (Lee 68 and S-100) and salt-sensitive genotypes (Dare and Glenn) under salt stress. However, the expression of GmUBC2 was higher in salt-tolerant genotypes than in salt-sensitive genotypes

while the expression of GmNHX1 was higher in salt-sensitive Dare than in the others. The differential expression among these cultivars further suggested their potential roles in conferring salt tolerance in soybean.