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ORIGINAL ARTICLE

Physiological and transcriptional responses of contrasting alfalfa (*Medicago sativa* L.) varieties to salt stress

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Abstract Alfalfa (*Medicago sativa* L.) acts as a most important legume forage crop and is widely cultivated in various environments. Salt stress is one of the major abiotic stresses in cultivation of alfalfa worldwide. Development of alfalfa cultivars for adaptable to salt environments can provide sustainable solutions. In the present study, we selected two varieties with contrasting salt tolerance—211609 and Xinjiang Daye—from 14 alfalfa varieties. Under salt stress condition, 211609 showed higher leaf water content, less severe cell membrane damage (Electrolyte leakage) and lower accumulation of reactive oxygen species than Xinjiang Daye which exhibited lower GSH content and less antioxidant enzyme activities. In addition, significantly higher expression levels of *NHX1*, *ZFG*, *CBF4* and *HSP23* genes were found in 211609 than those in Xinjiang Daye upon exposure to salt stress. Collectively, these results proved that 211609 showed higher tolerance to salt stress than Xinjiang Daye through regulation of physiological and transcriptional pathways. It could play an important role in breeding program of alfalfa varieties with improved stress tolerance in future.

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Introduction

Salt stress is one of the most important agricultural problems affecting plant growth, development and productivity. More than one-fifth of the world's arable land is now under the threat of salt stress (Li et al. 2011). In addition, with dramatic increasing in the human population and serious environmental problems, it will be difficult for world agriculture to meet the world's food and energy requirements (Tester and Langridge 2010). Therefore, it is urgent to utilize stress-affected land reasonably in sustainable agriculture.

The complex effects of salinity on the physiology and metabolism of plants include alterations in enzyme activity, osmotic balance, ion homeostasis, signal transduction, and relative gene expression (Li et al. 2014a, b). As a perennial and outcrossing species, alfalfa is one of the most valuable forage crops with high protein content and biomass production; and has been widely cultivated as an economic crop worldwide (Osborn et al. 1997). Although alfalfa can be grown under moderate saline-alkaline conditions, alfalfa plants are sensitive to high salt conditions. Salt stress imposed by 50–200 mM NaCl significantly limits productivity and growth range of alfalfa (Yang et al. 2005; Li and Brummer 2012).

To improve salt tolerance of alfalfa, transgenic alfalfa plants have been achieved by overexpression of foreign genes encoding compatible solutes (Li et al. 2014a, b), ion transporters (Zhang et al. 2014), protein kinase (Wang et al. 2014) and transcription factors (Jin et al. 2010; Tang

et al. 2014) in recent years. For example, overexpressing a bacterial *codA* in transgenic alfalfa plant's chloroplasts enhanced tolerance to NaCl on the whole plant level with higher chlorophyll content and lower MDA level than non-transgenic plants (Li et al. 2014a, b). Zhang et al. (2014) showed that transgenic alfalfa plants expressing *SeNHX1* gene exhibited lower MDA level, higher proline content and higher SOD, POD, and CAT activities than those in wild-type plants after 0.6 % NaCl stress treatment for 21 days. In addition, the salt tolerance of transgenic alfalfa plants expressing *GmDREB* gene was higher than that of wild-type plants as measured by ion leakage, chlorophyll fluorescence value, contents of free proline and total soluble sugars (Jin et al. 2010).

Nevertheless, salt tolerance of alfalfa can be improved based on the presence of physiological variation for salt tolerance within alfalfa varieties (Noble et al. 1984; Ashraf et al. 1986). Alfalfa has a large diversity of varieties with different tolerance to stress conditions. Seedling growth and productivity under light stress showed an increase in aerial biomass, while in severe drought and salinity stress, the varieties died except relative high tolerant ones (Castroluna et al. 2014). Wang et al. (2009) compared salt tolerance of six alfalfa varieties and indicated the importance of antioxidant enzymes in the establishment of alfalfa seedlings under salinity condition. Gene expression analyses and antioxidant enzyme assays in two alfalfa varieties after short term salt treatment showed that salt induced increases of enzyme activities and *CAT* gene expression (Mhadhbi et al. 2011). Comparative transcriptomic analysis of two contrasting alfalfa varieties showed that many genes encoding transcription factors (TFs) were differentially regulated between the two varieties in response to salt (Zahaf et al. 2012). However, only limited numbers of alfalfa varieties were used in these studies. Therefore, it is important to screen salt stress tolerant cultivars among various alfalfa varieties for the purpose of studying tolerant germplasm in future. Moreover, many studies focused on effect of salt stress on germination and seedling establishment (Johnson et al. 1992; Scasta et al. 2012; Anower et al. 2013). How alfalfa plants respond to long term salt stress was largely unknown. The aim of this study was to compare the difference of salt tolerance among 14 alfalfa varieties and evaluate contrasting salt stress responses of two alfalfa varieties—211609 and Xinjiang Daye—at physiological and transcriptional levels after 18 days of salt treatment.

Materials and methods

Plant materials and growth conditions

Fourteen alfalfa (*M. sativa* L.) varieties were used in this study, which were kindly provided by Northwest Plateau Institute of Biology, Chinese Academy of Sciences and The Agricultural Research Service, United States Department of Agriculture.

The alfalfa seeds were surface-sterilized with 5 % sodium hypochloride solution for 10 min, and then thoroughly rinsed five times with sterile Milli-Q water. After stratifying at 4 °C for 2 days in darkness, these seeds were germinated on half-strength Murashige and Skoog (MS) solid medium including 0.8 % agar (pH 5.7) under a 16/8 h light/dark cycle, with a light intensity of 350 μmol m^{-2} s^{-1} and a relative humidity of 64 % at 25 °C. After 3 days of germination, the same size seedlings of 14 alfalfa varieties were transferred once again to 1/2 MS solid medium including 0.8 % agar (pH 5.7) with 0 and 250 mM NaCl for experiment in MS medium.

For experiment in soil, the selected alfalfa varieties of 211609 and Xinjiang Daye were germinated on the 1/2 MS solid medium including 0.8 % agar (pH 5.7) for 1 week. Then, the seedlings of uniform size were transferred to plastic pots (10 cm side length at top and 7.5 cm side length at bottom and 8.5 cm high) filled with equal quantity pre-autoclaved vermiculite and soil. Nine seedlings were planted in each pot and irrigated with similar quantities of 0.2 % (w/v) nutrient solution (18–18–18 of nitrogen–phosphorus–potassium per 100 g fertilizer, plus 3 % magnesium and microelement) every 4 days for 3 weeks.

Salt stress treatment and sampling

For salt treatment on the MS medium grown seedlings, 3-day-old seedlings of 14 alfalfa varieties were treated with 0 and 250 mM NaCl, respectively. After growth for 14 days, the seedlings were sampled to measure electrolyte leakage (EL). Chlorosis/necrosis severity was rated as follows: 0, no yellow or purple cotyledons; 1, one cotyledon turns yellow or purple; 3, two cotyledons turn yellow or purple; 5, one cotyledon turns albinism; 7, two cotyledons turn albinism (plant die) under salt treatment 14 days. Severity index was evaluated as described previously (Piccinni et al. 2000): Severity index = Σ [(number of plants with each score × score value)]/(total number of plants × highest score value).

For salt treatment on the soil grown seedlings, 4-week-old plants were irrigated with 0.2 % (w/v) nutrient solution supplemented with NaCl, which was increased stepwise to 250 by 50 mM every other day. For each variety, twelve pots of seedlings were used as replicates in each independent experiment, which were conducted in a randomized complete block design under the same growth condition. At 6, 12 and 18 days after salt treatment, the leaf samples from the same position were used for physiological parameter measurement.

For real-time PCR analysis, 4-week-old plants were directly irrigated with 250 mM NaCl solution for 4 h; and then the leaves from the same position were sampled for gene expression assessment.

Measurement of leaf water content and electrolyte leakage

For leaf water content (LWC) assay, the detached leaves at the same part of the plants were sampled at different time points and quantified as initial fresh weight (FW). Then, the dry weight (DW) was measured after 16 h incubation at 80 °C. LWC was calculated according to the following formula: $LWC = (FW - DW)/FW \times 100$ (Shi et al. 2012).

For the determination of EL, about 0.1 g of detached leaves of control and salt-stressed plants were incubated in 15 ml deionized water. After gently shaking at room temperature for 6 h, the initial conductivity (C_i) was measured by a conductivity meter (Leici-DDS-307A, Shanghai, China). Then, the samples were boiled at 100 °C for 20 min. After cooling to room temperature, the conductivity of the killed tissues (C_{max}) was determined. Relative EL (%) = $(C_i/C_{max}) \times 100$. The ratio of NaCl/Control EL was calculated according to the following formula: Ratio of NaCl/Control EL = the EL under salt stress condition/the EL under control condition.

Determination of soluble protein content, ROS levels and antioxidant enzyme activities

About 0.3 g of fresh leaves were ground quickly with liquid nitrogen and then homogenized in 50 mM sodium phosphate buffer (pH 7.8). After centrifugation at 10,000 g for 15 min at 4 °C, the gained supernatant was used for the assessment of soluble protein content, ROS levels including H_2O_2, O_2^- and $HO^{\cdot}$, and antioxidant enzyme activities (Hu et al. 2010). The soluble protein content was measured by Bradford method with bovine serum albumin (BSA) as standard.

For the assessment of H_2O_2 content, the method was used as descried by Hu et al. (2010). Briefly, 1 ml of the above supernatant was homogenized in 1 ml of 0.1 % titanium sulfate [in 20 % (v/v) H_2SO_4] thoroughly for 10 min. After centrifugation at 12,000 g for 10 min, the absorbance of the gained supernatant was measured at 410 nm and the H_2O_2 content was calculated by the standard curve of H_2O_2.

The O_2^- and $HO^{\cdot}$ contents were assayed by Plant SOA Elisa Kit (10–40–421, Yinuotai, Wuhan, China) and Plant $HO^{\cdot}$ Elisa Kit (10–40–614, Yinuotai, Wuhan, China) based on antibody-antigen-enzyme-antibody complex following the manufacturer's instructions. Briefly, 50 µl standard substance and 50 µl sample solution (five times diluent) were added to standard orifice and sample orifice, respectively, and then incubated for 30 min at 37 °C. After discarding these solutions, the plate was washed using cleaning solution five times, and then injected 50 µl enzyme standard reagent into each hole except blank orifice following incubation for 30 min at 37 °C. Color agent A and B were added, respectively, into the same hole and reacted for 15 min at 37 °C in the dark after discarding the above solution in each orifice and thoroughly washing the plate as well. Finally, 50 µl stop buffer was quickly injected into every orifice, and then the absorbance of the mix was measured at 450 nm in <15 min.

The activities of SOD, CAT and GR were measured using Total SOD Assay Kit with WZT-1 (S0102, Beyotime, Shanghai, China), CAT Assay Kit (S0051, Beyotime, Shanghai, China), and GR Assay Kit (S055, Beyotime, Shanghai, China), respectively, according to the manufacturer's instructions. In addition, the POD activity was assayed with Plant POD Assay Kit (A084-3, Jiancheng, Nanjing, China) as the instruction described. For the determination of APX activity, Plant APX Elisa Kit (10–40–542, Yinuotai, Wuhan, China) was used on the basis of the manufacturer's instruction.

Determination of GSH content

The GSH content was assayed using GSH Assay Kit (A006-1, Jiancheng, Nanjing, China) in accordance with the described introduction as previously described (Shi et al. 2012).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from 450 mg leaves using TRIzol reagent (Invitrogen) and treated by RQ1 RNase-free DNase (Promega) to remove the possible contamination of genomic DNA. The first-strand cDNA was synthesized with 2 µg of above RNA using reverse transcriptase (TOYOBO). For quantitative real-time PCR (RT-qPCR), a CFX 96 Real Time System (BIO-RAD) with SYBR-green fluorescence and the comparative $^{\Delta\Delta}$CT method were used as previously described (Livak and Schmittgen 2001). Gene-specific primers used for RT-qPCR were as follows: for *SOS1* (5′-GCT GAC TTT CCC GTA TG-3′ and 5′-TGG CAC CCA GTT CTT TC-3′), for *SOS2* (5′-CCG TGG TAT CTT CTG TT-3′ and 5′-CAA GGG TTA GGT GTA TT-3′), for *NHX1* (5′-GCC TTC GTG CTT TAC TAT CAA C-3′ and 5′-GAT TAC CAT TGC GTT CAC TTG G-3′), for *ZFG* (5′-TCT GTC GTT TTC ACC ATC CAC-3′ and 5′-TCA ACT CAC AGG ACG CAA AG-3′), for *CBF4* (5′-GAT TGC ACT GAG AGG AAG GTC-3′ and 5′-CCG CCT TTT GAA TAT CCC TTG-3′), for

HSP23 (5′-CAT TCA ACA CCA ACG CCA TG-3′ and 5′-CGG ATC AAA CAC ATC TGA GAG G-3′). *MsActin* was used as internal control with primers (5′-TCC TAG GGC TGT GTT TCC AAG T-3′ and 5′-TGG GTG CTC TTC AGG AGC AA-3′). In addition, *MtUBI* gene which was not changed by salt stress (Gruber et al. 2009) was used as control using primers 5′-TTG GAG ACG GAT TCC ATT GCT-3′ and 5′-GCC AAT TCC TTC CCT TCG AA-3′. The expression levels of the above genes were standardized with *MsActin*. There were three technical replicates and the experiment was repeated three times.

Statistical analysis

All the experiments in the study were repeated at least three times of independent experiments and the results explained are the mean ± SE. The asterisk above the columns of figures indicated significant difference between two alfalfa varieties at $P < 0.05$ by student's t test.

Results

Comparative analysis of salt tolerance among 14 alfalfa varieties

To evaluate the salt tolerance of 14 alfalfa varieties, healthy seedlings were subjected to 1/2 MS solid medium containing 0 and 250 mM NaCl. The leaf EL and chlorosis/necrosis severity index were measured at salt stress 14 days. Under control condition, the EL of 14 varieties was relatively low and maintained at the same level. After salt stress treatment for 14 days, all varieties showed a largely increased EL, and significant differences in the ratio of NaCl/Control EL were observed among 14 varieties (Fig. 1a). The Leaf firing was known as the change of leaf chlorosis and always visually assayed by the numbers of the yellow and brown leaves as described (Carrow 1995). 14 alfalfa varieties showed significant differences in the chlorosis/necrosis severity index (Fig. 1b). Among them, 211609 had the lowest ratio of NaCl/Control EL and chlorosis/necrosis severity index, while Xinjiang Daye presented the highest ratio of NaCl/Control EL and chlorosis/necrosis severity index (Fig. 1). Meanwhile, Xinjiang Daye showed obvious senescence of cotyledons and young leaves than that of 211609 after 14 days salt treatment (Fig. 2). These results indicated that alfalfa varieties exhibited natural variations to salt. Variety 211609 was relatively tolerant to salt while Xinjiang Daye was relatively sensitive to salt. To further examine the possible mechanism of the salt tolerance variations, two alfalfa varieties (211609 and Xinjiang Daye) with different

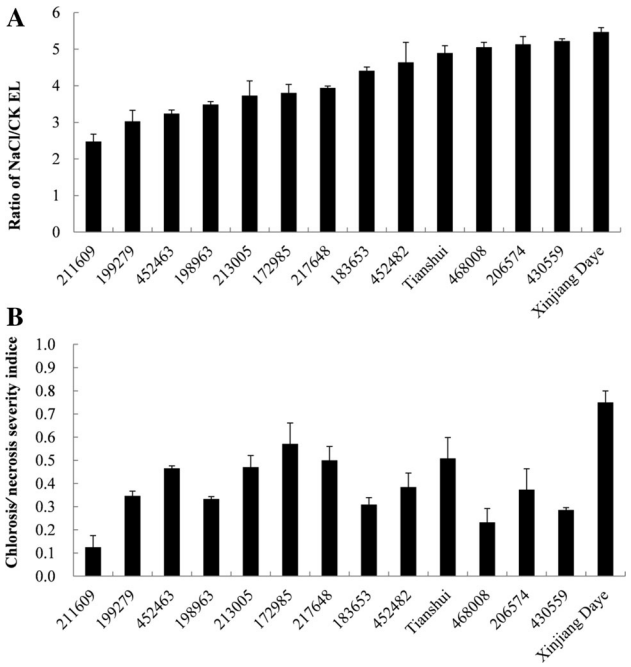

Fig. 1 Comparison of salt stress tolerance among 14 alfalfa varieties. Ratio of NaCl/Control EL (**a**) and chlorosis/necrosis severity index (**b**) of 14 varieties were showen here. Ratio of NaCl/Control EL was calculated based on the EL under control and 250 mM NaCl treatment for 14 days on 1/2 MS plate. Chlorosis and necrosis were rated as: 0, no yellow or purple cotyledons; 1, one cotyledon turns yellow or purple; 3, two cotyledons turn yellow or purple; 5, one cotyledon turns albinism; 7, two cotyledons turn albinism (plant die) after salt treatment for 14 days on 1/2 MS plate. Chlorosis/necrosis severity index was measured as described in Experimental Procedures. Values represent mean ± SE (n = 3)

sensitivity to salt stress were selected for the following experiments.

Comparative analysis of water status and leaf EL between 211609 and Xinjiang Daye

The 4-week-old plants grown in soil were irrigated with NaCl solution with a gradual increase from 50 to 250 mM every other day. Under control condition, the two varieties showed no leaf chlorosis phenomena; however, salt treatment for 18 days induced leaf firing at different extent in both varieties. Xinjiang Daye had more severe yellow and brown leaves than 211609 (Fig. 3), which was consistent with the phenotype on the 1/2 MS plates (Fig. 2).

To understand how the salt treatment affected plant water status and cell membrane integrity, the LWC and EL were measured and compared in 211609 and Xinjiang Daye. The two varieties maintained the similar LWC at about 85 % (Fig. 4a) and the similar EL at about 2 % under control condition (Fig. 4b). In the presence of salt, the LWC showed a gradual decline in both varieties; and the

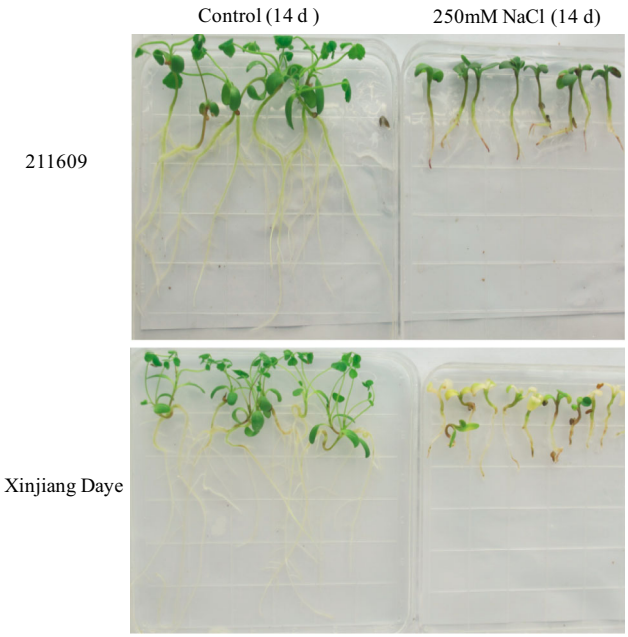

Fig. 2 Two alfalfa varieties response to salt stress on 1/2 MS solid medium. The young seedlings with uniform size were subjected to control condition and 250 mM NaCl condition for 14 days

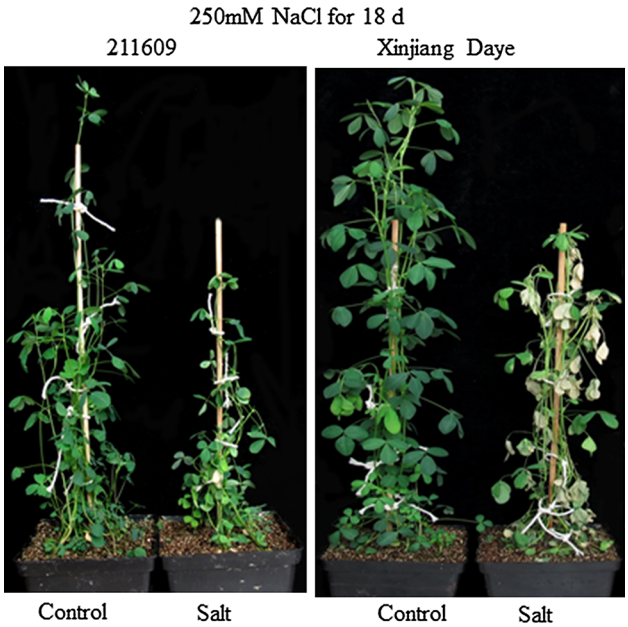

Fig. 3 Plants response to salt stress in soil. The healthy 4-week old alfalfa plants with similar size were watered by 0 mM NaCl (control) and 250 mM NaCl solution for 18 days, respectively

LWC of 211609 decreased from 84.3 to 77.5 %, while Xinjiang Daye from 83.3 to 69.8 %. 211609 showed significantly higher LWC than Xinjiang Daye at salt stress 18 days (Fig. 4a). Contrary to the change of LWC, the two varieties exhibited a largely increase in EL after salt tress

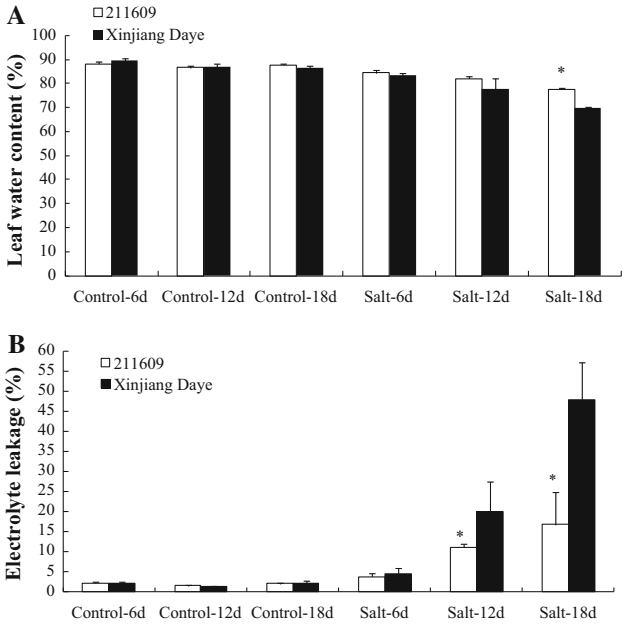

Fig. 4 Water status and cell membrane damage of two alfalfa varieties differing in salt tolerance during salt stress. LWC (**a**) and EL (**b**) of 211609 and Xinjiang Daye were shown here under salt stress for 6, 12 and 18 days. Values represent mean ± SE (n = 3). *Asterisk* symbols indicate significant difference with $P < 0.05$ (t test) in relative to Xinjiang Daye

treatment. The EL of Xinjiang Daye was significantly higher than that of 211609 at 12 and 18 days after salt tress treatment (Fig. 4b). These results indicated that tolerant 211609 exhibited a better capacity of maintaining water content in cells and suffered less cell membrane damage compared to sensitive Xinjiang Daye.

Comparative analysis of ROS levels between 211609 and Xinjiang Daye

Environmental stresses such as salinity induce the over-production of ROS, which damage lipid membrane and increase the content of MDA (Smirnoff 1993). Under control condition, no significant differences were found in ROS levels between 211609 and Xinjiang Daye. Salt treatment resulted in the higher contents of H_2O_2, $HO^{\cdot}$ and O_2^{-} in the two varieties, especially at salt stress 12 and 18 days. However, significantly higher ROS contents were observed in Xinjiang Daye after 12 and 18 days of salt treatment compared to 211609, except the content of H_2O_2 at salt stress 12 days (Fig. 5). These results showed that 211609 accumulated lower contents of ROS which lead to less oxidative injury for plants than Xinjiang Daye under salt stress condition.

Comparative analysis of soluble protein and GSH contents between 211609 and Xinjiang Daye

As compatible solutes play important roles for protecting protein structures, scavenging ROS and adjusting osmotic stress, we examined the content of soluble protein. Under control condition, the soluble protein content of 211609 was similar to that of Xinjiang Daye. Salt treatment leaded to the increased soluble protein content in both varieties. 211609 showed higher soluble protein content than Xinjiang Daye at 12 and 18 days after salt treatment; however, no significant differences were found between the two varieties (Fig. 6a).

Additionally, acting as a non-enzymatic antioxidant, the reduced form of glutathione (GSH) could eliminate hydrogen peroxide via glutathione peroxidase (Kumar et al. 2003; Schonhof et al. 2007; Erkan et al. 2008). Prior to the salinity treatment, the internal GSH level was very low and no significant differences between the two varieties were observed; however, salt treatment greatly increased GSH content in the two varieties, especially at salt stress 12 and 18 days. 211609 showed significantly higher content of GSH than that of Xinjiang Daye after salt treatment for 12 and 18 days (Fig. 6b).

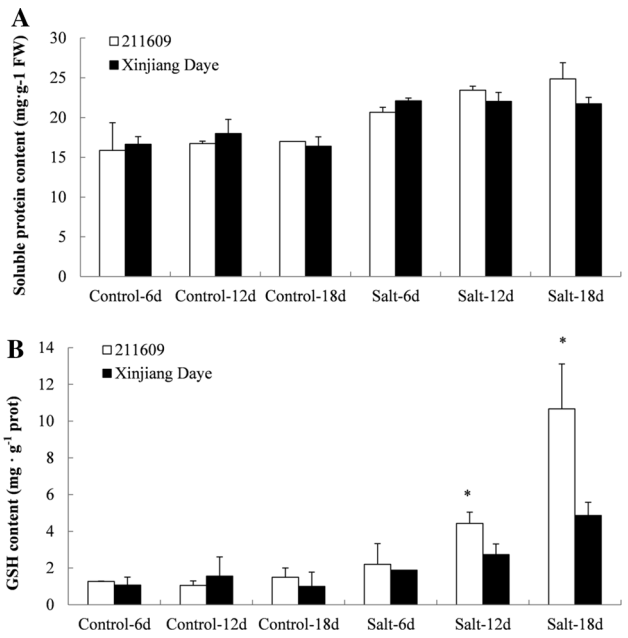

Fig. 6 Contents of soluble protein and GSH of two alfalfa varieties differing in salt tolerance during salt stress. Comparisons of soluble protein content (**a**) and GSH content (**b**) of 211609 and Xinjiang Daye were shown here under salt stress for 6, 12, and 18 days. Values represent mean ± SE (n = 3). *Asterisk* symbols indicate significant difference with $P < 0.05$ (*t* test) in relative to Xinjiang Daye

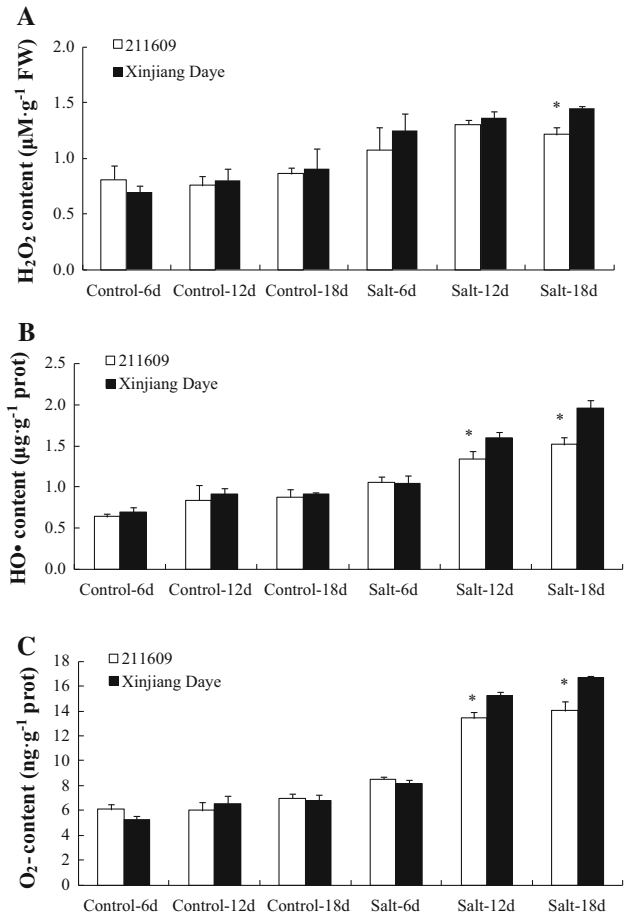

Fig. 5 ROS levels of two alfalfa varieties differing in salt tolerance during salt stress. Changes of H_2O_2 content (**a**), $HO^{\cdot}$ content (**b**) and O_2^{-} content (**c**) of 211609 and Xinjiang Daye were shown here under salt stress for 6, 12, and 18 days. Values represent mean ± SE (n = 3). *Asterisk* symbols indicate significant difference with $P < 0.05$ (*t* test) in relative to Xinjiang Daye

Comparative analysis of antioxidant enzyme activities between 211609 and Xinjiang Daye

Plants would activate antioxidant enzymatic defense system to avoid injury of ROS accumulation and membrane lipid peroxidation. Thus, we further evaluated the activities of antioxidant enzymes (SOD, POD, CAT, APX and GR) between 211609 and Xinjiang Daye in the absence and presence of NaCl. Without stress treatment, there were no significant differences in the activities of five antioxidant enzymes between the two varieties, except that CAT activity was significantly higher in 211609 than that in Xinjiang Daye at 12 days under control condition. Salt treatment induced the activities of antioxidant enzymes in both varieties compared to control condition. Moreover, 211609 showed significantly higher antioxidant enzyme activities than Xinjiang Daye after salt stress, including CAT, APX, SOD and GR at 12 days and POD, CAT at 18 days and CAT at 6 days (Fig. 7). These results

indicated that 211609 was more efficient to eliminate ROS accumulation and relieve oxidative damage than Xinjiang Daye.

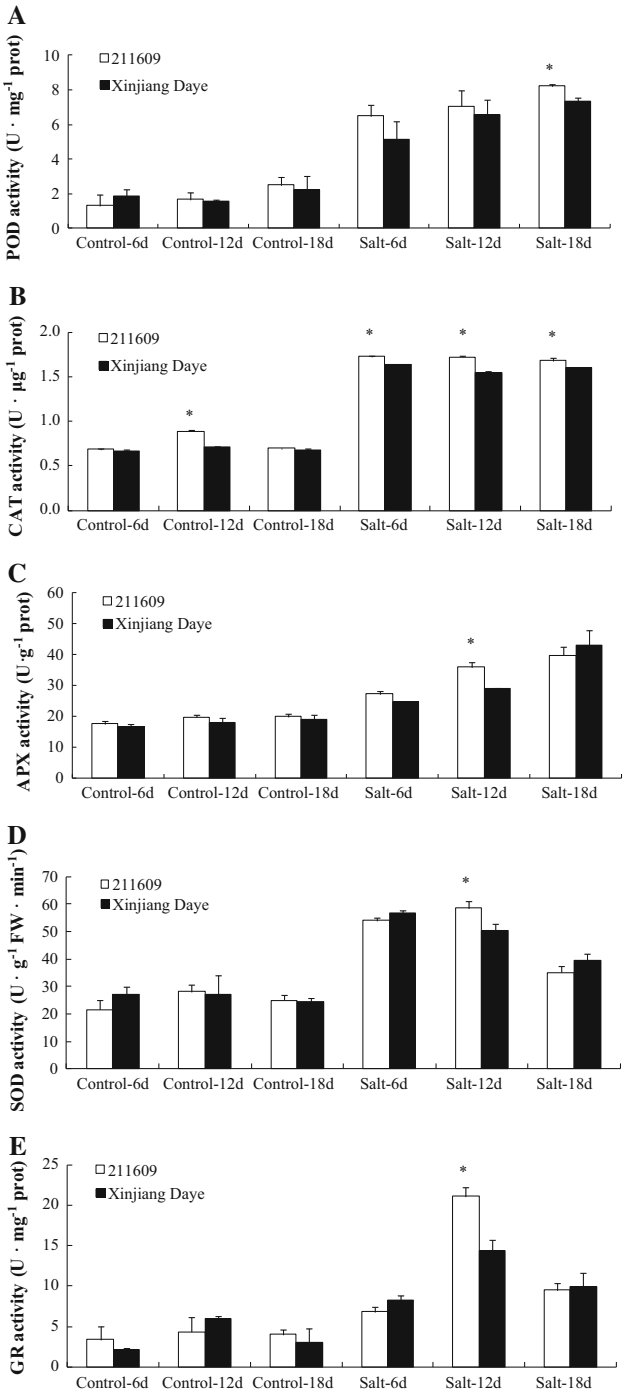

Fig. 7 Antioxidant enzyme activities of two alfalfa varieties differing in salt tolerance during salt stress. Changes of POD (**a**), CAT (**b**), APX (**c**), SOD (**d**) and GR (**e**) of 211609 and Xinjiang Daye were shown here under salt stress for 6, 12, and 18 days. Values represent mean ± SE (n = 3). *Asterisk* symbols indicate significant difference with $P < 0.05$ (*t* test) in relative to Xinjiang Daye

Gene expression variation of salt-stressed alfalfa plants

In order to investigate the changes in gene expression induced by salt stress, we performed a transcriptional expression analysis. The value of expression level for each gene was normalized by *MsActin*. *UBI* was not changed after salt treatment (Gruber et al. 2009) and was used as positive control in the present study. The results showed that *UBI* had the similar abundance of relative expression under control and salt conditions in each variety after normalization with *MsActin* (Fig. 8a), which was consistent with previous report (Gruber et al. 2009). Given the significant roles of SOS proteins in regulation of Na^+ acquisition (Zhu 2003), we studied the effect of salt stress on expression of *SOS1* and *SOS2* by RT-qPCR. Under control condition, the abundance of *SOS1* in 211609 was relatively lower than that of Xinjiang Daye, and the relative expression level of *SOS2* was similar in the two varieties (Fig. 8b, c). Treatment with NaCl leaded to higher increase in *SOS1* and *SOS2* transcripts of 211609 and Xinjiang Daye; however, no significant difference was found in the relative expression level of *SOS1* between the two varieties

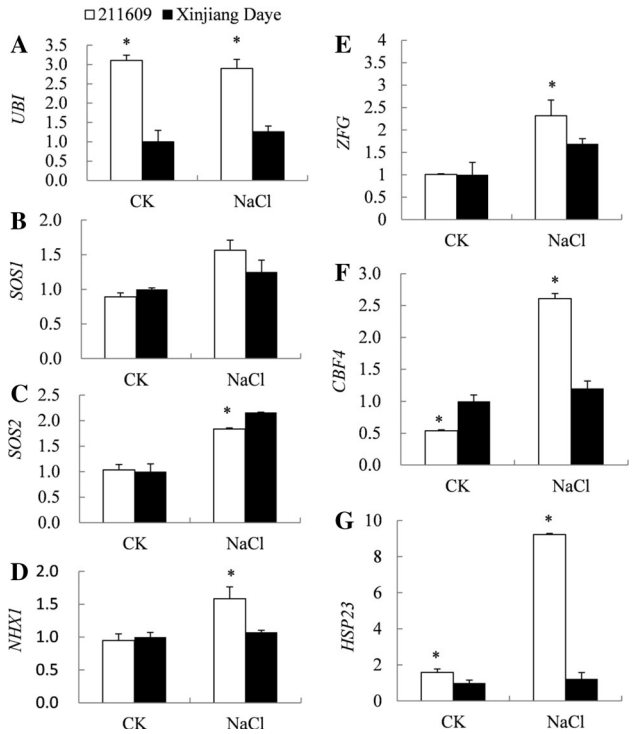

Fig. 8 Relative expression levels of salt-related genes of two alfalfa varieties under 250 mM NaCl stress for 4 h. *UBI* gene (**a**) was used as positive control in RT-qPCR. Relative expression variations of *SOS1* (**b**), *SOS2* (**c**), *NHX1* (**d**), *ZFG* (**e**), *CBF4* (**f**) and *HSP23* (**g**) genes of 211609 and Xinjiang Daye were shown here. Values represent mean ± SE (n = 3). *Asterisk* symbols indicate significant difference with $P < 0.05$ (*t* test) in relative to Xinjiang Daye

no matter under control or salt stress condition (Fig. 8b). However, Xinjiang Daye showed the significantly higher relative expression level of *SOS2* than 211609 under salt stress condition (Fig. 8c). In addition, acting as the vacuolar Na^+/H^+ exchanger, we measured the expression level of *NHX1* gene. The results indicated that significant increase of *NHX1* transcript was observed in 211609 under salt treatment compared to control condition, while the relative expression level of *NHX1* in Xinjiang Daye was almost constant under control and salt stress conditions (Fig. 8d). *ZFG*, *CBF4* and *HSP23* genes are all induced by environmental stresses and play important roles in abiotic stress responses according to previous researches (Chao et al. 2009; Li et al. 2011; Lee et al. 2012a, c). In the present study, we also found that salt treatment induced the expression of *ZFG*, *CBF4* and *HSP23* at varying extent in both 211609 and Xinjiang Daye. Under control condition, the relative expression level of *ZFG* was similar between the two varieties, *CBF4* of Xinjiang Daye was about 2.0-fold higher than 211609, and *HSP23* of 211609 was about 1.5-fold higher than Xinjiang Daye. When subjected to salt stress, 211609 showed 1.5-fold for *ZFG*, 2.2-fold for *CBF4* and 7.7-fold for *HSP23* higher relative expression levels than Xinjiang Daye, respectively. Significant differences were found in the three gene expression levels between the two varieties under salt stress condition (Fig. 8e–g). These above data showed that salt tolerant 211609 exhibited higher expression levels of stress responsive genes than Xinjiang Daye.

Discussion

Plants differ greatly in their tolerance of salinity, as reflected in their different growth responses (Munns and Tester 2008). In this study, we screened the salt tolerant 211609 and the salt susceptible Xinjiang Daye from 14 alfalfa varieties drastically differing in salt tolerance according to the ratio of NaCl/Control EL and the chlorosis/necrosis severity index (Fig. 1).

Since salt stress results in osmotic stress and reduces water availability to plants, maintenance of LWC indicates that the plants are able to prevent water loss (Anower et al. 2013). 211609 showed significantly higher LWC in leaf tissues at salt stress 18 days compared to Xinjiang Daye, suggesting that 211609 could keep higher water content in cells under stress condition (Fig. 4a). An alternative hypothesis could be that variety 211609 was able to accumulate higher amount of compatible solutes and reduce osmotic potential in plant cells, which was helpful for water uptake form soil, resulting in better plant growth and less chlorosis phenomenon in 211609 under salt stress (Figs. 2, 3). Enhanced accumulation of compatible solutes such as soluble sugars, proline and glycine betaine to prevent water loss is a common adaptive strategy in response to abiotic stress (Ashraf and Foolad 2007). In addition, the EL acts as one indicator of cell membrane stability and has been widely used to evaluate the extent of cell injury for the stress treated plants (Bouchabke et al. 2008). Significantly lower EL was found in 211609 at salt stress 12 and 18 days compared to Xinjiang Daye (Fig. 4b), indicating that 211609 suffered less severe cell injury induced by salt stress and maintained a better stability of membrane structures under adverse condition.

Salinity stress disturbs redox homeostasis in plant cells, and induces oxidative stress by causing a burst of ROS. ROS can directly attack membrane lipids, resulting in lipid peroxidation and oxidation of proteins and nucleic acids (Yin et al. 2009; Alhdad et al. 2013). Significantly lower H_2O_2 content at salt stress 18 days, O_2^- and $HO^{\cdot}$ contents at salt stress 12 and 18 days were found in 211609 than those in Xinjiang Daye (Fig. 5). The results demonstrated that variety 211609 suffered less oxidative injury from ROS than Xinjiang Daye in term of accumulation of ROS. To deal with oxidative damage under extremely adverse conditions, plants have developed an antioxidant defense system that includes antioxidants and antioxidant enzymes (Mittler 2002; Foyer and Noctor 2005). Glutathione, especially GSH, is the major non-enzymatic antioxidant contributing to plant antioxidant defense and abiotic stress responses (Miller et al. 2010). In the present study, 211609 showed significantly higher GSH content than Xinjiang Daye at salt stress 12 and 18 days (Fig. 6b). Meanwhile, we measured the activities of enzymatic antioxidants including SOD, POD, CAT, APX and GR. SOD is responsible for catalyzing O_2^- into H_2O_2; POD, CAT and APX participate in oxidation reactions by converting H_2O_2 to H_2O, which regulate H_2O_2 level in plants; and GR is responsible for the modulation of glutathione redox state through converting GSSG into GSH (Miller et al. 2010). The increased antioxidant enzyme activities were advantageous to adapt salt stress for alfalfa plants (Wang et al. 2009). Various cultivars had different levels of antioxidant enzymes which were higher in tolerant species than those in sensitive species under diverse environmental stresses (Bor et al. 2003; Demiral and Türkan 2005). Accordingly, significantly greater increases in CAT at 6 days, CAT, APX, SOD and GR at 12 days, and POD and CAT at 18 days were measured in tolerant 211609 during the salt treatment compared to sensitive Xinjiang Daye (Fig. 7). These above results suggested that 211609 possessed a better ability of ROS scavenging and protecting the plant cells from oxidative damage, which was consistent with less EL and lower ROS accumulation in the variety under salt stress (Figs. 4b, 5).

To further explore the mechanism underlying the greater salt tolerance of 211609 than Xinjiang Daye, we evaluated

the relative expression variations of salt-related genes between the two alfalfa varieties. Genes relative to abiotic stress responses have been identified and categorized into two types according to the proteins encoded (Ozturk et al. 2002). The first type of genes expressed functional proteins, such as membrane transporter proteins and water-channel proteins, and the second type was involved in signal transduction pathways and expression regulatory processes, such as transcription factors and kinases (Chinnusamy et al. 2005). SOS1, as a putative Na^+/H^+ antiporter in the plasma membrane, mediates Na^+ extrusion from plant cells and regulates the long-distance Na^+ transport (Yamaguchi et al. 2013). The expression of *SOS1* gene provides a helpful marker for adaptation to salt stress (Elmaghrabi et al. 2013). Overexpression of *SOS1* leads to improved salt tolerance in transgenic Arabidopsis (Shi et al. 2003). The higher expression level of *SOS1* might account for the greater tolerance to salt stress in *M. falcate* than in *M. truncatula* (Liu et al. 2015). In the present study, the relative expression level of *SOS1* was higher in 211609 than in Xinjiang Daye under salt stress condition; however, no significant difference was found between the two varieties (Fig. 8b). Activation of *SOS1* by salt stress is controlled by SOS2 and SOS3 which are a serine/threonine protein kinase and a myristoylated calcium-binding protein, respectively (Qiu et al. 2002). Previous research showed that the abundance of *SOS2* transcript was higher in salt susceptible *M. truncatula* than in salt tolerant *M. falcate*. Moreover, treatment with NaCl increased expression of *SOS2* in roots of *M. truncatula*, while SOS2 in roots of *M. falcate* was relatively constant (Liu et al. 2015). Our results exhibited that exposure to 250 mM NaCl for 4 h obviously enhanced the abundance of *SOS2* expression in both alfalfa varieties compared to control condition; and significantly higher transcript of *SOS2* in susceptible Xinjiang Daye was measured than that in tolerant 211609 (Fig. 8c). In addition, Na^+/H^+ antiporters belonging to NHX family are involved in compartmentalization of Na^+ from cytoplasm into vacuoles by using H^+ gradient as a driving force (Blumwald 2000). Therefore, *NHX1* gene is one of the important factors contributing to plant salt tolerance (Bassil et al. 2011). The significantly higher relative expression level of *NHX1* was found in 211609 upon exposure to NaCl (Fig. 8d), suggesting that 211609 had more efficient compartmentation of Na^+, and accumulated less Na^+ in cytoplasm than Xinjiang Daye.

Transcription factors play important roles in transcriptional regulation of plant response to signals from development and environment. The previous studies have identified many transcription factors involved in salt stress, such as Zinc finger proteins (ZFPs) and AP2/EREBP family, which overexpression in transgenic plants could improve plant stress tolerance to salt (Gao et al. 2005; Jin et al. 2010; Tang et al. 2013). The expression level of *ZFG* was highly induced after saline treatment in the two alfalfa varieties, which was consistent with previous research (Chao et al. 2009). Furthermore, significantly higher expression of *ZFG* was observed in 211609 than that in Xinjiang Daye under salt condition (Fig. 8e). For DREB/CBF belonging to AP2/EREBP subfamily, it could be induced by cold, salt and drought treatment in many species, such as in *Arabidopisis* (Liu et al. 1998; Nakashima et al. 2000), soybean (Li et al. 2005) and *M. truncatula* (Pennycooke et al. 2008). Overexpressing *MtCBF4* enhanced tolerance to salt stress, and activated expression levels of corresponding downstream genes in *M. truncatula* and transgenic *Arabidopsis* (Li et al. 2011). When subjected to salt treatment, 211609 showed significantly higher relative expression level of *CBF4* than Xinjiang Daye (Fig. 8f). These results indicated that variety 211609 could activate more transcripts of stress responsive downstream genes than variety Xinjiang Daye.

In addition, plant small heat shock proteins genes (*sHSPs*) are encoded in the nuclear genome and localized to different cellular compartments, including chloroplasts, mitochondria, the endoplasmic reticulum, and the cytosol (Vierling 1991). It has been reported that sHSPs have protective roles against a variety of stresses except high temperatures in recent years (Lee et al. 2012c). Alfalfa mitochondrial small heat shock protein (*MsHsp23*) is a multifunctional protein which overexpression could enhance the tolerance of transgenic plants to environmental stresses (Lee et al. 2012a, b). In our study, the transcription level of *HSP23* in 211609 was almost eightfold higher than that in Xinjiang Daye under saline treatment (Fig. 8g). In addition, we found that 211609 showed higher content of soluble protein than Xinjiang Daye after salt stress, although no significant difference was found between the two varieties (Fig. 6a). Accumulation of compatible solutes under stress condition is important for protection of plants by balancing osmotic pressure and modulating cell membrane stability (Li et al. 2013). These results indicated that 211609 might accumulate more compatible solutes and had a better capacity of protecting plants from adverse damage.

Conclusions

In this study, we found that 211609 showed higher tolerance to salt tress than Xinjiang Daye. The higher LWC, less EL, lower ROS and higher antioxidant enzyme activities in tolerant 211609 might explain the greater tolerance to salt stress in physiological aspect. Moreover, 211609 exhibited significantly higher expression levels of *NHX1*, *ZFG*, *CBF4* and *HSP23* stress responsive genes than Xinjiang Daye at transcriptional level. These results

highlighted that selection of salt tolerant variety is important for further breeding of alfalfa with enhanced stress resistance.

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