

Research Paper

Identification of novel loci for salt stress at the seed germination stage in soybean

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Salt tolerance in soybean [*Glycine max* (L.) Merr.] at the seed germination stage is a critical determinant of stable stand establishment in saline soil. This study examined one population of 184 recombinant inbred lines (RILs, F_{7:11}) derived from a cross between Kefeng1 and Nannong1138-2 and one natural population consisting of 196 soybean landraces. A total of 11 quantitative trait loci (QTLs) and 22 simple sequence repeat (SSR) loci associated with three salt tolerance indices were detected by linkage and association mapping. The SSR marker Sat_162 was found to be closely linked to the co-localized QTLs at a site 792,811 bp from the gene Glyma08g12400.1, which was verified in response to salt stress at the germination stage. Five SSR markers, Satt201, BE475343, CSSR306, Satt664 and Satt567, were co-associated with two of the salt tolerance indices, and two SSR markers, Satt156 and Satt636, were co-associated with all three salt tolerance indices. Furthermore, elite alleles and their carrier materials were identified by analyzing alleles at the loci associated with these salt tolerance indices. These results may be beneficial for the future breeding of soybean salt tolerance at the germination stage using marker-assisted selection and molecular pyramiding breeding.

Key Words: soybean, salt tolerance, germination, linkage mapping, genome-wide association mapping.

Introduction

Soil salinity is a key abiotic stress and adversely affects crop productivity and quality (Hamwiah and Xu 2008, Hamwiah *et al.* 2011, Wang *et al.* 2011). Due to excessive or poorly managed irrigation and fertilization (Flowers and Yeo 1995), areas of soil salinity are continuously increasing: approximately 20% of agricultural land worldwide is currently affected by salt (Flowers and Yeo 1995). According to the salinity tolerance (maximum salinity without yield loss), soybean (*Glycine max* (L.) Merr.) is classified as a moderately salt-tolerant crop (Ashraf and Wu 1994, Phang *et al.* 2008). However, salinity stress inhibits seed germination, and the soybean yield is reduced when soil salinity exceeds 5 dS/m (Ashraf and Wu 1994, Phang *et al.* 2008). Papiernik *et al.* (2005) reported that soybean production was reduced by 40% with increasing salinity stress (7 dS/m). Shao *et al.* (2009) found that the soybean yield were decreased by 52.5% and 61.0%, respectively, when they were

irrigated with 14–15 and 18–20 dS/m saline water in a field condition. The development of salt-tolerant soybean cultivars is an efficient way to utilize salinized land and maintain sustainable soybean production under conditions of high salinity (Hamwiah *et al.* 2011). Nonetheless, the distribution of salt is uneven in fields (Hamwiah *et al.* 2011), and salt tolerance is a complex trait that is affected by many genetic and non-genetic factors (Ashraf and Foolad 2013, DeRose-Wilson and Gaut 2011, Long *et al.* 2013, Pathan *et al.* 2007, Wang *et al.* 2011). Therefore, elucidating the genetic architecture of salt tolerance and implementing molecular marker-assisted techniques will advance the breeding of salt-tolerant soybeans (Pathan *et al.* 2007).

Quantitative traits are typically dissected by linkage analysis and association mapping. The former detects quantitative trait loci (QTLs) using mapping methods for experimental populations derived from bi-parental crosses, whereas the latter is based on linkage disequilibrium (LD) and identifies genotype-phenotype correlations in natural populations. Compared with linkage analysis, association mapping requires less research time to establish an associated population, provides higher mapping resolution and facilitates gene discovery (Hu *et al.* 2013, Long *et al.* 2013). Regardless, association mapping and linkage analysis are complimentary to each other in terms of providing prior

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knowledge, cross-validation, and statistical power (Yu and Buckler 2006). In recent years, salt tolerance has been analyzed in soybean mainly through linkage mapping yet rarely through association mapping. Two co-dominant polymerase chain reaction (PCR) markers closely linked to salt tolerance have been reported by Guo *et al.* (2000), and a number of major quantitative trait loci (QTLs) for salt (including alkaline salt) tolerance have been detected in previous studies (Chen *et al.* 2008, Ha *et al.* 2013, Hamwieh and Xu 2008, Hamwieh *et al.* 2011, Lee *et al.* 2004, Pathan *et al.* 2007, Tuyen *et al.* 2010, 2013). For example, Lee *et al.* (2004) detected a major salt tolerance QTL near the Sat_091 simple sequence repeat (SSR) of the N linkage group (LG) in the F_{2.5} population of a cross between cultivars S-100 (salt tolerant) and Tokyo (salt sensitive). In this case, the QTL and the Ncl locus initially reported by Abel (1969) were likely involved. According to pedigree tracking, the SSR markers Sat_091 and Satt237, which flank the major tolerance QTL, had been consistently associated with the salt tolerance of the descendants for over 60 years (Lee *et al.* 2004). This QTL was also confirmed in a different mapping population by different researchers (Guan *et al.* 2014, Ha *et al.* 2013, Hamwieh and Xu 2008, Hamwieh *et al.* 2011), and the region includes the cloned causal gene underlying this salt tolerance locus (Guan *et al.* 2014, Qi *et al.* 2014). A significant QTL for controlling alkaline salt tolerance was identified in LG D2, explaining 50.2% of the short tandem repeat (STR) variation in the F₆ population derived from the cross between Jackson and JWS156-1 (Tuyen *et al.* 2010). The QTL was then fine-mapped using a 3.30-cM interval between GM17-11.6 and Satt447, with a physical map length of approximately 771 kb (Tuyen *et al.* 2013). Utilizing F_{7.11} recombinant inbred lines (RILs; 184) derived from a cross between Kefeng No.1 and Nannong1138-2, Chen *et al.* (2008) detected a major salt tolerance QTL in the G LG during seedling growth in both field and greenhouse experiments. In addition, Qiu *et al.* (2011) mapped 23 QTLs related to salt tolerance at the germination stage in soybean backcross introgression lines constructed with Hong-feng11 as the recurrent parent and Harosoy as the donor parent. Association analysis of salt tolerance has also been reported in soybean (Kan *et al.* 2015, Zhang *et al.* 2014). Epistatic association mapping for salt tolerance was performed using 257 soybean cultivars with 135 SSR markers, and 83 QTL-by-environment (QE) interactions for the salt tolerance index were detected, with several soybean salt tolerance genes found in close proximity to frequently observed QTLs (Zhang *et al.* 2014). Association mapping of soybean seed germination under salt stress was also conducted in one natural population consisting of 191 soybean landraces with 1142 single-nucleotide polymorphisms (SNPs), with eight SNPs and 13 suggestive SNPs being associated with salt tolerance indices. In addition, nine possible candidate genes were obtained by blast analysis. Five genes, Glyma08g12400.1, Glyma08g09730.1, Glyma18g47140.1, Glyma09g00460.1, and Glyma09g00490.3, have been verified in response to

salt stress at the germination stage (Kan *et al.* 2015).

The above studies largely focused on salt tolerance at the soybean seedling stage but rarely at the germination stage. However, seed germination is critical for ensuring new seedlings and enhancing yield during the plant growth cycle (Wang *et al.* 2011). It is established that soybean germplasms have different degrees and mechanisms of salt tolerance at different developmental stages (Pathan *et al.* 2007, Phang *et al.* 2008). Although QTLs for salt stress at the germination stage have been identified by linkage mapping (Qiu *et al.* 2011) and association analysis (Kan *et al.* 2015, Zhang *et al.* 2014), little is known about the genetic mechanisms of salt tolerance at the germination stage using QTL and association mapping strategies (Kan *et al.* 2015, Qiu *et al.* 2011, Zhang *et al.* 2014). Moreover, the genetic mechanism of salt tolerance at the seed germination stage is very important for improving the salt tolerance of soybean. Thus, the objectives of this study were to determine the genetic architecture of soybean salt tolerance at the seed germination stage via linkage and association analyses and to identify major loci contributing to salt tolerance at the seed germination stage for the genetic improvement of soybean salt tolerance.

Materials and Methods

Plant materials and salt tolerance evaluation

The linkage analysis population included 184 RILs (F_{7.11}). The population (designated as NJRIKY) was derived from a cross between Kefeng1 and Nannong1138-2 and was developed by single-seed descent at the National Center for Soybean Improvement of China (Fu *et al.* 2006, Hu *et al.* 2013). An association mapping panel including 196 accessions was used in this experiment. The germplasm number, name and origin of all accessions are listed in **Supplemental Table 1**. The association mapping panel seeds were from four environments: the Jiangpu Experimental Station of the Nanjing Agricultural University (32.12° N 118.37° E), Nanjing, China, in 2012 (E1) and 2013 (E2); the Experimental Farm of the Jiangsu Yanjiang Institute of Agricultural Sciences (31.58° N 120.53° E), Nantong, China, in 2012 (E3); and the Experimental Farm of the Agricultural College of Yangzhou University (32.23° N 119.25° E), Yangzhou, China, in 2012 (E4) (Hao *et al.* 2012). The RILs population seeds were from two environments: the Jiangpu Experimental Station of the Nanjing Agricultural University (32.12° N 118.37° E), Nanjing, China, in 2012 (E1) and 2013 (E2).

The soybean seeds were germinated under a no-salt condition (0 mM NaCl solution) or salt stress (150 mM NaCl solution); the concentration of 150 mM NaCl used for the salt treatment was previously verified (Kan *et al.* 2015). The RILs population seeds from two environments and the association mapping panel seeds from four environments were germinated with three replications for both the salt-treated and control groups. For germination, the soybean seeds were first dried in an oven for three days and adjusted to a

moisture content of ~10%. Forty healthy dried seeds of uniform size, normal seed coats, no breakage or disease spots were selected, weighed and placed in sterilized Petri dishes (9 cm diameter) on two sheets of filter paper. A solution of 15 mL (0 or 150 mM NaCl) was added, and the seeds were incubated in the dark in the same temperature-controlled germination chamber at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After 24 h, the imbibed seeds were weighed, and we calculated the number of germinated seeds; germination was considered to have occurred when the root length and the shoot length reached one seed length and one-half of the seed length, respectively (Wang *et al.* 2011). Subsequently, we rinsed the seeds, placed them in new dishes with new filter paper, and added 5 mL of solution (0 or 150 mM NaCl). In the next six days, we calculated the number of germinated seeds; the seeds were then rinsed, and 5 mL of solution (0 or 150 mM NaCl) was added to the dishes every 24 h until the end of the experiment.

The following three germination related traits were evaluated: 1) the imbibition rate (IR) = $(W_2 - W_1)/W_1$, where W_2 represents the seed weight after imbibition for 24 h and W_1 represents the dry seed weight before imbibition (Wang *et al.* 2011); 2) the germination index (GI) = $\sum Gt/Dt$, where Gt represents the number of germinated seeds at N days and Dt represents N days of germinated seeds; and 3) the germination rate (GR) % = (the number of germinated seeds/seed number for the test) \times 100. Salt tolerance (ST) was defined as the ratio of germination-related traits (IR, GI and GR) under salt conditions to that under no-salt conditions (Long *et al.* 2013).

Phenotypic data analysis

Descriptive statistics and correlation analysis were performed based on the mean values of all phenotypic data from the RILs population across two environments and the natural population across four environments. Analysis of variance (ANOVA) for all traits was performed using the PROC GLM model, and Pearson's correlations between traits were calculated using the PROC CORR model with the software SAS 9.0 (SAS Institute 1999).

QTL mapping

The mean ST value of three replications for each RIL and a genetic linkage map (Fu *et al.* 2006) were used for the QTL analysis. In total, 224 polymorphic markers, including 221 SSR markers, three EST-SSRs and one R gene (resistance to soybean mosaic virus), were applied to this RILs population. The constructed linkage map covered 2,625.9 cM of the soybean genome and converged into 24 LGs, with an average distance of 11.8 cM between markers. The linkage mapping was performed with composite interval mapping (CIM) Model 6 using QTL Cartographer V 2.5_011 (<http://statgen.ncsu.edu/qtlcart/>). The control marker number and window size were set at 5 and 10 cM, respectively. The forward and backward regression method was selected, and empirical thresholds were computed using permutation test

analyses (1000 permutations, overall error level 5%) (Churchill and Doerge 1994). QTLs considered to be significant were those having LOD (logarithm of odds) peaks that exceed the genome-wide thresholds; suggestive QTLs were those having LOD peaks that did not exceed the genome-wide threshold but were higher than a threshold of 2.0 (King *et al.* 2013). Confidence intervals were set as the map interval corresponding to a 1-LOD decline on either side of the LOD peak.

Genotyping and association analysis

For each accession of the association mapping panel, genomic DNA was extracted from young leaves using the CTAB (cetyltrimethylammonium bromide) method described by Doyle and Doyle (1990), with slight modifications. We incubated the sample at 65°C and extracted twice with chloroform-isoamyl alcohol (24:1). In total, 205 SSR markers covering 20 soybean chromosomes were selected from published genetic maps (Hwang *et al.* 2009, Song *et al.* 2004). Certain SSRs selected (Hwang *et al.* 2009) were not available on the public USDA map (Song *et al.* 2004), and these positions on the public map were calculated based on the two SSR markers closest to the marker. The 10- μL PCR reaction mixture consisted of 20 ng total DNA, 0.4 μM forward and reverse primers, 200 μM each dNTP, $1 \times$ PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2 mM MgCl_2 and 0.5 U Taq DNA polymerase. PCR was performed with an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 54°C for 60 s and 72°C for 1 min, with a final extension at 72°C for 8 min. The reactions were performed using an MJ Research PTC 225 DNA engine thermal cycler (Bio-Rad, USA). The PCR products were separated on a 8% non-denaturing polyacrylamide gel with a 29:1 ratio of acrylamide: bisacrylamide and then silver-stained as described by Santos *et al.* (1993). The stained bands were analyzed based on their migration distance relative to the pBR322 DNA Marker (Tiangen Biotech Co., Ltd., Beijing, China) using Quantity One software Version 4.4.0 (Bio-Rad Laboratories).

The genetic structure of the panel was investigated using a Bayesian model based on STRUCTURE analysis and distance-based principal component analysis. The Bayesian model-based program STRUCTURE 2.2 (Pritchard *et al.* 2000) was employed to infer the population structure using a burn-in of 100,000, a run length of 100,000, and a model allowing for admixture and correlated allele frequencies. The number of groups (K) was set from two to ten, with seven independent runs each, and the most likely number of clusters was then determined using the Delta K method described by Evanno *et al.* (2005). The correct estimation of K from the Kinship matrix was calculated with SPAGeDi 1.2 software (Hardy and Vekemans 2002), which was used to estimate a kinship matrix for each pair of accessions; according to Yu *et al.* (2006), negative kinship values were set to zero. The level of LD between the pairs of SSR markers was calculated using TASSEL V2.1 software ([532](http://www.</p></div><div data-bbox=)

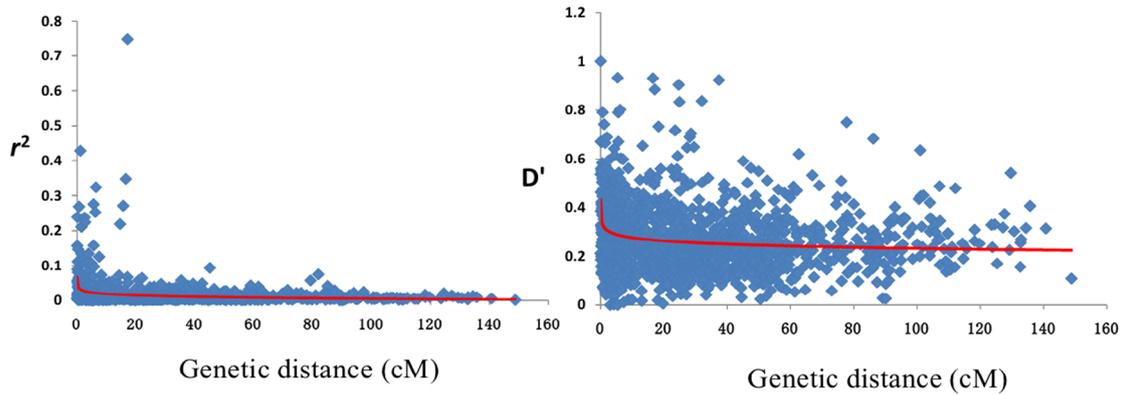


Fig. 1. Scatterplot of r^2 and D' of SSR pairs versus the syntenic marker genetic distance (cM) in the entire population.

maizegenetics.net/); D' and r^2 were used to measure LD for each pair of loci, and the significance (P -value) of each SSR pair was determined using 100,000 permutations (Fig. 1).

In this study, the mixed-model association mapping approach was used to identify the marker-trait associations Q (structure) + K (Kinship), where Q describes a fixed effect and K a co-variable. The association analysis was conducted using TASSEL V2.1 software (Bradbury *et al.* 2007). To identify significant associations, P -values were compared to the Bonferroni threshold ($-\text{Log}(0.05/205) = 3.61$), and SSRs significantly associated with salt tolerance were identified according to this threshold ($P \leq 0.05/205 = 2.44 \times 10^{-4}$ or $-\text{Log } P \geq 3.61$) (Hu *et al.* 2013). In addition, SSRs of interest that were significantly ($2 < -\text{Log } P < 3.61$) associated with salt tolerance indices were defined as suggestive SSRs (King *et al.* 2013).

Mining of elite alleles and carrier materials

The phenotypic effects of the alleles associated with the observed phenotypes were estimated by comparing the average phenotypic value between accessions with the specific allele and were based on the average value of all accessions.

The equations were as follows: $a_i = \sum X_{ij} / n_i - \sum X / n$,

$$AN = \frac{a_i}{\sum N_k / n_k} \times 100\%$$

where a_i represents the phenotypic effect of i allele, X_{ij} represents the phenotypic value of j material with i allele, n_i represents the number of materials with i allele, $\sum X/n$ represents the mean of the phenotypic value of all materials, and AN represents the ratio of an allele effect of the locus and a null allele effect of the locus (Bresghehlo and Sorrells 2006, Wen *et al.* 2008). A result of $a_i > 0$ corresponds to a positive allele, whereas $a_i < 0$ corresponds to a negative allele. The phenotypic allele effect was estimated through a comparison between the average phenotypic value over accessions with the specific allele and that of accessions with the “null allele”; the average positive (or negative) allele effect of a locus was then calculated over the estimated phenotypic effects of all positive (or negative) alleles (Bresghehlo and Sorrells 2006, Wen *et al.* 2008).

Results

Phenotypic variation and correlation analysis

The means, standard deviations and ranges of three germination-related traits (IR, GI and GR) and three salt tolerance indices (ST-IR, ST-GI and ST-GR) of the two parental lines, 184 RILs and 196 soybean accessions are shown in Table 1. Overall, higher values were observed for the three germination-related traits under no-salt conditions compared to under salt conditions. The mean IR value of Kefeng1 was lower than that of Nannong1138-2, and the mean GI and GR values and the mean ST-IR, ST-GI and ST-GR of Kefeng1 were higher than those of Nannong1138-2. There were statistically significant differences for GI and GR under salt stress as well as ST-GI and ST-GR between the two parents (t test, $P = 1.85 \times 10^{-2}$, $P = 2.99 \times 10^{-3}$, $P = 2.66 \times 10^{-2}$, $P = 2.42 \times 10^{-2}$, respectively). In contrast, no differences regarding IR, GI, and GR under no-salt conditions, IR under salt stress, and ST-IR were observed. The germination traits of the two populations under the salt-stressed conditions had larger variations than without salt stress. ANOVA indicated significant ($P < 0.001$) genetic variation for the three germination traits among the 184 lines, 196 accessions and treatments. Furthermore, the mean ST-IR, ST-GI and ST-GR varied from 0.73 to 1.11, from 0.14 to 1.14, and from 0.20 to 1.07, respectively, in the RILs population and from 0.83 to 1.08, from 0.16 to 0.70, and from 0.31 to 1.43, respectively, in the natural population; significant ($P < 0.001$) genetic variation for these indices in the two populations was found. In addition, there were significant ($P < 0.001$ and $P < 0.01$) genetic variations for the three germination-related traits and the salt tolerance indices in the two populations across the two and four environments, respectively. In contrast, there was no difference for IR and ST-IR in the RILs population across two environments. The phenotypic segregation of ST-IR, ST-GI and ST-GR in the two populations approximately fit a normal continuous distribution, indicating that these three salt tolerance indices are controlled by multiple-genes (Fig. 2).

Pearson’s correlations between the three germination-

Table 1. Descriptive statistics and ANOVA of three germination-related traits under 0 mM NaCl (C) or 150 mM NaCl (S) conditions and three salt tolerance indices based on the means of the traits in the parents, 184 recombinant inbred lines (RILs) and 196 soybean accessions

Trait	Treatment	Parents		RILs				196 soybean accessions					
		Kefeng No. 1	Nannong1138-2	Mean ± SD ^a	Range	G ^b	T ^c	E ^d	Mean ± SD ^a	Range	G ^b	T ^c	E ^d
IR ^e	C	130.63	135.62	136.40 ± 0.07	107.59–155.96	***	***	ns	134.68 ± 6.04	105.06–152.42	***	***	***
	S	125.94	128.16	127.05 ± 0.07	85.35–144.69				124.39 ± 4.70	100.84–136.09			
ST-IR ^f	S/C	0.97	0.94	0.93 ± 0.04	0.73–1.11	***		ns	0.92 ± 0.03	0.83–1.08	***		***
GI ^g	C	36.25	35.72	26.00 ± 11.10	2.72–52.00	***	***	***	35.94 ± 9.38	7.03–55.75	***	***	***
	S	19.93	12.26	12.01 ± 0.52	0.47–33.76				16.68 ± 6.63	2.32–36.09			
ST-GI ^h	S/C	0.58	0.36	0.46 ± 0.14	0.14–1.14	***		***	0.45 ± 0.10	0.16–0.70	***		***
GR ⁱ	C	75.42	69.17	55.68 ± 0.18	7.92–88.75	***	***	***	68.63 ± 13.22	15.83–94.58	***	***	***
	S	60.63	44.17	39.54 ± 0.18	1.67–85.00				51.39 ± 15.26	10.21–86.88			
ST-GR ^j	S/C	0.82	0.64	0.70 ± 0.17	0.20–1.07	***		**	0.74 ± 0.14	0.31–1.43	***		***

*** Significant at $P < 0.001$; ** Significant at $P < 0.01$; ns, not significant.

^a SD standard deviation.

^b Genotype.

^c Treatment.

^d Environment.

^e Imbibition rate.

^f Salt tolerance (ST) was defined as the ratio of the imbibition rate under salt conditions to that under no salt conditions.

^g Germination index.

^h Salt tolerance (ST) was defined as the ratio of the germination index under salt conditions to that under no salt conditions.

ⁱ Germination rate.

^j Salt tolerance (ST) was defined as the ratio of the germination rate under salt conditions to that under no salt conditions.

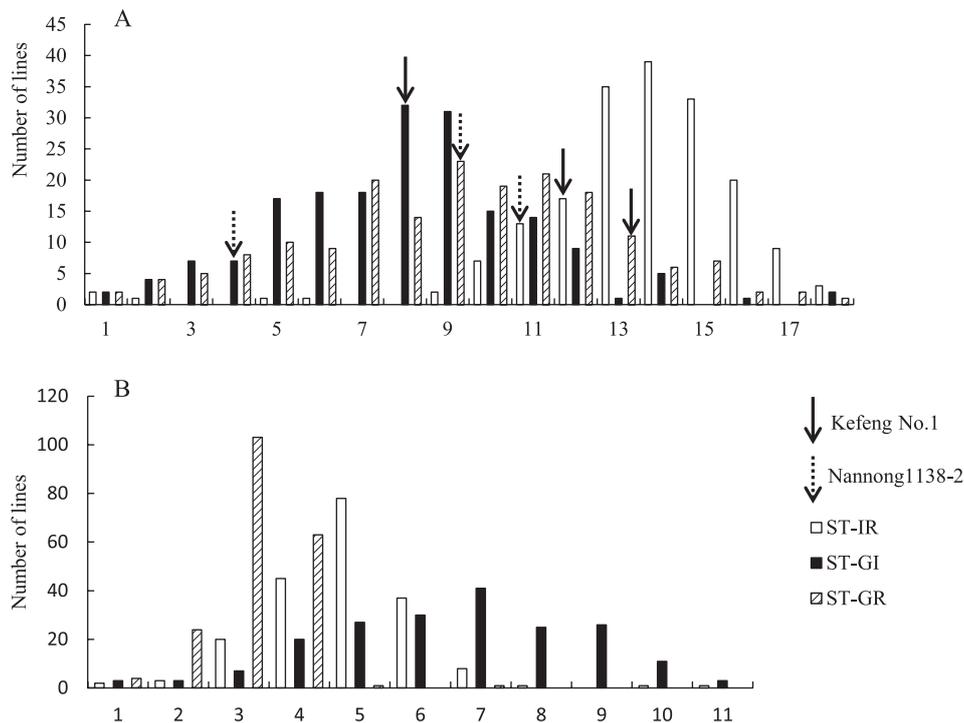


Fig. 2. Frequency distributions of three salt tolerance indices (ST-IR, ST-GI and ST-GR) in recombinant inbred lines (RILs) (A) and natural population (B) based on the means of the traits from two environments and four environments. ST-IR, salt tolerance was defined as the ratio of the imbibition rate under salt conditions to that under no salt conditions; ST-GI, salt tolerance was defined as the ratio of the germination index under salt conditions to that under no salt conditions; ST-GR, salt tolerance was defined as the ratio of the germination rate under salt conditions to that under no salt conditions. The white histogram represents the frequency distributions of ST-IR; the black histogram represents the frequency distributions of ST-GI; the patterned histogram represents the frequency distributions of ST-GR.

related traits and the salt tolerance indices were analyzed based on the means of two mapping populations (Table 2). For the two populations, ST-IR was significantly negatively

correlated with ST-GR ($P < 0.001$), whereas ST-GI and ST-GR were significantly positively correlated ($P < 0.001$). However, there was no correlation between ST-IR and

Table 2. Phenotypic correlations between three germination-related traits under 0 mM NaCl (C) or 150 mM NaCl (S) conditions and salt tolerance indices based on the means of the traits in 184 recombinant inbred lines (RILs) and 196 soybean accessions

Trait	Treatment	IR ^a		ST-IR ^b	GI ^c		ST-GI ^d	GR ^e		ST-GR ^f
		C	S	S/C	C	S	S/C	C	S	S/C
IR ^a	C		0.87***	-0.17*	0.16*	0.17*	0.07 ^{ns}	0.18*	0.16*	0.05 ^{ns}
	S	0.74***		0.12 ^{ns}	-0.12 ^{ns}	-0.06 ^{ns}	0.03 ^{ns}	-0.05 ^{ns}	-0.07 ^{ns}	-0.04 ^{ns}
ST-IR ^b	S/C	-0.22**	0.48***		-0.42***	-0.36***	-0.14*	-0.35***	-0.38***	-0.27***
GI ^c	C	0.34***	0.004 ^{ns}	-0.42***		0.86***	0.23**	0.91***	0.88***	0.38***
	S	0.22**	-0.10 ^{ns}	-0.42***	0.87***		0.60***	0.77***	0.93***	0.58***
ST-GI ^d	S/C	-0.10 ^{ns}	-0.18*	-0.12 ^{ns}	0.09 ^{ns}	0.51***		0.17*	0.51***	0.80***
GR ^e	C	0.23**	-0.06 ^{ns}	-0.38***	0.95***	0.79***	0.02 ^{ns}		0.84***	0.22**
	S	0.20**	-0.12 ^{ns}	-0.43***	0.90***	0.94***	0.36***	0.86***		0.65***
ST-GR ^f	S/C	0.03 ^{ns}	-0.19*	-0.30***	0.39***	0.67***	0.71***	0.27***	0.69***	

*, ** and *** Significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$; ns, not significant. The correlation coefficients of the natural population and RILs are listed at the top right and bottom left of the Table, respectively.

^a Imbibition rate.

^b Salt tolerance (ST) was defined as the ratio of the imbibition rate under salt conditions to that under no salt conditions.

^c Germination index.

^d Salt tolerance (ST) was defined as the ratio of the germination index under salt conditions to that under no salt conditions.

^e Germination rate.

^f Salt tolerance (ST) was defined as the ratio of the germination rate under salt conditions to that under no salt conditions.

Table 3. QTLs for salt tolerance indices based on the mean traits of three replications in 184 recombinant inbred lines (RILs)

Environment	Trait	QTLs	Chromosome	Marker	Position	LOD	Confidence interval	Additive effect	R ² (%)	Reference
E1	ST-IR ^a	<i>qST-IR-8</i>	8	Sat_162	25.8	7.19 (2.76)	21.4–33.0	+	25.94	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-IR-18</i>	18	satt217	77.1	2.61* (2.76)	66.7–95.0	+	5.28	
	ST-GI ^b	<i>qST-GI-2-1</i>	2	satt428	146.1	2.42* (2.95)	125.1–160.1	+	17.80	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-GI-2-2</i>	2	sat_351	291.5	3.37 (2.95)	287.5–291.8	+	7.60	
	ST-GR ^c	<i>qST-GR-8</i>	8	Sat_162	24.8	5.17 (3.01)	19.8–32.4	-	15.62	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
	<i>qST-GR-7</i>	7	satt245	48.4	2.85* (3.01)	36.2–59.4	+	6.10	Chen <i>et al.</i> 2008, Zhang <i>et al.</i> 2014	
E2	ST-IR ^a	<i>qST-IR-8</i>	8	Sat_162	22.8	5.11 (2.78)	9.0–31.8	+	15.87	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-IR-18</i>	18	satt130	84.0	2.78 (2.78)	72.1–99.0	+	7.80	
	ST-GI ^b	<i>qST-GI-17</i>	17	sat_292	96.5	2.25* (3.05)	61.7–108.51	-	5.41	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-GI-10-1</i>	10	sat_231	20.0	2.35* (3.05)	4.0–47.9	-	9.98	
	ST-GR ^c	<i>qST-GR-8</i>	8	Sat_162	24.8	5.15 (2.95)	6.0–31.8	-	18.45	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
	<i>qST-GR-7</i>	7	satt245	48.4	3.21 (2.95)	37.2–57.4	+	7.85	Chen <i>et al.</i> 2008, Zhang <i>et al.</i> 2014	
Combined	ST-IR ^a	<i>qST-IR-8</i>	8	Sat_162	25.8	5.90 (2.77)	17.4–32.0	+	20.94	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-IR-2</i>	2	Satt266	168.9	2.30* (2.77)	150.4–191.6	-	4.53	
	ST-GI ^b	<i>qST-GI-8</i>	8	Sat_294	169.9	2.12* (2.87)	158.7–188.6	-	4.49	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
		<i>qST-GI-10-2</i>	10	BE801128	222.9	3.19 (2.87)	220.8–233.6	-	9.25	
ST-GR ^c	<i>qST-GR-8</i>	8	Sat_162	23.8	3.60 (3.00)	9.6–36.2	-	9.57	Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014	

The genome-wide threshold is shown in parentheses; * suggestive QTLs, QTLs with LOD peaks that did not exceed the genome-wide threshold but were higher than a threshold of 2.0.

+, the positive additive effect is from Kefeng No. 1; -, the negative effect is from Nannong1138-2.

^a Salt tolerance (ST) was defined as the ratio of the imbibition rate under salt conditions to that under no salt conditions.

^b Salt tolerance (ST) was defined as the ratio of the germination index under salt conditions to that under no salt conditions.

^c Salt tolerance (ST) was defined as the ratio of the germination rate under salt conditions to that under no salt conditions.

ST-GI in the RILs population; conversely, a significant negative correlation was found between ST-IR and ST-GI in the natural population ($P < 0.05$). Furthermore, ST-IR was sig-

nificantly negatively correlated with GI and GR under both salt conditions ($P < 0.001$), and ST-GI and ST-GR were strongly associated with GI and GR under control and salt

conditions, except in the RILs population between ST-GI and GI under control conditions. The correlations between three salt indices (ST-IR, ST-GI, and ST-GR) and IR under control conditions were similar in the two populations. However, the correlations between the three salt indices (ST-IR, ST-GI, and ST-GR) and IR under salt conditions were different in the two mapping populations. Additionally, there were significant correlations between the three germination traits but no correlations between IR under salt stress conditions or GI and GR under control or salt stress conditions in the two populations.

QTLs for three salt tolerance indices

The salt tolerance indices data from two environments and the average data across two environments of the RILs population were used for QTL detection. A total of 11 putative QTLs, including three for ST-IR, six for ST-GI, and two for ST-GR, were mapped to six chromosomes (2, 7, 8, 10, 17 and 18) for the three salt tolerance indices, clustering on chromosome 8 (Table 3). These 11 QTLs, with LOD values ranging from 2.12 to 7.19, explained 4.49–25.94 % of the phenotypic variation. The positive alleles of five QTLs were from Kefeng1, and six QTLs were from Nannong1138-2.

For the three salt tolerance indices, six QTLs, including two for ST-IR, two for ST-GI, and two for ST-GR, were detected in E1. Three QTLs, *qST-IR-8*, *qST-GI-2-2* and *qST-GR-8*, were significant QTLs, and the other three QTLs, *qST-IR-18*, *qST-GI-2-1* and *qST-GR-7*, were suggestive QTLs. In E2, six QTLs, including two for ST-IR, two for ST-GI, and two for ST-GR, were detected in the NJRIKY population. Four QTLs, *qST-IR-8*, *qST-IR-18*, *qST-GR-8* and *qST-GR-7*, were significant, and the other two QTLs, *qST-GI-17* and *qST-GI-10-1*, were suggestive. Based on the average data for the RILs population across two environments, a total of five QTLs were identified, including two for ST-IR, two for ST-GI, and one for ST-GR, as shown in Table 3. Three QTLs, *qST-IR-8*, *qST-GI-10-2* and *qST-GR-8*, were

Table 4. Summary of allele number, gene diversity and PIC of the 205 SSR markers in the 196 cultivated soybean accessions

Index	Means ± SD	Max	Min
Allele number	10.20 ± 0.54	47.00	2.00
Gene diversity	0.68 ± 0.01	0.95	0.03
PIC	0.65 ± 0.01	0.95	0.03

PIC: Polymorphism information content.

SD: Standard deviation.

significant, and the other two QTLs, *qST-IR-2* and *qST-GI-8*, were suggestive.

In addition, *qST-IR-18*, *qST-GR-7*, *qST-IR-8* and *qST-GR-8* were detected in both E1 and E2. Moreover, *qST-IR-8* and *qST-GR-8* were both detected across two environments. QTLs clustering was also found; two QTLs, *qST-IR-8* and *qST-GR-8*, were co-localized, with both being closely linked to the same marker: Sat_162.

Genetic diversity and population structure

The set of 205 SSR markers with a genome-wide distribution detected a total of 2100 alleles among the 196 soybean accessions. The average number of alleles per locus was 10.20 ± 0.54 , ranging from 2 to 47. The polymorphic information content (PIC) ranged from 0.03 to 0.95, with an average of 0.65 ± 0.01 , and the genetic diversity ranged from 0.03 to 0.95, with an average of 0.68 ± 0.01 (Table 4).

Seventy SSR markers across the 20 chromosomes were selected, and model-based STRUCTURE analysis revealed three subpopulations (Fig. 3A). The first group, referred to as POP1, contained 47 accessions; the second group, POP2, included 61 accessions, and the third group, POP3, contained 88 accessions (Fig. 3B).

Genetic relatedness and linkage disequilibrium

Relative kinship estimates based on the 70 SSR markers showed that 54.93% of the pairwise kinship estimates were

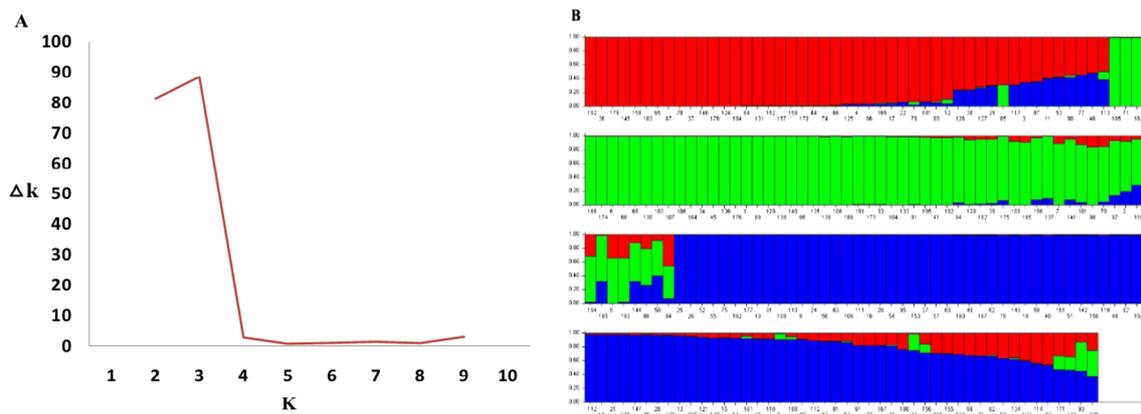


Fig. 3. Population structure of 196 cultivated soybean accessions. (A) The true K of the 196 cultivated soybeans according to Evanno *et al.* (2005). (B) The distribution of each line in the three subpopulations. Each individual is represented by a single vertical line that is broken into four colored segments, the lengths of these segments are proportional to each of the four clusters; the color is proportional in a single vertical line, indicating the proportion of the line that belongs to a subpopulation.

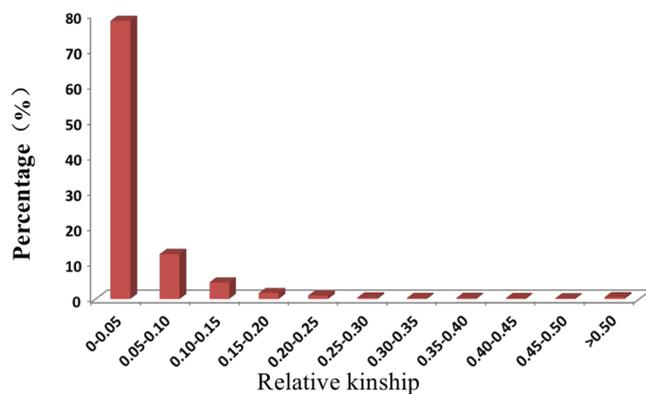


Fig. 4. The distribution of the pair-wise relative kinship coefficients for the 196 cultivated soybean accessions.

equal to zero, suggesting no relatedness between these pairs of lines. Of the pairwise kinship, 35.74% ranged from zero to 0.1, suggesting weak similarity; only 8.53% showed various degrees of relatedness, with relative kinship ranging from 0.1 to 0.50. For the remaining 0.50% of the entire coefficient matrix data set, relative kinship was higher than 0.50. This pattern of genetic relatedness revealed few lines with strong similarity, with most accessions being weakly related in this complex soybean association mapping panel (**Fig. 4**).

A K matrix was constructed for association analysis.

The extent of LD was detected among the 17,010 pairs of SSR loci for all accessions. Overall, 18.88% of those markers were in LD (based on $P < 0.05$). However, LD for pairs of markers from the same chromosome (within the same linkage group) was significantly higher (31.08%) than 18.88% (**Fig. 1**).

SSRs associated with the three salt tolerance indices

Based on the Bonferroni threshold ($P \leq 0.05/205 = 2.44 \times 10^{-4}$ or $-\log P \geq 3.61$), five SSR markers significantly associated with the three salt tolerance indices were identified (**Table 5**). Five SSR markers, including four SSR markers for ST-IR (Satt287, Sat_286, BE475343 and Satt156) and one SSR for ST-GR (Satt342), were found on chromosomes 16, 19, 2 and 1. However, no SSRs for ST-GI were detected in this study.

In addition, suggestive SSRs of salt tolerance were defined as SSRs significantly ($2 < -\log P < 3.61$) associated with salt tolerance indices (**Table 3**). As a result, four suggestive SSRs for ST-IR, five for ST-GI and eight for ST-GR were detected. Additional results were as follows: Satt201 was co-associated with ST-IR and ST-GI; BE475343 and CSSR306 were co-associated with ST-IR and ST-GR; Satt664 and Satt567 were co-associated with ST-GI and

Table 5. Markers with significant association signals ($P \leq 2.44 \times 10^{-4}$ or $-\log P \geq 3.61$) for salt tolerance indices based on the means of the traits in 196 soybean accessions

Trait	SSR	Chromosome	Position	$-\log P$	APAE	ANAE	Reference
ST-IR ^a	Satt277*	6	107.59	2.19	0 (0)	0 (0)	Cho <i>et al.</i> 2002, Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
	BE475343	2	30.74	6.20	+0.09 (+8.92)	-0.03 (-3.47)	
	Satt287	16	15.69	3.77	+0.04 (+4.12)	-0.15 (-15.55)	Lee <i>et al.</i> 2004
	Sat_286	19	87.42	5.90	+0.04 (+3.92)	-0.02 (-1.85)	
	Satt156	19	56.14	5.41	0 (0)	0 (0)	
	Satt201*	7	13.56	2.25	0 (0)	0 (0)	
	CSSR306*	7	8	2.55	+0.01 (+1.25)	0 (0)	
Satt636*	7	5	3.36	+0.02 (+1.62)	-0.01 (-0.75)		
ST-GI ^b	Satt664*	19	92.66	3.43	0 (0)	0 (0)	Lee <i>et al.</i> 2004
	Satt156*	19	56.14	3.33	0 (0)	0 (0)	
	Satt20*1	7	13.56	2.28	0 (0)	0 (0)	
	Satt567*	7	33.47	2.51	0 (0)	0 (0)	
	Satt636*	7	5	2.70	+0.11 (+30.26)	-0.03 (-8.33)	
ST-GR ^c	Satt363*	6	98.07	2.62	+0.05 (+6.61)	-0.06 (-12.05)	Cho <i>et al.</i> 2002, Kan <i>et al.</i> 2015, Zhang <i>et al.</i> 2014
	Satt342	1	48.14	4.07	+0.07 (+9.53)	-0.05 (-6.34)	
	BE475343*	2	30.74	2.46	+0.09 (+9.17)	-0.03 (-3.57)	Yue <i>et al.</i> 2001
	Satt664*	19	92.66	3.43	0 (0)	0 (0)	
	Satt156*	19	56.14	3.17	0 (0)	0 (0)	Lee <i>et al.</i> 2004
	CSSR306*	7	8	3.34	0 (0)	-0.08 (-9.65)	
	Satt567*	7	33.47	2.51	0 (0)	0 (0)	
	Satt636*	7	5	3.26	+0.07 (+10.97)	-0.09 (-13.26)	Guan <i>et al.</i> 2014, Ha <i>et al.</i> 2013, Hamwieh and Xu 2008, Hamwieh <i>et al.</i> 2011, Lee <i>et al.</i> 2004
	Sat_091*	3	79.51	3.00	+0.09 (+11.27)	-0.11 (-14.43)	

SSR, simple sequence repeat; LG, linkage group; APAE, the average positive allele effect; ANAE, the average negative allele effect.

SSR* was defined as a suggestive SSR significantly ($2 < -\log P < 3.61$) associated the salt tolerance indices.

^a Salt tolerance (ST) was defined as the ratio of the imbibition rate under salt conditions to that under no salt conditions.

^b Salt tolerance (ST) was defined as the ratio of the germination index under salt conditions to that under no salt conditions.

^c Salt tolerance (ST) was defined as the ratio of the germination rate under salt conditions to that under no salt conditions.

ST-GR; and Satt156 and Satt636 were co-associated with ST-IR, ST-GI and ST-GR.

Mining of elite alleles and carrier materials

On the basis of 22 SSR loci associated with the three salt tolerance indices, alleles of loci associated with the traits were further analyzed. As 11 SSR loci had no “null allele”, the phenotypic allele effects of these loci were not estimated. Elite alleles and their carrier materials of 11 SSRs are shown in **Supplemental Tables 2, 3**. Among the SSRs associated with ST-IR, BE475343 had the highest average positive effects (8.92%), whereas Satt287 had the highest average negative effects (15.55%). CSSR306 had only positive effects on ST-IR. Among the alleles associated with ST-IR, Sat_286-A133 and Sat_286-A152 had the most positive and negative phenotypic effects on ST-IR (19.10% and 6.65%, respectively), and their carrier materials were S134 and S12, respectively. For ST-GI, the proportion of the average positive allele effect of Satt636 was 30.26%, and the proportion of the average negative allele effect of Satt636 was 8.33%. In addition, Satt636-A185 and Satt636-A162 had the most positive and negative phenotypic effects on ST-GI (59.72% and 15.28%, respectively), and their carrier materials were S37 and S40, respectively. Among the SSRs associated with ST-GR, Sat_091 had both the highest average positive effects (11.27%) and the highest average negative effects (15.55%). Among the alleles associated with ST-GR, Satt636-A185 and BE475343-A250 had the most positive and negative phenotypic effects on ST-GR (31.85% and 58.51%, respectively), and their carrier materials were S15 and S134, respectively.

Discussion

Genetic diversity within cultivated soybean germplasm is of great value for ongoing soybean improvement efforts and association mapping. A variety of factors affect the amount and distribution of genetic diversity in soybean, including the extent of the domestication bottleneck, the breeding process and the amount of gene exchange between cultivated soybeans and their wild relatives. The genetic diversity of the soybean natural population has been reported in several previous studies, with the total number of alleles ranging from 826 to 1936 and the average alleles per locus ranging from 5.0 to 19.7 in different studies (Li *et al.* 2008, 2010, Wang *et al.* 2010, Wen *et al.* 2009). In this study, a total of 2,100 alleles and an average alleles per locus of 10.24 were identified in 196 accessions using 205 SSR markers. Such results inconsistent with previous studies may be due to a difference in the samples and markers used. Nonetheless, a population with abundant genetic diversity was used for association mapping in this study, and population structure can cause some allele frequencies to differ significantly between subpopulations, leading to Type I error in association mapping, especially in autogamous species, such as soybean (Ersoz *et al.* 2009). In an effort to reduce the false

positives caused by population structure, in the present study, the structure was controlled using 70 SSR markers across the 20 chromosomes, and association analysis was performed using a mixed-model (Q+K) by controlling for both population structure and cryptic familial relatedness, as described by Yu *et al.* (2006).

Seed germination is one of the plant growth stages that is highly susceptible to salt and is severely inhibited as salinity increases (Fredj *et al.* 2013). Inhibition of seed germination by salt stress can be due to toxic ion effects, decreases in water uptake, and reduced in nutrient mobilization efficiency (Long *et al.* 2013, Wang *et al.* 2011). Moreover, the three salt indices (ST-IR, ST-GI, and ST-GR) represent available traits that describe the mechanisms of soybean salt tolerance during seed germination based on correlations between the traits (Kan *et al.* 2015). We found a total of 11 QTLs and 22 SSR loci associated with the three salt tolerance indices using QTL mapping and association mapping, respectively. One SSR marker was closely linked to the co-localized QTLs, and seven SSR markers were co-associated with two or three of the salt tolerance indices. Because of the relatively high correlation between ST-GI and ST-GR, the number of co-localized or co-associated SSRs was the largest; therefore, the genetic mechanisms between ST-GI and ST-GR could be similar (Kan *et al.* 2015). Because no correlation was found between ST-IR and ST-GI in the RILs population, even though significant negative correlation were found between ST-IR and ST-GI in the natural population ($P < 0.05$), no co-localized SSRs and a fewer number of co-associated SSR markers were detected in the two populations. These results could dissect the genetic basis of soybean salt tolerance at the germination stage and be beneficial for developing marker-assisted selection in the future breeding of soybean salt tolerance.

Based on the genetic map, the Williams 82 physical map and the soybean whole-genome sequence in SoyBase (<http://www.soybase.org>), we compared the results for the three salt tolerance indices by QTL mapping with that by association mapping, and analyzed the results with previously reported QTLs. However, the loci for salt tolerance indices identified by QTL mapping were not consistent with those identified by association analysis. The possible reasons are as follows. In association analysis, a locus must have an effect in multiple lines to be detected, whereas a single locus may exhibit a major effect in a bi-parental cross population if other factors are not segregating (Hu *et al.* 2013, Wang *et al.* 2008). Therefore, it is possible that loci detected in a bi-parental population may go undetected using association mapping procedures (Wang *et al.* 2008). In addition, association analysis has limited power for detecting loci if alleles are rare in a natural population and subpopulation-specific; in contrast, QTL mapping can detect loci in a population constructed with lines belonging to different subpopulations (Famoso *et al.* 2011, Hu *et al.* 2013). In the present study, Kefeng1 and Nannong1138-2, the parental lines of the RILs population, are also two lines of the

natural population classified as pop1 from southern China and pop2 from northern China, respectively. Finally, different molecular markers were used in the analysis of the two population. For example, there were 14 SSR markers on chromosome 8 for QTL mapping and 6 SSR markers on chromosome 8 for association analysis in this study. Thus, lower marker density was the possible reason for no common genomic regions for these traits identified by linkage and association mapping. Although the loci detected by linkage mapping and association analysis had no cross-validation in our study, the results of the two methods were also complementary to each other based on the number of loci identified, providing a more robust understanding of the genetic architecture of salt tolerance than any single method (Hu *et al.* 2013). Among the association mapping results, Satt277, which was associated with ST-IR, and Satt363, which was associated with ST-GR, have previously been mapped (Cho *et al.* 2002, Zhang *et al.* 2014) to a location approximately 500 kb from BARC-025705-05001 (Kan *et al.* 2015). The SSR marker Satt363 was found to be associated with ST-GR, located in the same region as the QTL for resistance to multiple races of *Heterodera glycines* (Yue *et al.* 2001). The marker Satt664, co-associated with ST-GI and ST-GR, is approximately 1.88 cM from Satt513 in LG L, as reported by Qiu *et al.* (2011). The co-associated SSR marker Satt156 was found to be located in the same genomic region reported by Lee *et al.* (2004). Moreover, the SSR marker Sat_091, which was associated ST-GR, was previously identified to be closely linked to salt tolerance (Guan *et al.* 2014, Ha *et al.* 2013, Hamwieh and Xu 2008, Hamwieh *et al.* 2011, Lee *et al.* 2004), and the causal gene underlying this salt tolerance locus has been cloned (Guan *et al.* 2014, Qi *et al.* 2014). Regarding the QTL mapping results, the localizations of five SSR markers (Sat_162, satt217, satt130, satt245 and Satt266) closely linked to the QTLs (*qST-IR-8*, *qST-GR-8*, *qST-IR-18*, *qST-GR-7* and *qST-IR-2*) have been reported in previous studies (Chen *et al.* 2008, Cho *et al.* 2002, Kan *et al.* 2015, Zhang *et al.* 2014). The SSR marker Sat_162 was closely linked to the co-localized QTLs *qST-IR-8* and *qST-GR-8*, located in the same genomic regions reported by Kan *et al.* (2015) and Zhang *et al.* (2014) using association analysis. The candidate salt tolerance gene Glyma08g12400.1, localized to a site 550,651 bp from BARC-041663-08059, has been shown to respond to salt stress at the germination stage (Kan *et al.* 2015). Glyma08g12400.1 is homologous to the *SOS6* gene in *Arabidopsis*, which encodes the cellulose synthase-like protein AtCSLD5. Moreover, *SOS6* plays a critical role in osmotic stress tolerance and is likely involved in the regulation of reactive oxygen species (ROS) under stress (Zhu *et al.* 2010). The physical distance between the candidate gene Glyma08g12400.1 and the SSR marker Sat_162 was found to be 792,811 bp. These SSR markers located in previously reported QTLs reconfirmed the previous findings; these results might also indicate important targets for the identification of salt tolerance genes. Moreover, these findings

demonstrated that association mapping and linkage analysis are valid and provide further genetic information that is complementary for the improvement of breeding procedures. In addition, some of the loci reported here are newly identified. These novel SSRs will be important for furthering our understanding of the genetic basis of salt tolerance in soybean at the germination stage.

Elite alleles and their carrier materials were obtained using an analysis of the alleles at the loci associated with the three salt tolerance indices. As the phenotypic effects of positive (and negative) alleles were different from each other, the average positive (and negative) effects of a locus were also different from each other, indicating the potential of genetic recombination for breeding purposes (Wen *et al.* 2008). For Satt636, the phenotypic effects on ST-IR of positive (and negative) alleles varied from 0.13 to 7.06 (−0.13 ~ −1.62); the effects on ST-GI of positive (and negative) alleles varied from 17.50 to 59.72 (−1.39 ~ −15.28) and those on ST-GR of positive (and negative) alleles from 2.08–31.85 (−2.60 ~ −23.92). The average positive (and negative) effects for ST-IR, ST-GI, and ST-GR were 1.62 (−0.75), 30.26 (−8.33) and 10.97 (−13.26), respectively. In addition, the same marker locus could be associated with multiple traits, with the associated alleles performing distinctly in direction and size, and the covariation of the same allele in two related traits might be the genetic basis of their phenotypic correlation (Wen *et al.* 2008). These results may provide new salt tolerance gene accessions for salt tolerance pyramiding breeding in soybean and may significantly promote salt tolerance breeding in this important crop.

In conclusion, 11 QTLs and 22 SSR loci associated with three salt tolerance indices were obtained using linkage and association mapping. In addition, we identified one SSR marker closely linked to the co-localized QTLs and seven SSR markers co-associated with two or three salt tolerance indices, and elite alleles and their carrier materials were obtained using an analysis of alleles at the loci associated with the three salt tolerance indices. These results could lay the foundation for understanding the genetic basis of soybean salt tolerance at the germination stage and improve salt tolerance using marker-assisted selection (MAS) and molecular pyramiding breeding in soybean.

Acknowledgments

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