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Molecular and phenotypic comparisons of salt effects on soybean cultivars with differential chloride uptake capacities

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Molecular and phenotypic comparisons of salt effects on soybean cultivars with differential chloride uptake capacities

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ABSTRACT

Genetic manipulation of crop plants, through breeding or transgenic approaches, for enhanced tolerance to abiotic stress holds great promise for improving yields and promoting new methods for sustainable agriculture. This study examines the potential role that genes of the soybean, *Glycine max* L., encoding elongation factor-1 alpha (*EF-1*α) and glyoxalase I (*GlxI*) might play in response to salt stress. Previous reports have suggested a possible function for both *GlxI* and *EF-1*α in conferring enhanced salt tolerance in other plant species. In addition to other possible mechanisms, salt tolerance in soybeans can be regulated by plant uptake and transport of chloride ions. Soybean lines that transfer chloride to their foliage from the soil are termed "includers" and are considered to be more susceptible to salt stress than their counterparts, "excluders" that do not transport chloride into their leaves. We used chloride "includers", cv. Clark and Dare, and "excluders", cv. Lee68 and S100, to compare gene expression responses and plant susceptibility to chloride salts. Mineral analysis of Clark and Lee68 cultivars by inductively coupled plasma mass spectrometry was performed to verify the differences in chloride uptake. In an optimized greenhouse screening procedure, the excluder cv. Lee68 demonstrated fewer visual symptoms of salt stress when treated with the same salt concentrations as the includer, cv. Clark. RNA blots showed the soybean genes encoding *EF-1*α and I *GlxI* were equally induced in both includers and excluders following treatment with NaCl or $CaCl_2$. Although transcript levels for *EF-1*α and *GlxI* are induced by salt treatments, transcript profiles do not differ between salt-tolerant and susceptible soybean cultivars. This suggests that the cultivars respond to salt stress in similar ways, but that these genes are not responsible for the differential phenotypes.

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MEET THE STUDENT-AUTHOR

Sharon Faye Holifield

After I graduated from Mountain Home High School in 2007, I began my studies in Environmental, Soil, and Water Science at the University of Arkansas.

In my sophomore year, I took Biotechnology in Agriculture, taught by Dr. Ken Korth and immediately became interested in the work in his laboratory. Since working in Dr. Korth's lab, I have received awards from both the American Society of Plant Biologists and the Student Undergraduate Research Fellowship. I am also a member of the Arkansas Native Plant Society and the university's Crop, Soil, and Environmental Sciences Club.

I have enjoyed studying new techniques that improve the way that we use and take care of our environment. I will begin working on my master's degree in crop, soil and environmental sciences at the University of Arkansas in the summer of 2011.

INTRODUCTION

Salinity is an ever-present threat to crop yields, especially in countries where irrigation is an essential aid to agriculture. As global population increases, farmers will be forced to make use of marginal lands not suitable for today's food crops. The increase in land use for food production will also have a major impact on the environment and wildlife. Genetic manipulation of crop plants, whether through breeding or transgenic approaches, for enhanced abiotic stress tolerance holds great promise for improving yields in marginal lands and promoting new methods for sustainable agriculture.

Soybean, *Glycine max* L., is a legume that is an important staple crop grown worldwide, and susceptibility to abiotic stresses can be a factor in limiting overall production. One approach to generating plant lines that are more resistant to environmental stresses depends on understanding the plant's natural means of tolerance to stressful conditions. There are several possible mechanisms of salt tolerance in soybeans, including metabolic pathways and gene products that respond to environmental stresses (Phang et al., 2008). Salt sensitivity in soybean is often correlated with accumulation of chloride in the foliar tissues. Prevention of chloride transport from soil via the roots to foliar tissues has been shown to play a major role in salt tolerance (Luo et al., 2005; Valencia et al., 2008). Cultivars that have been characterized previously as either chloride includers or chloride excluders, which differ in systemic transport of the chloride ion, can be useful tools in breeding programs and in the study of mechanisms of salt tolerance (Table 1). Chloride includers transport the chloride ion from saline waters into leaves where it accumulates and damages the foliar tissue. Excluders take up chloride in roots, but the ion is not transported to foliar tissues (Figs. 1 and 2).

It is likely that multiple independent stress response pathways, and the genes that control them, are involved in plant strategies to cope with salt stress. In addition to chloride toxicity as a major factor in soybean, salt sensitivity in the wild species *G. soja* can be due to sodium accumulation in foliar tissues (Luo et al., 2005).

One approach to identifying genes that are employed by the plant to combat abiotic stresses is to seek out genes expressed at higher levels during a controlled stress treatment. In an attempt to identify such stress-induced genes, we searched an online soybean expressed sequence tag (EST) database for potential salt-induced genes and found ESTs encoding a putative *G. max* elongation factor-1 alpha (*GmElf1-*α) protein and a gene encoding a putative glyoxalase I (*GmGlxI*). Elongation factor-1 alpha (*EF-1*α), an essential component of the eukaryotic translational apparatus, is a GTP-binding protein that catalyses the binding of aminoacyl-transfer RNAs to the ribosome. It also may protect cellular proteins under salt-stress conditions in plants and yeasts by promoting the correct refolding of unfolded proteins *in vivo* (Shin et al., 2009). The glyoxalase system is comprised of two enzymes, glyoxalase I and II, with reduced glutathione as a catalyst (Fig. 3). Methylglyoxal, the primary physiological substrate for the glyoxalase-I reaction, is a potent cytotoxic compound that is produced as a byproduct of glycolysis, and can also be de-

rived from fatty acid metabolism or threonine degradation. Transgenic approaches with *GmEf-1α* and *GlxI* have been used previously to show that overexpression of these gene products can confer enhanced salt tolerance in *Arabidopsis* and tobacco plants, respectively (Shin et al., 2009; Singla-Pareek et al., 2003).

To gain a better understanding of the potential role of these genes in salt tolerance, one objective of these studies was to test the responsiveness of *GmEf-1α* and *GlxI* gene expression following salt treatments in soybean. We measured mRNA accumulation for each gene in cultivars characterized as either chloride includers or excluders. A second objective was to determine the potential role for salt components other than chloride for their impact on salt damage in soybean. To that end, we assessed phenotypic responses to salt treatments in four cultivars and assayed mineral accumulation in foliar tissues.

MATERIALS AND METHODS

Plant Materials and Growth Conditions. Soybean plants were grown in a greenhouse at the Rosen Center for Alternative Pest Control at the University of Arkansas, Fayetteville. The greenhouse was kept between 20 °C and 25 °C. Soybeans were grown under a 14-hour light and 10-hour dark cycle. Soybeans were germinated in a small-celled seed tray and then chosen for uniformity after emergence, five days after planting. The soybeans were then transplanted to 4-inch square pots, two plants per pot, and filled with sandy loam soil. Soybeans were grown without salt treatment for three weeks, and the initial salt treatment occurred after emergence of the first trifoliate leaf. The pots were placed in a shallow basin and flooded daily from below with one of four solutions: water, 80 mM NaCl, 40 mM $CaCl_2$,or 120mM NaCl. Soybeans in all groups were treated with Miracle Gro fertilizer every three days as per the manufacturer's instructions. For phenotypic scoring and mineral analysis, the plants were treated with one of three solutions: water, 80 mM NaCl or 40 mM $CaCl_2$; thus, equivalent amounts of chloride were provided in each salt treatment. For transcript measurements and glyoxalase assays, plants were treated with either water or 120 mM NaCl. The pots with soybean plants were flooded daily for one hour with water (control) or salt solution at around noon. After eight days of treatment, the soybeans were visually scored, foliar chlorophyll levels measured, and foliar tissue was collected for further analysis.

Plant Screening. Visual symptoms were recorded based on a scale of 1-6. The scale was defined as follows: 1 for healthy plant with no chlorosis, 2 for 25% of leaf chlorosis, 3 for 50% of leaf chlorosis, 4 for 75% of leaf chlorosis, 5 for 100% chlorosis, and 6 for complete leaf necrosis and plant death (Tamura and Chen, 2009).

Foliar chlorophyll levels were measured using a Minolta SPAD Chlorophyll Meter. The SPAD readings were taken from the 2nd trifoliate of each plant. Three readings were taken from the terminal leaflet, avoiding areas of necrosis, and then averaged to get the reading for the plant. Plants with a visual rating of >5 could not be accurately measured with the SPAD meter.

Mineral Analysis. Total Ca^{++}, Na^{+}, and other minerals were measured by digesting dried and ground leaf tissue that passed through a 1.18-mm mesh sieve in concentrated nitric acid and hydrogen peroxide followed by Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) analysis (Plank, 1992). All minerals except for chloride were analyzed using a Spectro Arcos ICP (Inductively Coupled Plasma Spectrophotometer, SPECTRO Analytical Instruments, Mahway, N.J. 07430). Chloride was extracted with deionized water and content of the leaves was measured using a digital chloridometer.

Glyoxolase Enzyme Activity. Extraction and analysis of glyoxalase I activity was performed according to the procedure of Ramaswamy et al. (1983). Terminal leaflets of soybean leaves at the V3 stage, from 1-month-old plants, were collected and immediately placed on ice. Tissue was homogenized in 2.0 ml of extraction solution at 4 °C. The extract was collected in 2-ml microcentrifuge tubes and the tissue was pelleted by centrifugation at 10,000 × g for 20 min. Supernatant(20 µl) was added to a quartz cuvette containing 1.0 ml of freshly prepared enzyme assay buffer and formation of thioester product was quantified by monitoring absorbance at 240 nm. This measured the activity of glyoxolase I enzyme in the tissue that could catalyze the formation of S-D-lactoylglutathione from methylglyoxal and reduced glutathione at 25 °C. Total protein concentration of extracts was determined with a BioRad Protein Assay kit (Bio-Rad Laboratories, Hercules, Calif.).

Gene Expression. Expressed sequence tags (EST) for *GmEf-1α* and *GlxI* were identified from the Dana Farber Cancer Institute *Glycine max* Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=soybean) and these sequences were used for oligonucleotide primer design. The sequence used for *GlxI* primer design is based on a cDNA that has been confirmed as encoding an active *GlxI* enzyme (Skipsey et al., 2000).

The primers used for amplification of *GmEf-1α* (Genbank accession AK246053) were:

EF-1αF 5'-TCTGTTTCTCCCTCACTCTGATCCAC-3' and

EF-1αR 5'-ACTCCACATACGAGCAAAAGACCCA-3'.

The primers used for amplification of GlxI (Genbank accession AJ010423) were:

GlxIF 5'-TCTGTTTCTCCCTCACTCTGATCCAC-3' and

GlxIR 5'- ACTCCACATACGAGCAAAAGACCCA-3'.

Gene expression was measured by RNA gel blots. Probes were prepared from cDNA via reverse-transcription polymerase chain reaction. RNA was extracted from the plant tissue using TriReagent (Molecular Research Center, Inc., Cincinnati, Ohio) according to manufacturer's instructions, and separated on denaturing formaldehyde gels (Sambrook et al., 1989). Flowers did not develop on Lee68 so we were unable to collect RNA for this tissue. Following transfer to nylon membranes, RNA was hybridized with ^{32}P-labeled probes as indicated (Figs. 7 and 8). Hybridization conditions were according to Church and Gilbert (1984) and signal was measured with a phosphorimager. Tissues assayed were the roots, shoots, leaves and flowers.

Data Analysis. Data for visual foliar plant health, glyoxolase I activity, SPAD chlorophyll levels, and mineral concentrations were subjected to an analysis of variance. Honest significant difference (Tukey) was used to compare means between each cultivar and each salt treatment, separately ($P = 0.05$). All statistical analysis was done using JMP version 9.0.0 (SAS Institute, Cary, N.C.) and graphs were constructed using GraphPad version 5.03 (GraphPad Software, Inc., La Jolla, Calif.) showing the statistical mean of each group and the standard error of the mean.

RESULTS AND DISCUSSION

Soybean Foliar Health. To confirm the phenotypic response of differential cultivars of soybean, plants were treated with salt solutions, and a visual scoring system (Tamura and Chen, 2009) was used to assess salt damage. Statistical analysis of the visual scoring showed a significantly greater reduction in plant foliar health in the Clark cultivar compared to Lee 68 when subjected to an 8-day treatment of 40 mM $CaCl_2$ or 80 mM NaCl (Fig. 4). These salt concentrations were chosen based on levels shown to be effective in previous studies (e.g., Valencia et al., 2008) and so that equivalent levels of chloride were applied in each case. Treatment with NaCl or $CaCl_2$ had severe effects on cv. Clark, whereas $CaCl_2$ treatment caused more damage than NaCl to the excluder Lee68. Although NaCl treatment did not lead to significantly different foliar ratings in Lee68 as compared with water-treated controls, the plants treated with salt solutions were visibly smaller (data not shown).

In addition to visual scoring, we measured chlorophyll content as a potential means to assess salt damage. There was a significantly lower level of chlorophyll in salt-treated Clark plants as compared to water-treated controls (Fig. 5). In addition, chlorophyll levels in Clark were lower than in Lee 68 following NaCl treatments. As in the visual scoring results, treatment with $CaCl_2$ had a slightly, but not significantly, greater negative impact on Lee68 than did NaCl, as compared to water-treated controls. These measurements were taken with a handheld Minolta SPAD meter (Konica Minolta Sensing, Inc., Osaka, Japan). This instrument is very simple to use and provides a rapid and non-destructive reading of chlorophyll content, taking just seconds to gather a single reading. Given that the trend of these data agree well with the visual ratings (Fig. 4), the measurement of chlorophyll with a SPAD meter could potentially provide an easy alternative method for screening soybean plants for salt tolerance. Decreased chlorophyll levels in response to salt stress have been shown to occur in other plant systems (Robinson et al., 1983). This method provides a quantitative output and avoids human bias that might impact visual ratings.

Mineral Accumulation in Salt-treated Plants. Measurement of leaf chloride levels confirms the differential uptake by the cvs. Clark (includer) and Lee 68 (excluder). Regardless of whether chloride was supplied as the sodium or calcium salt, Clark accumulated substantially higher levels of the chloride than did Lee 68 (Fig. 6a). In the includer Clark, chloride accumulated to higher levels when supplied to the plant as NaCl rather than $CaCl_2$. Not surprisingly, plants treated with $CaCl_2$ accumulated higher levels of Ca^{++}; however, we also noted that Ca^{++} accumulation was higher in cv. Clark, a chloride includer, than in the excluder Lee 68 (Fig. 6c). The same trend, i.e., higher sodium accumulation in the chloride includer, was observed in NaCl treatments (Fig. 6b). Taken together, the data suggest a possible parallel uptake of the cations with chloride when applied in high-salt conditions.

Transcript Analysis In Salt-Treated Leaves And Soybean Tissues. Transcript levels for genes encoding both the glyoxalase I and elongation factor-1 alpha (Fig. 7) were induced by NaCl treatment. This stress-induced molecular response appears to have been equivalent in both includer and excluder cultivars, suggesting that both types of soybean respond to salt stress with activation of defense mechanisms. Both includers and excluders accumulate higher levels of Na^+ and Cl^- after salt treatments, and even though excluder lines to not exhibit severe phenotypic damage, based on transcript induction they are also activating molecular defense responses. In addition, since both lines showed no difference in *GmEf1a* and *GlxI* expression in response to salt stress, it is unlikely that the different salt-tolerance phenotypes are due to the expression of either of these two genes.

Accumulation of transcripts encoding *GlxI* in plants has been shown previously to increase in response to salt treatments. In tomato, *GlxI* transcripts increased after 72 h of salt treatment (Espartero et al., 1995), and the orthologous gene was induced in wheat following infection with the fungal pathogen *Fusarium graminearum* or treatment with either $ZnCl_2$ or NaCl (Lin et al., 2010). These observations, along with reports of transgenic overexpression of *GlxI* in plants conferring enhanced salt tolerance (Singla-Parek et al., 2006; Veena et al., 1999), strongly suggest that this gene plays an important role in helping plants cope with salt stress. This

role of *GlxI* in salt tolerance might be widespread among organisms, as even the yeast *S. cerevisiae* responds to salt treatments and osmotic stress with enhanced *GlxI* transcripts and enzyme activity (Inoue et al., 1998). Induced transcription of gene family members of *GmEf-1α* has not been widely reported. Transcripts encoding *GmEf-1α* and the protein itself have been shown to accumulate in response to wounding in potato (Morelli et al., 1994), and expression levels of the gene in transgenic *Arabidopsis* correlate with salt tolerance (Shin et al., 2009). Therefore, this gene also likely plays a conserved role in stress responses in plants.

Transcripts for *GmEF-1α* are expressed throughout the plant but in different levels in various tissues (Fig. 8). Young leaves have the highest transcript levels, consistent with a role for *EF-1α* in growth and cell division. The mature leaves had the lowest transcript levels among tissues tested. Overall, there were no differences in transcript levels between the cultivars, suggesting that constitutive expression of *EF-1α* is not the cause of the differing tolerances to saline environments.

Glyoxalase Enzyme Assay. A glyoxalase-I enzyme assay of Lee68 and Clark under salt-stressed and control treatments was completed in order to further evaluate the relationship of the transcript level production, protein activity, and the phenotypic salt-stress tolerance. The enzyme analysis showed that although an increase may be detected on a transcript level, the *GlxI* activity in salt-treated soybeans was not significantly affected (Fig. 9).

This is in contrast with up-regulation of *GlxI* specific activity with a 20-fold increase noted in tomato plants that also correlated with an up-regulation of *GlxI* transcripts (Espartero et al., 1995). In contrast, tobacco cells stressed by the addition of NaCl demonstrated a decrease in the total amount of *GlxI* protein (Hoque et al., 2008). These contrasting studies suggest that the different species of plants (e.g., soybean, tomato, and tobacco) combat the negative effects of salt stress differently in relation to the glyoxolase pathway. The differences in change between the transcript level and the amount of protein activity in the soybean varieties could be due to post-transcriptional regulation within the plant cell.

CONCLUSIONS

We have further optimized a rapid and effective screening method for salt tolerance in soybean. Although transcript levels for *EF-1α* and *GlxI* are induced by salt treatments, transcript profiles do not differ between salt-tolerant and susceptible soybean cultivars. This suggests that the cultivars respond to salt stress in similar ways, but that these genes are not responsible for the differential phenotypes. As expected, the chloride-includer cultivar accumulated higher levels of chloride. Under the respective high-salt conditions, the chloride-includer also transported more Ca^{++} and Na^{+}, suggesting a possible parallel mechanism for transport of these elements with chloride.

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Table 1. Cultivars of soybean (*Glycine max*) with known sensitivites to salt and chloride-uptake capacities used in study.

Cultivar	Reaction to Salt	Classification
Lee 68	Tolerant	Cl-excluder
S-100	Tolerant	Cl-excluder
Dare	Sensitive	Cl-includer
Clark	Sensitive	Cl-includer

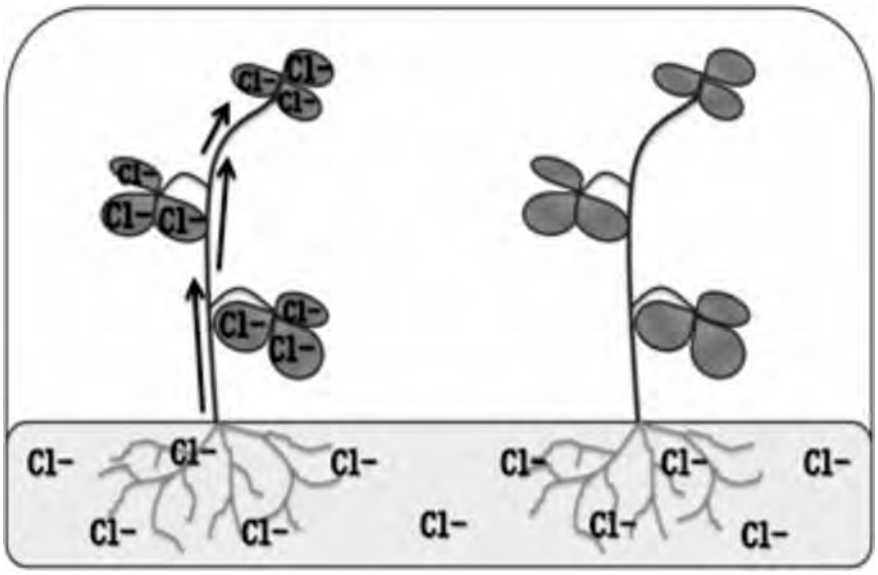

Fig. 1. The differential transport of chloride to foliar tissues in soybean by includers (left) and excluders (right).

Fig. 2. Soybean chloride includers exhibited severe damage after salt treatments. Soybeans were started from seed and flooded daily with 120 mM NaCl for eight days after emergence of the first trifoliate. An includer cultivar, Dare (left), and an excluder, Lee 68 (right) showed dramatically different phenotypes following 8 days of treatment with 120 mM NaCl.

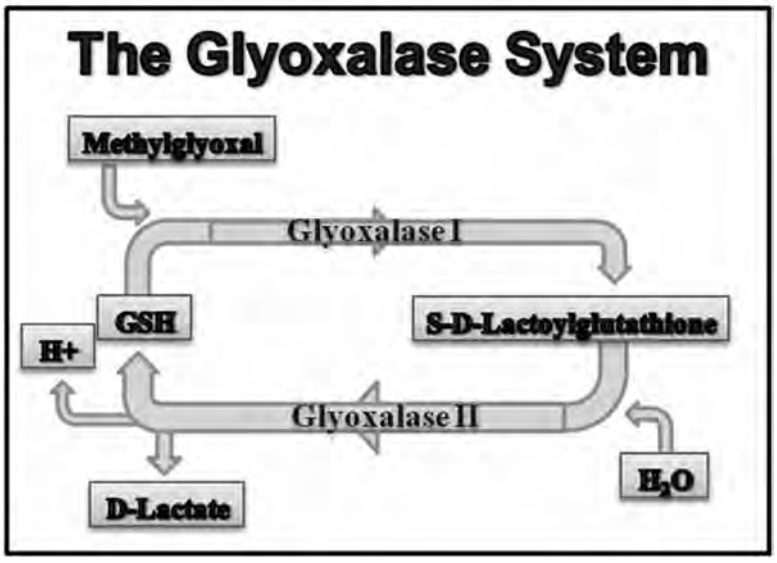

Fig. 3. The glyoxalase pathway is a ubiquitous detoxification pathway in prokaryotes and eukaryotes. Diagram of the glyoxalase system which includes glyoxalase I (*GlxI*) and glyoxalase II. *GlxI* converts methylglyoxal and reduced glutathione (GSH) to the product S-D-lactoylglutathione; *GlxII* forms D-lactate and glutathione.

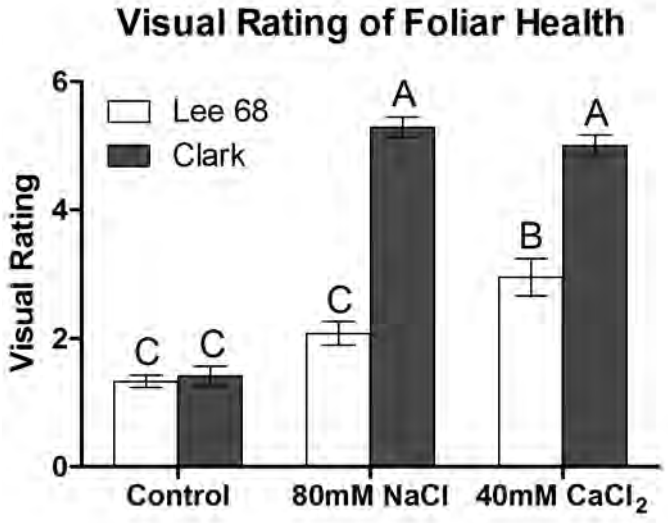

Fig. 4. Visual scoring of salt effects confirms tolerance of chloride excluders. After 8 days of salt treatments with 80 mM NaCl or 40 mM $CaCl_2$, soybean foliar health was rated on a scale of 1 (healthy plant with no chlorosis or necrosis) to 6 (complete necrosis). Similar letters indicate no significant difference. Error bars indicate standard error of the mean.

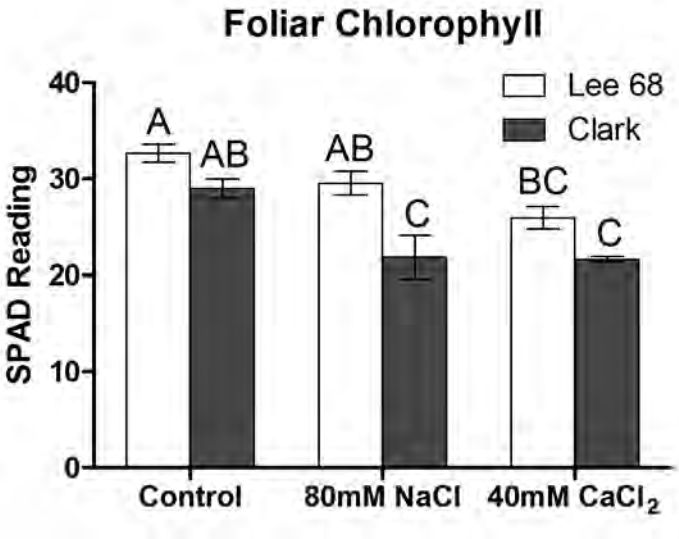

Fig. 5. Salt treatments lower chlorophyll levels in a chloride includer. Chlorophyll levels were determined with a Minolta SPAD 502 chlorophyll meter after 8 days of salt treatment. Similar letters indicate no significant difference. Error bars indicate standard error of the mean.

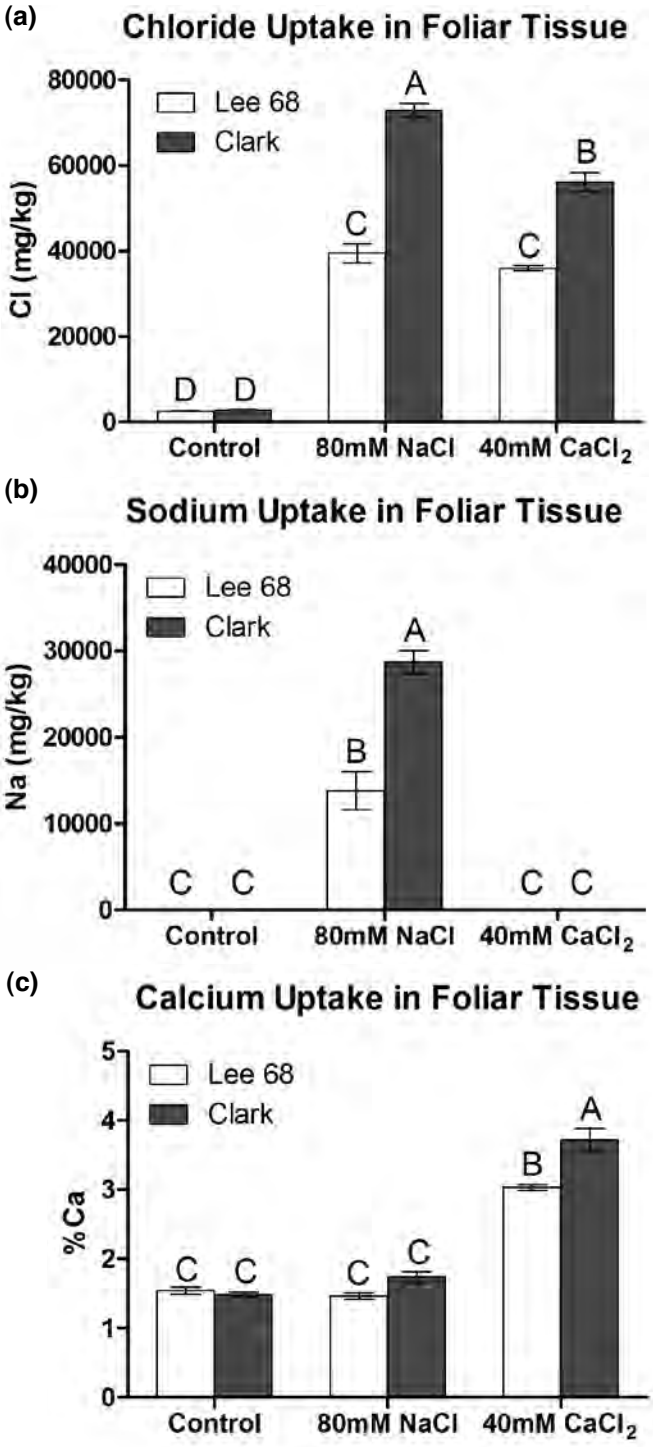

Fig. 6. Mineral levels differ in salt-treated Clark (includer) or Lee68 (excluder) cultivars. Soybeans were treated with 80 mM NaCl or 40 mM $CaCl_2$ for 8 days and then mineral concentrations were determined after foliar tissue was ground and analyzed. a) chloride uptake was greater in Clark than in Lee 68, verifying the includer and excluder classifications; b) sodium accumulated to significantly greater quantities in Clark than in Lee 68; c) calcium levels are highest in the $CaCl_2$-treated chloride includer.

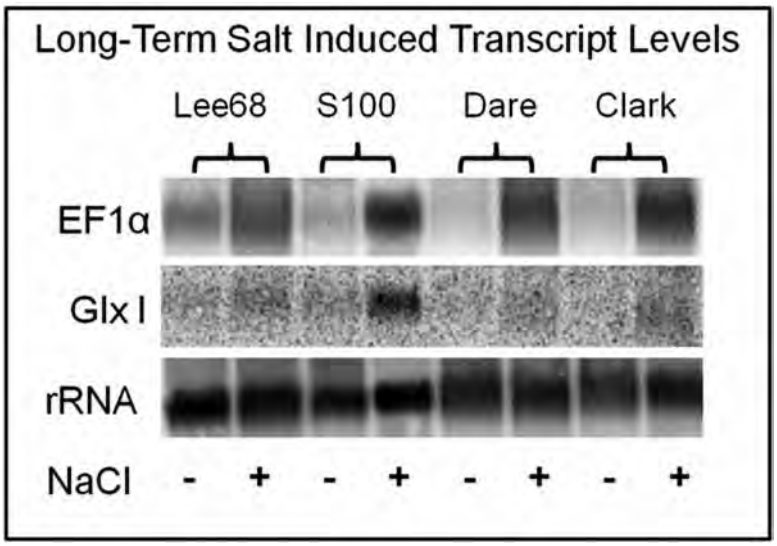

Fig. 7. Salt treatment increases transcript levels of both elongation factor-1 alpha (*EF-1*α) and glyoxalase I (*Glxl*) in both includers (Dare and Clark) and excluders (Lee68 and S100). The soybeans were treated with either water (-) or 80 mM NaCl (+) for 20 days before tissues were analyzed, and RNA probes were applied as indicated. rRNA probes were used as a control for measuring transcript levels.

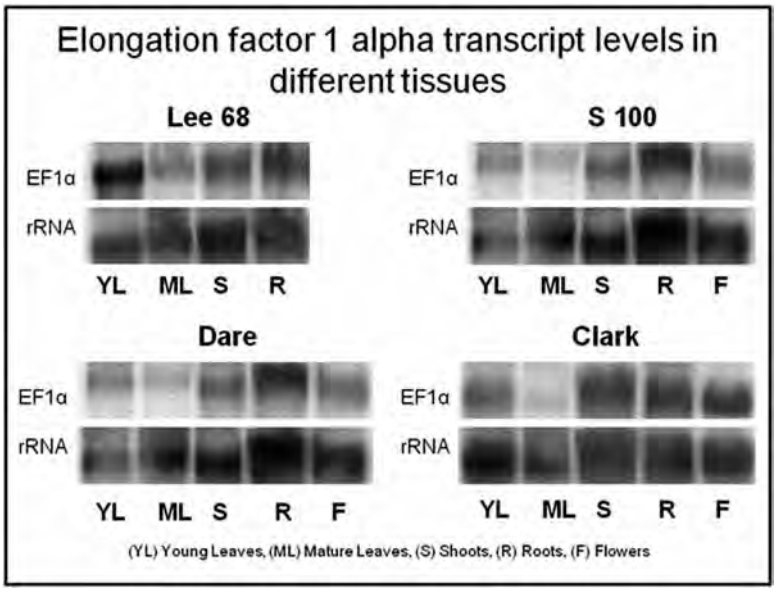

Fig. 8. Elongation factor 1 alpha (*EF-1*α) was expressed throughout the plant at different levels. Tissues tested were young leaves (YL,) mature leaves (ML,) stem (S,) roots (R,) and flowers (F). The transcript levels were lowest in the mature leaves in all soybean varieties. There were no noticeable differences in transcript levels between the excluders (Lee 68 and S 100) and the includers (Dare and Clark). Flowers did not form on Lee68 during the duration of this experiment. rRNA probes were used as a control for measuring transcript levels.

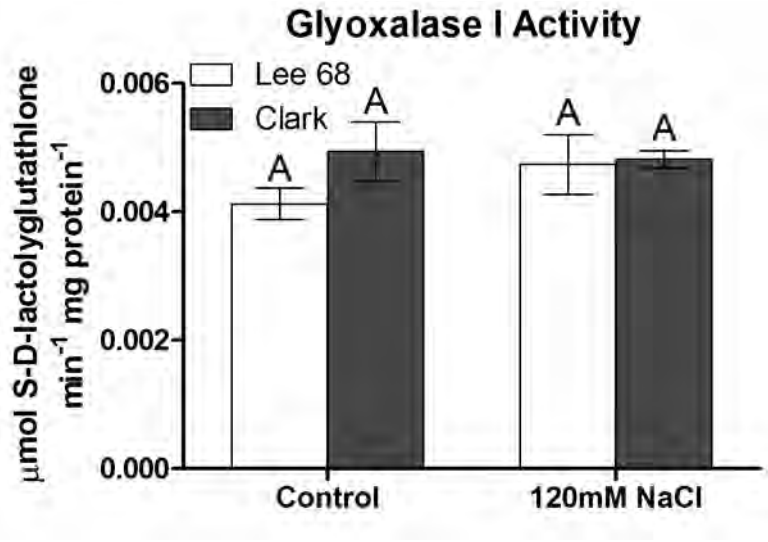

Fig. 9. Glyoxalase I activity is not affected by NaCl treatment. The amount of glyoxolase I enzyme activity in the tissue that could catalyze the formation of S-D-lactoylglutathione from methylglyoxal and reduced glutathione at 25 ºC was measured from tissue collected at day 8 of 120 mM NaCl treatment. Values were not significantly different. Error bars indicate standard error of the mean.