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ORIGINAL PAPER

Identification of a major QTL allele from wild soybean (*Glycine soja* Sieb. & Zucc.) for increasing alkaline salt tolerance in soybean

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Abstract Salt-affected soils are generally classified into two main categories, sodic (alkaline) and saline. Our previous studies showed that the wild soybean accession JWS156-1 (*Glycine soja*) from the Kinki area of Japan was tolerant to NaCl salt, and the quantitative trait locus (QTL) for NaCl salt tolerance was located on soybean linkage group N (chromosome 3). Further investigation revealed that the wild soybean accession JWS156-1 also had a higher tolerance to alkaline salt stress. In the present study, an F_6 recombinant inbred line mapping population ($n = 112$) and an F_2 population ($n = 149$) derived from crosses between a cultivated soybean cultivar Jackson and JWS156-1 were used to identify QTL for alkaline salt tolerance in soybean. Evaluation of soybean alkaline salt tolerance was carried out based on salt tolerance rating (STR) and leaf chlorophyll content (SPAD value) after treatment with 180 mM $NaHCO_3$ for about 3 weeks under greenhouse conditions. In both populations, a significant QTL for alkaline salt tolerance was detected on the molecular linkage group D2 (chromosome 17), which accounted for 50.2 and 13.0% of the total variation for STR in the F_6 and the F_2 populations, respectively. The wild soybean contributed to the tolerance allele in the progenies. Our results suggest that QTL for alkaline salt tolerance is different from the QTL for NaCl salt tolerance found previously in this wild soybean genotype. The DNA markers closely associated with the QTLs might be useful for marker-assisted selection to pyramid tolerance genes in soybean for both alkaline and saline stresses.

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Introduction

Salt-affected soils are classified into two main categories: sodic and saline. Sodic (alkaline) soils are dominated by excess sodium on exchange sites and a high concentration of carbonate/bicarbonate anions. They also have a high pH (>8.5–10.8) with a high sodium absorption ratio (SAR) and poor soil structure. Saline soils are also generally dominated by sodium ions, but the dominant anions are chloride and sulfate. Their pH values and SARs are much lower, and electrical conductivities are higher than in sodic soils. Salt-affected soils contain sufficient concentrations of soluble salts to reduce the growth of most plant species (Flowers and Flowers 2005). Table 1 shows the regional distribution of salt-affected soils based on the FAO/UNESCO Soil Map of the World. Saline soils cover an area of 397 million ha and sodic soils cover 434 million ha (FAO, AGL 2000). These areas are not necessarily arable but cover all salt-affected lands at the global level. However, there are regions where soybeans are grown that production is affected from excess salt. In the Songnen plain of northeast of China, a major soybean production area, there is a total of 2.39×10^6 hm^2 of sodic land (Ma and Liang 2007).

The most conspicuous symptom of alkaline salt stress on plants is the induction of leaf chlorosis and stunted growth.

Table 1 Regional distribution of salt-affected soils (FAO, AGL 2000)

Regions	Total area (million ha)	Saline soils (million ha)	%	Sodic soils (million ha)	%
Africa	1,899.1	38.7	2.0	33.5	1.8
Asia and the Pacific and Australia	3,107.2	195.1	6.3	248.6	8.0
Europe	2,010.8	6.7	0.3	72.7	3.6
Latin America	2,038.6	60.5	3.0	50.9	2.5
Near East	1,801.9	91.5	5.1	14.1	0.8
North America	1,923.7	4.6	0.2	14.5	0.8
Total	12,781.3	397.1	3.1	434.3	3.4

Plant growth affected by alkaline salt stress has been reported in many plant species such as wheat (Millar et al. 2007), rice (Yang et al. 1994), and tomato (Biatczyk et al. 1994). Some studies also revealed that alkaline salt stress could affect plant growth by decreasing the solubility of nutrients. Iron deficiency caused by high concentration of bicarbonate ions was reported in sunflower (Alcántara et al. 1988), peanut (Zuo and Zhang 2008), maize (Celik and Vahap Katkat 2008), sugar beet (Campbell and Nishio 2000), and soybean (Coulombe et al. 1984; Hansen et al. 2003; Norvell and Adams 2006; Zocchi et al. 2007). Rogovska et al. (2007) reported the relationship between soybean yield, soil pH and soil carbonate concentration. They indicated that an alkalinity stress index explained 45% of the yield variability across their experiment sites.

The annual wild soybean, *Glycine soja* Sieb. & Zucc., is believed to be the wild progenitor of the cultivated soybean [*G. max* (L.) Merr.]. Crosses between these two species are generally fertile. Wild soybean is mainly distributed in the East Asian countries such as China, Korea, and Japan. A number of studies with DNA markers revealed that the wild soybean had higher genetic diversity than the cultivated soybean (Xu et al. 2002; Xu and Gai 2003; Nichols et al. 2007; Lee et al. 2008). Several agricultural traits of interest have been identified in wild soybean, and some of them have been successfully introduced into the cultivated soybean (Sebolt et al. 2000; Wang et al. 2001; Concibido et al. 2003). These studies suggest that the wild soybean might be a potential genetic resource for improving the cultivated soybean.

At present, DNA marker-assisted selection (MAS) has been recognized as an efficient way to accelerate the development of new, more productive and better adapted cultivars (Xu and Crouch 2008). MAS is particularly useful for salt tolerance, which is difficult to evaluate in a conventional breeding program because the salt concentration has a gradient in either horizontally or vertically direction in a field. Several studies of quantitative trait locus (QTL) mapping for salt tolerance have been reported (Lee et al. 2004; Hamwieh and Xu 2008; Chen et al. 2008), and some DNA markers have been proposed for use in a soybean breeding program to select for salt tolerance. However, most of these studies were focused on saline stress. Little is known about the inheritance of alkaline salt tolerance in soybean. Our previous studies showed that the wild soybean accession JWS156-1 from the Kinki area of Japan had a high tolerance to NaCl salt, and that the QTL for NaCl salt tolerance was located in soybean linkage group N (chromosome 3) (Hamwieh and Xu 2008). Further investigation revealed that the wild soybean accession JWS156-1 also showed high tolerance to alkaline salt stress (Fig. 1). In this study, we used two mapping populations to identify and confirm QTL for alkaline salt tolerance in soybean from JWS156-1.

Fig. 1 Comparison of alkaline salt tolerance between the wild soybean accession JWS156-1 (*right*) and the soybean cultivar Jackson (*left*) under alkaline salt treatment (180 mM $NaHCO_3$)

Materials and methods

Plant materials

To verify the alkaline salt tolerance in wild soybean accession JWS156-1, a total of 51 cultivated soybean germplasm including Jackson (PI548657) were used to compare the

alkaline salt tolerance with JWS156-1 in the present study. The 51 cultivated soybean genotypes originated from the USA (24), Japan (13), China (7), Korea (6) and Brazil (1). Jackson is a saline-sensitive cultivar, which has been previously used in several studies (Abel and MacKenzie 1964; Abel 1969; Luo et al. 2005; Chen et al. 2008; Hamwieh and Xu 2008). Another wild soybean accession JWS556 which originated from the Kyushu area of Japan was also included.

A F_6 recombinant inbred line (RIL) ($n = 112$) derived from a cross between Jackson and JWS156-1 was used for QTL analysis. The RIL mapping population was developed, without any selection, by the single-seed descent (SSD) method from the F_2 generation.

To confirm QTL detected in the RIL population, a separate F_2 ($n = 149$) population derived from a cross between the same parents, Jackson and JWS156-1, was employed in this study.

Evaluation of alkaline salt tolerance

Experiment 1: verification of alkaline salt tolerance of the wild soybean JWS156-1

In this study, a salt-water flooding method was used for evaluating alkaline salt tolerance. In brief, the soybean seedlings for each variety/accession were grown in a 17 × 17 cm pot filled with upland field soil mixed with 1/5 vermiculite. After emergence, the pots were placed in a large plastic container (225 × 150 × 45 cm) containing $NaHCO_3$ solution to induce alkaline salt stress. The $NaHCO_3$ solution was introduced through small holes at the bottom of the pots. The solution level in the container was kept at the same level as the soil in the pots, so that soil could be saturated with $NaHCO_3$ solution, and the $NaHCO_3$ solution was constantly circulated by an air pump which applied oxygen to the growing soybean.

The experiment for comparison of the wild soybean accessions (JWS156-1, JWS556) with the 51 cultivated soybean varieties was carried out in 2009 in a greenhouse at the Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki, Japan. Six plants of each genotype were grown in a pot. Ten days after sowing, the soybean plants were initially treated with 60 mM $NaHCO_3$ for 2 days, and then the concentration was raised to 180 mM by adding 30 mM $NaHCO_3$ every 2 days. This concentration was maintained until the end of experiment. At the same time, the 53 soybean genotypes were treated with 0 mM $NaHCO_3$ (tap water) as a control. The experiment was carried out with two replications. The pH values for alkaline salt treatment and control were 9.5 and 6.4, respectively, and the electric conductivity values for alkaline salt treatment and control were 1.08 and 0.08 S/m, respectively. The greenhouse temperature was maintained at about 27°C. The ambient light in the greenhouse was supplemented by high pressure sodium lights for 14 h/day. When the most sensitive germplasm reached a salt toxicity symptom of complete death (about 3 weeks after treatment), a salt tolerance rating (STR) for each genotype was scored based on leaf scorching of the soybean plants. The STR scale was classified by five grades, ranging from 1 (complete death) to 5 (normal healthy leaves). Leaf chlorophyll content (SPAD value) for each genotype was measured using a chlorophyll meter (Konica Minolta SPAD-502). This SPAD value is proportional to the chlorophyll content in the leaves.

Experiment 2: evaluation of alkaline salt tolerance for the 112 F_6 RILs

Evaluation of alkaline salt tolerance for 112 F_6 RILs, as well as the two parents, Jackson and JWS156-1, was performed in two replications using the same method as described in the Experiment 1. After treatment with $NaHCO_3$ for 21 days, STR and SPAD values were recorded for each RIL and the two parents.

Experiment 3: evaluation of alkaline salt tolerance for the F_2 population

Evaluation of alkaline salt tolerance for the 149 F_2 plants, as well as the two parents, Jackson and JWS156-1, was performed using the same method as described in Experiment 1 except that only one plant was grown in each pot. After treatment with $NaHCO_3$ for 21 days, STR and SPAD values were recorded for each F_2 plant.

QTL analysis

DNA was extracted with the CTAB method using the extraction buffer containing 2% CTAB, 0.1 M Tris–HCl (pH 8.0), 20 mM EDTA, and 1.4 M NaCl. For the F_6 RIL population, DNA was extracted from a mixture of leaf samples from ten individuals for each RIL. For the F_2 population, DNA was extracted from the leaf sample of each F_2 plant. SSR markers from the 20 soybean linkage groups (Song et al. 2004) were used for QTL analysis. Amplification was carried out in a 20-μl reaction mixture containing 10 ng of DNA template, 10 pmol of each primer, 0.2 mM of each deoxynucleotide, 1× PCR buffer, and 0.5 units Ex*Taq* polymerase (TaKaRa Bio Inc., Shiga, Japan). PCR reaction was carried out for 35 cycles for 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, following an extension of 7 min at 72°C. PCR products were separated on 8% polyacrylamide gel and stained by ethidium bromide. The band pattern was visualized on a Typhoon 9410 Fluorescent Imager

Table 2 Comparison of salt tolerance rating (STR) and leaf chlorophyll content (SPAD) between wild soybean accession JWS156-1 and the 51 cultivated soybean genotypes

Sources	Treatment (180 mM $NaHCO_3$)		Control (0 mM $NaHCO_3$)	
	STR	SPAD	STR	SPAD
JWS156-1	5.0	28.4	5.0	34.1
Jackson	1.0	2.5	5.0	23.1
Mean of 51 cultivated soybeans	1.3	6.5	5.0	27.2
Range of 51 cultivated soybeans	1.0–4.0	0–21.3	5.0	17.5–37.9
F value	22.68**	5.69**	–	0.67^{ns}
LSD (0.05)	0.8	6.37	–	12.31

ns not significant

** Significant difference at 0.01 probability level

(Molecular Dynamics Inc., California, USA). Mapping was performed using JoinMap 4.0 software (Van Ooijen 2006). Loci were assigned to linkage groups based on an LOD score larger than 2.0 and a recombination frequency smaller than 0.36. Map distances in centimorgans (cM) were calculated using Kosambi's mapping function. QTL analysis was carried out using MapQTL 5.0 software (Van Ooijen 2004). Significance of QTL was estimated from a 2000-permutation test by random sampling of phenotypic data.

Table 3 Analysis of variance of salt tolerance rating (STR) and leaf chlorophyll content (SPAD) values for the 112 F_6 RILs

Sources of variation	STR		SPAD	
	df	Mean square	*df*	Mean square
Replications	1	0.20^{ns}	1	34.88^{ns}
Lines	111	4.08**	111	93.39**
Error	111	0.25	111	17.92

ns not significant

** Significant difference at 0.01 probability level

Results

Verification of alkaline salt tolerance of the wild soybean JWS156-1

Under alkaline salt treatment, the correlation coefficients between the two replications were $r = 0.87$** for STR and $r = 0.68$** for SPAD values. The alkaline salt tolerant wild soybean accession JWS156-1 showed the highest STR (5.0) and SPAD (28.4) values among the 53 genotypes tested (Table 2). In contrast, Jackson (sensitive) had low STR (1.0) and SPAD (2.5) values. As for the wild soybean accession, JWS556, STR and SPAD values were 4.0 and 21.7, respectively. These values were lower than several cultivated soybean genotypes. Under controlled condition, in which the 53 soybean germplasm were treated with tap water, no salt injury symptom was observed, although the SPAD values among them varied due to inherent variations in chlorophyll content of the soybean varieties and accessions (Table 2).

QTL analysis for alkaline salt tolerance in the F_6 RIL population

Analysis of variance indicated that there was significant variation for both STR and SPAD traits for the RILs, but not for the replications (Table 3). The correlation coefficient between the two replications was $r = 0.95$** for STR and $r = 0.91$** for SPAD values. The phenotypic distribution of RILs for the two traits showed continuous distributions (Fig. 2). The range in STR for the 112 RIL was from 1.0 to 5.0 with a mean of 3.2. The range in SPAD was from 1.0 to 34.2 with a mean of 18.5. There was a significant positive correlation ($r = 0.75$**) between STR and SPAD values. Wild soybean accession JWS156-1 (tolerant parent) showed higher alkaline salt tolerance than the cultivated soybean Jackson (sensitive parent) based on the two traits (Table 4).

A total of 236 SSR markers, which were polymorphic between the two parents (Jackson and JWS156-1), were used to construct the linkage map for the F_6 RILs. Of the 236 SSR markers, 230 were mapped on 20 linkage groups covering 3,278.7 cM of the whole genome. The average distance between two adjacent markers on the linkage groups was 14.26 cM. Most of the locations of the SSR markers were similar to those reported previously (Song et al. 2004). QTL analyses were carried out for STR and SPAD traits on the linkage map. For STR, a significant QTL was detected in linkage group D2 (chromosome 17) in each replication, and the combined results of the two replications (Table 5). Alleles from the wild soybean JWS156-1 contributed to the higher STR value of the tolerant progenies within the RIL population. For SPAD, a significant

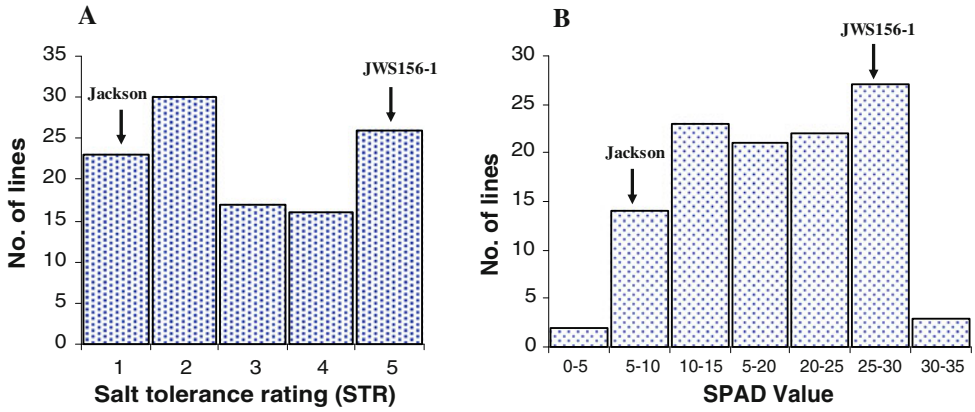

Fig. 2 Frequency distribution of salt tolerance rating (STR, **a**) and leaf chlorophyll content (SPAD, **b**) of the 112 F_6 RILs derived from a cross between the soybean cultivar Jackson and the wild soybean accession JWS156-1

Table 4 Variation of salt tolerance rating (STR) and leaf chlorophyll content (SPAD) values in the F_6 RIL population derived from a cross between the soybean cultivar Jackson and the wild soybean accession JWS156-1 grown under a 180 mM $NaHCO_3$ treatment

Sources	STR	SPAD
JWS156-1	5.0	27.3
Jackson	1.0	5.3
Mean of the 112 RILs	3.2	18.5
Range of the 112 RILs	1.0–5.0	1.0–34.2
F value	11 4**	12.5**
LSD (0.01)	1.69	8.25

** Significant difference at 0.01 probability level

QTL was also detected in the same region on linkage group D2 (chromosome 17) (Table 5). A LOD score chart for STR and SPAD based on combined data of the two replications is shown in Fig. 4. This QTL accounts for 50.2 and 43.0% of the total variation of STR and SPAD, respectively. No QTL was detected in other linkage groups for STR and SPAD.

QTL analysis for alkaline salt tolerance in the F_2 population

The alkaline salt tolerance of wild soybean accession JWS156-1 based on STR and SPAD was higher than the cultivated soybean cultivar Jackson. The frequency distribution of the STR and SPAD values of F_2 plants is shown in Fig. 3. Both traits showed a continuous distribution. However, the alkaline salt tolerance individuals (i.e. individuals with high STR and SPAD value) were predominant. A significant positive correlation ($r = 0.91$**) was shown between STR and SPAD values.

Seventy-five SSR markers were used to analyze the F_2 plants. Of these, 23 SSR markers were employed to construct a genetic map of linkage group D2 (chromosome 17) for the F_2 population. The order of the markers on the linkage group D2 (chromosome 17) was very similar to that for the RIL population. QTL analysis revealed significant QTLs for STR and SPAD on the linkage group D2 (chromosome 17) (Table 5; Fig. 4). The QTLs were located in the same region as that detected in the RIL population and accounted for 13.0 and 13.9% of the total variation of STR

Table 5 QTL analysis of salt tolerance rating (STR) and leaf chlorophyll content (SPAD) values for the F_6 RIL and F_2 populations derived from the crosses between soybean cultivar Jackson and the wild soybean accession JWS156-1

Populations	Traits	LOD	Expl.[a] (%)	Additive[b]	Dominance[c]	Nearest marker
F_6 RIL						
Replication 1	STR	13.2	46.9	−1.063	–	Satt447
	SPAD	11.2	41.2	−4.832	–	Satt447
Replication 2	STR	12.0	43.6	−0.989	–	Satt447
	SPAD	7.6	29.0	−3.637	–	Satt447
Average (Replications 1 and 2)	STR	14.5	50.2	−1.022	–	Satt447
	SPAD	11.8	43.0	−4.296	–	Satt447
F_2	STR	4.0	13.0	−0.735	−0.026	Satt461
	SPAD	4.6	13.9	−5.119	−0.221	Satt447

[a] The percentage of the variance explained by the QTL

[b] The estimated additive effect of alleles of maternal parent

[c] The estimated dominant effect

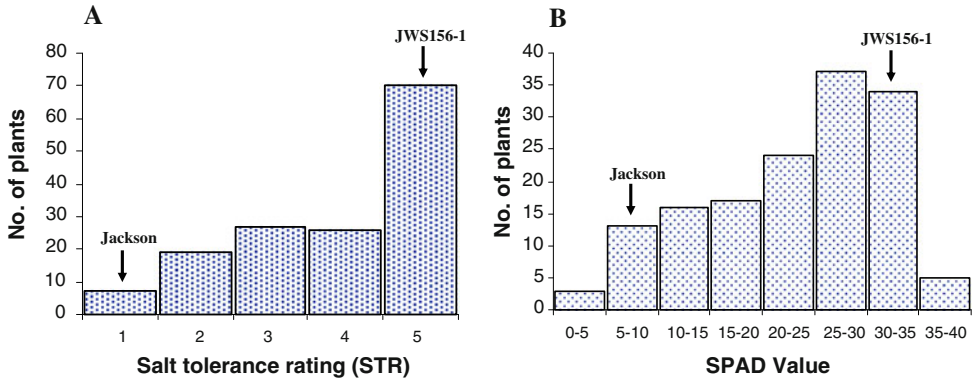

Fig. 3 Frequency distribution of salt tolerance rating (STR, **a**) and leaf chlorophyll content (SPAD, **b**) of the 149 F_2 plants derived from a cross between the soybean cultivar Jackson and the wild soybean accession JWS156-1

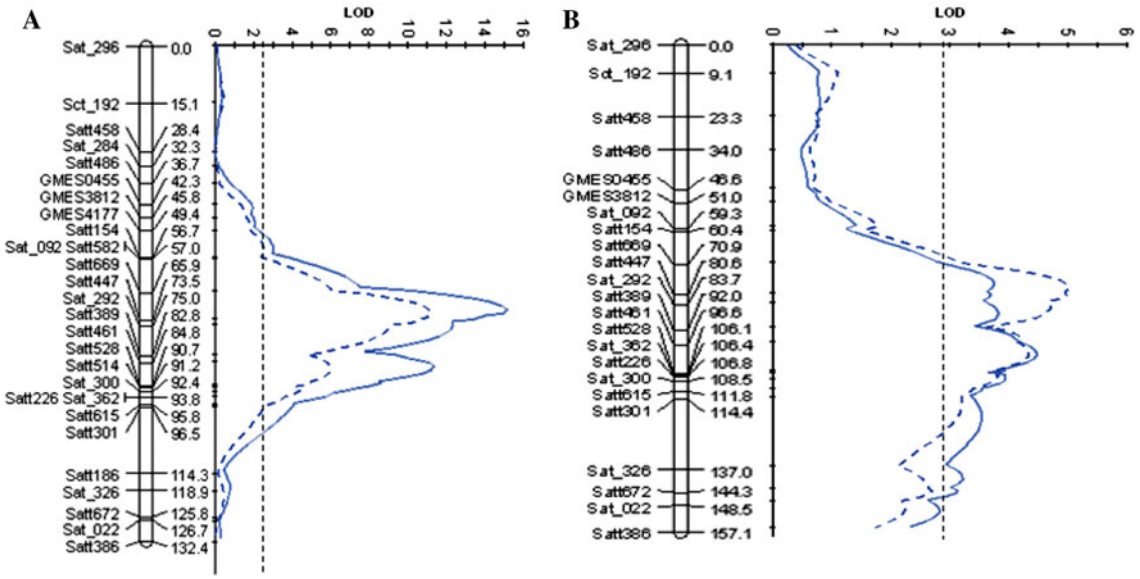

Fig. 4 Genetic map of linkage group D2 (chromosome 17) and QTL LOD score for salt tolerance rating (STR, *solid line*) and leaf chlorophyll content (SPAD, *dotted line*) values in the RIL population (**a**) and the F_2 population (**b**) derived from the crosses between soybean cultivar Jackson and the wild soybean accession JWS156-1. The *vertical dotted lines* indicate QTL significant levels ($p < 0.01$) for salt tolerance rating (STR) estimated from a 2000-permutation test by random sampling of phenotypic data

and SPAD, respectively. Alleles from the wild soybean JWS156-1 contributed to the higher alkaline salt tolerance in the progenies. In addition, the tolerant alleles showed a dominant effect over the sensitive alleles (Table 5).

Discussion

Our previous study showed that the wild soybean JWS156-1 had a higher tolerance to saline stress, and the tolerance QTL was located on linkage group N (chromosome 3) (Hamwieh and Xu 2008). In the present study, we showed that JWS156-1 was also tolerant to alkaline salt stress with the tolerant QTL located on linkage group D2 (chromosome 17). These results suggest that the gene for saline tolerance is different from the gene for alkaline salt tolerance in the wild soybean JWS156-1. In the experiment comparing alkaline salt tolerance between the wild soybeans and the 51 cultivated soybean varieties (Experiment 1), a cultivated soybean variety Lee (PI548656) was also included. NaCl tolerance in this variety is well documented (Abel and MacKenzie 1964; Abel 1969; Lee et al. 2004). However, the Lee variety did not show tolerance to alkaline salt stress (data not shown). Tolerance to NaCl may not always accompany tolerance to alkaline salt stress. On the other hand, MAS with DNA markers closely associated with the genes tolerant to the different salt stresses could be utilized to develop soybean cultivars with high tolerance to both saline and alkaline soils.

The most likely position of the alkaline salt tolerance QTL was in an interval region of around 26 cM between Satt669 and Sat_300. However, in both RIL and F_2 populations, two LOD score peaks were observed in this QTL region. One peak was located in the interval region between Satt669 and Satt389, and another peak in the interval region between Satt389 and Sat_300. To determine whether there

is more than one QTL for alkaline salt tolerance in this region, detailed QTL analysis with more DNA markers is necessary. In the RIL mapping population used in the present study, we found three lines that were heterozygous in the QTL region. These residual heterozygous RILs could be used as genetic material for fine-mapping of the alkaline salt tolerance allele as previously demonstrated in fine-mapping of other traits such as flowering time QTL (Yamanaka et al. 2005) and pod dehiscence QTL (Funatsuki et al. 2008) in soybean.

Although the alkaline salt tolerant QTL was detected in both RIL and F_2 populations, QTL effect and LOD score in the RIL population were higher than those detected in the F_2 population. This might be due to the fact that in the RIL population, evaluation for alkaline salt tolerance was based on performance of six plants in each line, while in the F_2 population evaluation was based on a single plant. Phenotypic evaluation based on several plants would be expected to provide more reliable results compared to evaluation based on a single plant. However, the dominant effect of alkaline salt tolerance was shown in the F_2 population and it was not detected in the RIL population.

In this study, a final concentration of 180 mM $NaHCO_3$ was used for alkaline salt treatment. The alkaline salt probably influences soybean growth based on three aspects: (1) high pH, (2) excess of Na^{1+}, and (3) decrease in solubility of nutrients (such as iron and calcium) caused by high pH. Iron deficiency problem caused by high pH has been reported in soybean (Coulombe et al. 1984; Hansen et al. 2003; Norvell and Adams 2006; Zocchi et al. 2007; Rogovska et al. 2007). However, the symptom observed in our study was not likely caused only by iron deficiency since iron deficiency generally would not cause the complete death of soybean plants. Charlson et al. (2003) carried out an analysis for associating SSR markers with soybean resistance to iron deficiency chlorosis. No SSR markers from the QTL region identified in our study were related to those reported in their study. Detailed studies on the physiological mechanism of alkaline salt tolerance are necessary in soybean.

In conclusion, a major QTL for alkaline salt tolerance in wild soybean JWS156-1 was detected on the soybean linkage group D2 (chromosome 17). The QTL for alkaline salt tolerance was different from the QTL for saline tolerance found previously in this genotype. DNA markers closely associated with the QTLs might be used for MAS to pyramid tolerance genes in soybean for both saline and alkaline stresses.

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