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ORIGINAL PAPER

Salt tolerance in soybean WF-7 is partially regulated by ABA and ROS signaling and involves withholding toxic Cl^- ions from aerial tissues

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Abstract Salt tolerance in plants is a complex trait involving multiple mechanisms. Understanding these mechanisms and their regulation will assist in developing novel strategies to engineer salt-tolerant crops. In the current study, we investigated salt-tolerant mechanisms in soybean (*Glycine max*) cultivar WF-7 in comparison to salt-sensitive Union. In vivo and in vitro salt assays demonstrated the salt tolerance of WF-7 at the seedling stage and during germination. After a 10-day 200 mM NaCl treatment, chlorophyll content in Union was reduced by 50 % compared to a 17 % reduction in WF-7. WF-7 was also less affected by abscisic acid (ABA) and NaCl during germination than Union. Upon ABA and NaCl treatment, the ABA-responsive genes *SCOF1*, *ASN1*, *bZIP44*, and *AAPK1* are differentially expressed in WF-7 and Union seedlings. These results suggest that salt tolerance in WF-7 is in part regulated through an ABA-dependent pathway. In addition, following a 4-day 200 mM NaCl treatment, WF-7 produced more H_2O_2 than Union indicating the involvement of reactive oxygen species (ROS) in regulating salt tolerance in WF-7. Yet another mechanism WF-7 employs is withholding toxic chloride (Cl^-) ions from aerial tissues. Following 200 mM NaCl treatment, Cl^- accumulation was mostly localized to the roots of WF-7. In contrast, most of the Cl^- in Union was transported into the stems and leaves. Taken together, our results demonstrated a role of ABA and ROS in regulating salt tolerance in WF-7, and the critical role of Cl^- in NaCl-induced mortality in soybean. *Key message* Withholding toxic Cl^- ions from leaves and, to a lesser extent, stems, confers salt tolerance to soybean WF-7. In addition, ABA and ROS may be involved in salt-stress signal transduction.

Keywords *Glycine max* · Salt stress · H_2O_2 · Cl^- accumulation · NaCl toxicity

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Introduction

Salt stress is one of the major environmental factors that significantly reduced the yield of agricultural crops. Enormous areas of formerly arable land are being removed from crop production due to increasing soil salinity (Epstein et al. 1980). Soil salinity is increased by application of high levels of fertilizers and use of saline water for irrigation (Epstein et al. 1980). Decreasing the acreage of usable land for crop production poses a severe threat to global food security as more food and fuel will be needed to support the growing population. Development of salt-tolerant crops would enable formerly arable land in addition to saline land in arid and semi-arid regions to be utilized for crop production, thus expanding global food production areas.

Soybean (*Glycine max* L.) is becoming an increasingly important cash crop due to its high oil and protein content.

The demand for soybean is continually increasing due to the growing awareness of the nutritional and nutraceutical properties associated with soybean phytochemicals (Amarowicz and Pegg 2008; Boschin and Arnoldi 2011). The high oil content of soybean also lends itself for use in biodiesel production (Hill et al. 2006). Furthermore, soybean plantations can satiate the demand for reduced nitrogenous fertilizer usage as they enrich the soil with nitrogen.

Soybean is considered a salt-sensitive glycophyte, with all developmental stages adversely affected by salinity stress (Phang et al. 2008). High salt levels generate a two-part stress on plants: an osmotic stress caused by reduced water availability in soil and an ionic stress due to solute imbalance in the cytosol (Blumwald et al. 2000; Conde et al. 2011). In soybeans, salt stress reduces plant height and leaf size (Essa 2002; Wang et al. 2001), decreases the number of internodes and pods (Phang et al. 2008), decreases protein content and seed quality (Bahmaniar and Sepanlou 2008), and causes a reduction in chlorophyll content (Lu et al. 2009), thus causing a significant reduction in yield. Salt stress also inhibits nitrogen fixation and causes a decline in nodule dry weight, leading to a decrease of shoot dry weight and nitrogen levels (Delgado et al. 1994). Soybean plants have developed several mechanisms to cope with salt stress, conferring a wide spectrum of salt tolerance among genotypes (Phang et al. 2008). Mechanisms of salt tolerance include maintaining ion homeostasis by withholding toxic ions from sensitive aerial parts, adjusting osmotic potential in cells by accumulating metabolites, and restoring oxidative balance to prevent further damage due to excess accumulation of reactive oxygen species (ROS) (Phang et al. 2008). In addition, salt tolerance mechanisms have been classified as abscisic acid (ABA)-dependent and ABA-independent pathways (Zhu 2002). H_2O_2 signaling may also play a critical role in osmotic stress signal transduction pathways (Dat et al. 2000; de Carvalho 2008; Desikan et al. 1999; Neill et al. 2002). Therefore, understanding the mechanisms and identifying genes involved in salt-stress tolerance of soybeans will enable breeders to develop new strategies to enhance salt tolerance.

At the molecular level, a major quantitative trait locus for salt tolerance was mapped to the same region on the linkage group LG-N in two different populations (Guan et al. 2008; Lee et al. 2004). The objectives of the current study are to characterize physiological salt tolerance traits and to understand the mechanisms that control the salt tolerance in WF-7, the salt-resistant parent of one of the mapping populations (Guan et al. 2008). Union was the salt-sensitive parent of the same mapping population and was thus selected for comparison. WF-7 is the progeny of Chinese *G. max* cultivars Ju Xuan 23 × Qi Huang 1 (Cui et al. 1999).

Materials and methods

Plant materials

Soybean variety WF-7 was kindly provided by Dr. Lijuan Qiu, Chinese Academy of Agricultural Science, Beijing, China. The Union was originally received from USDA Soybean Germplasm Collection (PI548622) and was stored at VSU soybean breeding program. Soybean plants were grown under ambient light and temperature in a greenhouse at the Randolph farm of VSU.

NaCl and ABA treatments

The effect of NaCl and ABA on seed germination of both WF-7 and Union seeds was analyzed using 20–30 seeds per treatment. The seeds were placed in Petri dishes containing a layer of filter paper saturated with solutions containing NaCl (0, 50, 100, 150, and 200 mM) or ABA (0, 5, 10, 50, and 100 μM). The Petri dishes were maintained at room temperature and irradiated at ~120 μmol/m^2 in a 14/10 h (light/dark) photoperiod. The number of germinated seeds was recorded after a 7-day germination period. The germination tests were repeated four times, and Student's *t* test was used for statistical analysis.

For in vitro analysis of the effect of NaCl on soybean leaves, approximately 2 cm sections were taken from leaves from the top third trifoliate stage (V3) of greenhouse-grown WF-7 and Union plants. The leaf sections were floated in either water (control) or 200 mM NaCl; photographs were captured following 4-day treatments. Salt tolerance of WF-7 and Union plants was also analyzed in vivo. Greenhouse-grown 1-month-old plants were treated with either 2 L water (control) or 2 L 200 mM NaCl to saturate pots. The plants were photographed 2 weeks after receiving the treatment. All treatments were biologically replicated four times.

Detection of H_2O_2 production

Salt-stress-induced H_2O_2 production was detected using 3,3′-diaminobenzidine (DAB) staining as described by Xia et al. (2009). Union and WF-7 plants were watered with 200 mM NaCl (treated) or water (untreated) as described above. 4 days following treatment, partially developed leaves from the top trifoliate at stage V3 were detached from salt-treated and untreated plants, placed in 1 mg/ml DAB, and incubated at room temperature for 14 h. Samples were de-stained in boiling absolute ethanol before the pictures were captured.

Chlorophyll, sodium, and chloride quantification

To examine how salt stress affects chlorophyll content in salt-tolerant and salt-sensitive varieties, both WF-7 and

Union plants at V3 stage were treated with 200 mM NaCl as described above. Chlorophyll content was measured at day 0, 2, 4, 6, 8, and 10 using the protocol described by Palta (1990) and relative reduction rate was calculated. Distribution of chloride (Cl^-) and sodium (Na^+) contents of 1-month-old WF-7 and Union plants were analyzed 10 days after saturating the pots with 200 mM NaCl or water (control). For measuring Cl^- and Na^+ contents, 1 g of leaf, stem and root tissues from both treated and untreated soybean varieties were extracted with 20 ml of Mehlich I solution (8 ml of 6 M HCl and 25 ml of 0.5 M H_2SO_4) for 10 min with shaking followed by 30 min centrifugation at 3,000 rpm. All samples were filtered through 0.45 μm nylon filters and the aliquots were loaded to Prodigy High Dispersion Simultaneous ICP Spectrometer (ICP) for Na^+ measurement, and water-extracted samples were analyzed for Cl^- content using a Dionex ICS-5000 Ion Chromatography (IC).

RNA extraction and RT-PCR analysis

Total RNAs were extracted from leaves using TRI® reagent as per the manufacturer's instructions (Sigma; St. Louis, MO). WF-7 and Union leaves were incubated in H_2O (as control) or 10 μM ABA for 3 h prior to RNA isolation. Total RNA was also extracted from leaves after 48 h treatment with 200 mM NaCl or H_2O (control). All treatments for gene expression analysis were biologically repeated. RNA was quantified using a NanoDrop (Thermo Scientific; Wilmington, DE) and its quality was monitored on 1.2 % agarose gel; 1 μg of total RNA for each sample was used for cDNA synthesis in a 20 μl reaction using SuperScript® III (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions followed by PCR of 1 μl of the RT products using *Taq* DNA polymerase (New England BioLabs; Ipswich, MA) with 30 cycles of amplification. RT-PCR was performed for soybean ABA or abiotic stress-responsive genes *AAPK1*, *ASN1*, *SCOF1*, and *bZIP44* using primer pairs 5′-CAGAA CTACTCCTAGAGACAAG-3′ (forward) and 5′-TGAAGC TGCATCAATCGAGGAG-3′ (reverse) for *AAPK*, 5′-CCC TTTGGTGTTTTGCTC-3′ (forward) and 5′- GCCCTTCTT CCTTTTTTATC-3′ (reverse) for *ASN1,* 5′-ATGGCTTT GGAAGCTCTCAAC-3′ (forward) and 5′-TCATTGAA ATTGGGGGATTTCG-3′ (reverse) for *SCOF1*, and 5′-GGT CCTGCGTCCTAATTAG-3′ (forward) and 5′-TCACCAC TGAAAAACGGGTTGG-3′ (reverse) for *bZIP44*. Soybean tubulin gene 5′-ATGAGAGAAATCTTGCACATCCAG-3′ (forward) and 5′-TAAGGCTCCACAACGGTATC-3′ (reverse) served as the loading control. To quantify the relative gene expressions comparing to soybean tubulin, all gel images were analyzed by UVP-3 imaging analyzing software (UVP, LLC, California, USA).

Results

In vitro and in vivo assays of soybean salt tolerance

The 2 cm leaf sections taken from soybean cultivars WF-7 and Union were incubated in 200 mM NaCl or water (control) for 4 days. WF-7 leaf sections showed no phenotypic difference when floated in water or 200 mM NaCl, while Union leaf sections showed damage symptoms when incubated in 200 mM NaCl (Fig. 1a). 2 weeks after the 1-month-old plants were watered with 200 mM NaCl, Union seedlings were stunted compared to control and had curling and browning leaves (Fig. 1b). WF-7 seedlings had mildly stunted growth with minimal browning on a few leaves 2 weeks following the 200 mM NaCl treatment (Fig. 1b). This demonstrates that WF-7 is highly salt tolerant, while Union is salt sensitive.

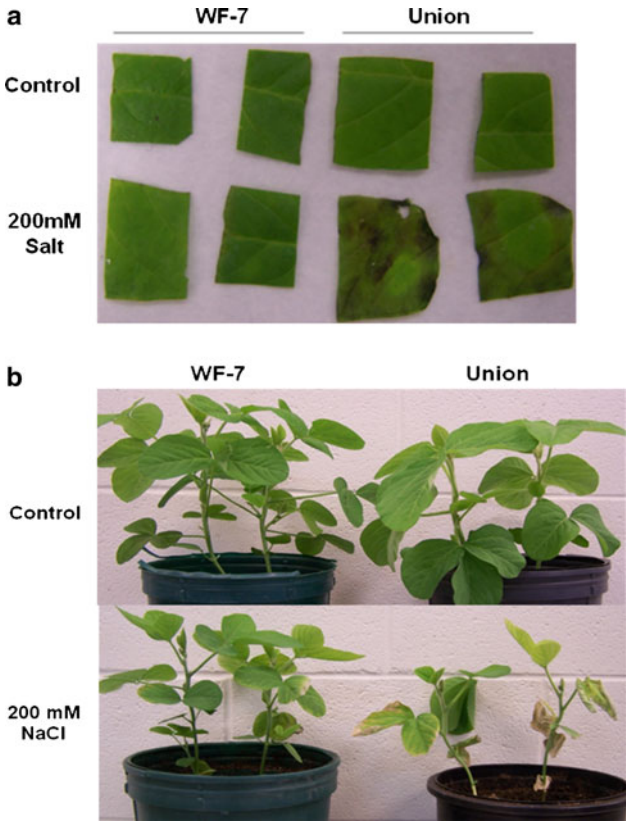

Fig. 1 In vitro (**a**) and in vivo (**b**) assays of salt tolerance for WF-7 and Union. **a** Approximately 2 cm sections from soybean leaves were floated in either water (*control*) or 200 mM NaCl. The photos were taken after 4 day treatment. **b** 4-week-old seedlings were watered with water (*control*) or 200 mM NaCl until the pots were saturated. The photos were taken 14 days after the treatment. All treatments were duplicated

Seed germination in response to NaCl and ABA

Sensitivity to salt stress and ABA during seed germination was measured by germinating seeds in the presence of 0, 50, 100, 150, and 200 mM NaCl or 0, 5, 10, 50, and 100 μM ABA. At 50 mM NaCl, the rate of seed germination in Union was reduced to 50 % while WF-7 maintained 95 % germination (Fig. 2a). At 100 mM NaCl, the germination rate of Union was further reduced to 25 % and WF-7 reduced to 47.5 %. At 200 mM NaCl, complete inhibition of seed germination occurred in Union while 27.5 % of WF-7 seeds germinated (Fig. 2a). Seed germination in Union was not as affected by ABA as it was in salt treatment, but it was still more sensitive to ABA than WF-7. Germination rates in Union were reduced to 45 and 12.5 % in the presence of 50 and 100 μM ABA, respectively, compared to 80 and 42.5 % in WF-7 (Fig. 2b). These results suggest that the salt tolerance of WF-7 may be partially through an ABA-dependent pathway.

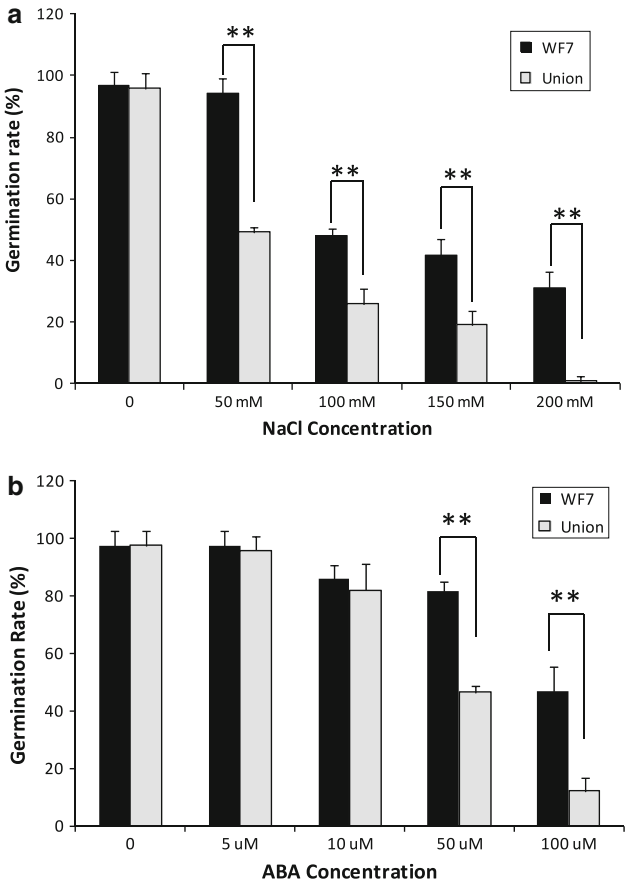

Fig. 2 Seed germination in response to NaCl and ABA. **a** Seed germination for WF-7 is significantly more tolerant to NaCl than Union. **b** WF-7 is more resistant to ABA than Union in seed germination. Each of four biological replicates assayed 20–30 seeds per variety per treatment. *Error bars* indicate standard deviation (SD). $^{**}p < 0.01$, as determined by Student's *t* test

ABA-responsive genes are differentially expressed in salt-tolerant and salt-sensitive cultivars

To further examine the involvement of ABA in salt tolerance, gene expression of ABA-responsive genes was analyzed using RT-PCR. The expression of ABA-responsive genes *AAPK1*, *ASN1*, *SCOF1*, and *bZIP44* was analyzed in Union and WF-7 seedlings before (control) and 3 h after ABA (10 μM) treatment (Fig. 3a, b) or 48 h after watering with 100 mM NaCl or H_2O (control; Fig. 3c, d). Gene expression of soybean tubulin was analyzed as a control for RNA quality and quantity. Control Union seedlings had high *AAPK1* expression, with decreased expression after exposure to ABA and no change after NaCl treatment. *ASN1* gene expression was faintly detectable in both Union and WF-7 seedlings before ABA treatment and induced following ABA treatment. *ASN1* was strongly induced in Union seedlings and, to a lesser extent, in WF-7 seedlings following NaCl treatment. *SCOF1* gene expression was undetectable in ABA-treated and control Union seedlings and untreated WF-7 seedlings but was induced in ABA-treated WF-7 seedlings and salt-stressed Union and WF-7 seedlings. Expression of *bZIP44*, a negative regulator of ABA signaling in soybean, was induced in both cultivars following ABA treatment, although changes in expression levels were more dramatic in WF-7 seedlings. *bZIP44* gene expression was undetectable in both cultivars following salt stress. These results, together with seed germination responses to ABA, suggest that salt tolerance in WF-7 is partially regulated by an ABA-dependent pathway.

Salt treatment reduces chlorophyll content in salt-sensitive cultivar

Chlorophyll content of leaves from WF-7 and Union plants (V3 stage) was measured over a 10-day period following NaCl treatment. Initially, WF-7 leaves had 5,734 μg chlorophyll per gram leaf tissue, while Union leaves had 4,701 μg chlorophyll (not shown); therefore, relative rates of chlorophyll reduction were calculated (Fig. 4). 2 days after NaCl treatment, WF-7 chlorophyll content dropped to 88 % of initial levels and maintained levels around 85 % during the following 10 days, ending at 83 % of original levels. The chlorophyll content in leaves from salt-sensitive Union plants fell to 73 % after 2 days, and decreased steadily to 43 % of unstressed levels, 10 days after salt stress. This further demonstrates the salt sensitivity of Union soybeans and the salt tolerance of WF-7 soybeans.

H_2O_2 production is greater in salt-tolerant cultivar WF-7

Following a 4-day salt treatment, leaves from WF-7 and Union plants were excised and assayed for H_2O_2 content

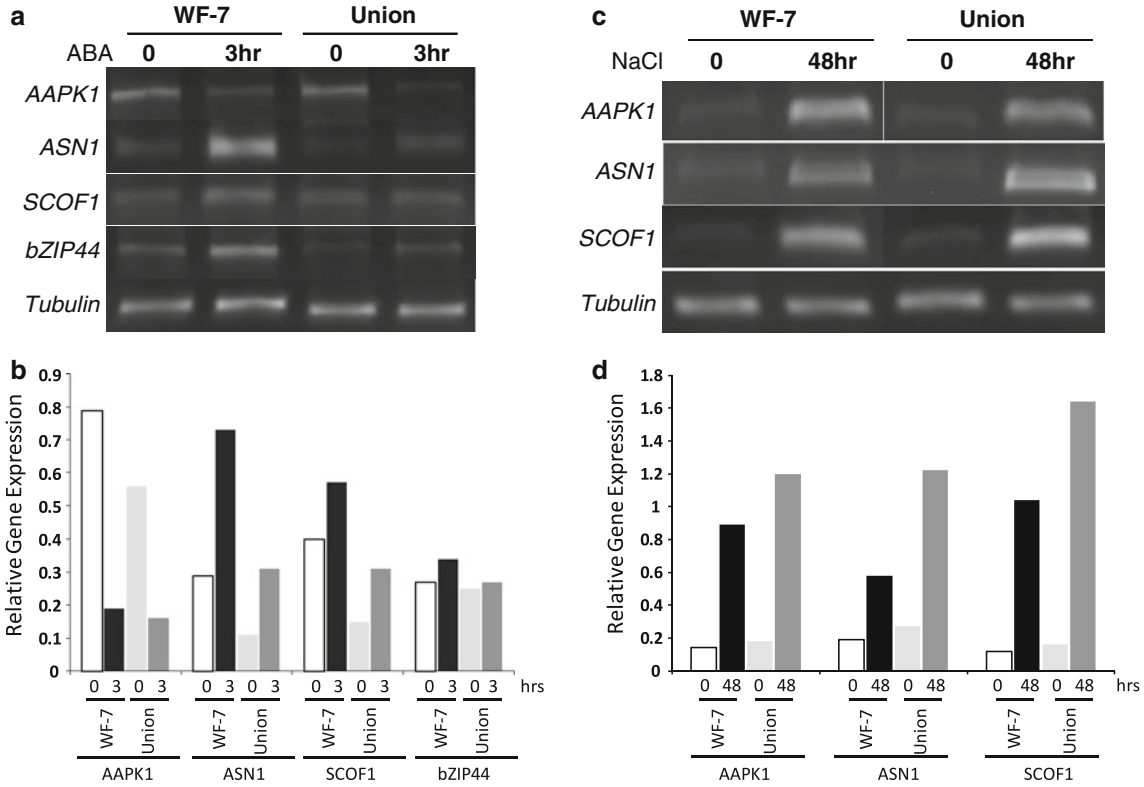

Fig. 3 Gene expression analysis of several ABA-responsive genes. Total RNAs were isolated from **a** 10-day-old seedlings treated or untreated with 10 μM ABA for 3 h, and **c** 48 h after watering seedlings with or without 200 mM NaCl. RT-PCR was performed for *AAPK1, ASN1, SCOF1* and *bZIP44*. Tubulin was used for loading control. Gene relative expressions comparing to the tubulin were analyzed for ABA treatments (**b**) and NaCl treatments (**d**)

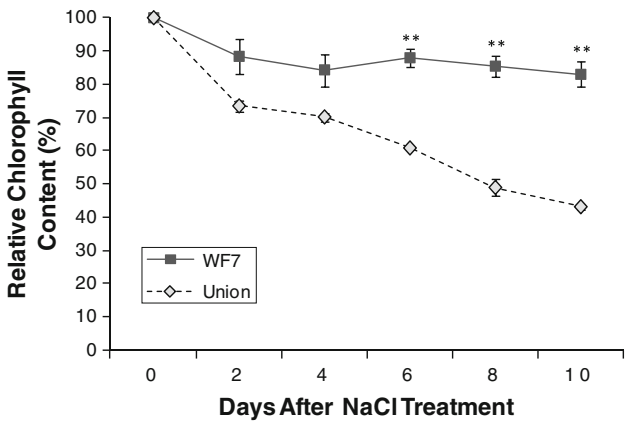

Fig. 4 Effect of NaCl treatment on chlorophyll contents of salt-tolerant and sensitive soybean cultivars. Both WF-7 and Union were grown to V3 stage, then, 200 mM NaCl was applied to saturate the pots, and samples were collected every other day until day 10. Chlorophyll contents were measured by spectrophotometer at wavelength of 654 nm and relative reduction rate was calculated. Experiments were biologically duplicated. *Error bars* indicate standard deviation (SD). $^{**}p < 0.01$, as determined by Student's *t* test

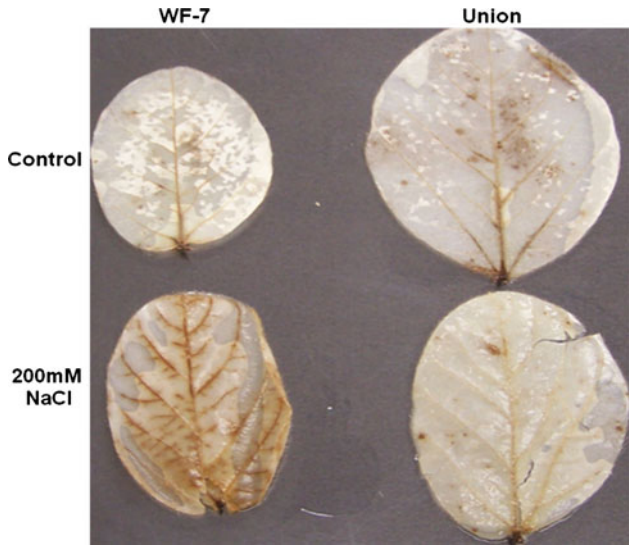

Fig. 5 Reactive oxygen species (ROS) production in WF-7 is induced by salt stress. Hydrogen peroxide was detected by incubating leaves excised from salt-stressed plants and stained by 1 mg/ml DAB solution for 14 h followed by de-staining

via DAB staining. Leaves from non-stressed plants were used as a control. Leaves from salt-stressed WF-7 produced more H_2O_2 than Union leaves (Fig. 5). This suggests that H_2O_2 may be involved as a signaling molecule in the stress signal transduction pathway conferring salt tolerance rather than an adverse reaction to salt stress in soybean.

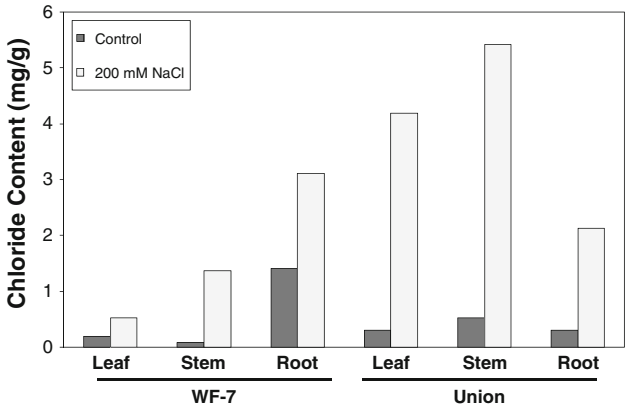

Fig. 6 Chloride content in soybean tissues. Tissue chloride content was quantified by ion-exchange chromatography (IC). Chloride content was measured in leaf, stem and root tissue of WF-7 and Union plants watered with 200 mM NaCl or water (*control*)

Chloride withheld from aerial tissue in salt-tolerant cultivar WF-7

Distribution of Na^+ and Cl^- ions was analyzed by measuring Na^+ and Cl^- levels in leaves, stems, and roots in WF-7 and Union plants watered with 200 mM NaCl or water (control). Chloride levels in WF-7 leaf tissue increased from 0.2 to 0.5 mg Cl^- per gram of tissue and in stems, increased from 0.08 to 1.5 mg Cl^- per gram of tissue. Cl^- content in WF-7 roots increased from 1.4 to 3.1 mg per g tissue following 200 mM NaCl treatment (Fig. 6). This Cl^- distribution pattern in WF-7 shows that the majority of Cl^- was maintained in the root, and not transported to aerial tissues, even with no salt treatment. On the other hand, Union tissue had low Cl^- content in controls (less than 0.5 mg per gram in each tissue type); however, following NaCl treatment, leaf and stem tissue contained 4 and 5.5 mg Cl^- per gram of tissue, respectively, while root tissue only contained 2 mg/g. No significant changes in Na^+ content of root, stem, and leaf tissue were found for both WF-7 and Union before and after salt treatment (data not shown). The results suggest that in soybeans, excess Cl^- accumulation in leaves during salinity stress is critical and toxic. Furthermore, another mechanism conferring salt tolerance may be in place in WF-7 that effectively withholds Cl^- in the roots thus minimizing toxic Cl^- ions in leaves.

Discussion

Salt-induced damage of many plants, including wild soybean (*Glycine soja*), is due to the accumulation of excess Na^+ in leaves, whereas salt toxicity in *G. max* is attributed to elevated Cl^- levels in leaves (Abel and MacKenzie 1964; Gao et al. 2007; Luo et al. 2005). Our findings were consistent with these studies. When plants received water containing 200 mM NaCl, WF-7 leaves and stems contained Cl^- levels considerably lower than levels found in root tissue prior to treatment. Conversely, Union leaf and stem tissue showed a 14- and 10-fold increase of Cl^- content. Chloride levels in root tissue more than doubled in WF-7 following NaCl treatment. Union root tissue showed a 7-fold increase in Cl^- content, but levels remained lower than stem and leaf tissues. Furthermore, there were no significant differences in Na^+ content of root, stem, and leaf tissue in either cultivar. This confirms the findings that salt-stress-induced toxicity is due to Cl^- ions in soybeans. In addition, one mechanism conferring salt tolerance to WF-7 plants involves withholding Cl^- from leaves by accumulating it in roots and, to a lesser extent, the stems.

Salt-tolerant plants often show enhanced sensitivity to ABA (Gao et al. 2007; Kang et al. 2002); however, WF-7 was less sensitive to ABA treatments than salt-sensitive Union. Our findings are more consistent with the involvement of a negative regulator of ABA signaling (Liao et al. 2008). Transgenic *Arabidopsis* plants over-expressing *GmbZIP*s, negative regulators of ABA, showed reduced sensitivity to ABA but enhanced tolerance to salt stress compared to wild type (Liao et al. 2008). *GmbZIP44* gene expression was not detected in salt-treated seedlings 48 h after treatment; however, *GmbZIP44* gene expression may peak 12–24 h after stress.

Interestingly, more H_2O_2 was produced in WF-7 leaves than Union leaves during salt stress. In general, H_2O_2 accumulation is considered a detrimental effect of biotic and abiotic stresses, exacerbating the damage caused by the stress itself. While the accumulation of H_2O_2 and other ROS can be damaging in excess quantities, they may play crucial roles as signaling molecules in stress signal transduction pathways (Dat et al. 2000; de Carvalho 2008; Neill et al. 2002). H_2O_2 induces a number of cellular responses including expression of defense genes (Desikan et al. 2000) and activation of MAPK signaling cascades (Desikan et al. 1999; Kovtun et al. 2000). Furthermore, H_2O_2 can induce salt tolerance in barley (Fedina et al. 2009) and alleviate drought stress in soybean (Ishibashi et al. 2011). H_2O_2 may function as a signaling molecule conferring salt tolerance in WF-7 plants. There must be a fine-tuned balance between ROS and antioxidant machinery, contingent upon both time and location, which dictates whether ROS are damaging, protective, or signaling factors (Gill and Tuteja 2010).

Salt tolerance in WF-7 may also involve cross-talk between H_2O_2 and ABA signal transduction pathways. ROS and ABA signaling may directly regulate salt-stress responses; alternatively ROS and ABA signaling may be induced in parallel to salt-stress signaling due to cross-talk of stress signal transduction pathways. In plants, cross-talk among signaling pathways plays a central role in many

biotic and abiotic stress events. Furthermore, H_2O_2 may play a direct role in ABA signaling as a second messenger (Kwak et al. 2003). The response of *Arabidopsis* suspension cells to ABA can be diminished by the addition of dimethylthiourea, a ROS scavenger (Böhmer and Schroeder 2011), indicating the involvement of ROS in ABA signaling. WF-7 seedlings had an enhanced resistance to salt stress and were less sensitive to ABA than salt-sensitive Union seedlings. The ABA-responsive genes *ASN1* and *GmbZIP44* were induced by ABA in both WF-7 and Union seedlings while *SCOF1* and *ASN1* were induced by salt-treated WF-7 and Union seedlings. These results indicate that salt tolerance in WF-7 is potentially regulated by both ABA-dependent and independent pathways. Indeed, WF-7 soybeans may effectively overcome salt stress by utilizing multiple mechanisms involving ROS, ABA, and sequestration of Cl^- away from aerial tissue.

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