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Regulation of root-to-leaf Na and Cl transport and its association with photosynthetic activity in salt-tolerant soybean genotypes

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ABSTRACT

Soil salinity is a major constraint to sustainable crop production. Genetic improvements are needed for growing soybean in salinity-prone environments. Salt-tolerant soybean genotypes alleviate a reduction in photosynthesis and growth under saline conditions; however, the detailed mechanisms involved remain unclear. Here, we aimed to clarify how Na and Cl root-to-leaf transport is quantitatively regulated, and to identify whether photosynthetic tolerance depends on traits associated with either stomata or with mesophyll tissues. Two pairs of pot-grown soybean near-isogenic lines (NILs) consisting of tolerant and susceptible counterparts, derived from a cross between salt-tolerant FT-Abyara and salt-sensitive C01, were subjected to salinity treatment in a rainout greenhouse. Comparison of photosynthetic responses between genotypes indicated that genotypic differences in salinity tolerance depended on the ability for sustained CO_2 assimilation in mesophyll tissues, rather than stomatal conductance. The ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) declined exponentially with increasing Na and Cl concentration regardless of genotype, but tolerant genotypes effectively kept both elements at significantly low levels. Under saline conditions, tolerant genotypes reduced Na and Cl content at the two transport pathways: from root to stem, and from stem to leaf, but the reduction of Cl at each pathway was only minor. These results suggest that integrating genetic capacity for Cl transport regulation and osmotic adjustment should be an important target in salinity-tolerance soybean breeding.

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Genotypic difference; Na and Cl concentration; near-isogenic line; osmotic adjustment; salinity tolerance; stomatal conductance

Soil salinity is a major constraint to sustainable crop production. Approximately, 10% of the world cropland is adversely affected by salinity (Shannon, 1997). Soil salinity occurs principally due to the native chemical composition of the soil, often made worse by inappropriate cultural practices, particularly irrigation and fertilization. Setting a drainage system and washing off salts from the soil with fresh water can help in mitigating soil salinity, but these methods are often not economically or environmentally feasible in many soybean-growing regions. Soybean has been evaluated to be relatively sensitive to salinity (Katerji et al., 2003). Therefore, genetic and/or cultivation improvements are needed for growing soybean in salinity-prone environments.

Performance of many plant species can be moderately to severely affected by soil salinity, which hampers plant physiological processes by multiple mechanisms, among which, ion toxicity induced by an excess uptake of specific saline ions – including Na and Cl – has been shown to be a major cause of damage (Bayuelo-Jiménez et al., 2003; Eisa et al., 2012; Jones, 1981; Lu & Vonshak, 2002; Mäkelä et al, 1999; Mishra et al., 1991; Munns & Tester, 2008; Ziska et al., 1990). In soybean, germination (Abel & Mackenzie, 1964; Wang & Shannon, 1999) and nodule formation (Song et al., 2017; Tu, 1981), – and thereby early growth – are impaired under saline conditions (Singleton & Bohlool, 1984; Song et al., 2017; Yasuta & Kokubun, 2014). Among salt-induced physiological dysfunctions, a substantial decline in photosynthetic activity was a dominant factor limiting growth and yield in soybean (Eisa et al., 2012; He et al., 2016; Jones, 1981; Munns & Tester, 2008; Parker et al., 1983; Yang & Blanchar, 1993; Yasuta & Kokubun, 2014).

Numerous studies have demonstrated considerable genotypic variability for salinity tolerance among soybean cultivars (Ghassemi-Golezani et al., 2009; Hakeem et al., 2012; Karim et al., 2012; Lee et al., 2008; Mannan et al., 2012; Parker et al., 1983; Shelkea et al., 2017; Song et al., 2017; Yang & Blanchar, 1993; Yasuta & Kokubun, 2014). Genetic analyses of genotypes differing in salinity tolerance led to the identification of quantitative trait loci (QTLs) conferring the level of tolerance (Chen et al., 2008; Hamwieh et al., 2011; Lee et al., 2004; Xu & Tuyen, 2012). Using a population derived from the salt-tolerant cultivar

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Tiefeng 8 and the salt-sensitive cultivar 85–140, Guen et al. (2014a) mapped a salt-tolerance gene (*GmSALT3*) from Tiefeng 8, which is a dominant gene associated with the ability to limit Na^+ accumulation in shoots. A subsequent study showed that salinity tolerance in soybean is modulated by natural variation in the *GmSALT3* gene (Guen et al., 2014b). On the contrary, Do et al. (2016) isolated gene *Ncl*, which regulates ion (Na^+, K^+, Cl^-) transport and accumulation, from salt-tolerant Brazilian soybean cultivar FT-Abyara, and demonstrated that higher expression of *Ncl* in the roots led to lower ion accumulation in the shoot. Furthermore, when *Ncl* was incorporated into the 'Kariyutaka' soybean cultivar, the transformed genotype showed significantly enhanced salt tolerance. In another study, using an RIL population of a *Glycine soja* accession, Qi et al. (2014) identified salt tolerance gene *CHX1*, which conferred the capacity of lowering leaf Na^+ content. Using this gene, a subsequent study developed high-throughput single nucleotide polymorphism (SNP) markers for salinity tolerance in soybean (Patil et al., 2016). These studies suggested the possibility to screen for or breed soybean genotypes with an improved capacity to regulate the accumulation of specific ions responsible for salt tolerance.

Under saline conditions, most plants are prone to a reduction in leaf water potential induced by osmotic stress, leading to a decline in stomatal conductance, and thereby, in photosynthetic rate (Huchzermeyer & Koyro, 2005). Generally, a combination of osmotic stress and ion toxicity can occur under complex environments (Jones, 1981; Munns & Tester, 2008; Shannon, 1997; Zhang et al., 2010). Salinity-tolerant genotypes selected in these studies revealed that the extent of tolerance was due to the specific capacity for maintaining normal photosynthetic activity under saline conditions (Lu et al., 2009; Yasuta & Kokubun, 2014). In previous studies, in which photosynthetic responses of salt-tolerant and salt-sensitive genotypes were compared under saline conditions, salt-induced decline in photosynthetic rate was primarily due to a concurrent decline in stomatal conductance both, in salt-tolerant and in salt-sensitive genotypes, indicating that the reduction in stomatal conductance caused by osmotic stress is a dominant factor limiting photosynthesis even in salt-tolerant cultivars (He et al., 2016; Lu et al., 2009). In contrast, salinity-tolerant cultivars exhibited higher leaf water potential than sensitive cultivars under saline conditions, suggesting that the capacity for osmotic adjustment may have contributed to the observed level of salinity tolerance (Karim et al., 2012; Mannan et al., 2012). Thus, there have been contrasting reports as to whether tolerance to osmotic stress is associated with genotypic differences in photosynthetic capacity under saline conditions. Therefore, the question arose as to whether the salinity-tolerance of recently-bred genotypes is due to a greater tolerance to osmotic stress than that of susceptible genotypes.

Saline-induced toxicity varies with plant species, plant growth stage, and duration of saline stress (Jones, 1981; Lenis et al., 2011; Munns & Tester, 2008; Ren et al., 2012; Zhang et al., 2010). Reportedly, salinity tolerance of soybean cultivars is associated with the capacity of controlling Na and/or Cl transport to shoots (Do et al., 2016; Essa, 2002; Umezawa et al., 2001; Valencia et al., 2008; Yasuta & Kokubun, 2014), or with the ability to direct to and accumulate saline elements into the vacuole (Dabuxilatu & Ikeda, 2005a, 2005b, Essa, 2002). Sequestration of saline ions in vacuoles after being transported to leaves has been observed in other plant species as well, a phenomenon which may mitigate ion toxicity in the cytoplasm (Flowers et al., 1991; Fricke et al., 1996; Leigh & Tomos, 1993). Previous studies, in which relative toxicity of Na and Cl was compared using soybean and its wild ancestors, indicate that plant growth was inhibited more substantially by Cl than by Na (Abel & Mackenzie, 1964; Lenis et al., 2011; Parker et al., 1983; Phang et al., 2008; Ren et al., 2012; Valencia et al., 2008; Yang & Blanchar, 1993). Plants subjected to saline soil conditions absorb saline ions through the roots, and transport them to the leaves through the stem and petiole. Roots of salinity-tolerant soybean genotypes are capable of selective ion absorption, which has been shown to confer the observed salinity tolerance in these genotypes (Dabuxilatu & Ikeda, 2004, 2005a, 2005b; Do et al., 2016). Do et al. (2016) demonstrated that higher expression of *Ncl* in the root resulted in lower ion accumulation in the shoot. Using tolerant soybean genotypes, near-isogeneic lines (NILs) have been bred (Do et al., 2016; Hamwieh et al., 2011). However, little information is available on how the recently-bred salt-tolerant genotypes are capable of regulating Na and Cl transport from the roots to the leaves via stem/petiole, which is closely associated with the photosynthetic activity under saline conditions.

Therefore, we aimed to clarify how transport of Na and Cl from roots to leaves via stem/petiole is quantitatively regulated in the recently bred salt-tolerant soybean genotypes, and to identify whether the photosynthetic tolerance of these tolerant genotypes depends on factors associated with stomata or rather, with factors associated with metabolic activity in mesophyll cells. The results obtained should help soybean breeding programs for greater salinity tolerance, as well as the promotion of physiological studies on salinity tolerance in plants.

Materials and methods

Plant materials

We used two sets of NILs: NILs18-S/NILs18-T and NILs72-S/NILs72-T, which are salt-sensitive (S) and salt-tolerant (T) progenies of a residual heterozygous line (RHL) from a recombinant inbred line (RIL) population derived from a cross between salt-tolerant FT-Abyara and salt-sensitive C01 (Do et al., 2016; Hamwieh et al., 2011). The tolerant lines (NILs18-T and NILs 72-T) proved to carry salt-tolerance QTL derived from FT-Abyara (Hamwieh et al., 2011).

The experiments were conducted at the Graduate School of Agricultural Science, Tohoku University (38° 16′N, 140°50′E) in 2015 and 2016, with the only difference being a 2-day shift in sowing time between the 2 years. Plants were potted in a greenhouse with four open sides. Prior to sowing, fertilizer was applied at fixed rates and mixed with soil: 0.5 g of N, 1.5 g of P_2O_5, 2.0 g of K_2O, 10 g of fused phosphate, 20 g of calcium superphosphate, and 20 g of slaked lime per pot (1/5,000-a Wagner pot) of low-humic Andosol field soil (N: 0.55%, cation exchange capacity [CEC]: 44.9 cmol kg^{-1}). The seeds were inoculated with J1065, a strain of *Bradyrhizobium japonicum* obtained from Tokachi Nokyoren (Obihiro, Japan). On 5 June 2015, and 7 June 2016, five seeds per genotype were sown per pot; seedlings were thinned to one plant per pot after emergence. Preventative insecticides were applied as needed during plant culture.

Saline treatment

Air temperature and solar radiation data during the experiments were obtained from the website of Sendai Regional Headquarters, Japan Meteorological Agency, located about 3 km away from the experimental site. Daily mean air temperature during the experiments (from sowing to harvest) was 22.7 and 22.2°C in 2015 and 2016, respectively. Daily mean solar radiation was 17.9 and 15.0 MJm^{-2} in 2015 and 2016, respectively. Air temperature was slightly higher, while solar radiation inside the greenhouse was about 10% lower than outside the greenhouse, respectively.

Saline treatment was imposed by placing the pots in quadrilateral water containers (135 cm length × 91 cm width × 20 cm depth) containing saline water to a depth of about 10cm, which allowed plants to take up water through the bottom hole in each pot. Plants were subjected to one of four levels of NaCl solution (0, 40, 80, or 120 mM) for 28 days, starting 28 days after sowing (DAS) in 2015, and 29DAS in 2016. Plants in the 80 and 120 mM treatments were pre-treated with 40 mM solution for two days. The saline solution was renewed at every several-day intervals. Tap water was applied to control plants in the same manner as the saline treatment. A total of 80 pots (two lines (NILs18, NILs72), two genotypes (S, T) ×4 saline treatments ×5 replicates) were employed each year. Each container contained two lines with respective two genotypes (NILs18-S, NILs18-T, NILs72-S, NILs72-T), and the pots in each container were placed randomly with adequate spacing without mutual shading and moved every several days to minimize position effects.

Measurements and data analysis

Four (2015) and three (2016) plants per plot were sampled at 28 days after initiation of saline treatment (DAT). The samples were separated into leaves, stems + petioles, roots, and nodules, then oven-dried at 80°C for 3 days, and weighed.

Photosynthetic rate of the recently expanded terminal leaflet of the five (2015) or three (2016) plants was measured using a portable photosynthesis system (LI6400; Li-Cor Inc., NE, USA). Measurements were carried out during the period from 1000 to 1200 at 28DAT in 2015, and 12DAT and 24DAT in 2016, respectively. Air flow rate into the leaf chamber was controlled at 500 μmol s^{-1}, and the CO_2 concentration supplied to the leaf chamber was maintained at 380 μmol mol^{-1}. Irradiance on the measured leaves (2 cm^2) was regulated at a photon flux density of 1500 μmol m^{-2} s^{-1}, and the temperature and relative humidity inside the chamber were maintained at 25°C and ca. 60%, respectively.

In order to evaluate the relative contribution of the size of stomatal aperture and intracellular activity to photosynthetic rate, stomatal conductance and the ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) was calculated from the data obtained during the photosynthesis measurements, using the built-in software.

Leaf chlorophyll content of the same leaves measured for photosynthesis was estimated, using a chlorophyll meter (SPAD 502, Konica Minolta Inc., Tokyo, Japan).

For the determination of Na and Cl contents, the dried samples of four plants per plot/genotype obtained at 28DAT in 2015 were used, which were the same plants sampled for DW measurements. Plant parts (leaf, stem + petiole, root (including nodules)) of individual plants were ground in a mill. For the determination of Na content, 100 mg of the milled samples were extracted with 1M HCl, and filtered with filter paper (No. 6, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), then Na

concentration of the filtrate was measured with an atomic absorption spectrophotometer (A-2000, Hitachi Corp., Tokyo, Japan). For the determination of Cl, 50 mg of the milled samples were extracted with hot water (Matsumaru, 1991), and filtered with filter paper (No. 6, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), then the concentration of Cl in the filtrate was measured with an ion chromatograph (ICS-900, DIONEX Corp., Osaka, Japan).

For the microscopic observation, roots of NILs18-S and NILs18-T grown under 80 mM were sampled at 28DAT in 2016. After drying the root samples at room temperature for several weeks, root slices were taken from three sections of the root system: taproot, 1.5 cm below cotyledonary node, secondary root directly connected with tap root [0 cm], and secondary root 10cm apart from the connection with taproot [10 cm], as shown in Figure 1. The root discs were vacuum-dried, and coated with carbon, then photographed with a scanning electron microscope (SU8000, Hitachi High-Technologies Corp., Tokyo, Japan). After photographing, Na and Cl in root cross section were mapped with an energy-dispersive x-ray spectrometer (EDAX Apollo XV, AMETEK Co., Ltd., Tokyo, Japan).

Experiments were repeated for 2 years (2015, 2016). An analysis of variance (ANOVA) for dry weight (DW), traits on photosynthetic traits, and Na and Cl content was performed to evaluate the effects of the saline treatment, genotype, and their interactions using JMP7.0.2 (SAS Institute Japan Ltd., Tokyo, Japan). For the analysis on DW, the data for the 2 years (2015, 2016) were considered as replications, since the only difference was a 2-day shift in sowing time between the 2 years (5 June 2015, 7 June 2016).

Results

Dry weight

Figure 2 shows DW of whole plants of the two pairs of NILs (NILs18-S, NILs18-T, NILs72-S, NILs72-T) subjected to the four levels of salinity tested (0, 40, 80, 120 mM NaCl). Analysis of variance (ANOVA) of the effect of line, genotype and saline treatment on DW revealed that the effect was significant between susceptible and tolerant genotypes (S vs. T), but not between the two lines (18 vs. 72), while significant effects of year and saline treatment were observed (Table 1). Interactions between lines/genotypes and saline treatments were significant. In the 0 mM NaCl plot, DW did not differ among lines and genotypes. Dry weight decreased significantly with increasing NaCl concentration, but the magnitude of DW reduction was smaller in the tolerant (T) than in the susceptible (S) genotype for both NILs tested (18 and 72) (Table 1, Figure 2).

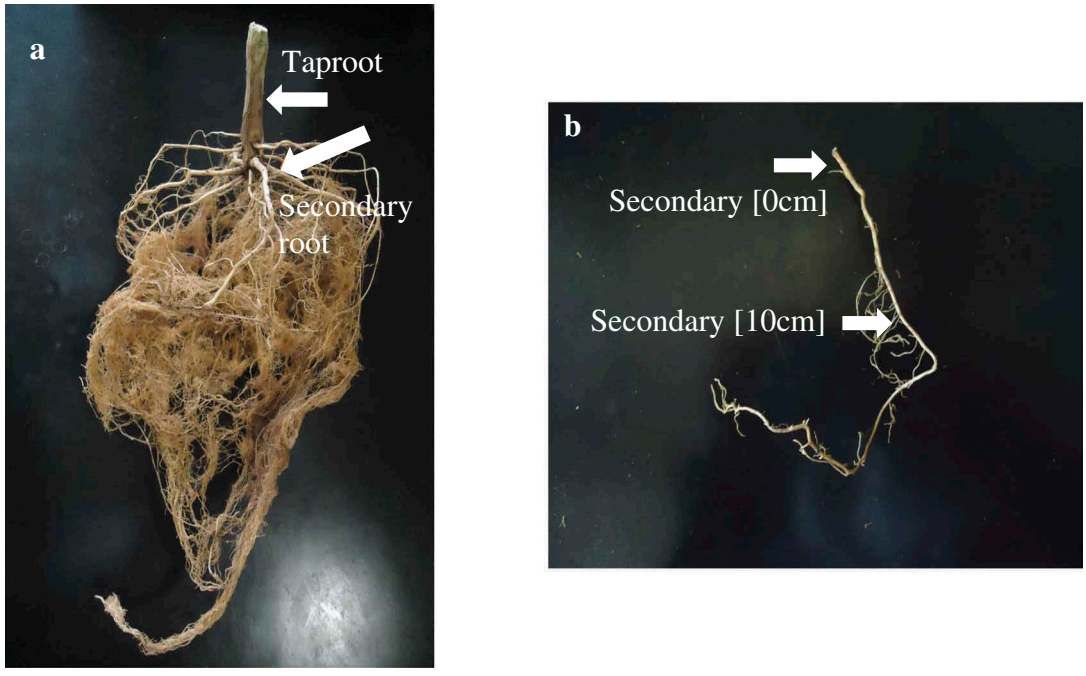

Figure 1. Photographs for roots sampled. (a): a whole root system below cotyledonary node. (b): Secondary root. Arrows on a indicate the position on the taproot and on a secondary root sampled for microscopic observation. Arrows on b indicate two positions on a secondary root directly connected with the taproot [0 cm], and 10 cm apart from the connection with taproot where root slices were taken for microscopic observation [10cm].

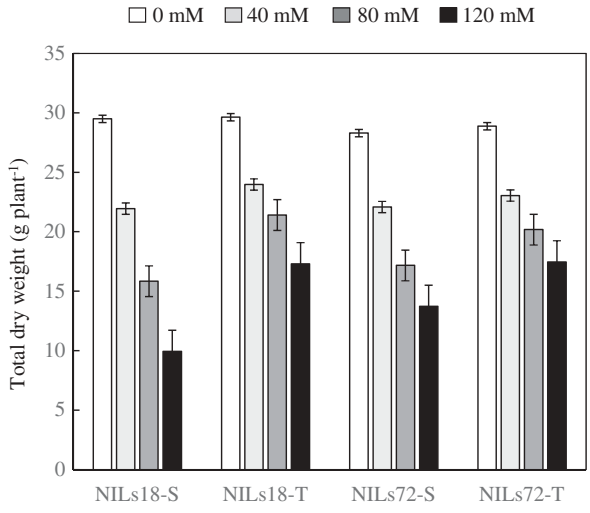

Figure 2. Total dry weight of soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Measurements were made at 28DAT in 2015 and 2016. Means with ± SE of the 2 years are shown.

Table 1. Summary of ANOVA for total dry weight of soybean NILs (NILs18-S/NILs18-T and NILs72-S/NILs72-T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl).

Source of variation	Total dry weight
Year (Y)	***
Genotype (S/T)	***
Line (18/72)	ns
Saline treatment (T)	***
S/T × T	***
18/72 × T	*
S/T × 18/72	ns
S/T × 18/72 × T	ns

Measurements were made at 28DAT in 2015 and 2016. Genotype (S/T): variation between susceptible (NILs18-S, NILs72-S) and tolerant (NILs18-T, NILs72-T) genotypes. Line (18/72): variation between NILs18-S/T and NILs72-S/T.
*, ***: Significant at P < 0.05 and 0.001, respectively. ns: Not significant at P < 0.05

Photosynthesis, stomatal conductance and chlorophyll content

Apparent photosynthetic rate (A) of the most recently expanded leaf was measured at 28DAT in 2015, and 12DAT and 24DAT in 2016. Since the results obtained at 28DAT in 2015 were similar to those at 24DAT in 2016, only the results obtained in 2016 are described here.

The effect of saline treatment was significant on A, stomatal conductance, and on the ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) in all the lines/genotypes both, at 12DAT and 24DAT (Table 2). The effect of genotype on A and A/Ci was significant at 24DAT, but not at 12DAT.

At 24DAT, A was significantly reduced by saline treatment regardless of genotype, and the reduction was greater at higher NaCl concentration (Figure 3).

Table 2. Summary of ANOVA for photosynthetic rate (A), stomatal conductance (Gs), and the ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) of soybean NILs (NILs18-S/Nils18-T and NILs72-S/NILs72-T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl).

	12 DAT			24 DAT		
Source of variation	A	Gs	A/Ci	A	Gs	A/Ci
Genotype (S/T)	ns	ns	ns	***	ns	***
Line (18/72)	ns	ns	ns	ns	ns	ns
Saline treatment (T)	**	**	***	***	***	***
S/T × T	ns	ns	ns	ns	ns	***
18/72 × T	ns	ns	ns	ns	ns	ns
S/T × 18/72	ns	ns	ns	ns	ns	ns
S/T × 18/72 × T	ns	ns	ns	ns	ns	ns

Measurements were made at 12 and 24DAT in 2016. Genotype (S/T): variation between susceptible (NILs18-S, NILs72-S) and tolerant (NILs18-T, NILs72-T) genotypes. Line (18/72): variation between NILs18-S/T and NILs72-S/T. *, ** and ***: Significant at P < 0.05, 0.01 and 0.001, respectively. ns: Not significant at P < 0.05

The magnitude of the reduction was significantly smaller in tolerant than in susceptible genotypes. Similarly, stomatal conductance and A/Ci were reduced by saline treatment in both genotypes under study, and the reduction was greater as NaCl concentration increased. When comparing T and S genotypes, the magnitude of reduction in stomatal conductance was not significantly different between the two genotypes, whereas that of A/Ci was significantly different between S and T genotypes, as observed for A.

Leaf chlorophyll content was evaluated by SPAD reading. Although it did not differ at 12DAT, regardless of line/genotype nor saline treatment, chlorophyll content was substantially reduced by saline treatment in S genotypes, but it was not in T genotypes (Figure 4).

Na and Cl contents in plant parts

Figure 5 shows Na and Cl content in plant parts of four genotypes. In control plants (0 mM NaCl solution), Na and Cl concentrations were very low, with the concentrations decreasing in the following order; root > stem + petiole > leaf. In control plants, Na concentrations were not different between S and T genotypes in all plant parts, whereas those of Cl in leaf and stem + petiole were higher in S than in T genotypes. ANOVA for Na and Cl content of plant parts revealed that the leaf content of Na was significantly affected by all sources of variation (genotype (S/T)), line (18/72), saline treatment, and their interactions), whereas that of Cl was significantly affected by genotype (S/T), saline treatment, and their interaction (Table 3).

As shown in Figure 5, the saline treatment increased both, Na and Cl concentrations in all plant parts, regardless of genotype; however, the concentration of Cl was substantially higher than that of Na. A comparison of genotypes revealed that Na and Cl concentrations in roots did not

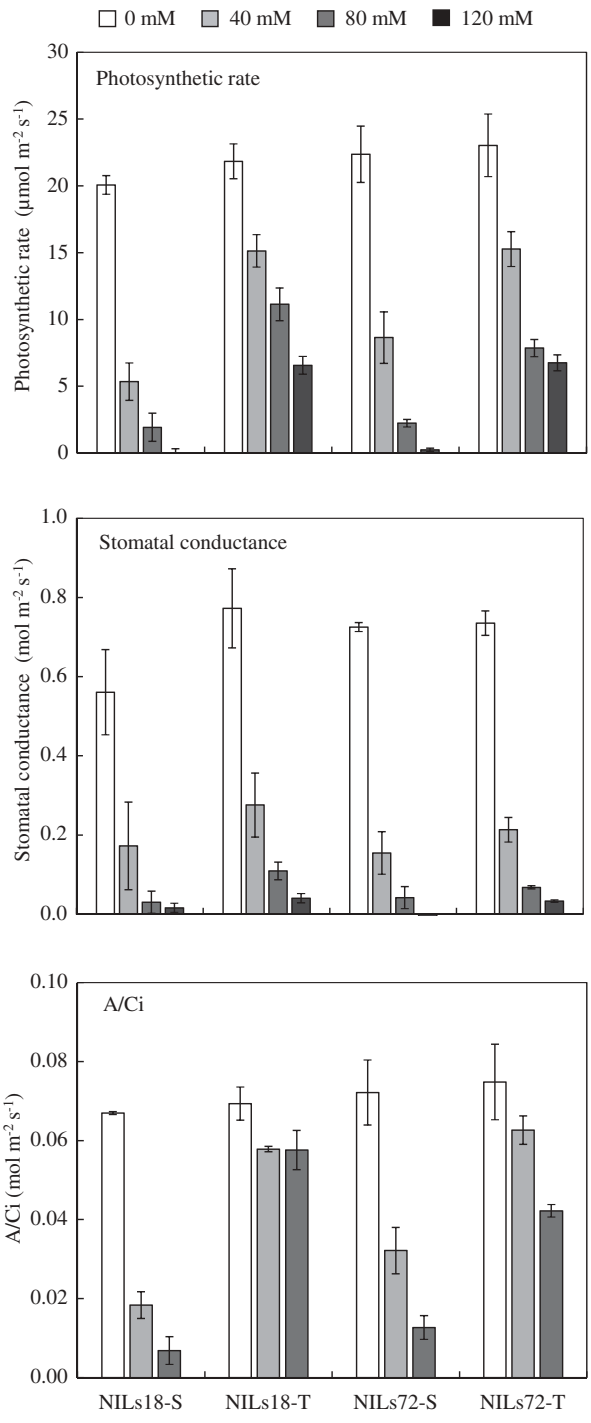

Figure 3. Photosynthetic rate, stomatal conductance, and ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) in leaves of soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Measurements were made 24DAT in 2016.Vertical bars indicate SE (n = 3).

significantly differ among genotypes, but genotypic differences were obvious for stem + petiole and leaves; concentrations being higher in S than in T genotypes. The concentration of Na was substantially lower in leaves than

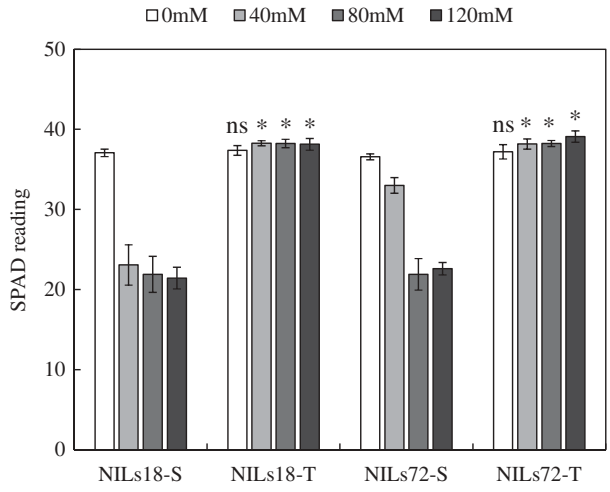

Figure 4. SPAD readings on leaves of soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Measurements were made 24DAT in 2016. Vertical bars indicate SE (n = 3). *: Significantly different between sensitive (S) and tolerant (T) genotypes of the respective NILs under the same saline condition ($P < 0.05$). ns: Not significant ($P < 0.05$).

in other plant parts regardless of genotype, whereas that of Cl in S genotypes was higher in leaves than in other plant parts. In T genotypes, the concentrations of both elements were greatly reduced through the route from root to leaf via stem + petiole, suggesting the involvement of a physiological mechanism regulating upward transport of the two elements.

Figure 6 shows the rate of Na and Cl content present in the three plant parts subjected to saline conditions. As for Na, in control plots (0 mM NaCl), most of Na was contained in roots, while the distribution to shoots was negligible. With increasing NaCl concentration, the rate of roots decreased, while it increased in the shoots, in this latter case being significantly lower in T than in S genotypes; further, the difference between S and T genotypes became larger with increasing NaCl concentration in the growing medium. In contrast, Cl accumulated to some extent in all plant parts even in control plots, and the change in the rate was smaller than that of Na, although the rate in the shoots was smaller in T than in S genotypes.

Distribution of Na and Cl in root

Figure 7 shows the X-ray mapping of Na and Cl on a root cross section. In the taproot and the secondary root [0cm], the two elements were distributed widely on cross section with no clear pattern of localization observed (data are not shown). In secondary roots [10 cm], the two elements are localized around the endodermis. A comparison of tolerant and susceptible

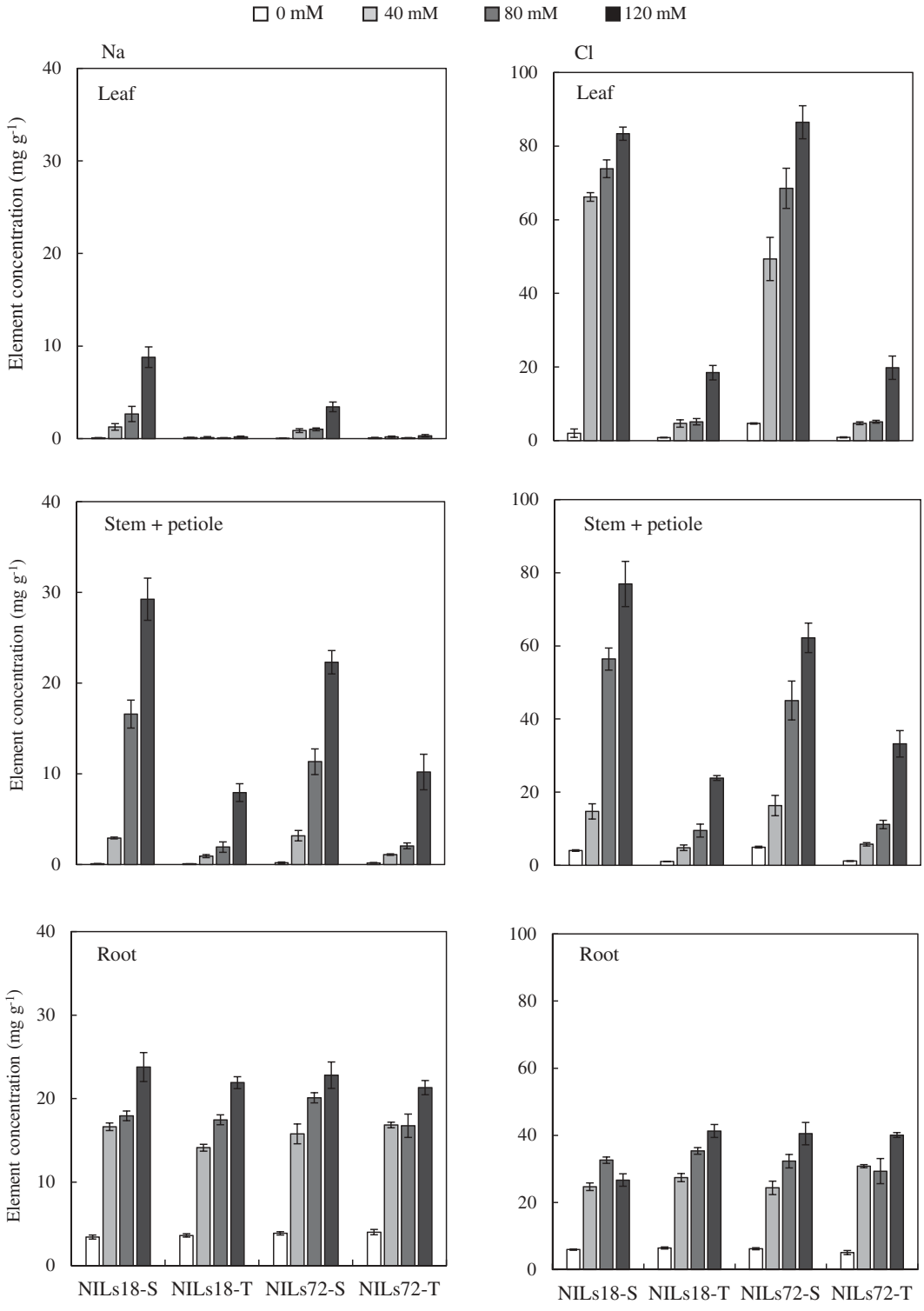

Figure 5. Sodium (Na) and chlorine (Cl) concentrations in different plant parts (leaf, stem + petiole, roots) of soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Left: Na. Right: Cl. Plants were sampled at 28DAT in 2015. Vertical bars indicate SE (n = 4).

genotypes showed that the localization of Na did not differ between the tolerant and susceptible genotypes, while Cl was more notably localized around the endodermis in the tolerant genotype (NILs18-T).

Discussion

Salt-induced inhibition of photosynthetic activity results from multi-faceted physiological dysfunction attributed primarily to direct toxicity and/or osmotic stress induced

Table 3. Summary of ANOVA for Na and Cl content of plant parts of soybean NILs (NILs18-S/NILs18-T and NILs72-S/NILs72-T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl).

	Na			Cl		
Source of variation	Leaf	Stem + petiole	Root	Leaf	Stem + petiole	Root
Genotype (S/T)	***	***	ns	***	***	ns
Line (18/72)	**	ns	ns	ns	ns	ns
Saline treatment (T)	***	***	***	***	***	***
S/T × T	***	***	ns	***	***	ns
18/72 × T	***	ns	ns	ns	ns	ns
S/T × 18/72	***	*	ns	ns	*	ns
S/T × 18/72 × T	***	*	ns	ns	*	ns

Measurements were made at 28 DAT in 2015. Genotype (S/T): variation between susceptible (NILs18-S, NILs72-S) and tolerant (NILs18-T, NILs72-T) genotypes. Line (18/72): variation between NILs18-S/T and NILs72-S/T. *, ** and ***: Significant at $P < 0.05$, 0.01 and 0.001, respectively. ns: Not significant at $P < 0.05$

by salt ions (Bayuelo-Jiménez et al., 2003; Bray et al., 2000; Lu & Vonshak, 2002; Mäkelä et al., 1999; Mishra et al., 1991; Ziska et al., 1990). Recently bred salinity-tolerant genotypes should exhibit a capacity for alleviating these dysfunctions. However, up to the present, there has been little information on how tolerant genotypes are capable of regulating Na and Cl transport from the roots to the leaves, which may be closely associated with the photosynthetic activity under saline conditions. In this study, we aimed to clarify how the transport of Na and Cl from root to leaf via stem/petiole is quantitatively regulated, and to determine whether photosynthetic tolerance to salinity depends on factors associated with stomata or rather factors associated with activity in mesophyll cells.

An objective of the present study was to clarify whether tolerance to osmotic stress is associated with genotypic differences in salinity tolerance, by using stomatal conductance as the indicator. A comparison of photosynthetic response between tolerant and susceptible genotypes revealed that saline treatment substantially reduced stomatal conductance regardless of genotype; additionally, the magnitude of this reduction did not differ between tolerant and susceptible

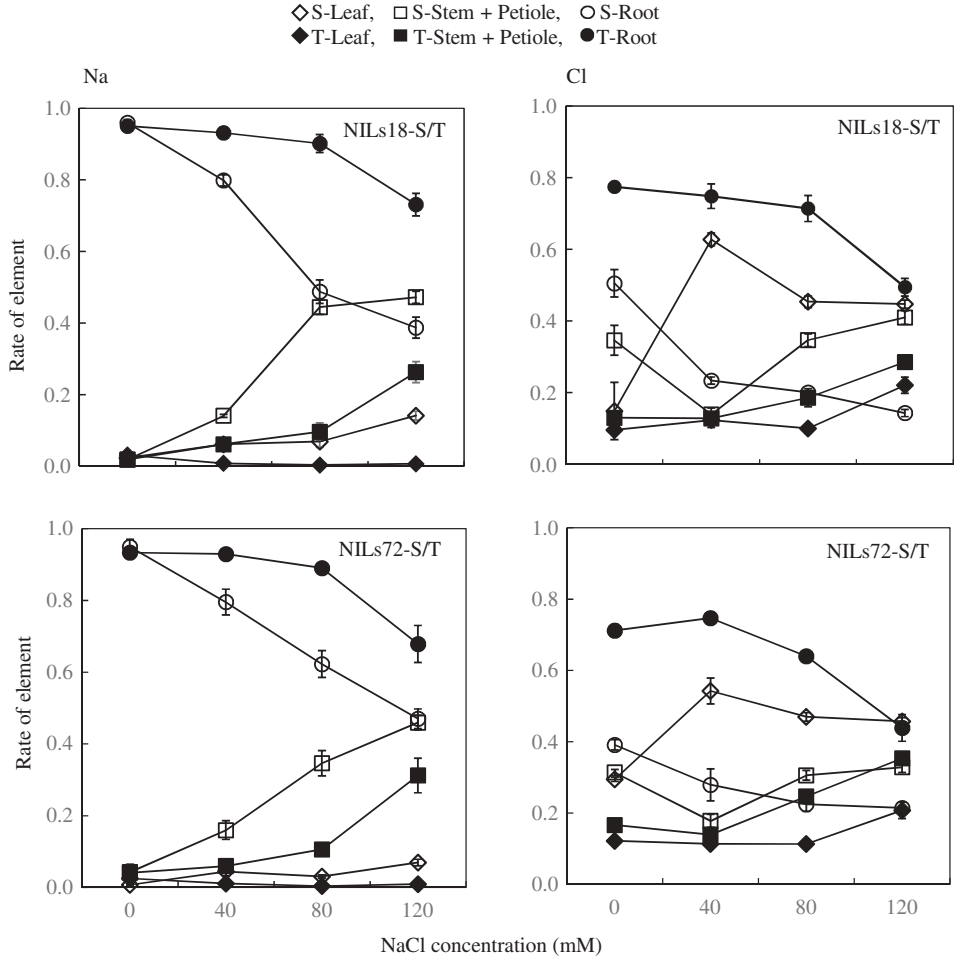

Figure 6. Partitioning of absorbed elements (Na and Cl) into the three plant parts (leaf, stem+ petiole, roots) in soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Plants were sampled at 28DAT in 2015. Vertical bars indicate SE (n = 4). Left: Na. Right: Cl. Open symbol: sensitive genotype (S). Closed symbol: tolerant genotype (T).

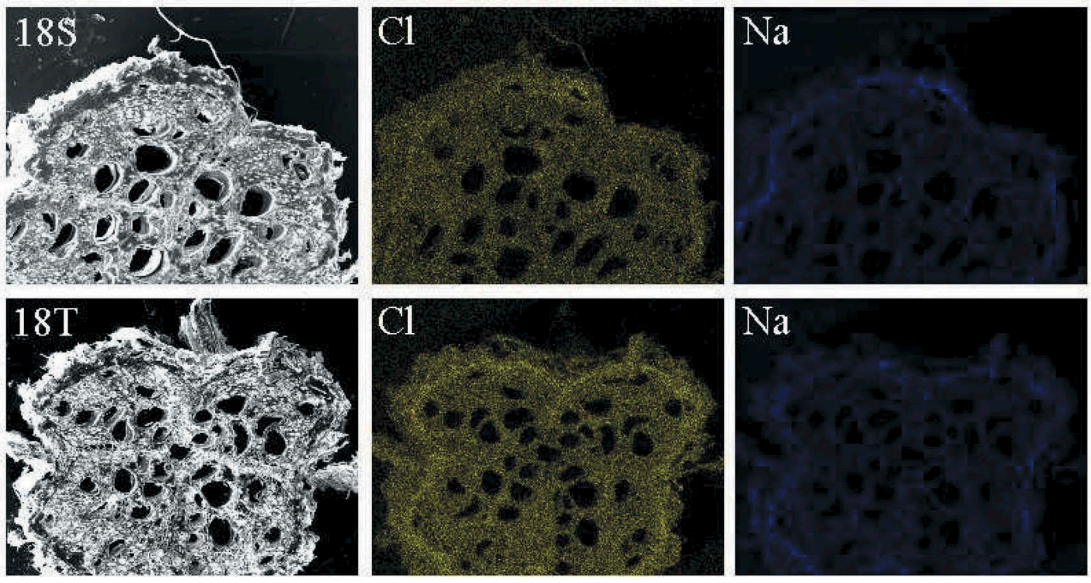

Figure 7. Mapping of Na and Cl in soybean genotypes (NILs18-S and NILs18-T) subjected to saline treatment (80 mM NaCl). Plant roots were sampled at 28DAT in 2016 and root slices were taken from a secondary root 10cm apart from the connection with the taproot (Secondary [10cm]). Na and Cl of the sections were mapped for electron microscopic observation. Upper: NILs18-S, Lower: NILs18-T. Left: without mapping, Center: mapped by Cl, Right: mapped by Na.

genotypes. In contrast, the saline-induced decline in photosynthetic rate and A/Ci was less severe in tolerant than in susceptible genotypes (Figure 3, Table 2). These results suggest that differences in photosynthetic rate between tolerant and susceptible genotypes under saline conditions were not due to a capacity for osmotic adjustment, but rather, to the capacity for sustained CO_2 assimilation under the stress. Regarding this issue, there have been reports that the salinity tolerance of soybean genotypes is due to a greater tolerance to osmotic stress (Karim et al., 2012; Mannan et al., 2012). Although our results suggested that the capacity for osmotic adjustment did not contribute to a greater salt tolerance in the tolerant genotypes used in this study, there is a possibility that the genetic improvement of osmotic adjustment enhances the salinity tolerance in soybeans, whether it is feasible through the conventional breeding or newer genomic methods.

In soybean, it is known that leaf Na or Cl content is closely correlated with leaf chlorosis (Lee et al., 2008; Parker et al., 1983; Phang et al., 2008). In the present study, SPAD reading, an indicator of leaf chlorophyll content, was not affected by saline treatment in the tolerant genotypes, whereas it was substantially affected in the susceptible genotypes (Figure 4). Thus, sustainment of leaf greenness is likely to be a visible indicator of the salinity tolerance of tolerant genotypes.

Salt-induced decline in photosynthetic rate in soybean has been reported by many studies (He et al., 2016; Karim et al., 2012; Umezawa et al., 2001; Yasuta & Kokubun, 2014). However, there has been no report on the quantitative relationship between saline content and photosynthetic rates in soybean leaf. Therefore, another objective of this study was to clarify how the transport of Na and Cl from roots to leaves via stem/petiole is quantitatively regulated in tolerant genotypes. Since the ratio of photosynthetic rate to intercellular CO_2 concentration (A/Ci) proved to be an indicator of salinity tolerance among genotypes, we analyzed the relationship between A/Ci and Na and Cl content in leaf. As shown in Figure 8, A/Ci declined exponentially over Na and Cl concentration. Estimating a threshold value at the point where the rate declined to a tenth of the maximum rate (ca. 0.08 mol m^{-2} s^{-1}), the concentration was ca. 0.65 and 23.9 mg g^{-1} for Na and Cl, respectively. Although it appears that A/Ci is more sensitive to Na than Cl concentration, the independent effect of the two ions on A/Ci is not clear in the present study. This analysis clearly revealed that tolerant genotypes were capable of maintaining salt concentration below the threshold value, which appears to be a critical trait conferring salinity tolerance in tolerant genotypes.

The physiological mechanisms on how Na and Cl in leaves affected A and A/Ci under saline conditions were not clear in the present study. Flexas et al. (2012) emphasized that mesophyll conductance to CO_2, which is closely associated with A/Ci, is a central player in photosynthesis; the mesophyll conductance can be the most significant photosynthetic determinant under

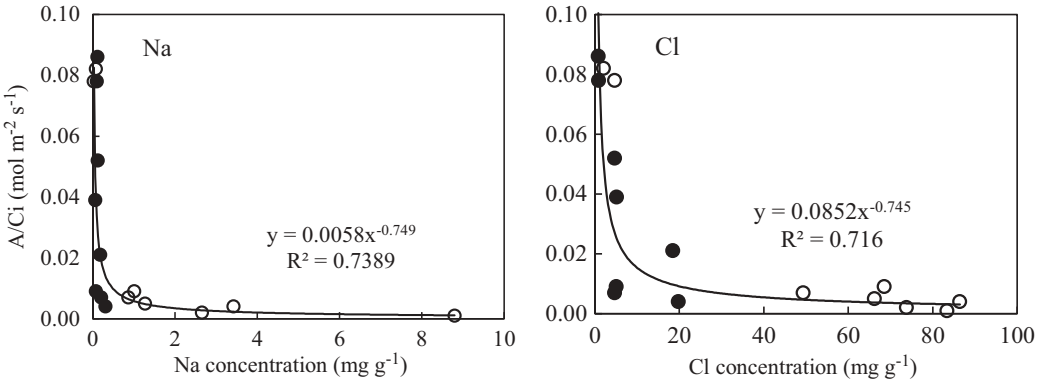

Figure 8. Relationship between A/Ci and leaf Na and Cl content in soybean NILs (NILs18-S/T and NILs72-S/T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl). Measurements were made 26DAT and 28DAT in 2015 for A/Ci and element content, respectively. Open symbols: susceptible genotype (S), Closed symbols: tolerant genotype (T).

certain conditions including drought and salinity. Previous studies revealed that salinity stress hampered normal development of photosynthetic organs, presumably leading to a reduced capacity of mesophyll conductance. For example, saline treatment induced deformation of chloroplast and rubisco-containing body in soybean (He et al., 2014), while it caused chloroplast destruction in rice (Yamane et al., 2008). In addition, antioxidant capacity and the expression of ABA-responsive genes were affected by saline-treated soybean (Chen et al., 2013). Further study is needed to identify which factors regulating mesophyll conductance are involved in the salinity-induced decline in A/Ci observed in the present study.

As described above, it is obvious that tolerant genotypes effectively limited both, Na and Cl accumulation in the leaf. However, which element contributed more to salinity tolerance remains unclear. Toxicity caused by Na and Cl varies with plant species, plant growth stage, and duration of saline stress (Jones, 1981; Lenis et al., 2011; Luo et al., 2005; Munns & Tester, 2008; Ren et al., 2012; Tuyen et al., 2010; Zhang et al., 2010). Previous studies comparing relative toxicity of Na and Cl to soybean and its wild ancestors indicated that plant growth was inhibited more severely by Cl than by Na (Abel & Mackenzie, 1964; Abel, 1969; Munns & Tester, 2008; Parker et al., 1983; Phang et al., 2008; Valencia et al., 2008; Yang & Blanchar, 1993). In several plant species, saline ions were found to be sequestrated in vacuoles after reaching the leaf. Such strategy for allocation may prevent or at least mitigate ion toxicity in the cytoplasm (Flowers et al., 1991; Fricke et al., 1996; Leigh & Tomos, 1993). Regarding the reason for the relative toxicity of Na and Cl in soybean, Dabuxilatu and Ikeda (2005a, 2005b) attributed it to the differential distribution of each element in the plant tissues: Na was primarily found in vacuoles, whereas Cl accumulated in the apoplast as well as in vacuoles. This may explain why the Cl content observed in our study substantially exceeded that of Na (Figure 5).

Do et al. (2016) characterized the saline tolerant genotypes (NILs18-T, NILs72-T) as having a greater capacity than their susceptible counterparts (NILs18-S, NILs72-S), to limit the extent of Na, Cl, and K accumulation in shoots. As Figure 5 shows, our study confirmed this finding since the leaf concentrations of Na and Cl were significantly lower in the tolerant than in the susceptible genotypes. However, Do et al. (2016) did not examine precisely how ion contents were reduced during the transport processes from root to leaf via stem + petiole. For a quantitative evaluation of Na and Cl present in different plant parts, we calculated the ratio (%) of the concentration of each element in aerial plant parts to that of root. As shown in Table 4, the change in the element concentration during its transport was contrasting between Na and Cl. In the case of Na, the percentage in plant parts substantially reduced in the movement from root to stem + petiole, and there to the leaf, regardless of genotype. Conversely, the percentage of Cl increased in aerial organs in the susceptible, but not in the tolerant genotypes. It is noteworthy that the tolerant genotypes used in the present study proved capable of controlling the transport of both Na and Cl through the two major pathways: from root to stem + petiole, and stem + petiole to the leaf. In the susceptible genotypes, Cl concentration increased during transport from the root to the leaf. Considering that leaf Cl concentration was ca. 100-fold greater than that of Na under the highest NaCl concentration regime (120 mM) even in the tolerant genotypes, the capacity for controlling Cl accumulation in leaves was apparently insufficient compared with that of Na, even in these tolerant genotypes (Figure 5). Recently, Wei et al. (2016) analyzed the function of soybean Cl^{-1}/H^{+} antiporter GmCLC1, and found that overexpression of *GmCLC1* in the roots of soybean enabled transformed plants to limit Cl transport

Table 4. Relative element concentrations (Na and Cl) in aboveground plant parts (petiole + stem, leaves) to that of roots of soybean NILs (NILs18-S/NILs18-T and NILs72-S/NILs72-T) subjected to saline treatments (0, 40, 80, or 120 mM NaCl).

Element	Genotype	Root	Stem + petiole	Leaf
Na	NILs18-S	100.0	92.7	15.1
	NILs18-T	100.0	10.9	0.3
	NILs72-S	100.0	56.7	5.0
	NILs72-T	100.0	11.9	0.5
Cl	NILs18-S	100.0	173.0	226.4
	NILs18-T	100.0	26.8	14.4
	NILs72-S	100.0	139.6	212.1
	NILs72-T	100.0	38.2	17.4

Relative values of aboveground plant parts compared to roots (= 100) are shown. Data used for calculations were on plants under 80 mM NaCl solution (28DAT, 2015).

to shoots by its sequestration in roots. Genetic improvement for this capacity is a feasible target in the future salinity-tolerance breeding programs.

The X-ray mapping of Na and Cl in root tissues revealed that Na and Cl were evenly distributed on root cross-sections of the taproot and a secondary root [0cm] (data are not shown). In contrast, the two elements were unevenly distributed on the cross section of secondary root [10cm]; in which case, they were localized around the endodermis (Figure 7). The endodermis, the innermost cell layer of the cortex, contains the Casparian strip encircling the stele (Lersten & Carlson, 2004). The Casparian strip, which contains lignin and suberin, hinders diffusion of water and solutes through cell-wall space (Grebe, 2011; Van Fleet, 1961). It appears that Na and Cl are blocked by the Casparian strip, probably resulting in the reduction of the concentration of these elements in the stele compared with the concentrations in the outer space. This may be particularly true for the secondary roots, since secondary roots [10 cm] are elongating young roots actively absorbing nutrients (Lersten & Carlson, 2004). As shown in Figure 7, the localization of Na did not differ between tolerant and susceptible genotypes, whereas Cl was more notably localized around the endodermis in the tolerant genotype (NILs18-T), suggesting that the capacity for blocking Cl flow into the stele is a trait conferring salinity tolerance in this tolerant genotype. A preliminary measurement of the concentration of Cl concentration by X-ray analysis showed that the reduction in Cl in the stele was greater in the tolerant genotype than in the susceptible genotype. In the present study, we sampled roots only at one point (28DAT). Since the root capacity for absorbing nutrient elements varies with growth stage, further measurements on roots at different growth stages are needed for a more precise evaluation of the whole process of mineral nutrient transport.

Conclusion

In the present study, we aimed to clarify how the transport of Na and Cl from roots to leaves via stem/petiole is quantitatively regulated, and to identify whether the photosynthetic tolerance depends on factors associated with stomata or rather with mesophyll cells. Under saline conditions, tolerant genotypes were capable of reducing Na content at the two transport pathways: from the root to the stem + petiole, and from the stem + petiole to the leaf, with the concentration declining to one tenth or less of that found in the lower plant parts. The concentration of Cl similarly declined at the two pathways in the tolerant lines, but the reduction at each pathway was very slight, allowing Cl concentration in the leaf to be more than 10 times higher than that of Na. In addition to the two pathways, a possible contribution to the reduction by the Casparian strip of the endodermis in roots was suggested. A comparison of photosynthetic responses between tolerant and susceptible genotypes indicated that genotypic differences in salinity tolerance appears to be due to the capacity for sustained CO_2 assimilation in mesophyll tissues under salinity, rather than to stomatal factors, which are likely to be associated with the capacity for osmotic adjustment. Nevertheless, salt-induced decline in photosynthetic rate was severe even in tolerant genotypes. From these results, we conclude that selection for concurring genetic capacities for regulating Cl transport from the root to the leaf, and for osmotic adjustment must be an important target in soybean breeding programs for salinity-prone environments.

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Disclosure statement

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