Plant Growth Regul (2016) 80:137–147
DOI 10.1007/s10725-016-0150-6

ORIGINAL PAPER

Ameliorative effects of inoculation with *Bradyrhizobium japonicum* on *Glycine max and Glycine soja* seedlings under salt stress

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Received: 25 May 2015 / Accepted: 13 January 2016 / Published online: 20 January 2016

Abstract In order to understand whether inoculation with *Bradyrhizobium japonicum* can enhance soybean's ability to cope with salt stress, *Glycine max* Lee68 cultivar (the salt-tolerant) and *Glycine soja* N23227 accession (the relatively salt-sensitive) were used as the experimental materials in this study. The morphological and anatomical characteristics (including whole plant, organic, cellular and subcellular levels), physiological parameters (containing contents of chlorophyll and carotenoid, value of Fv/Fm (maximum photochemical efficiency of PSII), relative electrolytic leakage and isoflavone contents) and transcriptional pattern of a few isoflavone synthesis-related key enzyme genes (such as *PAL1*, *CHS8*, *CHI* and *IFS2*) were investigated. The results showed that, inoculation with *B. japonicum* on soybean seedlings under 100 mM NaCl stress could obviously increase leaf area, contents of chlorophyll and carotenoid, value of Fv/Fm and the numbers of osmiophilic globule, starch grain and well-arranged stroma thylakoids and grana thylakoids in chloroplasts of salt-stressed soybean seedlings, decrease the relative electrolytic leakage in roots and leaves, and thus demonstrated the ameliorative effects on salt injury to soybean seedlings with different salt tolerance. In comparison, the protective function of *B. japonicum* inoculation on salt-stressed Lee68 seedlings is mainly related to the effects on leaves, while that on N23227 is mainly on roots, which might be attribute to the transcription promotion of isoflavone biosynthesis-related key enzyme genes, such as *CHI* and *IFS2*, and more rise of isoflavone content in roots. Thus, inoculation with *B. japonicum* for alleviating salt stress on various soybean species with diverse salt tolerance can reflect the genotype differences or organ specificity, and may give us a meaningful pathway for salt tolerance improvement of *G. max* by inoculation with *B. japonicum* and gene engineering on isoflavone synthesis.

Keywords *Glycine max* · *Glycine soja* · Inoculation with *Bradyrhizobium japonicum* · Salt stress · Soybean isoflavones

Introduction

Approximately 22 % of the world's agricultural lands are affected by salinity (Bhatnagar-Mathur et al. 2008). More than 800 million hectares of land throughout the world have been reported to be salt-affected, and NaCl is the most soluble and abundant salt released (Munns and Tester 2008). Salt stress may severely limit plant growth, development and production, thus developing salt-tolerant crops by salt hardening, chemical regulation and genetic engineering breeding has been a much desired scientific goal but not with so much success to date (Tian et al. 2014). The cultivated soybean (*G. max*) is the most important legume crop in the world, offering high-quality protein (about 40 % of seed) and oil (about 20 % of seed) for human food and animal feed, and increasing the input of combined nitrogen as well as carbon into the soil (Dolatabadian et al. 2012). According to the habitats, soybean also includes the

Electronic supplementary material The online version of this article (doi:10.1007/s10725-016-0150-6) contains supplementary material, which is available to authorized users.

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wild species (*G. soja*). The genetic base of *G. max* is extremely narrow, and *G. soja*, possessing the same number of chromosomes (2n = 40) with *G. max*, has been suggested as a unique resource for studying the impact of human selection on genetic variation in soybean genome (Lam et al. 2010; Zhang et al. 2011). Improving salt tolerance is an important goal for many soybean breeding programs (Chen et al. 2014).

Facing to adverse environments, plants not only develop morphological and physiological alterations, but also can use plant growth-promoting bacteria (PGPB) to alleviate stress injury (Mayak et al. 2004; Yang et al. 2009; Gamalero et al. 2010; Bashan et al. 2014). Soybean can form nitrogen-fixing root nodules with many rhizobial species, among which, *B. japonicum* is a kind of slow-growing endosymbiont. In the case of soybean, *B. japonicum* is the most-researched bacterial symbiont and widely used as inoculant around the world (Brechenmacher et al. 2008). Salt stress affects the nodulation process mainly by decreasing the number of deformed root hairs and colonization of root surfaces, limiting the sites for bacterial infection and inducing root hair death (Dolatabadian et al. 2012; Muñoz et al. 2012). Isoflavones are a group of plant natural compounds synthesized almost exclusively by legumes. As a kind of symbiotic plant signal, isoflavones play important roles in plant-microbial interactions such as the induction of *nod* gene expression in *B. japonicum* bacteria that form nitrogen-fixing nodules on soybean roots and the inhibition of pathogen growth upon infection (Subramanian et al. 2006; Du et al. 2010). Soybean plants contain three major isoflavone aglycones (daidzein, glycitein and genistein) and their three corresponding glycosides, acetyl and malonyl-ester glycosides, which are synthesized via a branch of the general phenylpropanoid pathway that is present in all higher plant species (Yi et al. 2010). These compounds are thought to have protection for plants against biotic infection (e.g. fungus, bacteria, viruses and insects) with roles in plant defense and nodulation, or abiotic stresses such as salinity, drought, cold, freezing and heat (Tian et al. 2014). The main enzymes for isoflavone biosynthesis involved in the phenylpropanoid pathway include chalcone synthase (CHS), isoflavone synthase (IFS), chalcone isomerase (CHI) and phenylalanine ammonia lyase (PAL) (Yi et al. 2010). The genes encoding these main enzymes are multigene families in soybeans, such as *IFS* (*IFS1* and *IFS2*) (Gutierrez-Gonzalez et al. 2010), *PAL* (*1–3*) (Dixon et al. 2002), *CHS* (1–8) (Yi et al. 2010, 2011) and CHI (type I and II) (Ralston et al. 2005). There are some evidences supporting the idea that infection of soybean roots with *B. japonicum* can enhance the isoflavone levels, which might be related with the enhanced transcription of genes that coded enzymes in the phenylpropanoid pathway, such as *PAL*, *CHS* and *IFS* (Richard et al. 2000; Subramanian et al. 2006). More importantly, there has been extensive research regarding isoflavone compounds can protect plants against abiotic stresses such as UV, aluminum, gamma irradiation and heat stress (Dixit et al. 2012; Khan et al. 2012). Recently, we found that seed soaking with exogenous isoflavones could improve the drought tolerance of *G. max* and *G. soja* (Tian et al. 2014).

Up to now, the studies on effects of inoculation with *B. japonicum* on seedlings growth, ultrastructure of photosynthetic leaf organs, isoflavone contents and its related synthesis enzyme gene transcription of *G. max and G. soja* seedlings under salt stress are still lacking. Lee68 cultivar has been widely regarded as a salt tolerant genotype and used to reveal soybean responses to salt stress (Luo et al. 2005; Ma et al. 2012). Our previous work reported that Lee68 cultivar (*G. max*) possessed higher salt tolerance than N23227 accession (*G. soja*) by germination index, salt injury index and salt tolerant efficiency (Yu et al. 2001). It can suggest that Lee68 is a salt-tolerant soybean genotype while N23227 is a salt-sensitive one. In this study, choosing Lee68 cultivar and N23227 accession as the representative experimental materials of *G. max* and *G. soja* with different salt tolerance, the morphological and physiological differences in effects of inoculation with *B. japonicum* on both soybean seedlings under salt stress were firstly investigated, then the changes in transcriptional pattern of isoflavone synthesis-related *PAL1*, *CHS8*, *CHI* and *IFS2* in roots of *G. max* and *G. soja* seedlings inoculated with *B. japonicum* was analyzed using RT-PCR. The objectives of this work are to explore the positive effects of inoculation with *B. japonicum* on salt tolerance of *G. max* and *G. soja* with diverse living habitats, and to elucidate its possible physiological and molecular mechanisms for future practical application of soybean salt tolerance improvement by soybean-rhizobia symbiosis.

Materials and methods

Plant materials

Two soybean genotypes, *Glycine max* Lee68 cultivar (the salt-tolerant, USA) and *Glycine soja* N23227 accession (the relatively salt-sensitive, Jiangsu, China), and the rhizobium (*B. japonicum* strain ACCC 15609, purchased from Agricultural Culture Collection of China, Beijing) were used in this work.

Growth conditions

Bradyrhizobium japonicum ACCC 15609 was cultured in yeast extract-mannitol broth at 180 rpm at 28 °C for 6 days, and the growth was monitored by measuring

optical density at 600 nm of approximately 0.8. Seeds were surface-sterilized by 0.05 % (w/v) $HgCl_2$ for 5 min, fully rinsed with distilled water, and then placed dishes on wet filter paper for germination in the dark for 2 days at 26 °C. Uniformly germinated seeds were placed in a controlled greenhouse with 25 ± 2 °C/18 ± 2 °C temperature (day/night) and about 14/10 h photoperiod and relative humidity of 60–80 %. When the first true leaves were fully expanded, the seedlings were immersed in suspensions of *B. japonicum* about 30 min, then transplanted to plastic pots containing autoclaved vermiculite, finally watered with free-nitrogen Hoagland solution (Zhang and Smith 1996). 200 mL nutrient solution was added per pot and all nutrient solutions were renewed every 2–3 days. Each treatment was replicated three times with two plants per pot.

NaCl stress treatment

When the first pair of trifoliate leaf was fully unfolded, the above-inoculated soybean seedlings (about 6 days later) were subjected to salt stress by adding NaCl to the nutrient solution at 0, 50, 100, and 140 mM, respectively. This experiment was to choose the suitable NaCl concentration, at which not only the soybean plants could grow but also *B. japonicum* colonization was not completely suppressed (Sharifi et al. 2007). At the initiation of the salt treatment, NaCl concentration was gradually increased by 25 mM at 1-day intervals until reaching the required salt concentration. Subsequently, using the selected suitable NaCl concentration (100 mM), four treatments with three replicates per treatment were carried as follows: (1) Control, non-inoculated with *B. japonicum* and no NaCl treatment. (2) NaCl, non-inoculated with *B. japonicum* but NaCl treatment. (3) Bj, inoculated with *B. japonicum* but no NaCl treatment. (4) NaCl + Bj, inoculation with *B. japonicum* and plus NaCl treatment.

Measurements of growth parameters, chlorophyll and carotenoid contents and Fv/Fm value

After inoculation for 28 days, the plant height was measured with a ruler. For determination of fresh weight, roots, over ground parts and nodules were separated from soybean plants and weighted after being washed with distilled water. Dry weight was obtained after they were oven dried at 100 °C for 30 min and then at 80 °C for 3 days until a constant dry weight. Leaf area was measured using leaf area meter (LI-3100, LI-COR, USA) from the leaf base to the tip (Wei et al. 2015). The relative chlorophyll content was measured as soil plant analysis development (SPAD) using a portable chlorophyll meter (SPAD-502, Minolta Co., Osaka, Japan, Japan). Content of carotenoid was determined using a spectrophotometer (UV-9100, Beijing, China) followed by the method of Tian et al. (2014). The second trifoliate leaf were cut into pieces and soaked with a mixture of ethanol and acetone at the volume ratio of 1:1 in the dark for 48 h. Absorbances of the extracts were measured at 450, 644 and 663 nm, respectively. Fv/Fm was measured with a plant efficiency analyzer (Handy-PEA, Fluorometer, Hansatech Instruments, UK). Prior to the measurement of Fv/Fm, plants were dark-adapted for approximately 30 min. Finally, plants were uprooted from pots to count nodule number. Above all measured leaves were the middle leaf of the second trifoliate leaf. Five replicates were performed.

Determination of relative electrolyte leakage

Relative electrolyte leakages (REL) of roots and leaves were determined by a modification of the method of Tian et al. (2014). Roots and leaves (1.5 g, respectively) were cut into fragments and placed in test tubes containing 15 ml distilled deionized water at a room temperature for 1 h, then the initial electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter (DDS-307, Shanghai, China). The samples were subsequently boiled at 100 °C water bath for 1 h to completely kill the tissues and release all the electrolytes. The samples were then cooled, and final electrical conductivity (EC_2) was measured. REL was calculated using the following formula: $EL = (EC_1 - EC_0)/(EC_2 - EC_0) \times 100\ \%$. EC_0 is the deionized water conductance.

Analysis of cellular ultrastructure

The mature leaf samples without veins were acquired at the area of about 2 cm^2 and immediately preserved in 2.5 % (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for at least 24 h, then immersed in 1 % (v/v) osmium acid for post-fixation about 2–3 h. Finally, resin embedding and ultrathin sectioning were conducted for transmission electron microscopy (TEM) (H-7650, Hitachi, Tokyo, Japan).

Measurements of total isoflavone contents

Total isoflavone extracts were measured by a slight modification of high performance liquid chromatography (HPLC) method of Wu et al. (2011). Root, stem and leaf were harvested separately from soybeans after inoculation for 28 days. The isoflavone were extracted from 1.0 g of fresh samples using 5 mL 80 % (v/v) alcohol by grinding with pestle and mortar and incubated in an 80 °C water bath for

6 h, finally 80 % (v/v) alcohol was added to a final volume of 10 mL. The paste samples were centrifuged for 20 min at 3000*g*, and the supernatant was collected, followed by filtration through a 0.45 μm Millipore filter prior to analysis and finally stored at −4 °C until analysis. The used isoflavone standards (daidzin, genistin, daidzein and genistein) were purchased from Shanghai Tongtian Biotech Co., Ltd (power, purity ≥99 %). These compounds were dissolved in 80 % (v/v) alcohol and mixed to form authentic mixture solutions of 1, 10, 20, 40, 60, 80, 100, 120 μg mL^{-1}. Triplicate repeats were performed for each extract.

Semi-qRT-PCR assay of *PAL1, CHS8, CHI* and *IFS2*

Roots of the tested soybean seedlings were harvested at each time point of 0, 12, 18, 24 and 48 h after inoculation, then frozen in liquid nitrogen and stored at −80 °C for total RNA extraction. The non-inoculated roots were collected at the same time point as controls. RNA extraction, cDNA synthesis and semi-qRT PCR analysis were performed as described by Zhang et al. (2013). To normalize gene expression, soybean *Tubulin* (accession number: XM_003550379, forward primer, 5′-AACCTCCTCCTCATCGTACT-3′; reverse primer, 5′-GACAGCATCAGCCATGTTCA-3′) was used as an internal standard. The primers were designed with the Primer Premier 5.0 based on the elucidated sequences. The forward and reverse primers for *GmIFS2* (GenBank: AF195799.1) were as follows : 5′-GGCAGAACTCATCAACAATCC-3′ and 5′-CATTCCCGAAGTAGCCAGATT-3′, respectively; those for *GmCHS8* (GenBank: AY237728.1) were 5′-GCCAAAGTCAAAGATTACCCA-3′ and 5′-GCTCATACAAAGGCTTCTCAAC-3′, respectively; those for *GmCHI* (GenBank: AF276302.1) were 5′-CGGCAAGACCTATTTCCTCG-3, 5′-GCATCACCGTAAGTCCCAAC-3′, respectively; those for *GmPAL1* (GenBank : X52953.1) were 5′-GTCAAGAACACCGTGAGCCA-3′ and 5′-CCAGTTAGCA ACCCAGTCCC-3′. PCR conditions were as follows: an initial denaturation (5 min, 94 °C) followed by 28 cycles of denaturation (30 s, 94 °C), annealing (30 s, 55 °C) and extension (30 s, 72 °C), and an additional extension at 72 °C for 10 min. The PCR products were separated on 1.0 % agarose gel. Semi-qRT-PCR was used for gene expression analysis. Each time point was replicated three times.

Statistical analysis

All data were presented as mean ± standard deviation (SD) of three or five replicates using SPSS software, ver. 17.0. Data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests were employed to detect differences between means at $P < 0.05$.

Results

Effects of inoculation with *B. japonicum* on growth parameters and nodule occurring of Lee68 and N23227 seedlings under salt stress

At first, in order to select the suitable NaCl concentration for growth of two kinds of soybean plants (Lee68 cultivar and N23227 accession) and their nodule occurring, NaCl with the increased concentration (0, 50, 100, 140 mM) was added to the culture medium. We found that, the growth of Lee68 and N23227 seedlings were severely inhibited with the rise of NaCl concentration, under 140 mM NaCl treatment, the growth of Lee68 and N23227 seedlings were obviously reduced, especially for N23227, whose seedlings were dwarf, leaves were etiolated, abolished and even defoliated (Supplement Fig. 1). The two kinds soybean plants could grow and *B. japonicum* colonization were not completely suppressed in the presence of 100 mM NaCl, therefore, 100 mM NaCl was selected for the following experiments.

Under 100 mM NaCl stress alone (NaCl), when compared with controls, leaf area of the two tested soybean seedlings were significantly decreased, and leaf color turned into yellowing, chlorosis and even necrosis (Fig. 1a, b). The growth of Lee68 seedlings was obviously inhibited, the values of plant height, biomass of roots and overground parts (in fresh or dry weight) per plant, and leaf area were significantly decreased, while no evident changes were displayed in the biomass of N23227 seedlings except for the significant drops in its plant height and leaf area. When inoculated with B. *japonicum* alone (Bj), the growth of Lee68 and N23227 seedlings were promoted for different degrees, the promotion on Lee68 (for less growth parameters) was weaker than that of N23227 (for more growth parameters) (Table 1). Just as for the above-mentioned reasons or differences in performance, the ameliorative effects of inoculation with B. *japonicum* on salt-stressed N23227 seedlings (NaCl + Bj) were much better than those on salt-stressed Lee68 seedlings. In terms of the nodules occurring, the inhibitory effects of salt stress on number and biomass (fresh or dry weight) of nodules per plant of Lee68 seedlings were reached significant level, however, the depressing effect on N23227 seedlings not significant (Table 1; Fig. 1).

Effects of inoculation with *B. japonicum* on contents of chlorophyll and carotenoid, Fv/Fm and relative electrolytic leakage (REL) of Lee68 and N23227 seedlings under salt stress

Under the sole salt stress, changes in the contents of chlorophyll and carotenoid in leaves of Lee68 and N23227

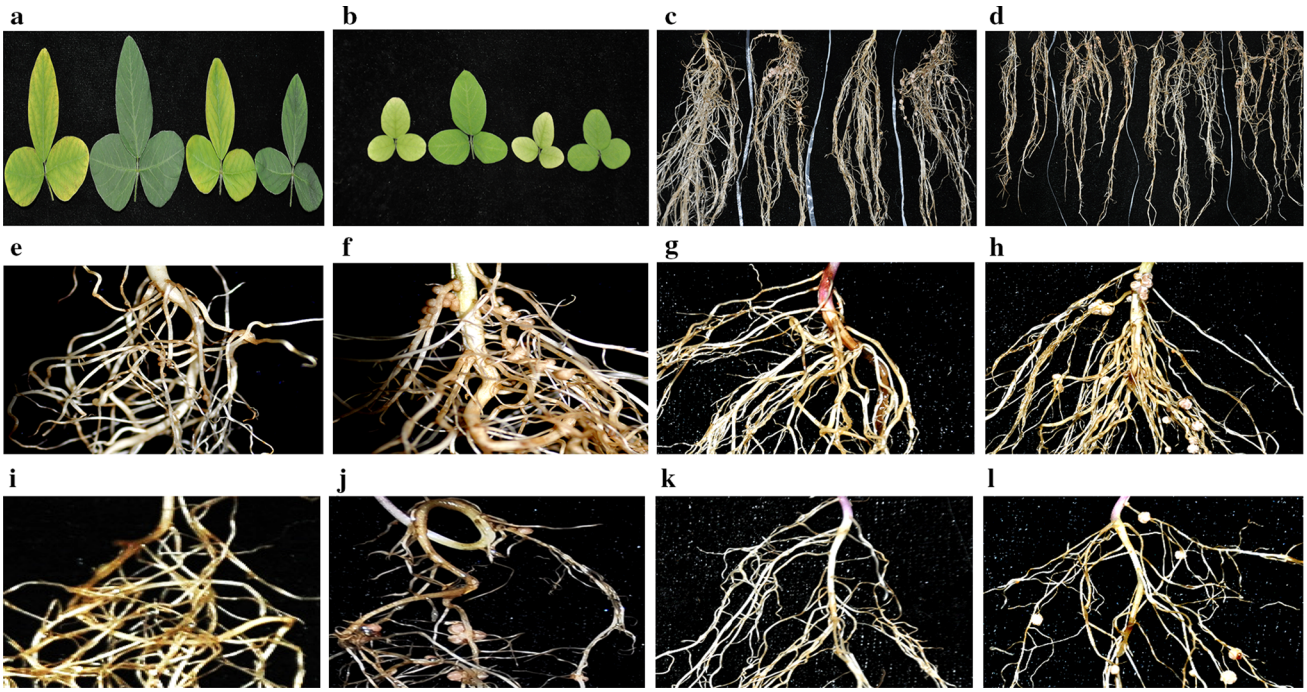

Fig. 1 The appearance of leaves (**a, b**), roots (**c, d**) and nodules (**e–l**) of Lee68 cultivar and N23227 accession plants under inoculation and non-inoculation with *B. japonicum* in the absence and presence of 100 mM NaCl treatment. *Note* From *left to right*, **a**, **c**, **e–h**: Lee68 (Control, Bj, NaCl, NaCl + Bj); **b**, **d**, **i–l**: N23227 (Control, Bj, NaCl, NaCl + Bj). The pictures were taken after inoculation for 28 days

seedlings were on the contrary when compared to their controls, the rise was found in Lee68 whereas drop in N23227 (Fig. 2a, b). This is also consistent with the differences in their salt tolerance (Yu et al. 2001). Especially, variation in the contents of chlorophyll was reached the significant level, while that of carotenoid not. This may be related to the more stability of carotenoid in leaves under normal, salt or other adverse environments than that of chlorophyll. When inoculated with *B. japonicum* alone, the contents of chlorophyll and carotenoid in leaves of Lee68 and N23227 were both significantly increased compared to their controls, the rises in Lee68 were higher than those in N23227. As for the inoculation with *B. japonicum* and subsequent salt treatment, the contents of leaf chlorophyll and carotenoid of Lee68 were increased significantly than its control, but displayed no accumulatively enhanced effect of both inoculation and salt stress; the contents of leaf chlorophyll and carotenoid of N223227 were also increased significantly than its control, but showed the jointed ameliorative effect on salt injury (Fig. 2a, b). As for leaf Fv/Fm value and compared to control, no obvious change was found in the salt-tolerant Lee68 cultivar seedlings under salt stress alone, and that of the salt-sensitive N23227 decreased significantly, but they were all significantly improved when inoculated with *B. japonicum* alone. When inoculated with *B. japonicum* and subsequently salt-stressed, non-obvious change in Fv/Fm value of Lee68 under sole salt stress was ascended to the value between control and sole inoculation, the salt stress-inhibited Fv/Fm value of N23227 was significantly restored to the control level (Fig. 2c). Under salt stress alone, except no significant change in REL in roots of N23227 seedlings, REL in roots and leaves of Lee68 and that in leaves of N23227 were all significantly increased as compared to the controls. When inoculated with *B. japonicum* alone, REL in roots and leaves of Lee68 and that in leaves of N23227 displayed different alleviated effects, with regard to that in roots of N23227, it indicated non-significant rise. The compound treatment of salt stress and *B. japonicum* inoculation showed the most obviously alleviated effect for leaves of Lee68 seedlings, as for the others, the reliefs not reach significant levels (Fig. 3).

Effects of inoculation with *B. japonicum* on ultrastructure of leaf mesophyll cell, chloroplast and thylakoid of Lee68 and N23227 seedlings under salt stress

Except for Lee68 seedlings under NaCl + Bj condition, whose leaf mesophyll cells were completely filled with chloroplasts by TEM, the mesophyll cells of Lee68 seedlings under NaCl stress and N23227 seedlings under NaCl or NaCl + Bj treatments were filled with a large central vacuole, and the chloroplasts were squeezed to near the cell

Table 1 Comparison of growth parameters between Lee68 cultivar and N23227 accession seedlings at 28 days after inoculation with *B. japonicum* under 100 mM NaCl stress

	Plant		Roots		Overground parts		Nodules			Leaf area (cm^2)	Plant height (cm)
	FW (g)/plant	DW (g)/plant	FW (g)/plant	DW (g)/plant	FW (g)/plant	DW (g)/plant	FW (g)/plant	DW (g)/plant	Nodule No./plant		
Lee68-Control	4.9 ± 0.36^{b}	0.75 ± 0.40^{c}	1.29 ± 0.21^{ab}	0.1 ± 0.01^{c}	3.58 ± 0.17^{b}	0.66 ± 0.03^{b}	–	–	–	14.46 ± 1.14^{c}	101.4 ± 3.5^{c}
Bj	4.66 ± 0.95^{b}	0.65 ± 0.18^{bc}	1.62 ± 0.33^{b}	0.09 ± 0.02^{ab}	2.98 ± 0.88^{b}	0.52 ± 0.14^{ab}	0.24 ± 0.07^{c}	0.05 ± 0.02^{c}	57.67 ± 10.22^{a}	19.22 ± 2.25^{d}	112.0 ± 5.8^{d}
NaCl	2.3 ± 0.03^{a}	0.39 ± 0.01^{a}	0.83 ± 0.07^{a}	0.06 ± 0.01^{a}	1.47 ± 0.07^{a}	0.33 ± 0.01^{a}	–	–	–	7.12 ± 1.57^{a}	70.8 ± 11.5^{a}
NaCl + Bj	2.46 ± 0.33^{a}	0.42 ± 0.06^{a}	1.06 ± 0.12^{ab}	0.07 ± 0.01^{ab}	1.41 ± 0.2^{a}	0.33 ± 0.05^{a}	0.14 ± 0.01^{b}	0.03 ± 0.0^{b}	40.0 ± 4.0^{b}	9.31 ± 0.52^{b}	90.6 ± 11.3^{b}
N23227-Control	0.42 ± 0.05^{a}	0.06 ± 0.01^{a}	0.25 ± 0.05^{a}	0.02 ± 0.0^{a}	0.18 ± 0.02^{a}	0.04 ± 0.0^{a}	–	–	–	3.48 ± 0.27^{b}	53.7 ± 7.5^{c}
Bj	0.78 ± 0.08^{b}	0.09 ± 0.01^{b}	0.34 ± 0.06^{a}	0.03 ± 0.01^{a}	0.44 ± 0.02^{c}	0.07 ± 0.0^{c}	0.04 ± 0.01^{a}	0.01 ± 0.0^{a}	18.33 ± 2.89^{c}	5.79 ± 0.43^{c}	57.1 ± 5.8^{c}
NaCl	0.4 ± 0.04^{a}	0.08 ± 0.0^{ab}	0.25 ± 0.04^{a}	0.02 ± 0.0^{a}	0.15 ± 0.01^{a}	0.04 ± 0.01^{a}	–	–	–	2.61 ± 0.14^{a}	31.1 ± 3.0^{a}
NaCl + Bj	0.53 ± 0.08^{a}	0.07 ± 0.01^{a}	0.22 ± 0.03^{a}	0.02 ± 0.0^{a}	0.31 ± 0.05^{b}	0.05 ± 0.01^{b}	0.02 ± 0.01^{a}	0.01 ± 0.0^{a}	13.33 ± 2.22^{c}	3.53 ± 0.15^{b}	44.7 ± 4.9^{b}

Values are mean ± SD (n = 5). Measurements in the same column followed by different letters are significantly different at $P < 0.05$ level between treatments according to Duncan's multiple-range test

FW fresh weight, *DW* dry weight, – no growth

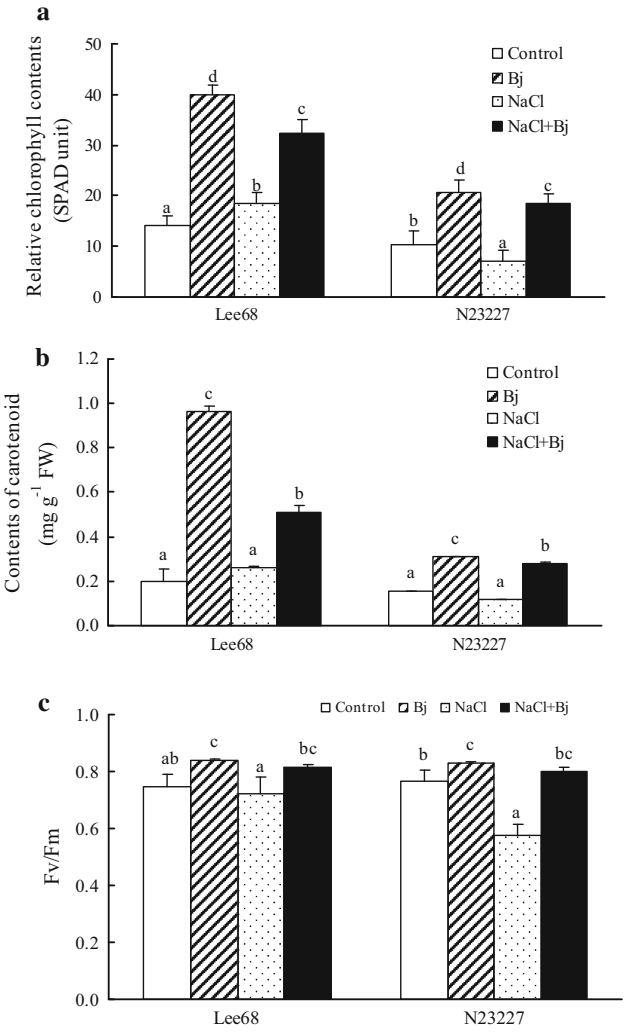

Fig. 2 Comparison of the relative chlorophyll contents (**a**), contents of carotenoid (**b**) and Fv/Fm value (**c**) between Lee68 cultivar and N23227 accession seedlings under inoculation and non-inoculation with *B. japonicum* in the absence and presence of 100 mM NaCl treatment. Statistical data are expressed as mean ± SD of five replicates. Means in bars followed by *different letters* indicate significant differences ($P < 0.05$) between treatments according to Duncan's multiple-range test

walls (Fig. 4a–d). More and larger starch grains were found in leaves of the inoculated Lee68 and N23227 seedlings, especially for Lee68 cultivar, whose chloroplasts were almost full of starch grains (Fig. 4e–h). When compared to the non-inoculated NaCl-treated seedlings, larger amount of osmiophilic globules and more closely arranged stroma thylakoids and grana thylakoids were showed in the inoculated plants of two soybean materials. While for the non-inoculated soybean seedlings under salt stress, the mesophyll cells were seriously twisted and deformed, the stroma thylakoids and grana thylakoids were reduced and arranged loosely (Fig. 4a, c, i–l), especially for N23227 seedlings.

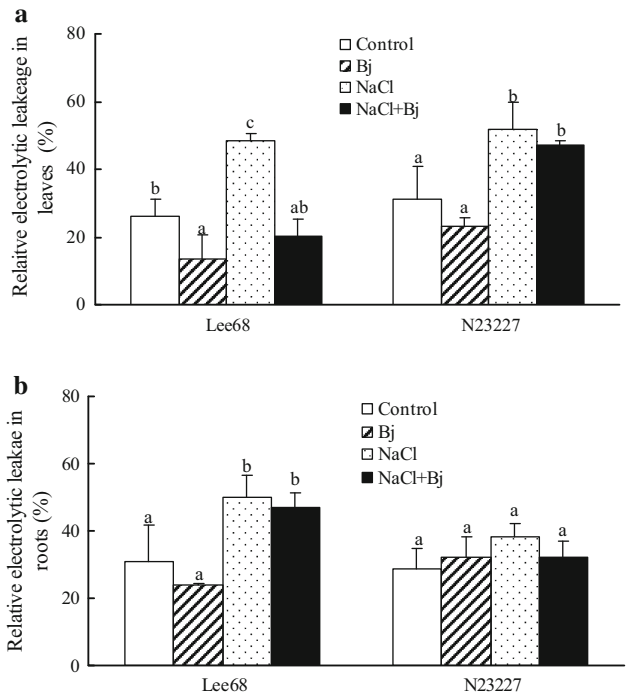

Fig. 3 Comparison of the relative electrolytic leakage in leaves (**a**) and roots (**b**) between Lee68 cultivar and N23227 accession seedlings under inoculation and non-inoculation with *B. japonicum* in the absence and presence of 100 mM NaCl treatment. Statistical data are expressed as mean ± SD of three replicates. Means in bars followed by *different letters* indicate significant differences ($P < 0.05$) between treatments according to Duncan's multiple-range test

Effects of inoculation with *B. japonicum* on isoflavone contents of Lee68 and N23227 seedlings under salt stress and transcription patterns of *PAL1*, *CHS8*, *CHI* and *IFS2* in roots

HPLC analysis showed isoflavones were found in all vegetative parts of two soybean genotypes in the presence of *B. japonicum* and under 100 mM NaCl treatment, and the mean concentration ranged from 252.9 to 10,143.83 μg g^{-1} DW. In general, under four kinds of culture conditions (Control, Bj, NaCl, NaCl + Bj), the contents of isoflavone in roots were obviously much higher than those in stems or leaves of the two soybean plants. As for Lee68, except no significant isoflavone content change in leaves of seedlings under sole inoculation compared to the control, the isoflavone contents in roots or stems of seedlings under sole inoculation, salt and their compound treatment were all displayed an obvious downward trend ($P < 0.05$), and this was quite clear under the sole salt stress (Fig. 5a). In comparison with the control, the isoflavone contents were evidently raised in leaves of N23227 seedlings under sole inoculation, salt and their composite treatment, those in stems were decreased whereas with no significant difference among the three above treatments. Specially, the isoflavone contents in roots of N23227 seedlings under two kinds of inoculation conditions (without or with salt) were significantly increased, especially the latter, which displayed the dramatically enhanced effect of inoculation and salt stress on the isoflavone synthesis. In view of the huge rise of isoflavone contents in roots of N23227 seedlings under *B. japonicum* inoculation, we checked the transcription levels of some key genes related to isoflavone synthesis involved in the phenylpropanoid pathway (Yi et al. 2011). After inoculation with *B. japonicum* for 12, 18, 24 and 48 h, the transcription levels of *PAL1*, *CHS8*, *CHI* and *IFS2* genes in Lee68 were relatively stable or not seen obvious ups and downs, but all of them in N23227 showed gradual increase trend with the extension of inoculation time, especially the quicker increase of *CHI* and *IFS2* genes, which were directly related to isoflavone synthesis (Fig. 5b).

Discussion

Generally, the main injury symptoms of plants suffered from salt stress may include: plant growth is restrained, leaves are etiolated with reduced pigment contents, structures of photosynthetic organs such as chloroplasts and thylakoids are damaged and therefore photosynthetic function is dropped, cell membrane permeability of roots and leaves are increased and resulted in the huge intracellular material leakage (Omoto et al. 2010; Liu et al. 2012; Roy et al. 2014). As for leguminous plants exposed to salinity stress, the nodules and nitrogen fixation are reduced (Dolatabadian et al. 2012). In this work, under sole salt stress, growth of the tested Lee68 and N23227 seedlings was significantly inhibited, and according to lots of measured morphological, physiological parameters and observed leaf chloroplast ultrastructure changes (Table 1; Figs. 1a, b, 2, 3, 4), salt stress effects on Lee68 plants was relatively slighter, and the salt injury was mainly focused on roots, while for N23227 plants, the salt injury was heavier especially on leaves. On the one hand, it can reflect the consistence with results of our previous study, namely Lee68 cultivar is the salt tolerant, while N23227 accession is the salt-sensitive (Yu et al. 2001), and further suggests that, the plant organ difference in salt injury on both soybean species is also existed. Certainly, the difference may be related to the free-nitrogen Hoagland solution culture condition for the two salt-stressed soybean seedlings.

Symbiosis with rhizobia and nodule occurrence are the intrinsic characteristics of leguminous plants (Brechenmacher et al. 2010). A precise exchange of molecular signals between the host leguminous plant (involving the secretion of phenolic compounds, isoflavones and flavones)

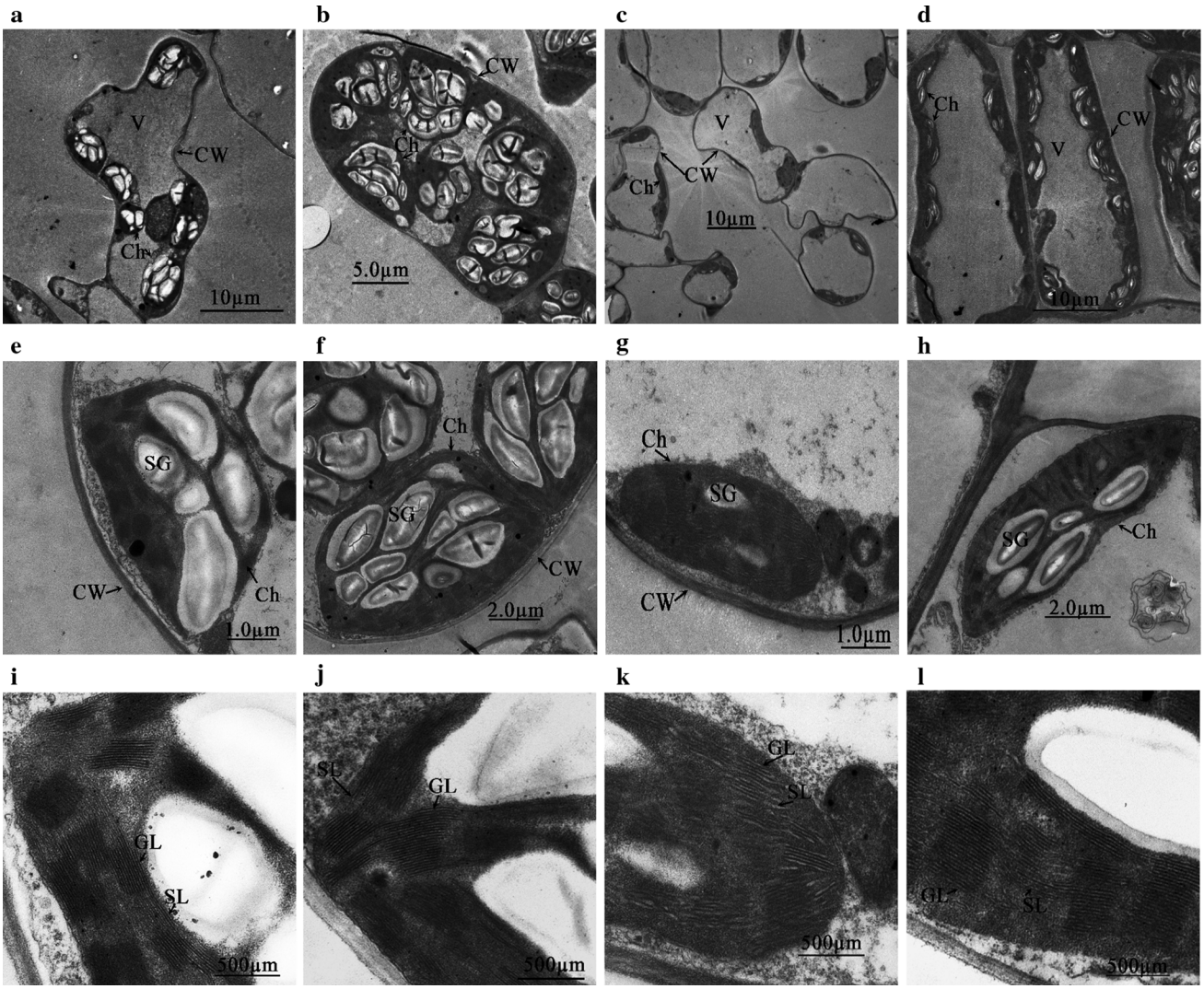

Fig. 4 Transmission electron micrographs of leaf mesophyll cells, chloroplasts and thylakoids of Lee68 cultivar and N23227 accession plants inoculated and non-inoculated with *B. japonicum* in the presence of 100 mM NaCl treatment. *Note* Leaf mesophyll cells (**a–d**), chloroplasts (**e–h**) and thylakoids (**i–l**). From *left to right* Lee68 (NaCl, NaCl + Bj), N23227 (NaCl, NaCl + Bj). *Abbreviations*: *Ch* chloroplast, *CW* cell wall, *OG* osmiophilic globule, *SG* starch grain, *V* vacuole, *SL* stroma lamella, *GL* granum lamella. The pictures were taken after inoculation for 28 days. Specific scale in micrometer (μm) shown for each panel

and rhizobia (activating the expression of *nod* genes and stimulating production of nod factor) over space and time is essential to the development of effective root nodules (Zhang and Smith 1996). Under sole inoculation with *B. japonicum*, most of morphological and physiological parameters of Lee68 and N23227 plants were obviously improved as compared to those under salt stress alone, which should be related to the greatly improved nitrogen nutrition, more evident enhancement was displayed for Lee68 plants, the nodule number per plant was about 3.15 times that of the N23227 (Table 1). When two soybean seedlings inoculated with *B. japonicum* were subjected to salt stress, the salt injury symptoms (such as leaf area, plant height, REL values in roots and leaves, number and arrangement of chloroplasts and thylakoids in mesophyll cells, etc.) were much alleviated than those under sole salt stress, and partial indicators, such as REL values in Lee68 leaves and N23227 roots were most obviously restored to control levels (Fig. 3a), the nodule number per plant of both soybean species were clearly reduced by contrast with the sole inoculation with *B. japonicum*, the reduction in Lee68 seedlings was significant whereas N23227 not, but the nodule number per plant of Lee68 was still about three times of N23227 (Table 1). Thus, it indicates that, the ameliorative effects of *B. japonicum* inoculation on salt-stressed soybean seedlings may reflect the plant organ differences or specificity, the alleviative role in REL values in leaves of Lee68 or roots of N23227 are more

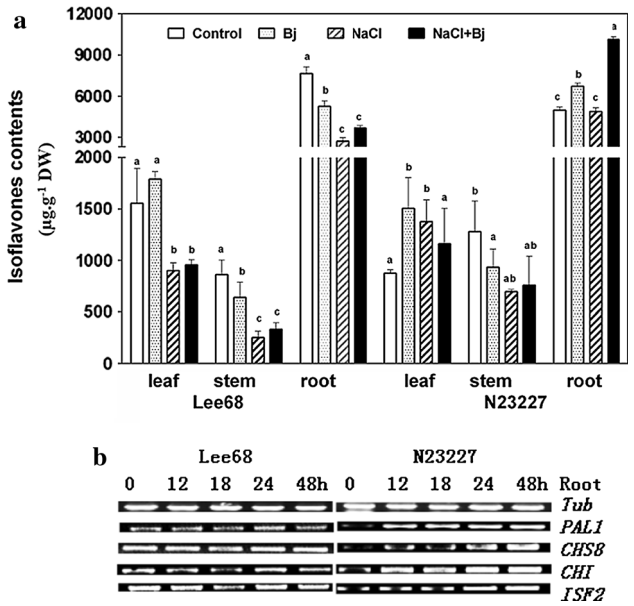

Fig. 5 **a** Comparison of total isoflavone contents in leaves, stems and roots in Lee68 cultivar and N23227 accession plants under inoculation and non-inoculation with *B. japonicum* in the absence and presence of 100 mM NaCl treatment. Statistical data are expressed as mean ± SD of three replicates. Means in bars followed by *different letters* indicate significant differences ($P < 0.05$) between treatments according to Duncan's multiple-range test. **b** Expression patterns of *PAL1*, *CHS8*, *CHI* and *IFS2* in roots of Lee68 cultivar and N23227 accession plants inoculated with *B. japonicum* for 0, 12, 18, 24 and 48 h by Semi-qRT-PCR. The expression pattern of the non-inoculated control plants at each time points was similar to those at 0 h of inoculation and thus picture not showed. The transcript levels have been normalized against those of *GmTubulin*

outstanding. Certainly, for the change in REL in roots of salt-stressed N23227 seedlings is slighter than that of Lee68, this effect on roots of N23227 seedlings is relatively weaker. This kind of plant organ differences or specificity is also consistent with different protective effects of *B. japonicum* inoculation on chloroplast and thylakoid ultrastructure of leaf mesophyll cells of the salt-stressed Lee68 and N23227 seedlings (Fig. 4).

Once the symbionts were formed between soybean seedlings and rhizobial bacteria, thousands of secondary substances were produced in the root hairs (Brechenmacher et al. 2010). Isoflavones, which are synthesized via a branch of the phenylpropanoid pathway, are ubiquitous secondary products present mostly in leguminous plants, and are essential for the establishment of symbiosis between soybean and *B. japonicum* (Subramanian et al. 2006). In soybean, isoflavones both attract rhizobia and induce nod gene expression to initiate nitrogen-fixing root nodule formation (Yu et al. 2003). In this study, under four kinds of culture conditions (Control, Bj, NaCl, NaCl + Bj), the contents of isoflavone in roots were obviously much higher than those in stems or leaves of Lee68 and N23227 plants. The reason for this phenomenon might be that, the main isoflavone biosynthesis is constitutively occurred in roots for vegetative seedlings and in seeds for reproductive plants, at the same time, isoflavones and the glycosylated derivatives are small molecules and compatible solutes so that they can be transported through xylem mobility from a site of synthesis to a site of accumulation (Dhaubhadel et al. 2003; Yu et al. 2003; Subramanian et al. 2009). Under salt stress alone, isoflavone contents in roots, stems and leaves of Lee68 plants were significantly decreased than the control, while those in roots, stems and leaves of N23227 plants showed little changes (Fig. 5a). This was not consistent with a few reports that isoflavone contents were increased in soybean plants under salt stress (Zhou et al. 2007; Wu et al. 2011). The inconsistent research results might be related to the free-nitrogen solution culture condition in this work. When the inoculated Lee68 and N23227 seedlings were exposed to salt stress, the isoflavone contents in roots, stems of Lee68 and N23227, that in leaves of N23227 were not clearly changed in contrast with those under sole salt stress, but the isoflavone content in roots of N23227 under two kinds of *B. japonicum* inoculation conditions (with or without NaCl stress) were significantly increased than the control, especially the former, displayed the remarkably enhanced effect of compound salt stress and *B. japonicum* inoculation on isoflavone synthesis. In addition, a certain positive relevance may be found between the isoflavone content enhancement and the promoted transcription level of *PAL1*, *CHS8*, *CHI* and *IFS2* genes encoded the key enzymes for isoflavone synthesis, especially the promotion of *CHI* and *IFS2* gene transcription (Fig. 5b). Moreover, when compared with the control, the change in biomass of roots, reduction of nodule number per plant (Table 1) and increase of REL in roots (Fig. 4b) of *B. japonicum* inoculated N23227 seedlings under salt stress were all not reached the significant difference, whether the above-mentioned are attribute to the *nod* genes induction of *B. japonicum* and protective role of hugely raised isoflavones in roots, further research is yet needed in future.

In conclusion, *B. japonicum* inoculation for *G. max* Lee68 cultivar and *G. soja* N23227 accession seedlings under salt stress could obviously improved the plant growth, increased leaf contents of chlorophyll and carotenoid and value of Fv/Fm, maintained the ultrastructure of thylakoid and chloroplast of mesophyll cells, decreased REL values in roots and leaves, and thus demonstrated the ameliorative effects on salt injury to the two soybean seedlings with different salt tolerance. In comparison, the protective function of *B. japonicum* inoculation on salt-stressed Lee68 seedlings is mainly related to the effects on leaves, while that on N23227 is mainly on roots, which may be attribute to the transcription promotion of

isoflavone biosynthesis-related key enzymes genes, such as *CHI* and *IFS2*, and more rise of isoflavone content in roots. Thus, inoculation with *B. japonicum* for alleviating salt stress on various soybean species with diverse salt tolerance can reflect the genotype difference or organ specificity, and may also give us a meaningful pathway for salt tolerance improvement of *G. max* by inoculation with *B. japonicum* and gene engineering on isoflavone synthesis.

Acknowledgments This research was jointly funded by the National Natural Science Foundation of China (No. 30871462) and the Transgenic Engineering Crops Breeding Special Funds of China (No. 2009ZX08004-008B) to Yu BJ.

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