Salinity Tolerance of Super-Nodulating Soybean Genotype En-b0-1

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Abstract: Salinity stress causes various physiological dysfunctions in soybean (Glycine max (L.) Merr.). For example, reduced nitrogen (N) uptake due to salt-induced depression of nodule formation severely limits soybean growth and yield. Super-nodulating soybean genotypes were previously identified by their superior N₂ fixation and photosynthesis. Here, we have tested our hypothesis that the super-nodulating En-b0-1 genotype is more salinity tolerant than a normal-nodulating genotype. The super-nodulating genotype and its parental normal-nodulating cultivar Enrei were grown in pots and subjected to saline conditions during the pre-flowering and reproductive growth stages. Under saline conditions imposed during pre-flowering, En-b0-1 formed heavier nodules, resulting in greater N uptake, higher photosynthetic activity, and greater biomass production compared with Enrei. Saline treatment increased the concentrations of sodium (Na) and chlorine (Cl) in all plant parts regardless of genotype; but in En-b0-1, the concentrations of these elements in shoots were significantly lower, while those in roots and nodules were higher than in Enrei. When the salinity treatment was imposed during the reproductive growth stages, En-b0-1 maintained higher N uptake, leading to better alleviation of salinity-induced yield reduction than in Enrei. The super-nodulating genotype En-b0-1 was more tolerant to salinity than its parental normal-nodulating cultivar, due to its superior nodulation and prevention of excessive accumulation of Na and Cl in shoots, which were retained in roots and nodules, thus supporting our hypothesis.

Key words: Chlorine, Nodulation, Photosynthesis, Salinity tolerance, Sodium, Soybean, Super-nodulation.

Soil salinity is a major constraint for crop production; about 10% of the world’s cropland is detrimentally affected by salinity (Shannon, 1997). Soil salinity arises principally due to the native chemical composition of soil, but salinity may be exacerbated by inappropriate irrigation or fertilization practices. Soil salinization also occurs due to inundation by seawater. For example, the recent tsunami that struck the coast of the Tohoku region of Japan salinized a large coastal area in the region (Nanzyo et al., 2012). Installing drainage systems and leaching salts from the soil with fresh water can reduce soil salinity; however, this method is often not economically or environmentally feasible for amelioration of excess salinity in many arid and semi-arid regions.

Soybean has been classified as moderately salt sensitive, together with maize, potato, tomato and other crops (Katerji et al., 2003). Saline conditions hamper germination, growth (Abel and Mackenzie, 1964; Wang and Shannon, 1999), and nodule formation (Singleton and Bohlool, 1984) in soybean, resulting in significant reductions in seed yield (Parker et al., 1983; Yang and Blanchar, 1993). Studies comparing genetically diverse cultivars have found significant differences in salt tolerance (Parker et al., 1983; Yang and Blanchar, 1993; Lee et al., 2008; Tuncturk et al., 2008; Ghassemi-Golezani et al., 2009; Hakeem et al., 2012; Karim et al., 2012; Mannan et al., 2012).

Soil salinity impairs physiological functions by multiple mechanisms, including water stress, specific ion toxicity, ion imbalance stress, and induced nutrient deficiency (Jones, 1981; Shannon, 1997; Munns and Tester, 2008; Zhang et al., 2010). The relative importance of these detrimental stresses may vary according to crop species, growth stages, and the duration of the stress imposed (Jones, 1981; Munns and Tester, 2008). In soybean, genotypic differences in tolerance were particularly associated with the ability to prevent aboveground parts of plants from accumulating sodium (Na) and chlorine (Cl) (Essa, 2002).

Soybean assimilates significant amounts of nitrogen (N) through N₂ fixation in symbiosis with rhizobia. Successful establishment of legume–Rhizobium symbiosis is dependent on salinity (Rao et al., 2002). N metabolism is also affected by salinity, probably through the depression of the activity of enzymes involved in N metabolism (Mansour et al.,
Addition of genistein (a nod gene inducer) enhances soybean nodulation and growth under saline conditions (Miransari and Smith, 2007, 2009). In chickpea, salt-tolerant genotypes have greater nodulation and symbiotic N₂ fixation capacity than sensitive genotypes (Rao et al., 2002). These studies indicate that salt-induced reduction in growth and yield in legume crops including soybean is partly due to depressed N₂ fixation activity (van Hoorn et al., 2001).

The improved super-nodulating soybean genotypes form more nodules, and thereby exhibit greater N₂ fixation capacity, regardless of soil N content, compared with their parental normal-nodulating cultivar (Takahashi et al., 1995; Maekawa et al., 2003, 2005; Takahashi et al., 2005). The super-nodulating genotypes also recover nodulation capacity better following waterlogged conditions (Kokubun, 2013). The super-nodulating genotype En-b0-1 exhibits greater biomass production under elevated CO₂ concentrations, owing to its superior nodulation and N₂ fixation (Otera et al., 2010). These traits raise the question of whether super-nodulating soybean genotypes are more tolerant to salinity. In the present study, we test the hypothesis that the super-nodulating genotype En-b0-1 is more tolerant to salinity than the normal-nodulating genotype.

Materials and Methods

1. Plant materials

Two soybean genotypes with contrasting nodulation capacities were used: the super-nodulating genotype En-b0-1, and its parental normal-nodulating cultivar Enrei. En-b0-1 was selected from the progeny of Enrei × En6500, an EMS (ethyl-methyl-sulfonate)-induced mutant of Enrei (Akao and Kouchi, 1992). The super-nodulating genotype was previously found to form a significantly larger number of nodules and to thereby exhibit higher N₂ fixation activity than its parental genotype (Takahashi et al., 2005).

Plants were grown in pots placed in a greenhouse with four open sides; solar radiation and temperature inside the house were 10% lower and slightly higher, respectively, than outside the house. Prior to sowing, fertilizer was applied at fixed rates: 0.5 g of N, 1.5 g of P₂O₅, 2.0 g of K₂O, 10 g of fused phosphate, and 10 g of slaked lime per pot (1/5000-a Wagner pots) of low-humic Andosol field soil (N: 0.55%, CEC: 44.9 cmol kg⁻¹). The fertilizers and soil were mixed prior to sowing. The seeds were inoculated with a strain (J1065) of Bradyrhizobium japonicum obtained from Tokachi Nokyoren (Obihiro, Japan). Four to five seeds per pot were sown on 20 June 2011 and seedlings were thinned to one plant per pot after emergence. Plants were regularly irrigated with tap water when the saline treatment was not imposed. Preventative insecticides were applied several times during plant cultivation. The experiment was conducted in the experimental facility of Tohoku University (38º16N, 140º50E).

2. Saline treatment

Saline treatment was imposed by placing the pots in quadrilateral water containers (135 cm × 91 cm × 20 cm depth) in which saline water was maintained at a depth of 5 – 10 cm in order to supply water to plants through the bottom hole of each pot. The saline treatment was imposed at two growth stages: the pre-flowering stage (from 24 to 51 days after sowing (DAS)) and reproductive stages (52 to 101 DAS). The salt concentration was adjusted by adding salt gradually to raise the concentration to 20 – 30 mM over two weeks to reach the final concentration of 100 and 70 mM NaCl for pre-flowering and reproductive stages, respectively. The final NaCl concentration was determined according to previous studies in which cultivar differences in salinity tolerance were most clearly distinguished at 100 or 120 mM NaCl (Lee et al., 2008; Valencia et al., 2008). However, the final concentration of the salinity treatment for the reproductive stage was adjusted to a lower level than that for the pre-flowering stage, because salt-induced damage during the reproductive stage became apparent at a concentration of 70 mM NaCl. For control plots, tap water was applied in the same manner as the saline treatment. The saline water solution was renewed at several-day intervals. Each container contained two genotypes, and the pots in each container were placed randomly with adequate spacing without mutual shading and moved every several days to minimize position effects. There were two replications (two treatment containers) per treatment (100 / 70 mM NaCl or control).

3. Measurements

Five plants per plot were sampled at 27 days after the pre-flowering stage saline treatment (DAT), and at 49 DAT (101 DAS) after the reproductive stage saline treatment. The samples were separated into various plant parts such as leaves, stems and petioles (also including pods at reproductive stage), roots, and nodules, and were oven-dried at 80°C for 3 days, and weighed. Prior to drying, the sampled leaves were photocopied and the leaf areas were measured on the copies. Nodules were detached from the roots, counted, and weighed. The dried samples were ground in a mill and the N concentration was analyzed with an atomic absorption spectrophotometer (A-2000, Hitachi Corp., Tokyo) and an ion chromatograph (ICS-900, DIONEX Corp., Osaka).

The apparent photosynthetic rate of the recently
expanded terminal leaflet of five plants from each pre-flowering stage treatment was measured using a portable photosynthesis system (LI6400; Li-Cor Inc., NE). Measurements were carried out at 1000 and 1200 during the saline treatment. The flow rate of air in the leaf chamber was controlled at 500 $\mu$mol s$^{-1}$, and the CO$_2$ concentration supplied to the leaf chamber was maintained at 380 $\mu$mol mol$^{-1}$. Irradiance on the measured leaves (6 cm$^2$) was regulated at a photosynthetic photon flux density of 1500 $\mu$mol m$^{-2}$ s$^{-1}$, and the chamber temperature was maintained at 25°C. Leaf chlorophyll content of the same leaves measured for photosynthesis was estimated at 3- to 5-day intervals after each saline treatment, using a chlorophyll meter (SPAD 502, Konica Minolta Inc., Tokyo, Japan).

At maturity, the plants that had received the saline treatment during reproductive stages, and control were harvested, and yield and yield components were measured using five plants per plot.

4. **Statistical analysis**

Analysis of variance (ANOVA) was performed for dry weights of plant parts, their N, Na, and Cl concentrations at 51 and 101 DAS, and their yield and yield components at maturity to evaluate the effects of and interactions between genotype and saline treatments using JMP version 5.1 (SAS Institute Inc., NC). Tukey’s test was applied to determine any significant differences between the means of these traits.

**Results**

1. **Effects of saline treatment imposed during the pre-flowering stage**

   (1) Nodulation, N uptake, and leaf color

   In control plants, nodule number at 27 DAT (51 DAS) was significantly greater in En-b0-1 than in Enrei, while per-nodule weight was similar in the two genotypes, leading to heavier per-plant nodule weight in En-b0-1 (Fig. 1). In salt-treated plants, nodule number was substantially reduced regardless of genotype, while weight per nodule was markedly heavier in En-b0-1 than in Enrei, resulting in heavier nodule weight per plant in En-b0-1. The N concentration of plant parts at 27 DAT was generally higher in En-b0-1 than in Enrei, except in nodules (Table 1). The saline treatment did not significantly affect the N concentration, except in nodules.

   Figure 2 shows changes in leaf chlorophyll content during the period from 0 to 27 DAT. Leaf chlorophyll contents during this period were greater in En-b0-1 than in Enrei, regardless of saline treatment. However, in saline conditions, the values declined sharply in Enrei, but only slightly in En-b0-1.

   (2) Sodium and chlorine accumulation

   In control plants, Na was detected only in roots and nodules, while Cl concentrations were very low in all plant parts (Fig. 3). 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 the two genotypes revealed that the Na and Cl concentrations in either leaves or stems and petioles were significantly higher in Enrei than in En-b0-1, whereas those in roots and nodules were higher in En-b0-1 than in Enrei.
Table 1. Nitrogen concentrations (mg g\(^{-1}\)) in different plant parts of two soybean genotypes subjected to saline treatment during the pre-flowering growth stage (24 – 51 DAS). Plants were sampled at 27 DAT (51 DAS).

| Genotype | Treatment | Leaf | Stem + petiole | Root | Nodule |
|----------|-----------|------|----------------|------|--------|
| Enrei    | Control   | 28.3 c | 9.2 b         | 19.1 bc | 45.6 a |
|          | Saline    | 30.1 c | 11.6 b        | 17.5 c | 34.7 b |
| En-b0-1  | Control   | 36.5 a | 13.6 a        | 22.1 a | 42.9 a |
|          | Saline    | 34.4 b | 13.7 a        | 20.5 ab | 37.1 b |

Genotype (G) *** *** * ns
Treatment (T) ns ns ns ***
G × T * ns ns ns ns

Values followed by the same letter are not significantly different at \(P=0.05\) as determined by Tukey’s means comparison test. *, **, ***: Significantly different at \(P<0.05\), 0.01, 0.001. ns: Not significantly different at \(P<0.05\).

(3) Leaf growth, photosynthesis, and biomass production

Figure 4 shows leaf area measured at 27 DAT (51 DAS). In control plots, leaf area was not significantly different between the two genotypes. The saline treatment markedly reduced leaf area regardless of genotype, but the reduction was greater in Enrei. The saline treatment initially caused discoloring of leaves, followed by retardation of growth.

Photosynthetic rates at the same stage as leaf area measurements (27 and 28 DAT) were markedly reduced by the saline treatment regardless of genotype (Fig. 4). The reduction in photosynthetic rate was more substantial in Enrei than in En-b0-1.

Dry weights of plants parts at 27 DAT (51 DAS) are shown in Table 2. In control plots, stems and petioles or nodules of En-b0-1 were significantly heavier, compared to those of Enrei, while roots were lighter in En-b0-1. The saline treatment reduced the weights of most plant parts in Enrei, whereas the salt treatment did not reduce stem and root weights but reduced the weights of leaves and nodules only slightly in En-b0-1.

2. Effects of saline treatment imposed during reproductive growth stages

(1) N uptake and leaf color
The N concentrations in seeds, and in stems and
seeds and in roots and nodules regardless of genotype, but N concentrations increased in pod walls and stems and petioles.

In the control plot, leaf chlorophyll content during the reproductive growth stage was fairly constant until ca. 28 DAT (80 DAS) with a gradual decline afterwards regardless of genotype (Fig. 5). In the salt-treated plot, however, the leaf chlorophyll content declined sharply after ca. 18 DAT, and this decline was steeper in Enrei than in En-b0-1.

(2) Yield and yield components
There were no significant differences in yield and yield components between Enrei and En-b0-1 in control plots, although the saline treatment substantially reduced yield regardless of genotype (Table 4). The yield reduction was markedly smaller in En-b0-1 than in Enrei, primarily due to the larger number of pods in En-b0-1.

Discussion
The objective of this study was to test the hypothesis that the super-nodulating genotype En-b0-1 is more tolerant to salinity than the normal-nodulating genotype from which it was derived. Under saline conditions imposed during the pre-flowering growth stage, the super-nodulating genotype En-b0-1 had heavier nodule weight, resulting in elevated N uptake, higher photosynthetic activity, and greater biomass production compared to its normal-nodulating parental cultivar Enrei (Fig. 1 and 4, Table 1 and 2). When the saline treatment was imposed during reproductive growth stages, En-b0-1 maintained a higher percent N in leaves or stems and petioles than Enrei, alleviating the yield reduction caused by salinity (Fig. 5, Table 3 and 4). These results supported our hypothesis that the super-nodulating genotype would be more salt tolerant.

In the normal-nodulating cultivar Enrei, the saline treatment during the pre-flowering stage substantially reduced the weight of roots and nodules, whereas the salt treatment reduced the weight of roots and nodules only slightly in the super-nodulating genotype En-b0-1 (Fig. 1, Table 2). The superior nodulation capacity of En-b0-1

| Genotype | Treatment | Leaf | Stem + petiole | Root | Nodule | Total |
|----------|-----------|------|----------------|------|--------|-------|
| Enrei    | Control   | 14.8 ab | 11.4 c   | 5.7 a | 1.0 b  | 32.9 b |
|          | Saline    | 5.3 d   | 8.6 cd    | 4.7 ab | 0.2 c  | 18.8 c |
| En-b0-1  | Control   | 16.0 a  | 15.9 ab   | 4.2 c | 1.8 a  | 38.0 a |
|          | Saline    | 10.8 c  | 16.4 a    | 4.7 ab | 0.7 b  | 32.7 b |

ANOVA

| Genotype (G) | *** | *** | ** | *  | *** |
| Treatment (T) | *** | ns  | ns | *** | *** |
| G × T         | *  | ns  | ** | ns | *  |

Values followed by the same letter are not significantly different at \( P = 0.05 \) as determined by Tukey’s means comparison test. *, **, ***: Significantly different at \( P < 0.05, 0.01, 0.001 \). ns: Not significantly different at \( P < 0.05 \).
Table 3. Nitrogen concentrations (mg g\(^{-1}\)) in different plant parts of two soybean genotypes subjected to saline treatment during reproductive growth stages (52 – 101 DAS). Plants were sampled at 49 DAT (101 DAS).

| Genotype | Treatment | Grain  | Pod wall | Stem + petiole | Root + nodule |
|----------|-----------|--------|----------|---------------|--------------|
|          |           | (mg g\(^{-1}\)) |          |               |              |
| Enrei    | Control   | 69.6 ab | 5.8 c    | 4.8 d         | 17.1 a       |
|          | Saline    | 64.9 c  | 13.2 a   | 10.9 ab       | 15.4 b       |
| En-b0-1  | Control   | 71.2 a  | 7.0 b    | 6.3 c         | 17.9 a       |
|          | Saline    | 69.5 ab | 7.7 b    | 13.8 a        | 14.8 b       |

ANOVA

| Genotype (G) | Treatment (T) | G x T |
|--------------|---------------|-------|
| ***          | ***           | ns    |

Values followed by the same letter are not significantly different at \(P = 0.05\) as determined by Tukey’s means comparison test. *, **, ***: Significantly different at \(P<0.05, 0.01, 0.001\). ns: Not significantly different at \(P<0.05\).

under saline conditions. Under saline conditions, En-b0-1 exhibited larger individual nodule mass, which contributed to its greater nodule mass per plant than in Enrei (Fig. 1). King and Purcell (2001) found that individual nodule mass was greater in the drought-tolerant cultivar Jackson than in the drought-susceptible genotype KS4895 under water-deficit conditions. They proposed that larger nodule size was likely to be advantageous for maintaining higher relative water content (RWC) in nodules. The larger nodules in En-b0-1 may also have contributed to maintaining RWC in nodules, as plants must adapt to lower water potentials under both drought and salt stress (Bray et al., 2000).

The salt tolerance of soybean appeared to be associated with the capacities for both osmotic adjustment and N uptake, because the leaf water potential of a salt-tolerant genotype AGS313, identified by screening 170 cultivars and lines, was not affected by saline stress (50 mM NaCl), and maintained photosynthetic activity and biomass production (Karim et al., 2012; Mannan et al., 2012). Regarding the association of water status in plants with photosynthetic capacity, Lu et al. (2009) found that

Table 4. Yield and yield components of two soybean genotypes subjected to saline treatment during reproductive growth stages (52 – 101 DAS).

| Genotype | Treatment | Pod number (plant\(^{-1}\)) | Seed number (plant\(^{-1}\)) | 100-seed weight (g) | Yield (g plant\(^{-1}\)) |
|----------|-----------|-----------------------------|------------------------------|---------------------|------------------------|
| Enrei    | Control   | 63.2 a                      | 115.2 a                      | 31.0 a              | 35.7 a                 |
|          | Saline    | 46.2 b                      | 81.2 b                       | 12.5 c              | 9.9 c                  |
| En-b0-1  | Control   | 66.0 a                      | 113.8 a                      | 29.5 a              | 33.4 a                 |
|          | Saline    | 56.6 a                      | 96.0 ab                      | 17.6 b              | 16.7 b                 |

ANOVA

| Genotype (G) | Treatment (T) | G x T |
|--------------|---------------|-------|
| ns           | ***           | ns    |

Values followed by the same letter are not significantly different at \(P = 0.05\) as determined by Tukey’s means comparison test. *, **, ***: Significantly different at \(P<0.05, 0.01, 0.001\). ns: Not significantly different at \(P<0.05\).
photosynthesis decreased less in a salt-tolerant genotype S111-9 than in a sensitive genotype, which was mainly due to decreased stomatal conductance. Whether the higher photosynthetic activity in En-b0-1 under saline conditions (Fig. 4) was associated with osmotic adjustment in leaves needs to be examined further.

Plant responses to the toxicity of ions such as Na+ or Cl may vary with the crop species, growth stage, and the duration of the stress imposed (Jones, 1981; Munns and Tester, 2008; Zhang et al., 2010). In soybean, genotypic differences in salt tolerance were associated with the capacity of plants to prevent the aboveground parts from accumulating both Na and Cl (Essa, 2002). For example, when grown with hydroponics and 120 mM NaCl for 14 days, average leaf Na and Cl contents of susceptible soybean cultivars were 2.64 and 1.96 times those of tolerant cultivars, resulting in a substantial reduction in biomass production in the susceptible cultivars (Valencia et al., 2008). The present study revealed that the salt tolerance of En-b0-1 could be ascribed to its ability to accumulate less Na and Cl in shoots (including leaves and stems and petioles), while accumulating more of these elements in roots and nodules (Fig. 3). Therefore, the salinity tolerance in these soybean cultivars was associated with tolerance to both Na and Cl, although several previous studies had suggested that salt-induced inhibition of growth was more closely associated with Cl than Na contents in aboveground plant parts (Abel and Mackenzie, 1964; Abel, 1969; Parker et al., 1983; Yang and Blanchar, 1993; Valencia et al., 2008; Ren et al., 2012). In this respect, Dabuxilatu and Ikeda (2005) ascribed the greater toxicity of Cl to its allocation within the plant: Cl was stored in both vacuoles and apoplastic spaces in both leaves and roots, whereas Na was predominantly stored in vacuoles in roots. In the present study, the saline conditions stimulated the accumulation of Cl more substantially than Na, particularly in shoots (Fig. 3), which suggests that Cl is more responsible for salt-induced physiological dysfunction in soybean.

Regarding the differences in salt tolerance among Glycine species, G. soja was found to be more tolerant to salt stress than G. max (Kao et al., 2003, 2006; Luo et al., 2005; Lenis et al., 2011). In a study comparing G. soja with G. max in solutions with 150 mM Na+, Cl−, or NaCl, Luo et al. (2005) found that the salt tolerance of G. soja was mainly dependent on its ability to withhold Na in the roots and stems and thereby decrease Na content in the leaves. A single dominant gene for salt tolerance was identified in the G. soja genotype PI483463 (Lee et al., 2009), and was found to be different from the gene identified in a G. max tolerant line S-100. In subsequent studies comparing several Glycine species, the salt tolerance of G. angustifolia, G. tomentella, G. tabacina was greater than that of G. soja, and was associated with the capacity of these species to prevent excessive accumulation of Na or Cl in leaves (Kao et al., 2003, 2006; Lenis et al., 2011). In the present study, the distribution pattern of Na and Cl in En-b0-1 was similar to that found in these salt-tolerant Glycine species (Fig. 3), suggesting that a similar mechanism regulating salt tolerance operates in several Glycine species.

QTLs conditioning salt tolerance in soybean have been identified using recombinant inbred lines (RIL) derived from crosses between salt-tolerant and -susceptible genotypes, (Lee et al., 2004; Chen et al., 2008; Hamwieh and Xu, 2008; Hamwieh et al., 2011; Xu and Tuyen, 2012). A gene conditioning supernodulation has been identified (Arai et al., 2005; Kim et al., 2005). Incorporating these genes conditioning salt tolerance and supernodulation into elite cultivars may alleviate reductions in growth and yield caused by salt stress in soybean.

In conclusion, the present study clarified that the supernodulating genotype En-b0-1 was more tolerant to salinity than its parental normal-nodulating cultivar, due to its higher capacity for nodulation and better ability to prevent excessive accumulation of Na and Cl in shoots, while withholding these toxic elements in roots and nodules.

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