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Mapping and confirmation of loci for salt tolerance in a novel soybean germplasm, Fiskeby III

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Abstract

Key message **The confirmation of a major locus associated with salt tolerance and mapping of a new locus, which could be beneficial for improving salt tolerance in soybean.**

Abstract Breeding soybean for tolerance to high salt conditions is important in some regions of the USA and world. Soybean cultivar Fiskeby III (PI 438471) in maturity group 000 has been reported to be highly tolerant to multiple abiotic stress conditions, including salinity. In this study, a mapping population of 132 F_2 families derived from a cross of cultivar Williams 82 (PI 518671, moderately salt sensitive) and Fiskeby III (salt tolerant) was analyzed to map salt tolerance genes. The evaluation for salt tolerance was performed by analyzing leaf scorch score (LSS), chlorophyll content ratio (CCR), leaf sodium content (LSC), and leaf chloride content (LCC) after treatment with 120 mM NaCl under greenhouse conditions. Genotypic data for the F_2 population were obtained using the SoySNP6K Illumina Infinium BeadChip assay. A major allele from Fiskeby III was significantly associated with LSS, CCR, LSC, and LCC on chromosome (Chr.) 03 with LOD scores of 19.1, 11.0, 7.7 and 25.6, respectively. In addition, a second locus associated with salt tolerance for LSC was detected and mapped on Chr. 13 with an LOD score of 4.6 and an R^2 of 0.115. Three gene-based polymorphic molecular markers (Salt-20, Salt14056 and Salt11655) on Chr.03 showed a strong predictive association with phenotypic salt tolerance in the present mapping population. These molecular markers will be useful for marker-assisted selection to improve salt tolerance in soybean.

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Introduction

Salinity is an important abiotic stress factor which negatively impacts 60 million ha or approximately 20% of the total irrigated agricultural land area in the world (FAO and ITPS 2015; Squires and Glenn 2011); global crop losses are estimated at 27.3 billion US dollars, annually (Qadir et al. 2014). Salinity stress in agriculture results from application of irrigation water, which causes yield reductions of crops. The severity of the problem appears to be on the rise in agriculture, with affected farmland increased by an estimated 0.3–1.5 million ha annually (FAO and ITPS 2015). The trend toward increasing abiotic stress problems, including salinity stress, may be exacerbated in the future as a result of global climate change, leading to increasing environmental concern (Batlle-Sales 2011; Várallyay 2010).

In soybean, salt build-up in the soil can severely affect germination, seedling emergence, vegetative growth and development, as well as final seed yield and quality at maturity, resulting in modest to total crop loss (Blanco et al. 2007; Phang et al. 2008; Wang and Shannon 1999). In

addition, salt stress reduces efficiency of nitrogen fixation and decreases the number and biomass of root nodules (Delgado et al. 1994; Elsheikh and Wood 1995; Singleton and Bohlool 1984). Salt stress may also decreases protein, free amino acids, sucrose, and starch content in mature soybean seed (El-Sabagh et al. 2015; Rabie and Kumazawa 1988). Visual symptoms of salt stress include leaf chlorosis and eventually a necrotic bleached appearance known as leaf scorching (Fig. 1).

Not all soybean cultivars are equally sensitive to salt stress. Although most cultivars will accumulate excess salt in a soil or nutrient solution medium, leading to toxicity, others are able to effectively exclude Cl^- accumulation from the shoot. Previous genetic studies reported a dominant gene for salt exclusion or salt tolerance in soybean. Analysis of eight soybean populations subjected to sodium chloride-induced salt stress revealed that F_2 progenies segregated for Cl^- accumulation in a ratio of three excluders (tolerant by excluding chloride ions from the stems and leaves) to one includer (sensitive in which chloride ions are transported to the stems and leaves resulting in injury) (Abel 1969; Abel and MacKenzie 1964). In addition, progenies of BC_1F_1 crosses segregated in a ratio of 1 excluder to one includer, consistent with a single gene hypothesis (Abel 1969). Leaf

necrosis and Cl^- accumulation ratings were highly correlated in these studies and appeared pleotropic.

Using salt-induced leaf chlorosis or leaf scorching as a measure of salt tolerance in soybean, Lee et al. (2004) detected and mapped a major locus on chromosome (Chr.) 03, which explained 41 and 60% of the total phenotypic variation under greenhouse and field conditions, respectively. Subsequently, a similar major locus for salt tolerance was also identified and mapped on Chr. 03 in three additional mapping populations (Hamwieh et al. 2011; Hamwieh and Xu 2008). The locus for salt tolerance on Chr. 03 was also confirmed in a recombinant inbred line (RIL) population derived from a wild soybean [*Glycine soja* Siebold & Zucc.], accession, PI 483463, (Lee et al. 2009). A gene at this major locus, *Glyma03g32900,* was identified and cloned (Do et al. 2016; Guan et al. 2014; Patil et al. 2016; Qi et al. 2014), the functionality of this gene in controlling salt tolerance-related traits, such as leaf sodium (Na) and chloride (Cl) content still warrants further investigations.

Soybean cultivar Fiskeby III (PI 438471), originating from Sweden, has been reported to be highly or partially tolerant to drought, iron deficiency chlorosis, aluminum toxicity, salt stress, and atmospheric ozone pollution (Burton et al. 2016; Pathan et al. 2007; USDA 2011). In previous

Fig. 1 Comparisons of salt tolerance based on leaf scorch between cultivars Fiskeby III (tolerant parent), Lee (tolerant), Williams 82 (moderately sensitive parent), and Hutcheson (sensitive check) grown under 120 mM NaCl treatment

studies, Fiskeby III exhibited higher tolerance to salt stress than other salt-tolerant soybean genotypes under greenhouse conditions (Lenis et al. 2011; Patil et al. 2016). The objectives of this study were to identify and map genomic location(s) for salt tolerance in cultivar Fiskeby III, and to suggest DNA markers for marker-assisted selection (MAS) for the improvement of salt tolerance in soybean. As a part of the second objective, we assessed the efficacy of three developed gene-based polymorphic molecular markers (Salt-20, Salt14056 and Salt11655) in predicting phenotypic salt tolerance in the Williams 82 × Fiskeby III population.

Materials and methods

Plant materials

Soybean cultivar Fiskeby III (PI 438471), from Sweden, is highly tolerant to salt stress (Lenis et al. 2011; Patil et al. 2016). Cultivar Williams 82 (PI 518671) is salt sensitive (Do et al. 2016; Patil et al. 2016). However, it is less sensitive to salt than cultivar Hutcheson (PI 518664, Supplementary Table S1) that was included as a standard sensitive check in our study.

A cross of Williams 82 (moderately sensitive) and Fiskeby III (tolerant) was accomplished at the University of Missouri, Columbia, MO, in the summer of 2012. The true F_1 hybrids were advanced to the F_2 generation in the winter nursery in Costa Rica in the winter of 2012. In summer of 2014, the F_2 seeds of the population were planted at the Bay Farm Research Facility, Columbia, MO, USA. Leaf tissue of F_2 seedlings was collected for DNA extraction and genotyping and individual F_2 plants were harvested separately to obtain $F_{2:3}$ families. Subsequently, $F_{2:3}$ families were utilized to screen for salt tolerance in a greenhouse. One-hundred and thirty-two $F_{2:3}$ families from this population were used to detect and map genomic location(s) associated with salt tolerance.

DNA extraction and marker analysis

DNA was extracted from each F_2 plant using a standard CTAB method (Doyle and Doyle 1987). Briefly, extraction buffer containing 2% CTAB, 0.1 M Tris–HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl was added to the samples. The DNA pellet was dissolved in 150 µl of 1 × TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). DNA concentration was quantified with a spectrophotometer (NanoDrop Technologies Inc., Centreville, DE, USA) and was normalized at 50 ng/µl for marker genotyping.

Single-nucleotide polymorphism (SNP) genotyping was performed at Washington University in St. Louis using the SoySNP6K Illumina Infinium BeadChips (Illumina, Inc.

San Diego, CA, USA). The assay consisted of a series of standard protocols, such as incubation, DNA amplification, hybridization of samples to the bead assay, extension, and imaging of the bead assay. The SNP alleles were called using the GenomeStudio Genotyping Module (Akond et al. 2013; Song et al. 2013).

Genotyping of gene-based molecular markers

A previously identified gene sequence on Chr. 3 controlling salt tolerance in soybean (*Glyma03g32900*) was analyzed reference lines such as Hutcheson, Lee and Holladay including the parents in this study (Do et al. 2016; Guan et al. 2014; Qi et al. 2014; Valliyodan et al. 2016). Selected SNPs were used to develop Kompetitive Allelic-Specific PCR (KASP) assays (http://www.lgcgroup.com). Three KASP assays were used to genotype the mapping population, including Salt-20 (M1), Salt14056 (M2), and Salt11655 (M6) at the University of Missouri, Columbia, MO that were designed on SNPs of promotor intron 3 and exon 5, respectively (Patil et al. 2016). The reaction mixture was prepared according to the protocol described by LGC Genomics, LLC (http://www.lgcgroup.com). Briefly, KASP assays were run in a 10 µl final reaction volume containing a 5 µl KASP master mix, 0.14 µl primer mix, 2 µl of 10–20 ng/µl genomic DNA, and 2.86 µl water. The following cycling conditions were used: 15 min at 95 °C, followed by ten touchdown cycles of 20 s at 94 °C, 1 min at 61–55 °C (dropping 0.6 °C per cycle); and then 26 cycles of 20 s at 94 °C, 1 min at 55 °C. The fluorescent end-point genotyping method was carried out using a Roche LightCycler 480-Instrument II (Roche Applied Sciences, Indianapolis, IN, USA) as described (Patil et al. 2016).

Salt tolerance phenotyping

Leaf scorch score (LSS) and chlorophyll content ratio (CCR)

Phenotypic evaluation of the $F_{2:3}$ population for salt tolerance was conducted using the plastic cone-tainer method as previously described by Lee et al. (2008). Seven seedlings of each $F_{2:3}$ family were grown per cone-tainer and evaluated in two replications over time in a greenhouse at the University of Missouri, Columbia, MO, USA, using artificial lights and a 13 h photoperiod in 2015. Three soybean genotypes, Hutcheson (highly sensitive check), Fiskeby III (tolerant parent), and Williams 82 (moderately sensitive parent), were included in all phenotypic assays to ensure the consistency of the experimental conditions. At the vegetative growth stage V2 (Fehr et al. 1971), soybean seedlings in cone-tainers were treated with salt water (120 mM NaCl) such that the salt solution filled the bottom one-third (7 cm) of each cone-tainer for 7 h/day. Electrical conductivity (EC) of salt water was monitored daily (Hamwieh et al. 2011).

Individual soybean plants were visually rated for salt sensitivity or tolerance when Hutcheson showed severe leaf scorch (approximately 2 weeks after the treatment imposition). Leaf scorch was scored using a 1–5 scale, where 1 = no apparent chlorosis; 2 = slight (25% of the leaves showed chlorosis); 3 = moderate (50% of the leaves showed chlorosis and some necrosis); 4 = severe chlorosis (75% of the leaves showed chlorosis and severe necrosis); and 5 = dead (leaves showed severe necrosis and were withered) for each plant and recorded as mean for each cone-tainer as previously described (Lee et al. 2008).

Leaf chlorophyll content was quantified on individual plants of each $F_{2:3}$ family, the parents and the Hutcheson check for the topmost fully expanded leaf 1 day before and about 14 days after the initiation of the salt treatment using a chlorophyll meter (Chlorophyll meter SPAD-502, Konica Minolta). Chlorophyll content ratio (CCR) was calculated as the ratio of leaf chlorophyll content after treatment dividing leaf chlorophyll content before treatment is an indicator of an indirect measure of the integrity and maintenance of the photosynthetic apparatus in response to stress (Ghassemi-Golezani et al. 2011; Patil et al. 2016; Weisany et al. 2011).

Leaf sodium (LSC) and chloride (LCC) content analysis

After leaf scorch and chlorophyll determinations, leaves were harvested and dried at 60 °C for 7 days. The dried leaf tissue of seven seedlings from each $F_{2:3}$ family was ground using a Thomas Model ED-5 laboratory Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and then analyzed for leaf chloride (LCC) and leaf sodium (LSC) contents at the Delta Research Center, University of Missouri, Portageville, MO as previously described (Lenis et al. 2011). Briefly, the LCC assay was accomplished by dissolving ground leaf tissue (0.15 g) in 30 ml of distilled water and agitating on an Eberbach Corporation orbital shaker (Eberbach Corporation, Ann Arbor, MI, USA) at 60 cycles per min for 1 h. Standards for calibration of 25, 50, 100 and 500 mg/kg of chloride (Cl^-) were made using Ricca Chemical Company's Primary Cl^- solution of 1000 mg/kg (Arlington, TX, USA). A standard curve was established using an ion-specific electrode attached to a Fisher Scientific AR 50 dual channel pH, ion, conductivity meter (Fischer Scientific, Pittsburg, PA, USA). After standard reference curves were established, the Cl^- in solution extracted from samples of leaves was determined for $F_{2:3}$ lines. The Cl^- quantity (LCC) in the solution was converted to Cl^- concentration by multiplying the mg/kg chloride in solution by volume of distilled water and dividing by weight of the plant sample.

Ground, dry leaf tissue (0.25 g) was used to determine leaf sodium content (LSC) by means of a modified wet acid dilution procedure with a Hach Digesdahl™ Digestion Apparatus, 115VAC, 50/60 Hz (Hach Company, Loveland, CO, USA) using H_2SO_4 and H_2O_2. Tissue concentrations of Na^+ were determined using a Perkin-Elmer™ (Wellesley, MA, USA) atomic absorption spectrophotometer as previously described (Lenis et al. 2011).

Data analysis

The experimental design was a randomized block employing two replications. Phenotypic variation of LSS, CCR, LSC, and LCC was analyzed using ANOVA procedure and Duncan's multiple range test in IBM® SPSS® Statistics Version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). The frequency distribution plots for the $F_{2:3}$ families plots were developed for the four measures of salt tolerance using Minitab 17 software (Minitab, Inc., 1829 Pine Hall Road State College, PA 16801 USA).

The midpoint of leaf scorch score (LSS) scale (2.5) was used as the cutoff to classify salt tolerance of F_2 populations into tolerant and sensitive groups. The results indicated that segregation of LSS for evaluating salt tolerance fitted a 3:1 (tolerant:sensitive) ratio (Hamwieh and Xu 2008; Lee et al. 2009). It is easier to classify salt tolerance of F_2 populations from tolerant and sensitive parents but more difficult to classify traits of F_2 populations from a cross of tolerant and moderately sensitive parents as used in the present study and unable to use for other traits. Therefore, $F_{2:3}$ families were classified into tolerant, intermediate and sensitive categories based on parental means plus/minus standard deviations as a cutoff. Phenotypic segregation of a 1:2:1 (tolerant:intermediate:sensitive) ratio for all traits in $F_{2:3}$ families was tested using Chi square analysis. Similarly, segregation of gene-based markers in the F_2 population was also evaluated for a goodness-of-fit to a 1:2:1 ratio.

A genetic linkage map was constructed in the F_2 population using the Kosambi mapping function described in the JoinMap 4.0 software (van Ooijen 2006). A likelihood of odds (LOD) threshold score of 3.0 and a maximum genetic distance of 50 cM were utilized for an initial linkage grouping of markers. For genetic analysis, a comprehensive analysis approach, including initial interval mapping (IM), cofactor selection, genome-wide permutation test, and multi-QTL method (MQM), was performed using the MapQTL5.0 software (van Ooijen 2004). The significant threshold of the LOD score was calculated by permutation test with a large set of 1000 iterations. The epistatic interaction between genomic regions significantly associated with salt tolerances was analyzed using a mixed model described in the QTLnetwork 2.1 program (Yang et al. 2008).

Results

Phenotypic variance of salt tolerance

The Hutcheson check was the most salt-sensitive genotype in the study (Table 1). In contrast, Fiskeby III exhibited salt tolerant superior to that of Williams 82 or Hutcheson, based on means of the four measures of salt tolerance employed in the study (Table 1; Fig. 1). The two replications were highly correlated for $F_{2:3}$ families and control

genotypes ($r > 0.80$ for all traits), indicating a high degree of repeatability in the phenotypic data. The heritability in four salt tolerant traits showed the highest value for CCR and the lowest values for LSC (Table 1). In addition, the genotypic means of the four measures of salt tolerance were highly correlated (r at least 0.40, $P < 0.01$), with the highest correlation between the two traits involving color, LSS and CCR ($r = 0.78$, Table S2).

The range for LSS, CCR, LSC, and LCC among the $F_{2:3}$ families showed that some $F_{2:3}$ families had numerically higher tolerance than the salt tolerant parent, Fiskeby III

Table 1 Variation in leaf scorch score (LSS), chlorophyll content ratio (CCR), leaf sodium content (LSC) and leaf chloride content (LCC) of the parents and 132 $F_{2:3}$ families derived from a cross between Williams 82 and Fiskeby III grown under 120 mM NaCl treatment in a greenhouse

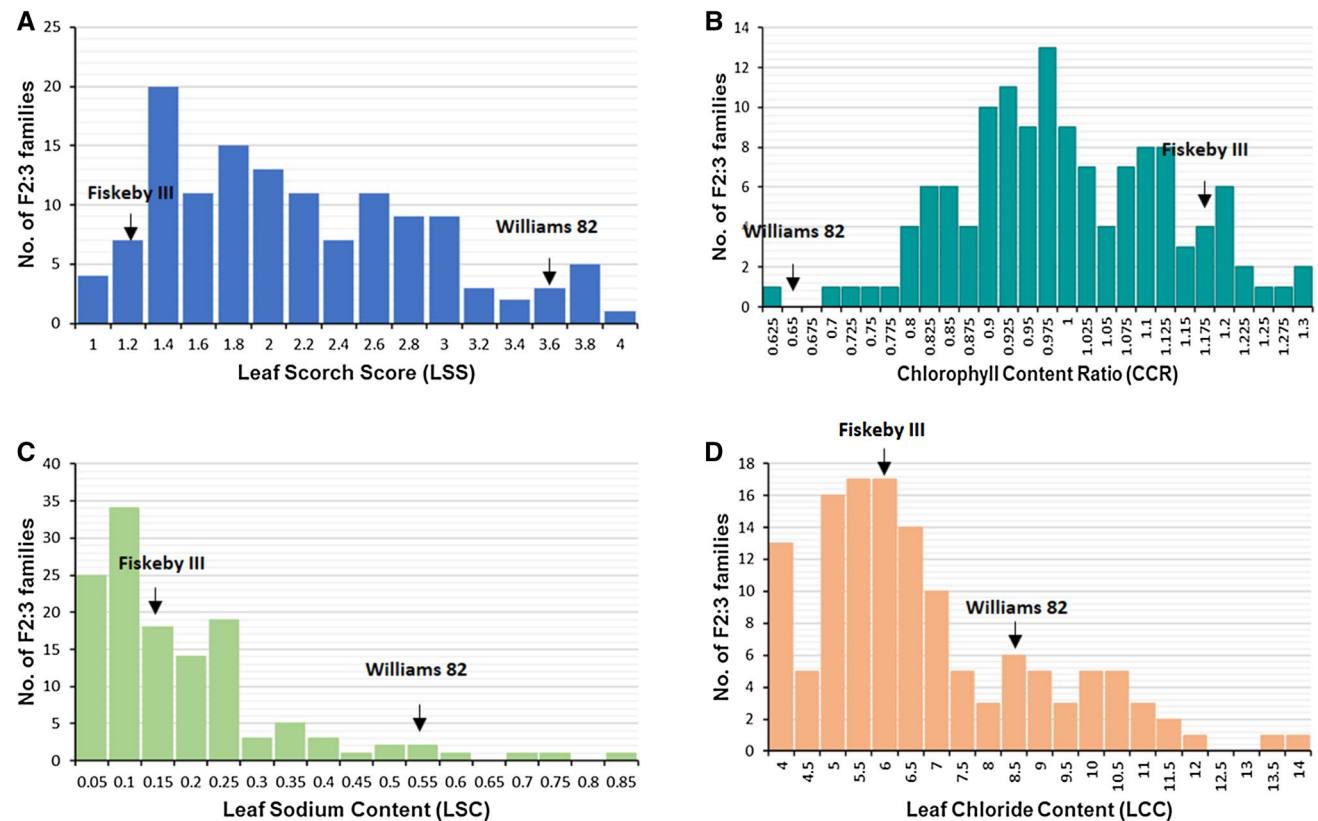| Sources	LSS	CCR	LSC (g/kg)	LCC (g/kg)
Hutcheson	5.0	0.55 ± 0.02	0.614 ± 0.21	10.67 ± 0.91
Fiskeby III	1.14 ± 0.10	1.17 ± 0.02	0.153 ± 0.021	5.76 ± 0.41
Williams 82	3.67 ± 0.26	0.67 ± 0.05	0.568 ± 0.038	8.34 ± 0.06
Mean of $F_{2:3}$ families	2.07	0.98	0.177	10.40
Range of $F_{2:3}$ families	1.00–4.20	0.67–1.35	0.005–0.85	3.10–13.6
H^2 ($F_{2:3}$ families)	0.38	0.49	0.12	0.47

H^2, broad sense heritability

Fig. 2 Distribution of leaf scorch score (LSS) (**a**), chlorophyll content ratio (CCR) (**b**), leaf sodium content (LSC) (**c**), and leaf chloride content (LCC) (**d**) of 132 $F_{2:3}$ families derived from a cross between Williams 82 and Fiskeby III grown under 120 mM NaCl treatment

(Table 1; Fig. 2). All traits showed a discontinuous distribution, with salt tolerant lines (low LSS, high CCR, low LSC and low LCC) being predominant and skewed toward salt tolerance. Phenotypes of these traits were also classified into salt-tolerant, intermediate, and salt-sensitive groups (Table 2). Based on Chi square analyses, the phenotypic segregations well fit an F_2 ratio of 1 tolerant: 2 intermediate: 1 sensitive for LSS ($\chi^2 = 2.28$ and $Pr = 0.68$), CCR ($\chi^2 = 3.32$ and $Pr = 0.79$), and LCC ($\chi^2 = 2.69$ and $Pr = 0.74$), indicating that these three traits were controlled by a single gene. The LSC trait fit an F_2 ratio of 9 tolerant: 6 intermediate: 1 sensitive ratio with $\chi^2 = 3.21$ and $Pr = 0.79$, suggesting that this trait was controlled by two genes (Table 2).

Genetic linkage map

A total of 2158 polymorphic SNP makers between the two parents, Williams 82 and Fiskeby III, were used to construct a genetic linkage map in the F_2 population. Of these 2148 SNP loci (99.5%) were successfully mapped on the 20 linkage groups that covered 2834 cM of the whole soybean genome and corresponded to 20 soybean chromosomes (Supplementary Fig. S1). The smallest number of SNP markers (71) was on Chr. 10 and the largest number (149) on Chr. 13. The average distance between two adjacent SNP markers was 1.38 cM across all chromosomes. However, largest gaps (around 40 cM) were near the center of Chrs. 10 and 11 (Table 3; Supplementary Fig. S1).

Loci associated with salt tolerance

The significant thresholds of the LOD values determined by the genome-wide permutation tests for each trait, LSS, CCR, LSC and LCC, were 2.7, 2.6, 2.4 and 2.7, respectively. The genomic region significantly associated with LSS, CCR, LSC, and LCC was identified with high LOD and high R^2 values at the same position on Chr. 03. The closest marker with the highest LOD value and R^2 for means of LSS, CCR

Table 3 Summary of the genetic map for number of markers, chromosome length, average space between markers, and maximum space between markers in centi-morgans (cM) for each of the 20 chromosomes in the F_2 population derived from a cross between Williams 82 and Fiskeby III with SNP markers

Chromosome	No. of markers	Length of chromosome (cM)	Average space (cM)	Maximum space (cM)
1	107	127	1.20	10.4
2	127	158	1.20	14.7
3	126	130	1.00	16.3
4	122	146	1.21	14.3
5	110	145	1.33	22.8
6	134	165	1.24	8.9
7	88	146	1.69	13.9
8	118	169	1.45	12.0
9	106	150	1.43	11.3
10	71	147	2.09	40.4
11	80	141	1.78	40.0
12	75	114	1.55	13.6
13	149	180	1.22	15.5
14	99	113	1.15	12.1
15	118	142	1.22	24.7
16	96	104	1.10	11.9
17	94	146	1.57	10.5
18	142	139	1.00	10.5
19	104	143	1.39	24.9
20	82	138	1.71	15.6
Overall	2148	2843	1.38	40.4

and LSC was Gm03_40727780 (ss715585963), located at the position 38716240 (Table 4; Fig. 5). A new genomic region associated with LSC was also identified with high LOD value (4.56) and R^2 (11.5%) on Chr. 13 (Table 4; Fig. 3). The closest marker with the highest LOD value, Gm13_38988256 (ss715616164), was located at the position 40167119 of the soybean physical map. No significant

Table 2 Chi square tests for salt-tolerant traits in an $F_{2:3}$ families from a cross between Williams 82 and Fiskeby III

Groups	LSS			CCR			LSC			LCC		
	Obs	Cut-off	Mean	Obs	Cut-off	Mean	Obs	Cut-off	Mean	Obs	Cut-off	Mean
Tolerant	40	< 1.51	1.29	37	> 1.07	1.16	69	< 0.13	0.07	41	< 5.3	4.35
Intermediate	64	1.51–2.7	2.09	71	0.86–1.07	0.96	58	0.13–0.62	0.25	62	5.3--8.3	6.30
Sensitive	28	> 2.7	3.25	24	< 0.86	0.79	5	> 0.62	0.93	29	> 8.3	9.97
Size	132			132			132			132		
χ^2 (1:2:1)	2.28			3.32			–			2.69		
χ^2 (9:6:1)	–			–			3.21			–		
Pr	0.68			0.81			0.79			0.74		

Obs, observed number of $F_{2:3}$ families, Cut-off, cut-off value based on parental means plus/minus standard deviations, –, no significant fit with expected ratio

Table 4 Mapping of leaf scorch score (LSS), chlorophyll content ratio (CCR), leaf sodium content (LSC) and leaf chloride content (LCC) in an F_2 population from the cross of Williams 82 and Fiskeby III

Trait	Chr.	Genetic position	LOD	R^2 (%)	Flanking markers	Interval (cM)	Additive effect	Dominance effect	h^2
LSS	Chr. 03	86.9	19.08	48.2	Salt-20[a], Gm03_41135466	3.6	0.71**	− 0.50**	0.38
CCR	Chr. 03	86.9	11.01	31.3	Salt-20[a], Gm03_41135466	3.6	− 0.10**	0.08**	0.24
LSC	Chr. 03	86.9	7.69	20.6	Salt-20[a], Gm03_41135466	3.6	0.12**	0.03**	0.14
LCC	Chr. 03	86.9	25.59	58.9	Salt-20[a], Gm03_41135466	3.6	2.36**	− 0.67**	0.56
LSC	Chr. 13	143.7	4.56	11.5	Gm13_37204738, Gm13_38988256	10.3	− 0.09**	− 0.07*	0.10

h^2, narrow sense heritability

*Significant effect at 0.05 probability level

**Significant effect at 0.01 probability level

[a]Salt-20 was mapped in the same position with Salt14056, Salt11655, Gm03_40600088, Gm03_40613405 and Gm03_40663609 on Chr.03
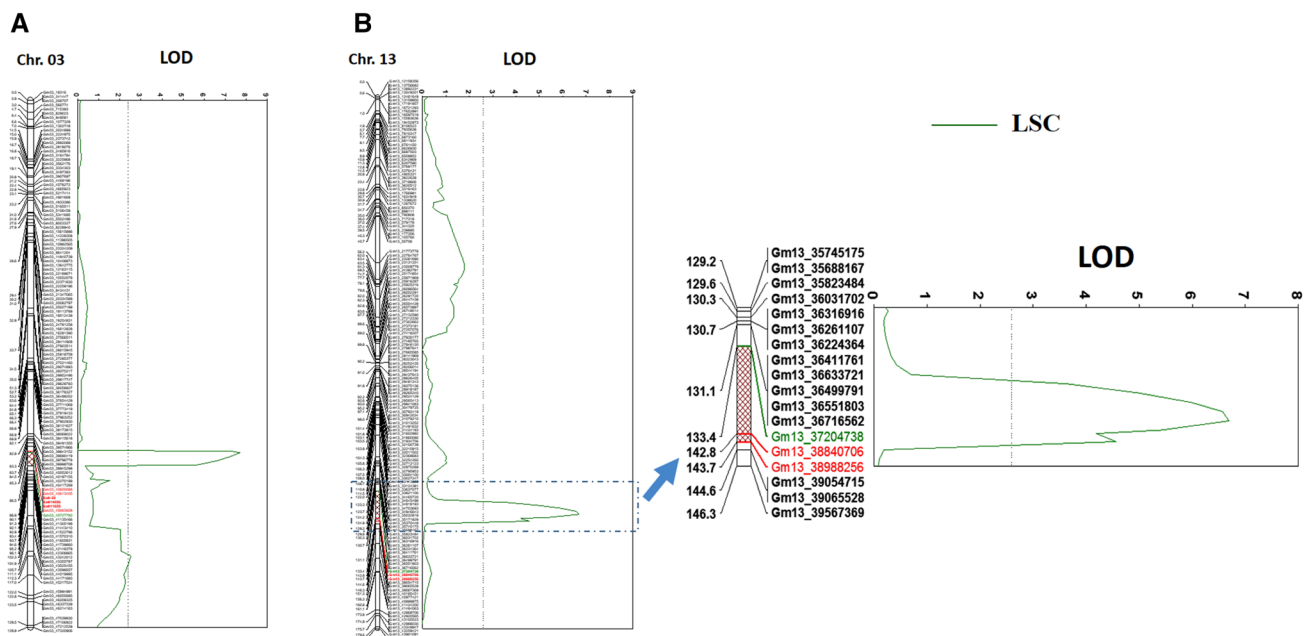

Fig. 3 A logarithm of the odds (LOD) plot showing the location of locus for leaf sodium content (LSC) on Chr. 03 (**a**) and a putative locus for leaf sodium content (LSC) on Chr. 13 (**b**) in $F_{2:3}$ families derived from a cross between Williams 82 and Fiskeby III

epistatic interactions (either additive × additive or additive × dominant) were detected among two loci for LSC mapped on Chrs. 03 and 13 (data not shown).

Evaluation of gene-based molecular markers in F_2 population

Results of genotyping 132 F_2 plants with gene-based molecular markers (GBM) showed a clear differentiation between homozygous and heterozygous genotypes (Fig. 4). Because recombination between GBMs was not found in the 132 plants of the F_2 population, symbolic genotypes, AA, aa, and Aa (A allele from Fiskeby III and an allele from Williams 82), were labeled Fiskeby III, Williams 82

and heterozygote for all GBMs. The segregation of GBMs fits a 1:2:1 ratio with $\chi^2 = 3.32$ and $P = 0.81$ (Table 5).

Phenotypic data were grouped by AA, Aa, and aa categories of GBM, resulting in a significant association between GBMs markers and the four phenotypic measures of salt tolerance (Table 5). The AA and Aa genotypes with lower LSS, higher CCR, lower LSC and lower LCC showed higher salt tolerance compared to aa genotype. Heterozygotes clearly showed dominance for tolerance in all four measures of salt tolerance. In addition, the results were more highly significant when GBMs (Salt-20, Salt14056 and Salt11655) are located in significant intervals containing the locus for salt tolerance with high LOD and R^2 values (Table 4; Fig. 5).
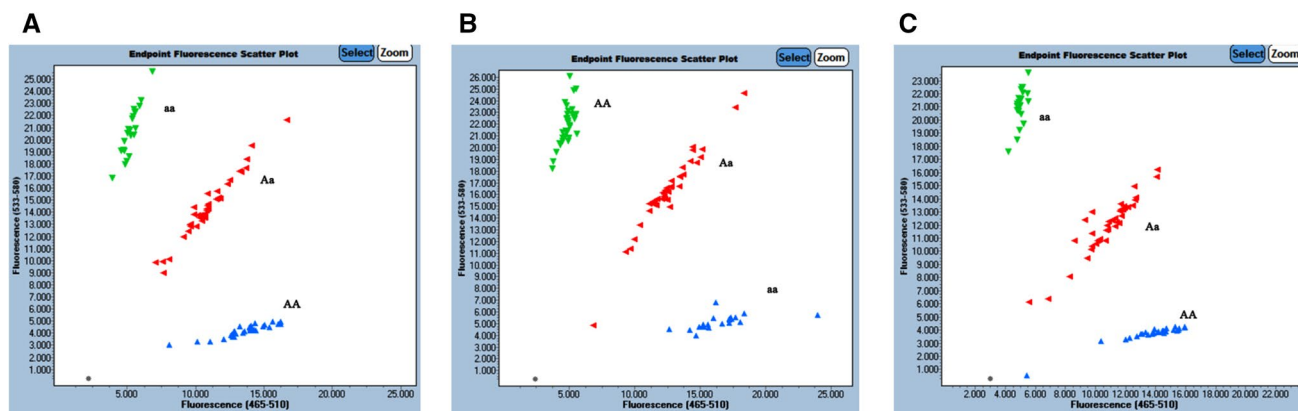
Fig. 4 Genetic segregations of three gene-based markers, Salt-20 (**a**), Salt14056 (**b**) and Salt11655 (**c**) in an F_2 population from a cross between Williams 82 and Fiskeby III

Table 5 Chi square tests for gene-based markers (GBMs) in an F_2 population and phenotype of groups for 132 $F_{2:3}$ families from a cross between Williams 82 and Fiskeby III

Phenotype of groups by GBM					Genotypes of GBM		
Groups	LSS**	CCR**	LSC (g/kg)**	LCC (g/kg)**	GBM	Observed	Expected
Tolerance	1.69[a]	1.04[a]	0.13[a]	4.69[a]	AA	42	33
Intermediate	1.91[a]	1.02[a]	0.14[a]	6.37[b]	Aa	61	66
Sensitive	3.05[b]	0.84[b]	0.35[b]	9.40[c]	aa	29	33
					Size	$n = 132$	
					χ^2 (1:2:1)	3.32	
					Pr	0.81	

**The different letters with mean of traits are different groups according to Duncan's multiple range test ($P < 0.01$)

Discussion

By classifying $F_{2:3}$ families into tolerant, intermediate, and sensitive groups based on parental means, standard deviations and using the Chi square analysis, the phenotypic segregation of LSS, CCR and LCC showed a good fit a 1:2:1 ratio while LSC best fit a 9:6:1 ratio (Table 2). These results suggested that LSS, CCR and LCC were controlled by a single gene while LSC was controlled by two genes. Similarly, a major locus was identified for LSS, CCR and LCC by gene mapping while two loci were mapped for LSC.

The quality and marker resolution of a genetic linkage map affect the accuracy of quantitative trait loci (QTL) mapping and the identification of candidate genes. Increasing marker density is one way to obtain higher resolution of genetic maps (Gutierrez-Gonzalez et al. 2011; Li et al. 2014). Recently, the development of high-throughput genotyping assays, such as the SoySNP6K Illumina Infinium BeadChip and SoySNP50K BeadChip, provided a powerful tool for constructing high-resolution linkage maps in soybean (Akond et al. 2013; Song et al. 2013).

In this study, the F_2 population was genotyped using the SoySNP6K Illumina Infinium BeadChip assays to obtain 2158 polymorphic SNP makers. A high-resolution genetic map with the average distance of 1.38 cM between two markers was constructed and used for mapping salt tolerance genes. The mapped locus for salt tolerance on Chr. 03 with high peak and a narrow marker interval reflected the efficiency of using a high-resolution map. In addition, this added precision may have allowed the detection of a new putative locus for salt tolerance on Chr. 13, based on leaf analysis of shoot sodium content.

Leaf scorching, a visual trait, has been used as a main assessment for salt tolerance in many studies (Hamwieh and Xu 2008; Lee et al. 2004). Decreased chlorophyll content under salt stress has also been useful for evaluating salt tolerance (Ghassemi-Golezani et al. 2011; Hamwieh et al. 2011; Lenis et al. 2011; Patil et al. 2016). However, many non-genetic factors can generate 'noise' in these traits, such as variation within an experiment for light intensity, nutrient supply, air temperature, air movement, and the presence of heavy metals and alkaline salt in the medium (Hu et al. 2014; Kumar Tewari and Charan Tripathy 1998; Resurreccion et al. 2002; Tuyen et al. 2010; Zhao et al. 2005).
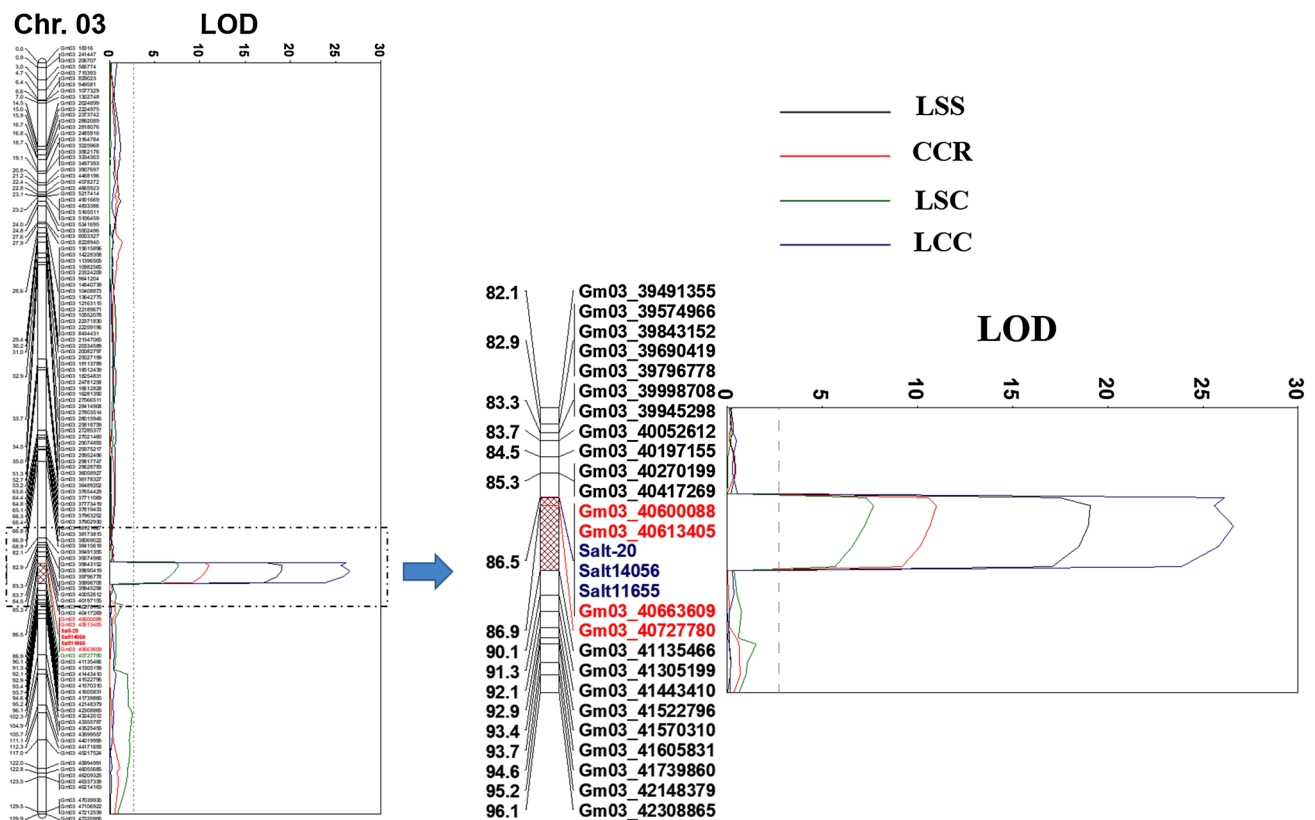
Fig. 5 A logarithm of the odds (LOD) plot showing the location of a gene for leaf scorch score (LSS), chlorophyll content ratio (CCR), leaf sodium content (LSC), and leaf chloride content (LCC) in $F_{2:3}$ families derived from a cross between Williams 82 and Fiskeby III

Thus, other traits such as leaf or shoot sodium and chloride content have been used to assess salt tolerance more directly for genetic mapping in crop plants, such as in rice (Bonilla et al. 2002; Hossain et al. 2015; Koyama et al. 2001; Lin et al. 2004; Qiu et al. 2015), wheat (Genc et al. 2010; Lindsay et al. 2004), and barley (Nguyen et al. 2013; Xue et al. 2009). Leaf sodium and chloride content are highly correlated with leaf scorching and chlorophyll content as measures of salt tolerance in soybean (Do et al. 2016; Lenis et al. 2011), and are used here in genetic mapping study for the first time. The results suggest that the major gene on Chr. 03 may control salt tolerance as measured by all four measures of tolerance.

Earlier genetic studies and QTL mapping in soybean suggested that salt tolerance is controlled by a single dominant gene (*Ncl*) by measuring chloride exclusion and leaf scorching and a major QTL is mapped to Chr. 03 (Abel 1969; Abel and MacKenzie 1964; Lee et al. 2004). The major QTL on Chr. 3 for salt tolerance was identified and confirmed in mapping populations derived from different salt-tolerant sources (Ha et al. 2013; Hamwieh et al. 2011; Hamwieh and Xu 2008; Qi et al. 2014). Recently, a candidate gene, *Glyma03g32900* underlying salt tolerance, was isolated and the gene function was related to a sodium transporter (Guan

et al. 2014; Qi et al. 2014). Results of a separate study found that this gene controls both sodium and chloride content based on the analyses of gene expression and testing near-isogenic lines (NILs) under field conditions (Do et al. 2016; Liu et al. 2016). In this study, the gene *Glyma03g32900* controlling leaf sodium and chloride content was verified by a high correlation between gene-based markers and the two traits, LSC and LCC. Further, the dual functions of this gene (*Glyma03g32900*) controlling two traits (LSC and LCC) should be considered for future studies. In addition, a classical genetic analysis was also performed for LSC using flanking markers (Gm13_37204738 and Gm13_38988256) of the putative locus on Chr. 13 and LSC was controlled by another dominant gene on Chr. 13 (data not shown).

Although several studies detected and consistently mapped the major locus for salt tolerance on Chr. 03, some evidence showed that there are additional genes underlying salt tolerance in the soybean genome. For instance, eight putative QTL significantly associated with salt tolerance under greenhouse and field conditions were reported (Chen et al. 2008). Comparison of salt tolerance performance of three sets of NILs suggested that there might be another gene that affects or interacts with the salt tolerant gene on Chr. 03 (Hamwieh et al. 2011). By association mapping

of soybean seed germination under salt stress, five candidate genes located on Chrs. 08, 09, and 19 were verified in response to salt stress (Kan et al. 2015). Four soybean accessions were reported to be new sources for novel determinants of salt tolerance in genes other than the cloned gene, *Glyma03g32900* (Guan et al. 2014). Correlations between gene-based markers and phenotype showed that a new soybean accession might carry novel gene(s) for salt tolerance (Patil et al. 2016). A putative locus associated with LSC mapped on Chr. 13 in the present study appears to be a novel locus that may harbor other genes underlying salt tolerance in soybean. However, a favorable allele for low LSC was from the moderately sensitive parent, Williams 82. Some $F_{2:3}$ families showed higher tolerance than salt-tolerant parent (Fiskeby III) suggesting that elevated salt tolerance may be contributed by favorable alleles derived from both parents. Therefore, a greater level of salt tolerance observed in Fiskeby III might be from a different allele or was not expressed in a cross with Williams 82. Developing NILs from a cross of Williams 82 and Fiskeby III will be useful for a further investigation of the importance of the gene(s) located on Chr. 13 in providing salt tolerance.

The interval of a genetic map significantly associated with a trait from QTL analysis and fine-mapping provided basic information to predict candidate genes (Bargsten et al. 2014). This process was more efficient with a high-density genetic map, genomic sequence data, and predicted genes in a physical map (Zhang et al. 2016). Sixty-eight candidate genes for alkaline salt tolerance were predicted in a 771.7 kb interval of a physical map on Chr. 17 (Tuyen et al. 2013). For salt tolerance, the genes located in a significant interval were predicted and cloned by map-based cloning (Do et al. 2016; Ha et al. 2013; Qi et al. 2014). Based on the genomic region significantly associated with LSC on Chr. 13, three coding sequences, including *Glyma.13g305700*, *Glyma.13g305800*, and *Glyma.13g305900*, with salt stress response/antifungal function were close to significant markers for LSC (Supplementary Fig. S2) (http://www.soybase.org). A further study of these new candidate genes could be useful for salt tolerance improvement in soybean.

The availability and accessibility of whole-genome sequencing data, high-resolution genetic linkage maps, candidate genes, and gene annotations are valuable genomic resources to develop functional genetic markers for genetic and breeding studies in plants (Chin et al. 2010; Galeano et al. 2012; Reinprecht and Pauls 2016). SimpleProbe and TaqMan genotyping assays developed for SNPs and mutant alleles of genes controlling oleic acid and linolenic acid content were successfully applied to genetic analysis and breeding in soybean (Shi et al. 2015). Six KASP assays developed based on the gene *Glyma03g32900* were used to study variation of gene structural variation and salt tolerance in soybean, in which three SNP markers Salt-20 (M1), Salt14056

(M4) and Salt11655 (M6) were shown to be highly correlated with salt tolerance phenotypes in 106 diverse soybean lines and an RIL mapping population (Patil et al. 2016). In this study, those markers were also integrated into a genetic linkage map that was employed for genetic mapping of salt tolerance. The segregations of those markers well fitted a 1:2:1 ratio and showed a high correlation with salt tolerance (Table 5).

In conclusion, the validation of the gene-based markers in an F_2 mapping population suggests that these markers are useful tools for tracking and selecting the salt-tolerant gene on Chr. 03. By analyzing the association with salt tolerant phenotypes, gene-based markers could be used to identify promising soybean lines with novel salt tolerant gene(s). The putative locus for LSC on Chr. 13 suggests the presence of a novel gene(s) controlling salt tolerance and may be useful to stack with the known gene on Chr. 03 for improving salt tolerance in soybean.

Author contribution statement TDD designed the study, conducted experiments, performed salinity assays and data analysis, and wrote the manuscript; TDV produced crosses, developed mapping population, contributed to data analysis, manuscript reviewing and editing; DD and SS analyzed leaf sodium and chloride content; GP contributed to gene-based marker analysis; DCY contributed to greenhouse assays and oversaw seed harvest; PC, AS, DX and TEC reviewed and edited the manuscript; HTN and JGS conceived the study, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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