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SALINITY STRESS

Differential Cl^-/Salt Tolerance and NaCl-Induced Alternations of Tissue and Cellular Ion Fluxes in *Glycine max, Glycine soja* and their Hybrid Seedlings

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Abstract

The salt-sensitive *Glycine max* N23674 cultivar, the salt-born *Glycine soja* BB52 population, and their hybrid 4076 strain (F_5) selected for salt tolerance generation by generation were used as the experimental materials in this study. First, the effects of NaCl stress on seed germination, tissue damage, and time-course ionic absorption and transportation were compared. When qualitatively compared with seed germination appearance in culture dishes, and tissue damages on roots or leaves of seedlings, or quantitatively compared with the relative salt injury rate, the inhibition on N23674 was all the most remarkable. After the exposure of 140 mM NaCl for 1 h, 4 h, 8 h, 12 h, 2 days and 4 days, the content of Cl^- gradually increased in the roots and leaves of seedlings of BB52, 4076 and 23674. Interestingly, the extents of the Cl^- rise in roots of the three experimental soybean materials were BB52 > 4076 > N23674, whereas those in leaves were just on the contrary. Secondly, by using the scanning ion-selective electrode technique (SIET), fluxes of Na^+ and Cl^- in roots and protoplasts isolated from roots and leaves were also investigated among the three experimental soybean materials. After 140 mM NaCl stress for 2, 4 and 6 days, and when compared with N23674, slighter net Cl^- influxes were observed in root tissue and protoplasts of roots and leaves of BB52 and 4076 seedlings, especially at the cellular protoplast level. The results indicate that with regard to the ionic effect of NaCl stress, Cl^- was the main determinant salt ion for salt tolerance in *G. soja*, *G. max* and their hybrid, and the difference in their Cl^-/salt tolerance is mainly attributed to the capacity of Cl^- restriction to the plant aboveground parts such as leaves.

Introduction

Approximately 22 % of the world's agricultural lands are affected by salinity (Bhatnagar-Mathur et al. 2008). It is reported that more than 800 million hectares of land throughout the world, which account for more than 6 % of the world's total land area, have been reported to be affected by salinity (Munns and Tester 2008). In China, there are 36 million hectares saline lands, which account for 4.88 % of the total available lands; and 9.2 million hectares cultivated lands have become salinization, which account for 6.62 % of the total cultivated lands (Yang 2008). The saline lands are expanding with the modernization of industry, increase in irrigated agrarian lands and greenhouse for vegetables and flowers in agriculture. Therefore, raising the salt tolerance of crops, biological improvement and comprehensive exploitation of saline soils are the most important approaches in future (Liu and Wang 1998).

The soybean comes of China, and is one of the main crops in the world (including China). It includes the cultivated species (*Glycine max* L.) and the wild species (*Glycine soja* L.) according to their habitats. Our previous studies have shown that *G. max* seedlings were more

sensitive to Cl^- than to Na^+ under NaCl stress, whereas *G. soja* showed more Cl^- tolerance (Luo et al. 2003, 2005). At present, although *G. max* belongs to one of the moderate salt-tolerant crops, great achievements in conventional breeding among *G. max* cultivars for improving its salt tolerance are difficult to obtain due to its narrowing basis of genetic germplasm and the relative limited Cl^-/salt tolerance, as well as the lower productivity and economic value in *G. soja*. But *G. soja* possess many kinds of notable stress resistance (e.g. higher drought tolerance, resistance to plant diseases and insect pests) and quality characteristic (e.g. higher protein content and lower fat content), and is the near relative ancestor species of *G. max*. Both have the same number of chromosomes (2 n = 40), the hybridization between them is very easy to perform, and their hybrid offspring not only have good fecundity but also have the same genetic characteristics as the hybrid offspring among the *G. max* cultivars.

A hypersaline environment, most commonly constituted by high NaCl, mainly results in perturbation of ionic steady state for Na^+, Cl^-, K^+ and Ca^{2+} at both the cellular and the whole plant levels, thus, the re-establishment of ionic homeostasis and maintenance of favourable K^+/Na^+ ratio can play very essential roles in plant salt tolerance (Hasegawa et al. 2000, Teakle and Tyerman 2010). Comparative studies have shown that the salt-tolerant *G. soja* (BB52 population) and their hybrid 185 strain (F_5) (crossed with *G. max* Jackson cultivar) seedlings exposed to salt stress accumulate less Na^+ and Cl^- in root and shoot tissues than the salt-sensitive Jackson cultivar (Wu and Yu 2009). The hybrid strain 4076 (F_5) was selected for salt tolerance generation by generation from the cross-assembly N23674 × BB52. Its salt-tolerant coefficient (an index that is correlated positively with the salt tolerance), dry matter and relative growth rate of seedlings under salt stress were obviously enhanced when compared with the female parent (*G. max* N23674 cultivar). In the field test, its seed quality (such as contents of proteins, fatty acids and amino acids) was obviously improved compared with the male parent (*G. soja* BB52 population), and its agronomic traits (such as the plant height, branch no., node no. of stem, pod no. per plant and weight per 100 seeds) were all between their parents (Du and Yu 2010). So in theory and practice, it is feasible to improve or enhance the Cl^-/salt tolerance in *G. max* by its hybridization with the salt-born *G. soja*. It is suggested that the greater capacity to exclude NaCl in BB52 and the hybrid is likely the result of salt uptake and transport restriction in roots. However, this needs further investigations, e.g. by electrophysiology, to clarify. In this study, on the basis of the comparative study on the effects of NaCl stress on seed germination, tissue damage and the time-course ionic absorption and transportation, we used a noninvasive ion flux technique to measure the fluxes of Na^+ and Cl^- in roots and protoplasts isolated from roots and leaves of the salt-tolerant BB52 population (*G. soja*), salt-sensitive N23674 (*G. max*) and their hybrid strain 4076 (F_5). The aim is to compare the NaCl-induced alternations of ion fluxes and physiological mechanisms of differential Cl^-/salt tolerance in *G. max*, *G. soja* and their hybrid, and to provide a theoretic basis for innovation of salt-tolerant soybean germplasms and improvement of salt tolerance in *G. max* by using the salt-born *G. soja*.

Materials and Methods

Plant materials and treatments

The experimental soybean materials were: (i) *G. max* N23674 cultivar (the salt-sensitive one from Jiangsu, China); (ii) the salt-born *G. soja* BB52 population (the salt-tolerant one from Shandong, China); and (3) their hybrid 4076 strain (F_5) selected for salt tolerance generation by generation.

In the seed germination phase, the above seeds were surface-sterilized with 1 g l^{-1} $HgCl_2$ for 5 min, then fully rinsed in distilled water. Twenty seeds were put in each culture dish, lined with two layers of filter paper and moistened with 20 ml each of the appropriate treatment solutions: 1/2 Hoagland (Control), 1/2 Hoagland +140 mM NaCl (salt treatment). Seeds were germinated at 25 °C in the dark. After 6 days, the germinated seeds were counted and used for measurement of the germination percentage and the relative salt injury rate. Three replicates were made for each treatment.

In the seedling phase, the above seeds were surface-sterilized as described above, and soaked in distilled water for 8 h, then germinated at 25 °C in the dark. About 40 germinated seeds (each with about 0.5–1 cm epicotyl) were planted in each plastic pot (25 × 18 cm) containing vermiculite and maintained in a greenhouse. The temperature of the greenhouse was maintained at 25–30 °C, photoperiod about 14/10 h (day/night), and watered with 1/2 Hoagland solution every 2 days. When the 1 week young seedlings were formed, they were secured using foam boards (with similar holes) and transferred to plastic pots filled with 1/2 Hoagland solution. When the first pair of unifoliolate leaves fully expanded, the seedlings were treated with 1/2 Hoagland solution (Control) and 140 mM NaCl solution (prepared with 1/2 Hoagland solution) for 6 days. All the solutions listed above were replaced every 2 days, and their pH values were adjusted to 6.0 with either 0.1 mM HNO_3 or KOH. Young roots with apices of 2–3 cm and leaves were sampled from the control and stressed plants of the three soybean materials

and used for content analysis of Cl^- and flux measurements of Na^+ and Cl^- at the tissue and cellular levels.

Measurement of the relative salt injury rate

The relative salt injury rate was determined according to Li (2008) by the following formula: relative salt injury rate (%) = (Control − T)/(Control) × 100 %. where 'Control' is germination percentage of no salt stress and 'T' is germination percentage in NaCl treatment.

Analysis of Cl^-

The extraction and measurement of Cl^- in roots and leaves were performed according to Yu et al. (2001). Twenty-five millilitres of deionized H_2O was added to 100 mg of dried plant powder and boiled together for 3 h, then filtered and deionized H_2O was added to make a final volume of 50 ml. The Cl^- content was assayed by the method of Zhou and Yu (2009). 0.5 ml of boiled water extraction from dried plant powder was mixed with 25 ml distilled water in 50 ml volumetric flask plus 1 ml gelatin-ethanol aqueous solution [gelatine : water : ethanol (w : v : v) = 0.025g : 70 ml : 30 ml], 0.5 ml HNO_3 solution (~30 %, v/v) and 0.5 ml 20 g l^{-1} $AgNO_3$ solution; then the distilled water was added to make a final volume of 50 ml. These solutions were mixed and held still in a dark cassette for 10 min. Finally, the optical density was measured at 300 nm. The amount of Cl^- was calculated from a standard curve prepared against pure NaCl solutions (0–0.16 mM) added to the above mixed solution (without the sample).

Estimation of tissue damage

Cell death was detected histochemically by trypan blue staining according to the method described by Joo et al. (2005). Detached leaves or roots were covered with an alcoholic lactophenol trypan blue mixture (30 ml ethanol, 10 g phenol, 10 ml H_2O, 10 ml glycerol, 10 ml of 10.8 M lactic acid and 10 mg of trypan blue), placed in a boiling water bath for 3 min, left at room temperature for 1 h, then transferred to a chloral hydrate solution (2.5 g ml^{-1}), and boiled for 20 min for destaining.

Isolation of root and leaf protoplasts

The protoplasts of roots and leaves of soybean seedling were isolated and purified according to our previous method (Zhang and Yu 2009). A brief description is given below. A suitable volume of CPW (cell and protoplast washing solution) (Cocking and Peberdy 1974) consisting of 0.2 mM KH_2PO_4, 1 mM KNO_3, 10 mM $CaCl_2{\cdot}2H_2O$ and 1 mM $Mg_2SO_4{\cdot}7H_2O$ was prepared first. Root tips (2–3 cm) were washed with deionized H_2O for three to five times and chopped into 0.5 mm in length and incubated in 5 ml of CPW-13M (13 % [w/v] mannitol, pH 5.8 was adjusted with Tris) supplemented with the following enzymes (in w/v): 3 % cellulase R-10 (Onozuka; Yakult Honsha Co., Ltd, Tokyo, Japan), 1.1 % Macerozyme R-10 (Yakult Honsha Co., Ltd), and 1.0 % Hemicellulase (Sigma, USA). Segments of roots were gently shaken at 60 rpm in the enzyme solution at 28 °C for 16 h. Protoplasts were then filtered through a nylon mesh with 50 μm diameter pores. Undigested tissues were placed in a small beaker containing 3 ml of a holding solution (CPW-13M, pH 5.8 was adjusted with Tris). Osmotic potentials of CPW and holding solutions, 290–300 mOsmol kg^{-1}, were adjusted with 13 % [w/v] mannitol by an automatic freezing-point depression osmometer (FM-8P type; Shanghai Medical University Instrument Plant, Shanghai, China). By gentle agitation with a glass rod, additional protoplasts were released from the undigested tissues and collected by filtering through the nylon mesh. Thereafter, a duplicate dilution method was adopted to minimize mechanical damage caused by the enzyme solution. The collected filtrate was centrifuged at 100 ***g*** for 10 min to remove the enzyme solution. The leaves were also chopped into 0.5 mm in length and incubated in 5 ml of holding solution (10 mM $CaCl_2{\cdot}2H_2O$, 0.1 % [w/v] bovine serum albumin, 9 % [w/v] mannitol) supplemented with the following enzymes (in w/v): 1 % cellulase R-10 and 0.20 % pectolaseY-23 (Yakult Honsha Co., Ltd, Tokyo, Japan), pH 5.8 at 28 °C for 4 h. Protoplasts were then resuspended and washed with 2–3 ml of a holding solution. Finally, the root or leaf protoplast suspensions (1 ml) were diluted with a 2-ml holding solution and used for ion flux measurements.

Measurements of net Na^+ and Cl^- fluxes with SIET

Net fluxes of Na^+ and Cl^- were measured noninvasively using the scanning ion-selective electrode technique (SIET) (the SIET system BIO-001A; Younger USA Sci. & Tech. Corp.; Applicable Electronics Inc.; and Science Wares Inc.; Xu et al. 2006) method as described in Sun et al. (2009) with slight modifications. The concentration gradients of the target ions were measured by moving the ionselective microelectrode between two positions close to the plant material in a preset excursion (20 μm for excised roots and 10 μm for protoplasts in our experiment) at a programmable frequency in the range of 0.3–0.5 Hz. The SIET can measure ionic fluxes down to picomolar levels but must be measured slowly at approximately 1–2 s per point. This is mainly due to the mechanical disturbance of the gradient by the electrode movement, although the time constant of the liquid ion

exchanger (LIX) electrodes is also a factor. The electrode is stepped from one position to another in a predefined sampling routine while also being scanned with the three-dimensional micro stepper motor manipulator (CMC-4). Pre-pulled and silanized glass micropipettes (2–4 μm aperture, XYPG120-2; Xuyue Sci. and Tech. Co., Ltd., Beijing, China) were first filled with a backfilling solution (Na: 100 mM NaCl; Cl: 100 mM KCl, pH 7.0) to the root cap, meristematic zone, elongation zone and mature zone from the apex. The micropipettes were then front filled with approximately 15 μm columns of selective liquid ion-exchange cocktails (LIXs; Na: Fluka 71178; H: Fluka 95293; Cl: Fluka 24902; K: Fluka 60398; Ca: Fluka 86542). An Ag/AgCl wire electrode holder (XYEH01-1; Xuyue Sci. and Tech. Co., Ltd.) was inserted in the back of the electrode to make electrical contact with the electrolyte solution. DRIREF-2 (World Precision Instruments, Sarasota, FL, USA) was used as the reference electrode. Ion-selective electrodes of the following target ions were calibrated prior to flux measurements: (i) Na^+: 0.9, 2.0, 5.0 mM (Na^+ concentration was usually 0.9 mM in the measuring buffer for root and cell samples); (ii) Cl^-: 0.25, 0.5, 2.0 mM (Cl^- concentration was usually 0.5 mM in the measuring buffer for root and cell samples). Only electrodes with Nernstian slopes >50 mV/decade (−50 mV/decade for Cl^- electrodes) were used in our study. Ion flux was calculated by Fick's law of diffusion:

$$J = -D(\mathrm{d}c/\mathrm{d}x)$$

where J represents the ion flux in the x direction, $\mathrm{d}c/\mathrm{d}x$ is the ion concentration gradient and D is the ion diffusion constant in a particular medium. Data and image acquisition, preliminary processing, control of the three-dimensional electrode positioner, and stepper-motor-controlled fine focus of the microscope stage were performed with ASET software, part of the SIET system.

Results

Effects of salinity on seed germination of *G. max*, *G. soja* and their hybrid seedlings

Under 140 mM NaCl stress for 6 days, the different inhibitory effects on seed germination were observed for *G. max* N23674 cultivar, *G. soja* BB52 population and their hybrid 4076 strain. When qualitatively compared with the seed germination appearance in culture dishes, or quantitatively compared with the relative salt injury rate, the inhibition on seed germination of N23674 was the most remarkable, the relative salt injury rate reached 73.6 %, while those on BB52 and 4076 were slight, the relative salt injury rates were only 7.1 % and 13.5 % respectively (Fig. 1a,b).

Effects of salinity on seedling growth and tissue damage of *G. max*, *G. soja* and their hybrid seedlings

No apparent salt injury symptom was found among the unifoliolate leaves of the three experimental soybean seedlings when stressed with 140 mM NaCl for 2 days, but when the treated time prolonged, the obvious and serious salt injury appeared in the unifoliolate leaves of N23674, especially after the onset of a 6-day exposure to salt stress, and the damages on BB52 and 4076 were relative slighter (Fig. 2a). Similar results were also obtained when tissue damage was monitored in leaves and roots by vital dye staining with trypan blue during the exposure to 140 mM NaCl stress for 2, 4, and 6 days respectively (Fig. 2b,c).

Changes in contents of Cl^- in roots and leaves of *G. max*, *G. soja* and their hybrid seedlings under salinity stress

During the exposure of 140 mM NaCl for 1 h, 4 h, 8 h, 12 h, 2 days and 4 days, the content of Cl^- increased with the treatment time in roots and leaves of each soybean seedlings, and the remarkable rises were displayed after the second day. Interestingly, the extents of the Cl^- rise in seedling roots were BB52 > 4076 > N23674, whereas those in leaves were just on the contrary (Fig. 3a,b). The contents of Na^+ also increased gradually in roots and leaves of the three experimental soybean seedlings when compared with each control, while those of K^+ decreased. In general, all the variations in BB52 and 4076 were obviously smaller than those in 23674, but no differential changes in their roots and leaves were observed (data not shown).

Comparison of Na^+ and Cl^- fluxes in roots of *G. max*, *G. soja* and their hybrid seedlings under salinity stress

The SIET data showed that the pattern of Na^+ and Cl^- fluxes in roots of *G. max* (N23674 cultivar) seedlings differed from those in *G. soja* (BB52 population) and their F_5 hybrid (4076 strain) after exposure to 140 mM NaCl for 6 days. In comparison with the control, the salt stress caused a drastic net Na^+ influx, ranging from 350 to 3000 pmol cm^{-2} s^{-1} in the measured regions of N23674 roots axes (0–30 mm from the apex) (Fig. 4a,d). However, Na^+ fluxes in roots of BB52 and 4076 varied slightly within the root cap and mature zone, and displayed small Na^+ effluxes at no significant level (Fig. 4b–d). Concerning the Cl^- fluxes, the rate of Cl^- efflux was found with a marked difference in the three experimental soybean materials under salt stress. In contrast to N23674, the remarkable NaCl-induced Cl^- effluxes were observed in

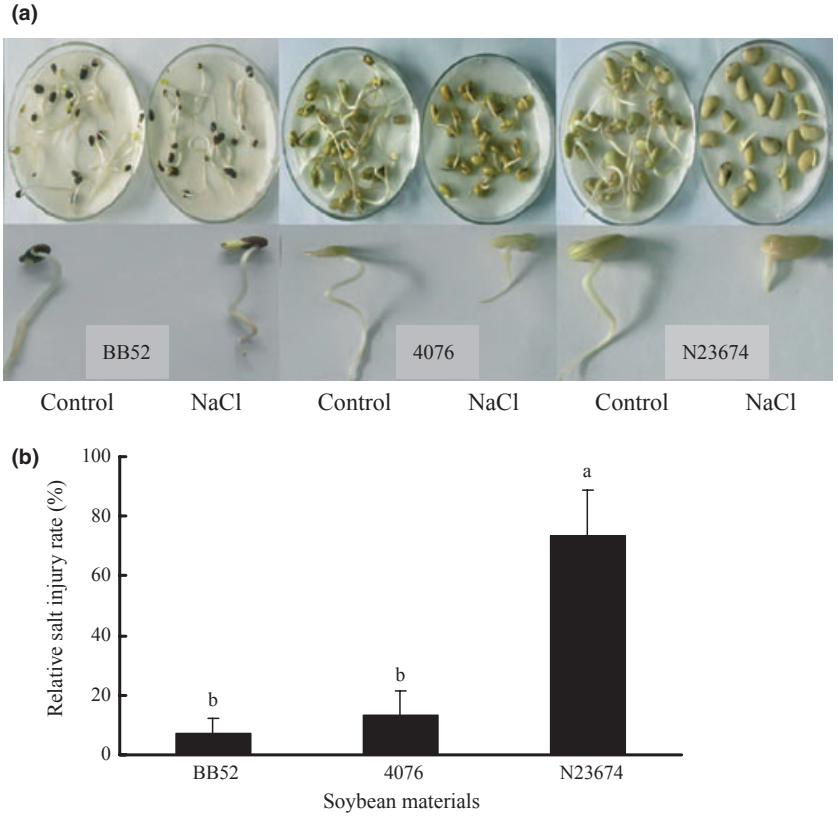

Fig. 1 Effects of salinity stress (140 mM NaCl for 6 days) on seed germination (a) and its relative salt injury rate (b) of *Glycine soja* (BB52 population), *G. max* (N23674 cultivar) and their F_5 hybrid (4076 strain).

roots of BB52 and 4076 seedlings under salt stress, especially along the elongation zone and mature zone, and thus showed significantly enhanced Cl^- effluxes when compared with their controls (Fig. 4e–h).

Comparison of Na^+ and Cl^- fluxes in protoplasts of roots and leaves of *G. max*, *G. soja* and their hybrid seedlings under salinity stress

Na^+ and Cl^- fluxes in root protoplasts

Under the control condition, cellular fluxes of Na^+ in protoplasts of roots of N23674, BB52 and 4076 seedlings all showed some equivalent influxes during the continuous flux recording for 10 min, while those of Cl^- all displayed certain effluxes. When the seedlings were exposed to 140 mM NaCl for 2, 4 and 6 days, the initial influx of Na^+ in root protoplasts of N23674 was weakened gradually, and its efflux of Cl^- was reversed gradually to a remarkable increase of net influx. With regard to BB52 and 4076 as exposure to salt stress for 4 and 6 days, the reverse Na^+ effluxes were caused in root protoplasts of both soybean materials, and its initial net Cl^- effluxes were transferred gradually to relatively slighter net influxes when compared with that of N23674 (Fig. 5a–h).

Na^+ and Cl^- fluxes in leaf protoplasts

Under the no salt stress condition, cellular fluxes of Na^+ in protoplasts of leaves of N23674, BB52 and 4076 seedlings also showed some equivalent influxes during the continuous flux recording for 10 min, while those of Cl^- all displayed obvious effluxes. When the N23674 seedlings were stressed with 140 mM NaCl for 6 days, a drastic increase of Na^+ influx and a remarkable conversion of Cl^- influx in leaf protoplasts were observed. With regard to BB52 and 4076, the initial net Na^+ influxes in leaf protoplasts were decreased first and then reversed to obvious Na^+ effluxes during the salt stress process for 6 days; their original net Cl^- effluxes in leaf protoplasts were decreased gradually when stressed for 2 and 4 days, and then reversed to slight net influxes when stressed for 6 days (Fig. 6a–h).

Discussion

Differential Cl^-/salt tolerance in *G. max*, *G. soja* and their hybrid seedlings

As NaCl is the most soluble and abundant salt released from salinized soil, Na^+ and Cl^- certainly become the two important toxic components of ionic stress (Munns and

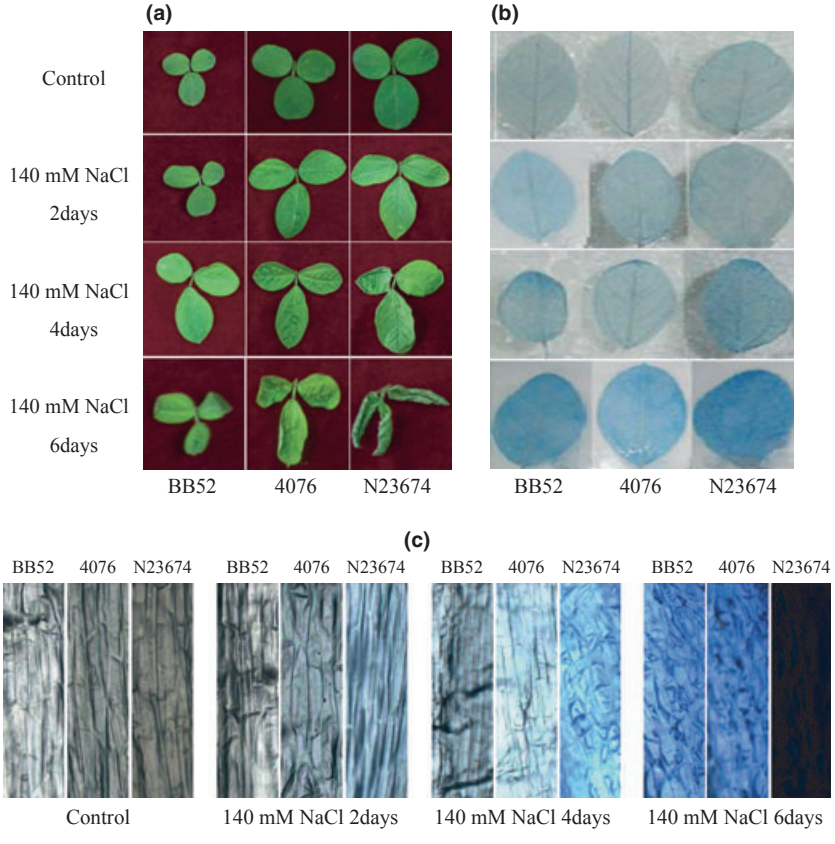

Fig. 2 Effects of salinity stress (140 mM NaCl for 6 days) on seedling growth showed as leaf appearance (a) and tissue damage in leaf (b) and root (c) staining with trypan blue of *Glycine soja* (BB52 population), *G. max* (N23674 cultivar) and their F_5 hybrid (4076 strain).

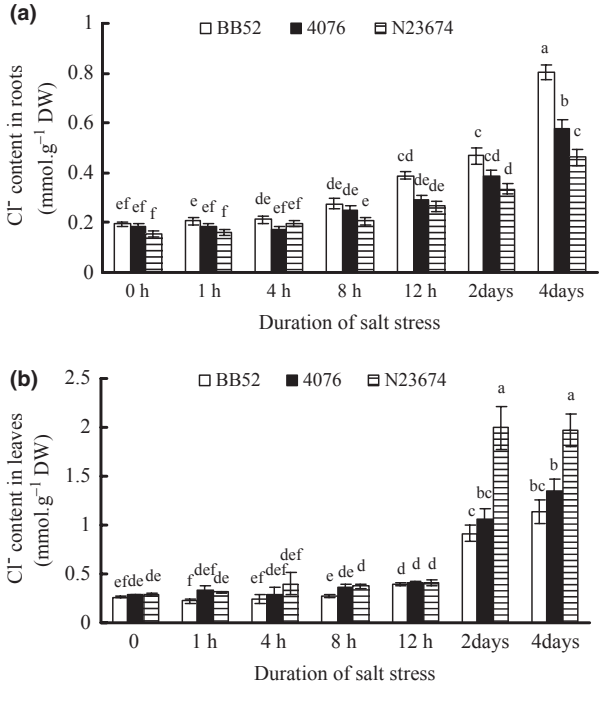

Fig. 3 Changes in Cl^- contents in roots (a) and leaves (b) of *Glycine max* (N23674 cultivar), *G. soja* (BB52 population) and their F_5 hybrid (4076 strain) seedlings under 140 mM NaCl for 4 days. Columns labelled with different letters are significantly different at $P < 0.05$.

Tester 2008). The plant integral tolerance to sodium and chloride ions, although a relative value, often varies with plant species. Crops, such as cotton, sugar beet and barley, are known to be salt tolerant, while the cultivated soybean is looked as a moderately salt-tolerant one (Maas and Hoffman 1977). In general, crops such as cotton, wheat and rice are more sensitive to Na^+ than to Cl^-. However, there are quite a few crops or woody species (tobacco, tomato, barley, grapevines, citrus, *G. max*, *Lotus* and poplar) that have been reported to be more sensitive to Cl^- than to Na^+. The genetic differences in the control of Cl^- transport from roots to shoots or maintenance of low shoot Cl^- is a major source of salt tolerance (Moya et al. 2003, Munns and Tester 2008, Sun et al. 2009, Teakle and Tyerman 2010, Tregeagle et al. 2010). Whereas *G. soja*, a relative species of *G. max*, and their selected hybrids crossed with *G. max*, all demonstrated strong Cl^-/salt tolerance (Luo et al. 2003, 2005, Wu and Yu 2009).

In this study, under NaCl stress condition, whether in the stage of seed germination (estimated by the index of relative salt injury rate) or in the stage of seedling growth (compared by the salt injury symptoms on leaves, and/or monitored by tissue damage in leaves and roots staining with trypan blue), the *G. soja* BB52 population and the hybrid 4076 strain selected from its cross with *G. max*

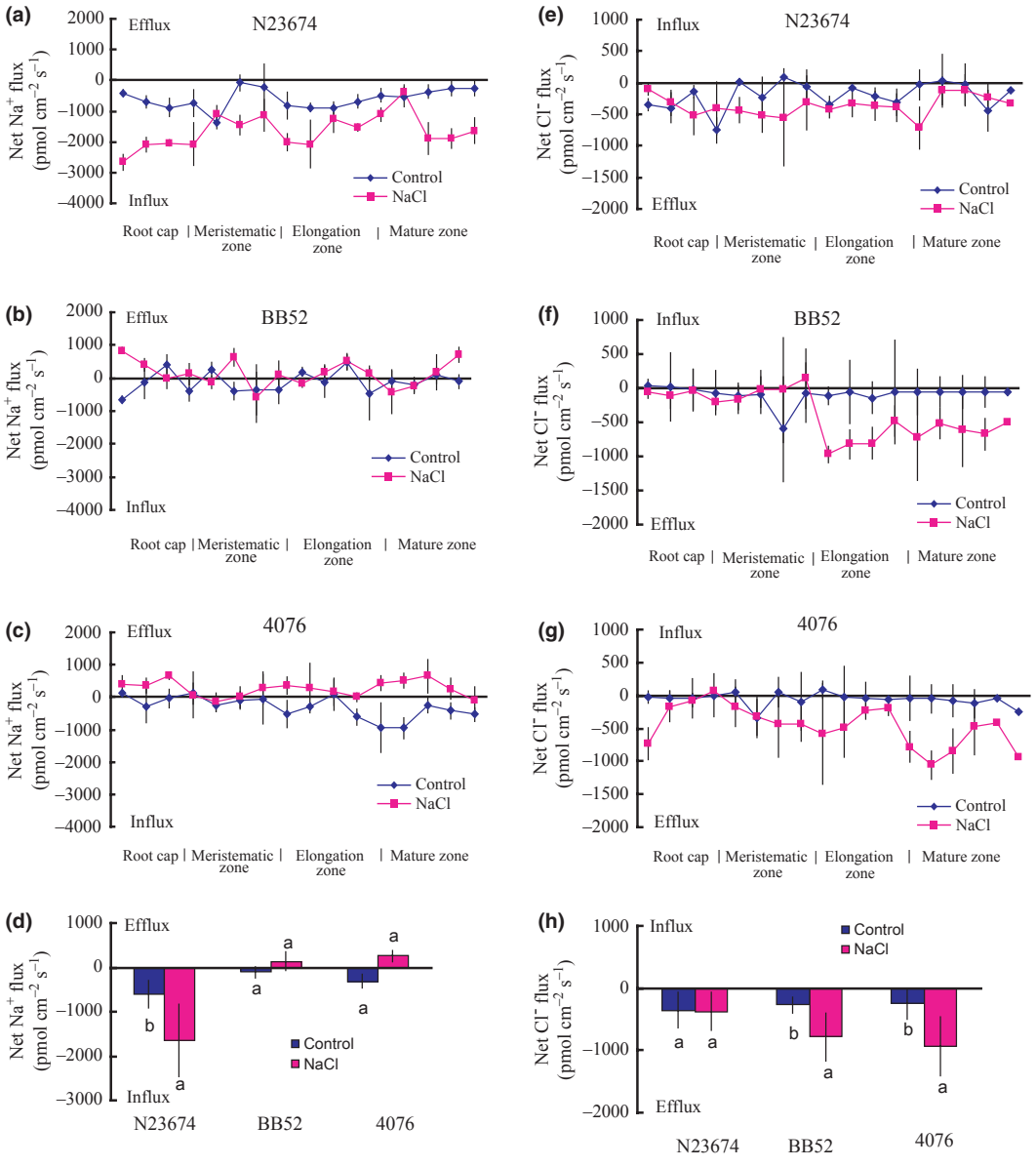

Fig. 4 Effects of salinity stress (140 mM NaCl for 6 days) on net Na^+ (a–c) and Cl^- (e–g) fluxes in roots of *Glycine max* (N23674 cultivar), *G. soja* (BB52 population) and their F_5 hybrid (4076 strain) seedlings. Control roots were treated without NaCl. Na^+ and Cl^- fluxes were measured along the root axes (0–30 mm from the apex, including the root cap, meristematic zone, elongation zone, mature zone). The mean fluxes of Na^+ and Cl^- within the measuring parts are shown (d, h). Each point is the mean of five to six individual plants and bars represent the standard error of the mean. Columns labelled with different letters are significantly different at $P < 0.05$.

N23674 cultivar all showed higher salt tolerance or less salt injury when compared with the female parent N23674 cultivar (Figs 1 & 2). Though the gradual rise in content of Cl^- was firmly observed in roots and leaves of seedlings of BB52, 4076 and 23674 after the exposure of 140 mmol·L^{-1} NaCl for 1 h, 4 h, 8 h, 12 h, 2 days and 4 days, and significant changes were displayed after the second day, it is very worthy to notice that the extents of the Cl^- rise in roots of the three experimental soybean materials were BB52 > 4076 > N23674, whereas those in their leaves were just on the contrary (Fig. 3a,b). Brumós et al. (2010) reported that, under 4.5 mM Cl^- application, the net Cl^- uptake rate was about four times lower in the citrus rootstocks Cl^--excluding genotype Cleopatra mandarin (CM) correlated with a lower root-to-shoot transport capacity when compared with the Cl^--including

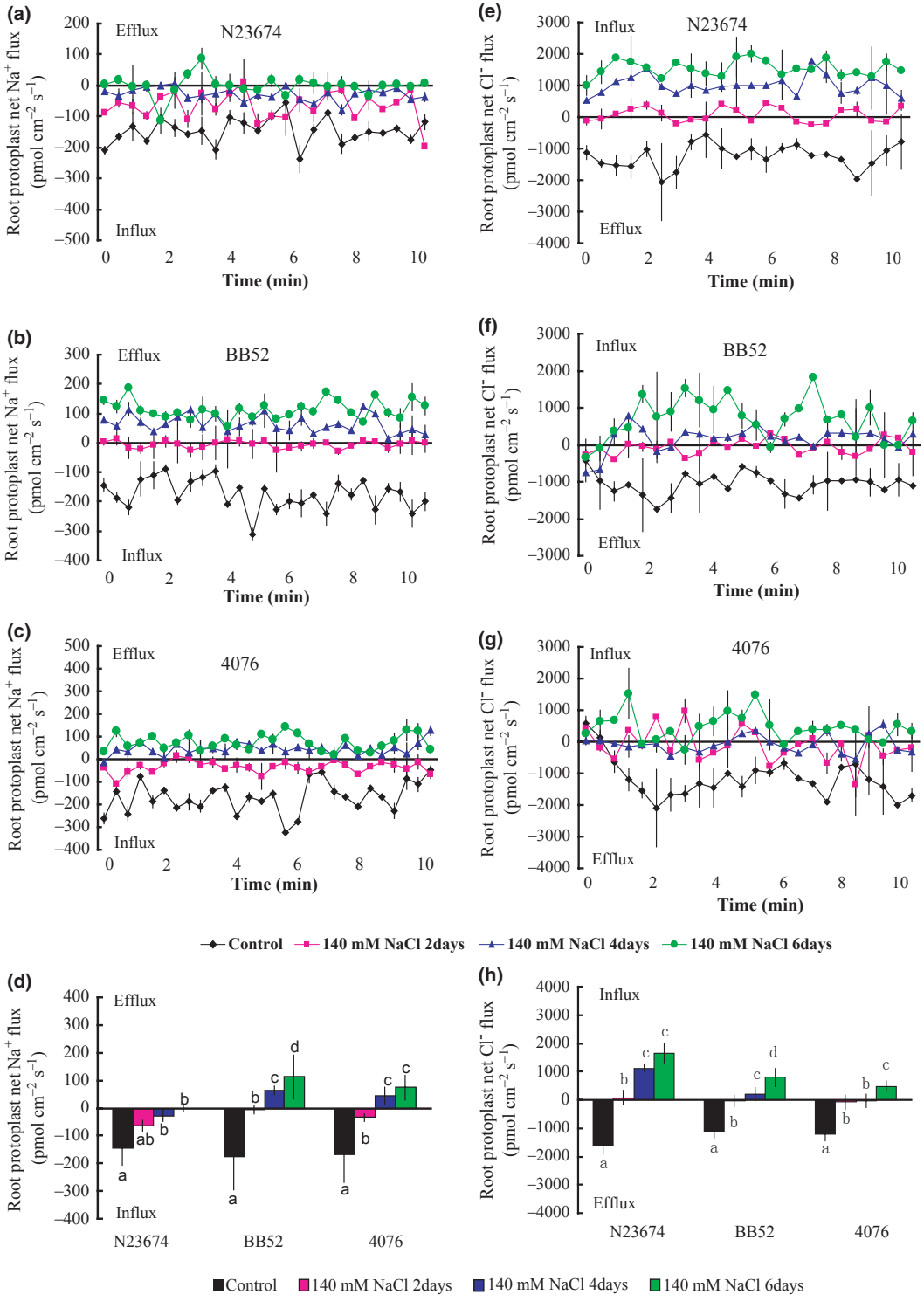

Fig. 5 Net fluxes of Na^+ (a–c) and Cl^- (e–g) in protoplasts isolated from roots of control and NaCl-stressed (140 mM NaCl for 2, 4, 6 days) seedlings of *Glycine max* (N23674), *G. soja* (BB52) and their F_5 hybrid (strain 4076) seedlings. A continuous flux recording of 10 min was conducted for each protoplast in corresponding measuring solutions (pH 6.0). Each point represents the mean of five to six individual protoplasts and bars represent the standard error of the mean. The mean fluxes of Na^+ within the measuring periods are shown (d, h). Columns labelled with different letters are significantly different at $P < 0.05$.

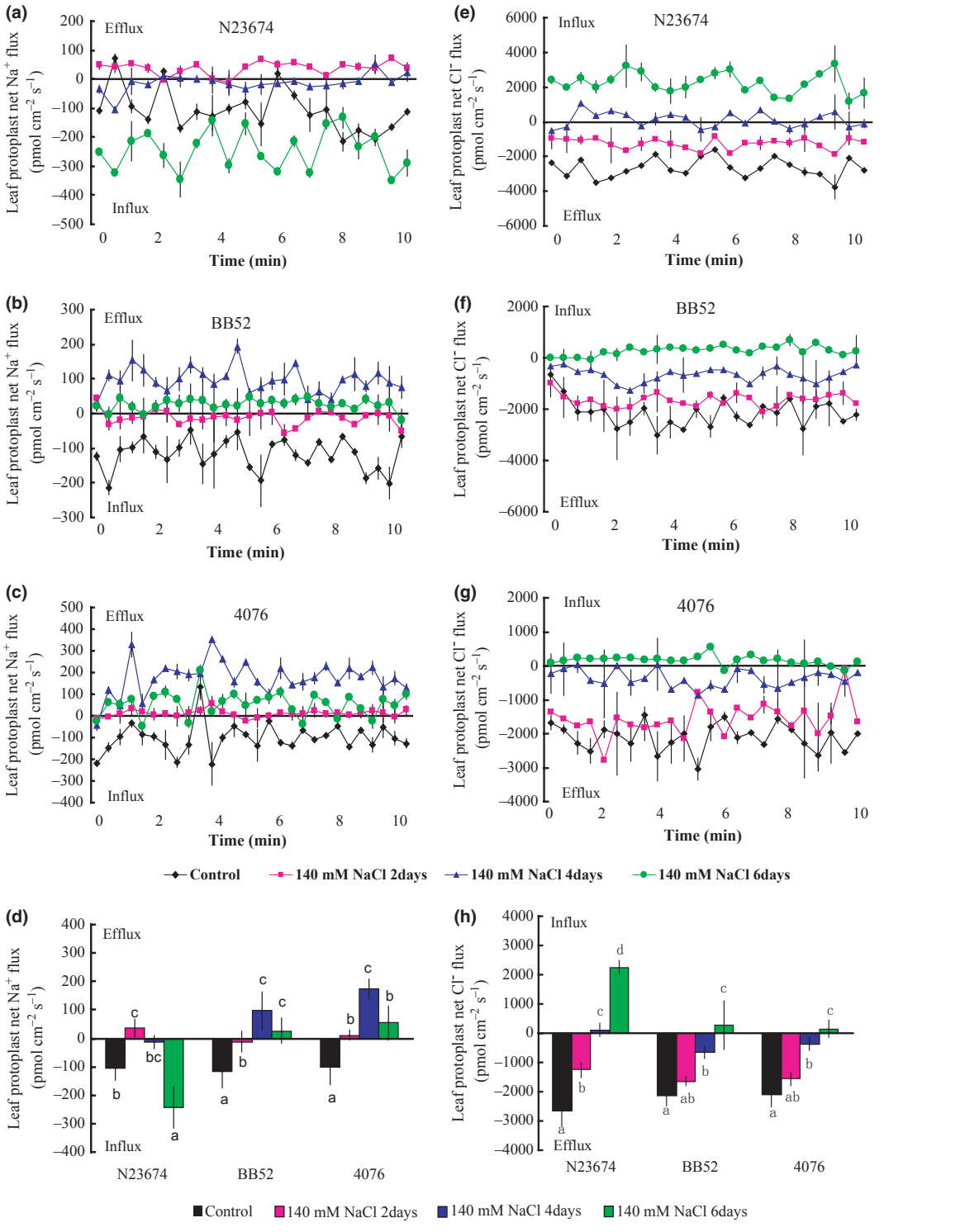

Fig. 6 Net fluxes of Na^+ (a–c) and Cl^- (e–g) in protoplasts isolated from leaves of control and NaCl-stressed (140 mM NaCl for 2, 4, 6 days) seedlings of *Glycine max* (N23674), *G. soja* (BB52) and their F_5 hybrid (strain 4076) seedlings. A continuous flux recording of 10 min was conducted for each protoplast in corresponding measuring solutions (pH 6.0). Each point represents the mean of five to six individual protoplasts and bars represent the standard error of the mean. The mean fluxes of Na^+ within the measuring periods are shown (d, h). Columns labelled with different letters are significantly different at $P < 0.05$.

citrus rootstocks Carrizo citrange (CC). It is suggested that most of the absorbed Cl^- by seedlings of the salt-tolerant BB52 and 4076 was mainly accumulated in their roots, and the transportation to the above-ground parts such as leaves was firmly restricted, but the salt-sensitive N23674 showed the reverse. Similar results were also found in our previous work on cross of *G. max* (Jackson cultivar) × *G. soja* (BB52 population) and their F_5 hybrid (185 strain) (Wu and Yu 2009). Therefore, the differential salt tolerance in *G. max*, *G. soja* and their hybrid, which was observed both in the stages of seed germination and seedling growth, mainly resulted from the differences in Cl^- absorption, extrusion and transportation within the plants under salinity stress. On this basis, further investigations were then conducted electrophysiologically to compare the NaCl-induced alternations of tissue and cellular ion fluxes in seedlings of *G. max*, *G. soja* and their hybrid.

NaCl-induced alternations of tissue and cellular ion fluxes in seedlings of *G. max*, *G. soja* and their hybrid seedlings

The salt tolerance of plants requires compartmentalization of Na^+ and Cl^- at the tissue, cellular and/or intracellular levels to avoid toxic concentrations within the cytoplasm, especially in leaf mesophyll cells. An early plant response to NaCl is the influx of Na^+ and Cl^- into root epidermal cells through non-selective cation channels at the plasma membrane (PM) (Munns and Tester 2008) and anion channels (such as chloride channels, CLCs) in various biomembranes (White and Broadley 2001, Teakle and Tyerman 2010). When the seedlings of *G. max* (N23674 cultivar), *G. soja* (BB52 population) and their hybrid (4076 strain) were exposed to 140 mM NaCl for 6 days, the different patterns or variations of Na^+ and Cl^- fluxes in their roots were displayed by the SIET. In comparison with the control, a drastic net NaCl-induced Na^+ influx and a weaker Cl^- efflux were observed in the salt-sensitive N23674, while a slight NaCl-induced Na^+ efflux and a remarkable Cl^- efflux was found in the salt-tolerant BB52 and 4076 (Fig. 4). Gradually and obviously increased cellular Cl^- influxes with the prolongation of salt stress time were only found in root and leaf protoplasts of the salt-sensitive N23674 (Figs 5h & 6h), and Na^+ influx in its leaf protoplasts showed the same as the above (Fig. 6d), while a definite extent of Na^+ efflux and minor Cl^- influx were displayed in the salt-tolerant BB52 and 4076 (Figs 5d,h & 6d,h). Especially, the salt-shocked N23674 root and leaf cells all exhibited typically greater Cl^- influxes than BB52 and 4076 over the period of recording (Figs 5h & 6h).

In contrast to the detailed knowledge on molecular regulation of intracellular K^+ and Na^+ homeostasis and metabolism of osmoprotective compounds, the regulation of Cl^- transport systems and the role of Cl^- homeostasis in plants under salt stress have been scarcely investigated yet (White and Broadley 2001, Zhu 2003, Yu and Liu 2004, Brumós et al. 2010). Voltage-dependent CLCs are found from bacteria to animals and plants, and mediate passive Cl^- transport that is driven by the electrochemical gradient (Moya et al. 2003, Diedhiou and Golldack 2006, Teakle and Tyerman 2010). Plant homologous CLC channels have been identified in tobacco, *Arabidopsis*, tomato, rice, spinach, corn and soybean, and this may suggest its wide existence in the plant kingdom. Li et al. (2006) had first cloned a putative CLC gene (*GmCLC1*) from soybean, and hypothesized that, at the cell level, overexpression and function of *GmCLC1* may control the sequestering of Cl^- into vacuoles to reduce ionic toxicity and/or physiological drought experienced by the cytoplasm. However, to date, there is no study on CLCs in the PM or other subcellular organs of soybeans including *G. soja*, *G. max* and their hybrids.

In summary, the above NaCl-induced alternations of tissue and cellular ion fluxes suggest that, with regard to the ionic effect of NaCl stress, Cl^- was the main determinant salt ion for salt tolerance in soybean. The difference in Cl^-/salt tolerance among *G. soja*, *G. max* and their hybrid is mainly attributed to the capacity of Cl^- restriction to the plant above-ground parts such as leaves. In other words, for the salt-sensitive *G. max* N23674 cultivar seedlings under NaCl stress, in comparison with Na^+, more Cl^- was absorbed by its roots and highly transported to its above-ground tissues after a longer duration of salt stress, and this caused high content of Cl^- to be retained in leaves. While the salt-tolerant *G. soja* BB52 population and the hybrid 4076 strain seedlings showed the contrary, and possessed the trait of strong Cl^-/salt tolerance. Moreover, it may also indicate that, through the cross between *G. max* N23674 cultivar and the salt-born *G. soja* BB52 population, and selection generation by generation, the obviously improved salt tolerance of the F_5 hybrid 4076 strain was mainly related to compartmentation of Cl^- in plants at the cellular and/or tissue levels. This good agronomic trait for salt tolerance in soybean needs to be highly emphasized and further investigated in future work.

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