Research Paper

Fine mapping of *qSKC-1*, a major quantitative trait locus for shoot K^+ concentration, in rice seedlings grown under salt stress

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Shoot K^+ concentration (SKC) is an important physiological parameter used to evaluate salt tolerance at the seedling stage in rice (*Oryza sativa* L.). *qSKC-1*, a major quantitative trait locus for SKC in rice under salt stress, was detected on chromosome 1 using three F_2 populations constructed by crossing ‘Nipponbare’ and its two salt-sensitive mutants (*rss2* and *rss4*) with an *indica* cultivar ‘Zhaiyeqing8’ (‘ZYQ8’). In this study, the chromosomal location of *qSKC-1* was determined precisely by fine mapping. First, the presence of *qSKC-1* was confirmed by QTL analysis of a re-constructed ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 population. Then, F_2 plants in which recombination events had occurred near *qSKC-1* were identified from the ‘Nipponbare’ $\times$ ‘ZYQ8’ and *rss4* $\times$ ‘ZYQ8’ F_2 populations, and their phenotypic values were confirmed by progeny tests. Eventually, by analyzing recombination events in these recombinants, the *qSKC-1* locus was mapped precisely to 445 kb between markers RM578 and IM8854. These results will facilitate map-based cloning of the *qSKC-1* locus.

Key Words: rice, salt tolerance, shoot K^+ concentration, quantitative trait locus, fine mapping.

Introduction

Salinity is a major abiotic stress for rice (*Oryza sativa* L.), often caused by excessive accumulation of Na^+ in cells. Although Na^+ is a beneficial element for some plants, such as C_4 species, high levels of cytosolic Na^+ are toxic to rice; excess Na^+ can destabilize membranes and proteins and negatively affect many cellular and physiological processes (Horie *et al.* 2012, Munns and Tester 2008, Negrão *et al.* 2011). Intracellular K^+ deficiency and K^+/Na^+ imbalance, caused by Na^+ over-accumulation, are strongly associated with salt sensitivity in plants (Horie *et al.* 2012, Munns and Tester 2008, Negrão *et al.* 2011). Control of Na^+ accumulation and maintenance of a high K^+/Na^+ ratio in plants, especially in shoots, may be essential for improving plant salt tolerance (Hauser and Horie 2008, Horie *et al.* 2012, Ren *et al.* 2005).

In attempts to understand the genetic factors controlling Na^+ and K^+ concentrations, and the Na^+/K^+ ratio, quantitative trait loci (QTLs) associated with these parameters under salt stress have been identified using mapping populations in rice. As a result, more than 100 QTLs, distributed widely along all 12 rice chromosomes, have been identified.

Among them, a few of the QTLs were mapped to the short arm of chromosome 1.

Using a population of F_8 recombinant inbred lines (RILs), derived from a cross between ‘Pokkali’ and ‘IR29’, Gregorio (1997) identified a major QTL (*saltol*) for K^+ and Na^+ absorption and the Na^+/K^+ absorption ratio, flanked by the AFLP markers P3/M9-8 and P1/M9-3 on chromosome 1, and this QTL explained 64.3–80.2% of the total phenotypic variation. Subsequently, Bonilla *et al.* (2002) re-mapped this QTL to a chromosomal region between the simple sequence repeat (SSR) markers RM23 and RM140, by integrating fragment length polymorphism (RFLP) and simple sequence length polymorphism markers onto the linkage map. Furthermore, Niones (2004) and Thomson *et al.* (2010) re-identified and fine mapped the *saltol* locus using the RILs, backcross lines, and near-isogenic lines (NILs) developed from a cross between ‘Pokkali’ and ‘IR29’. This QTL has now been confirmed to be located between the SSR markers RM1287 and RM7075 (Alam *et al.* 2011). In addition, Lin *et al.* (2004) detected several QTLs for Na^+ and K^+ uptake in shoots and roots using an F_2 population derived from a cross between ‘Nona Bokra’ and ‘Koshihikari’, including a major QTL responsible for shoot K^+ concentration (SKC) on chromosome 1 (*SKC1*), which was flanked by the RFLP markers C1211 and S2139 and accounted for 40.1% of the total phenotypic variance. Recently, Haq *et al.* (2010) identified a cluster of QTLs involved in leaf Na^+ concentration and the K^+/Na^+ ratio on

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chromosome 1 in a region flanked by the RFLP markers RG811 and RZ276, using F₈ and F₉ RILs from a cross between the rice varieties ‘Co39’ and ‘Moroberekan’.

Beyond these major QTLs [*saltol*, *SKC1*, and those detected by Haq *et al.* (2010)], there are several QTLs with lesser effects on shoot Na⁺ concentration (SNC), SKC, and the shoot Na⁺/K⁺ ratio (SNKR), which have been mapped to the short arm of chromosome 1 in rice, most in the same general region as *saltol* and *SKC1* (Koyama *et al.* 2001, Pandit *et al.* 2010, Zheng *et al.* 2015). Although it seems likely that these reported major and minor QTLs for the Na⁺ and K⁺ concentrations, and Na⁺/K⁺ ratio are located in adjacent or even the same chromosomal regions, it remains difficult to determine whether they are within the same locus, because most have only been mapped by a preliminary QTL analysis. Further fine mapping is required to confirm the (allelic) relationships among these loci.

Previously, we repeatedly mapped QTLs for SKC and SNC to the short arm of chromosome 1, using three F₂ mapping populations constructed by crossing two salt-sensitive mutants (*rss2* and *rss4*) and their wild-type cultivar ‘Nipponbare’ with an *indica* cultivar ‘Zhaiyeqing8’ (‘ZYQ8’; Deng *et al.* 2015b, Zhou *et al.* 2013). The QTLs, designated *qSKC-1* and *qSNC-1*, are located between SSR markers RM283 and RM312, and explained 8.0–40.6% and 11.5–28.3% of the total phenotypic variation, respectively. For the two loci, the allele derived from ‘ZYQ8’ increased SKC but decreased SNC in each mapping population. Based on those results, we performed fine mapping of *qSKC-1* using extreme individuals from the ‘Nipponbare’ × ‘ZYQ8’ and *rss4* × ‘ZYQ8’ F₂ populations in this study.

Materials and Methods

Plant materials

Two F₂ populations were used. One (*n* = 506) was derived from the cross between ‘Nipponbare’ (*Oryza sativa* L. ssp. *japonica*) and ‘ZYQ8’ (*Oryza sativa* L. ssp. *indica*), from which 204 randomly chosen individuals were used for primary mapping. The other population (*n* = 890) was generated previously by crossing *rss4* with ‘ZYQ8’ (Deng *et al.* 2015b). *rss4* is a salt-sensitive mutant derived from ethyl methanesulfonate-treated ‘Nipponbare’, the decreased salt tolerance of which was conferred by a locus on chromosome 6 but not by *qSKC-1* or *qSNC-1* (Deng *et al.* 2015b).

Seedling cultivation and salt treatment

The methods of seedling cultivation and salt treatment were described previously (Deng *et al.* 2015a, 2015b, Zhou *et al.* 2013). Briefly, uniformly germinated seeds were sown into 96-well plates with removed well bottoms, which had been floating in a black container filled with distilled water for 5 days and were then transferred into Yoshida’s solution (Yoshida *et al.* 1976). Seedlings were cultured in an artificial growth chamber under 28/24°C day/night temperatures, 70% relative humidity, and a 14-h photoperiod with

800 μmol m⁻² s⁻¹ photosynthetically active radiation.

To analyze the salt tolerance of ‘Nipponbare’ and ‘ZYQ8’, 14-day-old seedlings were exposed to Yoshida’s solution containing 120 mM NaCl. After 8 days of treatment, six seedlings each were sampled to determine the ion concentrations, and approximately 48 seedlings each were transferred to Yoshida’s solution to recover for 10 days, after which the survival rates were determined. Five replicates were performed for each analysis.

To evaluate the salt tolerance of the F₂ population derived from ‘Nipponbare’ × ‘ZYQ8’, 12–16 14-day-old seedlings from each F_{2:3} line and their parents were exposed to Yoshida’s solution containing 120 mM NaCl for 8 days, and then the shoots of each line were sampled and mixed to determine ion concentrations.

Progeny tests of the recombinants were performed using 20–24 F_{2:3} individuals from each F₂ plant. After 8 days of exposure to 120 mM NaCl, the shoots of each F_{2:3} seedling were sampled separately for ion concentration analysis.

Determination of K⁺ and Na⁺ concentrations

The K⁺ and Na⁺ concentrations were measured as described previously (Deng *et al.* 2015a, 2015b, Zhou *et al.* 2013). Briefly, the samples were oven-dried for 3 days at 80°C and then extracted in 100 mM nitric acid for 2 h at 90°C after weighing. The K⁺ and Na⁺ concentrations of the extracts were quantified using inductively coupled plasma-optical emission spectrometry (ICP-OES; Perkin-Elmer, Norwalk, CT, USA).

Linkage map construction, QTL analysis

In total, 204 randomly chosen individuals from the ‘Nipponbare’ × ‘ZYQ8’ F₂ population were genotyped using 14 SSR and three insertion/deletion (InDel) markers, and a linkage map of chromosome 1 was constructed from the genotype data using MAPMAKER/EXP 3.0 (Lander *et al.* 1987). The previously reported 184 individuals of the *rss4* × ‘ZYQ8’ F₂ population were genotyped using three InDel markers (IM7685, IM9146, and IM7719), and a linkage map was constructed using the 14 existing SSR markers supplemented with the three InDel markers.

SSR markers were obtained from Gramene (<http://www.gramene.org/>), and the InDel markers were developed based on genomic sequences by BLAST searching the sequences of ‘Nipponbare’ and ‘93-11’ (<http://www.ncbi.nlm.nih.gov/>). The procedures for SSR and InDel analysis, including DNA template preparation, polymerase chain reaction (PCR) amplification, and electrophoresis and silver staining of the PCR products, were described previously (Jing *et al.* 2007).

Composite interval mapping (CIM) using Windows QTL Cartographer 2.5 was used for QTL analysis. The significant logarithm of the odds (LOD) score for detection of QTLs was calculated based on 1,000 runs of randomly shuffling the trait values at an experiment-wise significance level of 0.05 (Churchill and Doerge 1994). In this study, the threshold values of LOD for SKC and SNC were 2.63 and 2.78,

Fig. 1. Comparison of the salt tolerance of 'Nipponbare' ('Nipp') and 'Zhaiyeqing 8' ('ZYQ8'). Fourteen-day-old seedlings were treated with 120 mM NaCl for 8 days, followed by a recovery period of 10 days. (A) Performance of 'Nipp' and 'ZYQ8' under salt stress. (B) Survival rates of 'Nipp' and 'ZYQ8' subjected to salt stress for 8 days. The values are expressed as means $\pm$ standard error of the mean (SEM; $n = 5$). ** indicates significant differences at the 0.01 probability level.

respectively, in the 'Nipponbare' $\times$ 'ZYQ8' F_2 population and 2.66 and 2.57, respectively, in the *rss4* $\times$ 'ZYQ8' F_2 population.

Results

Comparison of salt tolerance in 'Nipponbare' and 'ZYQ8'

At the seedling stage, 'ZYQ8' showed significantly higher salt tolerance than did 'Nipponbare'. After treatment with 120 mM NaCl for 8 days, most 'Nipponbare' plants were severely wilted and could not recover after salt removal, whereas 'ZYQ8' showed only slightly wilting, with >70% of the plants surviving (Fig. 1A, 1B). There was no significant difference in the Na^+ or K^+ concentration between shoots of 'Nipponbare' and 'ZYQ8' before salt treatment (Fig. 2A, 2B). By contrast, 'ZYQ8' accumulated significantly less Na^+ and more K^+ in shoots than 'Nipponbare' under salt stress (Fig. 2A, 2B). After exposure to NaCl stress for 8 days, the concentrations of Na^+ and K^+ in 'ZYQ8' were 0.74 and 0.89 $\mu\text{mol mg}^{-1}$ DW (dry weight),

respectively, whereas those in 'Nipponbare' were 1.34 and 0.73 $\mu\text{mol mg}^{-1}$ DW, respectively (Fig. 2A, 2B).

Identification of the QTL responsible for salt tolerance on chromosome 1

In total, 204 randomly chosen individuals from the 'Nipponbare' $\times$ 'ZYQ8' F_2 population were evaluated for salt tolerance and genotyped with 17 markers covering the whole of rice chromosome 1 for the QTL analysis. Both SKC and SNC in the F_2 population showed continuous distributions after salt stress for 8 days (Fig. 3A, 3B). A major QTL involved in SKC was detected in the interval between the InDel markers IM7685 and IM9146, with IM7719 as the closest marker to the peak (Fig. 4A, Table 1). This QTL explained 23.2% of the phenotypic variance, and the additive effect of the 'ZYQ8'-derived allele increased the SKC by 0.06. One QTL responsible for SNC was mapped to the region flanked by RM283 and IM9146, which explained 19.0% of the phenotypic variance and had an additive effect of 0.21 in terms of reducing the SNC associated with the

Fig. 2. K^+ and Na^+ concentrations in the shoots of 'Nipponbare' ('Nipp') and 'Zhaiyeqing 8' ('ZYQ8') under salt stress. Fourteen-day-old seedlings were treated with 120 mM NaCl for 3 or 8 days. (A) Shoot K^+ concentration (SKC). (B) Shoot Na^+ concentration (SNC). The values are expressed as means $\pm$ standard error of the mean (SEM; $n = 5$), and each replicate consisted of a pool of six plants. ** indicates significant differences at the 0.01 probability level.

Fig. 3. Frequency distribution of K⁺ and Na⁺ concentrations in shoots of the 'Nipponbare' ('Nipp') × 'Zhaiyeqing 8' ('ZYQ8') F₂ population (*n* = 204) under 120 mM NaCl treatment for 8 days. (A) Shoot K⁺ concentration (SKC). (B) Shoot Na⁺ concentration (SNC).

Fig. 4. Chromosomal positions of identified QTLs responsible for shoot K⁺ concentration (SKC) and shoot Na⁺ concentration (SNC) in two F₂ populations. (A) Logarithm of the odds (LOD) score curves for the QTLs responsible for SKC and SNC in the 'Nipponbare' ('Nipp') × 'Zhaiyeqing 8' ('ZYQ8') F₂ population. (B) LOD score curves for the QTL for SKC and SNC in the *rss4* × 'ZYQ8' F₂ population. The continuous and dashed black vertical lines indicate the LOD thresholds for the presence of a quantitative trait locus responsible for SNC and SKC, respectively.

Table 1. Quantitative trait loci (QTLs) for shoot K⁺ concentration (SKC) and shoot Na⁺ concentration (SNC) identified in two F₂ populations by composite interval mapping on chromosome 1

F ₂ population	Trait	QTL	Flanking markers	LOD score	PVE (%)	Additive effect ^a
'Nipp' × 'ZYQ8'	SKC	<i>qSKC-1</i>	IM7685–IM9146	8.2	23.2	−0.06
	SNC	<i>qSNC-1</i>	RM283–IM9146	3.9	19.0	0.21
<i>rss4</i> × 'ZYQ8'	SKC	<i>qSKC-1</i>	IM7685–IM9146	7.9	30.7	−0.06
	SNC	<i>qSNC-1</i>	RM283–IM9146	5.4	19.2	0.17

'Nipp', 'Nipponbare'; 'ZYQ8', 'Zhaiyeqing 8'; LOD, logarithm of the odds; PVE, percentage of phenotypic variance explained.

^a Positive values indicate that the allele increased the SKC or SNC score was derived from 'Nipponbare' or *rss4*, and negative one from 'Zhaiyeqing 8'.

'ZYQ8'-derived allele (Fig. 4A, Table 1). Both of the QTLs were located between RM283 and RM580, where *qSKC-1* and *qSNC-1* had been mapped in the F₂ population

of *rss4* × 'ZYQ8' (Deng *et al.* 2015b).

To confirm the location of *qSKC-1* and *qSNC-1*, three additional InDel markers, IM7685, IM7719, and IM9146, were used to genotype the 184 previously reported individuals from the *rss4* × 'ZYQ8' F₂ population (Deng *et al.* 2015b). As a result, *qSKC-1* and *qSNC-1* were detected between IM7685 and IM9146, and between RM283 and IM9146, respectively, consistent with the results of QTL mapping in the 'Nipponbare' × 'ZYQ8' F₂ population (Fig. 4B, Table 1).

To assess the genetic effects of *qSKC-1* and *qSNC-1*, ANOVA was performed using the genotypes of the InDel marker IM7719, the closest marker to both the *qSKC-1* and *qSNC-1* loci in the 'Nipponbare' × 'ZYQ8' and *rss4* × 'ZYQ8' F₂ populations. In both populations, plants homozygous for the 'Nipponbare' or *rss4* genotype had a significantly lower SKC than did those homozygous for the

Table 2. Shoot K⁺ concentration (SKC) and shoot Na⁺ concentration (SNC) in each group classified by genotypes of the nearest marker to *qSKC-1* and *qSNC-1* (IM7719) in two F₂ populations

F ₂ population	Trait	Mean (μmol mg ⁻¹ DW)		
		1/1 ^a	1/2 ^b	2/2 ^c
'Nipp' × 'ZYQ8'	SKC	0.95c ^d	1.00b	1.06a
	SNC	1.38a	1.24b	1.17b
<i>rss4</i> × 'ZYQ8'	SKC	0.69b	0.75a	0.78a
	SNC	1.48a	1.35b	1.25b

DW: dry weight; 'Nipp', 'Nipponbare'; 'ZYQ8', 'Zhaiyeqing 8'.

^a 1/1 denotes genotype of 'Nipp' and *rss4* in the 'Nipp' × 'ZYQ8' and *rss4* × 'ZYQ8' F₂ population, respectively.

^b 1/2 denotes genotype of 'Nipp' × 'ZYQ8' and *rss4* × 'ZYQ8' F₁ hybrid in the 'Nipp' × 'ZYQ8' and *rss4* × 'ZYQ8' F₂ population, respectively.

^c 2/2 denotes genotype of 'ZYQ8' in two F₂ populations.

^d Mean values with the same letters within a row did not differ significantly according to Tukey's test at P < 0.05.

'ZYQ8' genotype (Table 2). The heterozygous plants had SKC levels similar to those of the plants homozygous for the 'ZYQ8' genotype in the *rss4* × 'ZYQ8' F₂ populations; whereas they had significantly higher and lower SKC than plants homozygous for the 'Nipponbare' and 'ZYQ8' genotypes, respectively, in the 'Nipponbare' × 'ZYQ8' F₂ population (Table 2). The genetic effects of *qSNC-1* in the two F₂ populations were similar to that of *qSKC-1* in the *rss4* × 'ZYQ8' F₂ population, but not in the 'Nipponbare' × 'ZYQ8' F₂ population. In both populations, plants homozygous for the 'ZYQ8' genotype had SNC levels similar to those of the heterozygous plants, and both of them had a significantly lower SNC than the plants homozygous for the 'Nipponbare' or *rss4* genotype (Table 2).

Fine mapping of *qSKC-1*

Given that *qSKC-1* was the only and main-effect QTL for SKC detected in three F₂ populations ('Nipponbare' × 'ZYQ8', *rss2* × 'ZYQ8' and *rss4* × 'ZYQ8'), *qSKC-1*, but not *qSNC-1*, was selected for fine mapping using the extreme sampling strategy. In total, 100 individuals with

extremely low or high levels of SKC from the 'Nipponbare' × 'ZYQ8' F₂ population, containing 506 individuals (including the previous 204 individuals), were selected for recombinant screening using IM7685 and IM9146. Ten and four recombinants were identified between IM7685 and *qSKC-1*, and between IM9146 and *qSKC-1*, respectively (Fig. 5, Table 3). Of these 14 recombinants, 9 and 5 had extremely low and extremely high SKC levels, respectively (Fig. 5, Table 3). To confirm the phenotypes of the 14 recombinants, progeny tests were performed on 20–24 F_{2:3} individuals from each recombinant F₂ plant. All F_{2:3} individuals of the nine recombinants (group N1–N4) with low-extreme phenotypic values of SKC had fixed, low levels of SKC, and those of the five recombinants (group N5–N8) with high-extreme phenotypic values of SKC had fixed, high levels of SKC (Table 3). In addition, progenies from group N1–N4 generally had significantly higher levels of SNC and leaf damage than progenies from group N5–N8, although the progenies from some F₂ recombinants (group N1, N4, N5 and N8) showed continuous distributions across a wide range of phenotypes in terms of SNC (Supplemental Table 1).

To further delimit the region encompassing the *qSKC-1* locus, the InDel marker IM7719 and six additional SSR or InDel markers developed within the region between IM7685 and IM9146 were used to genotype the 14 recombinants (Supplemental Table 2). These analyses identified one *qSKC-1* co-segregating marker (IM8748), along with single recombinant events between RM578 and *qSKC-1* and between IM8854 and *qSKC-1* (Fig. 5, Table 3). The recombinant events increased gradually at markers flanking RM578 and IM8854 (Fig. 5, Table 3). From these data, the *qSKC-1* locus was determined to be within a 445-kb region between the markers RM578 and IM8854 (Fig. 5, Table 3).

Fine mapping of the *qSKC-1* locus was also performed using the previously constructed *rss4* × 'ZYQ8' F₂ population (Deng *et al.* 2015b). In total, 120 individuals with extremely low or high levels of SKC from this F₂ population of 890 individuals were selected for genotyping using IM7685 and IM9146. Ten recombinant events between

Fig. 5. Physical map of the *qSKC-1* locus. Numbers below the linkage map indicate the number of recombination events detected between the *qSKC-1* locus and the respective markers in the 'Nipponbare' ('Nipp') × 'Zhaiyeqing 8' ('ZYQ8') and *rss4* × 'ZYQ8' F₂ populations. Arrows indicate the region encompassing *qSKC-1*.

Table 3. Genotypes of F₂ recombinants in two F₂ populations and the shoot K⁺ concentration (SKC) of their F_{2,3} progenies

F ₂ population	Group ^a	Markers and genotype ^b										SKC (μmol mg ⁻¹ DW) ^c		Frequency distribution of SKC in F _{2,3} plants (μmol mg ⁻¹ DW) ^d					No. ^e
		IM7685	IM7719	IM7852	IM8284	RM578	IM8748	IM8854	IM8916	IM9146	DW ^c	≤0.60	0.61–0.70	0.71–0.80	0.81–0.90	0.91–1.00	1.01–1.10	≥1.11	
'Nipp' × 'ZYQ8'	N1	H	H	H	N	N	N	N	N	N	0.71	2	26	53	10			4	
	N2	H	H	H	H	N	N	N	N	N	0.72	1	21	19	5			2	
	N3	H	H	H	H	H	N	N	N	N	0.74		7	13	2			1	
	N4	N	N	N	N	N	N	H	H	H	0.73		19	24	4			2	
	N5	H	H	H	Z	Z	Z	Z	Z	Z	1.01					18	22	3	2
	N6	H	H	H	H	Z	Z	Z	Z	Z	1.01				1	9	13		1
	N7	Z	Z	Z	Z	Z	Z	H	H	H	0.99				1	8	10	1	1
	N8	Z	Z	Z	Z	Z	Z	Z	Z	H	1.02					13	11		1
rss4 × 'ZYQ8'	'Nipp'	N	N	N	N	N	N	N	N	N	0.78								
	'ZYQ8'	Z	Z	Z	Z	Z	Z	Z	Z	Z	0.97								
	R1	H	H	H	R	R	R	R	R	R	0.71	4	21	17	2				2
	R2	H	H	H	H	R	R	R	R	R	0.69	1	40	26	1				3
	R3	H	H	H	H	H	R	R	R	R	0.73	1	13	26	2				2
	R4	R	R	R	R	R	R	H	H	H	0.70		12	9					1
	R5	R	R	R	R	R	R	R	R	H	0.68	1	8	10	1				1
	R6	H	H	Z	Z	Z	Z	Z	Z	Z	1.01					12	10		1
rss4	R7	H	H	H	H	Z	Z	Z	Z	Z	0.99				2	22	20		2
	R8	Z	Z	Z	Z	Z	Z	H	H	H	1.02					7	12	1	1
	R9	Z	Z	Z	Z	Z	Z	Z	Z	H	1.02				1	27	34	5	3
	'ZYQ8'	R	R	R	R	R	R	R	R	R	0.74	1	4	8	1				
			Z	Z	Z	Z	Z	Z	Z	Z	0.98					8	6		

'Nipp', 'Nipponbare'; 'ZYQ8', 'Zhaiyeqing 8'.

^a Group indicates genotype category.

^b The genotype of the markers are represented by N ('Nipponbare' homozygous), Z ('ZYQ8' homozygous), H (heterozygous), and R (*rss4*), respectively.

^c The value was calculated from the F_{2,3} progenies of all F₂ recombinants with the same genotype category.

^d The number in each range was calculated from F_{2,3} progenies of all F₂ recombinants with the same genotype category.

^e No. represents the number of F₂ recombinants.

IM7685 and *qSKC-1* and six between IM9146 and *qSKC-1* were identified (Fig. 5, Table 3). Of these 16 recombinants, nine (group R1–R5) and seven (group R6–R9) had extremely low and extremely high phenotypic values of SKC, respectively, which were confirmed in progeny tests using $F_{2:3}$ individuals (Table 3). In addition, the progenies from most F_2 recombinants with low-extreme phenotypic values of SKC except group R1, had generally low levels of SNC and leaf damage, and those with high-extreme phenotypic values of SKC except group R6, had generally high levels of SNC and leaf damage (Supplemental Table 1).

These 16 recombinants were used for genotyping employing the seven markers located between IM7685 and IM9146. Consequently, the *qSKC-1* locus was delimited to the interval between RM578 and IM8854, with two recombinant events between RM578 and *qSKC-1* and between IM8854 and *qSKC-1*, consistent with the results of the fine mapping using the ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 population (Fig. 5, Table 3).

Discussion

Rice is a salt-sensitive crop, and its low salt tolerance is associated with excessive Na^+ accumulation and the resulting K^+ deficiency and K^+/Na^+ imbalance in plants (Horie *et al.* 2012, Munns and Tester 2008, Negrão *et al.* 2011). Recent genetic and molecular analyses of Na^+ and K^+ concentrations and the Na^+/K^+ ratio in rice under salt stress suggest that they are generally governed by polygenes. More than 100 QTLs for these physiological parameters of salt tolerance have been detected in rice, but only a few have been cloned or fine-mapped. In the present study, one major QTL (*qSKC-1*) for SKC was confirmed and delimited to a 445-kb region flanked by the markers RM578 and IM8854.

In previous studies, the QTLs for SKC and SNC (*qSKC-1* and *qSNC-1*) were detected in the interval between SSR markers RM283 and RM312 on chromosome 1, using the F_2 populations derived from crosses of *rss2* $\times$ ‘ZYQ8’, ‘Nipponbare’ $\times$ ‘ZYQ8’, and *rss4* $\times$ ‘ZYQ8’ (Deng *et al.* 2015b, Zhou *et al.* 2013). In the present study, based on the linkage maps constructed with 17 SSR and InDel markers using the reconstructed ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 populations and the previously reported *rss4* $\times$ ‘ZYQ8’ F_2 population, the QTLs for SKC and SNC were mapped to the interval between IM7685 and IM9146, and between RM283 and IM9146, respectively. Further association analysis indicated that the marker IM7719 was significantly associated with SKC and SNC in both F_2 populations. According to the linkage map of chromosome 1 (Fig. 4A), the markers IM7685, IM7719 and IM9146 are located between the markers RM283 and RM312. Therefore, both QTLs are located in the same chromosomal region as those identified previously (Deng *et al.* 2015b, Zhou *et al.* 2013). These results confirmed the presence of both *qSKC-1* and *qSNC-1* on chromosome 1. Nevertheless, it was noteworthy that the genetic effects of *qSKC-1* in different F_2 populations were not ex-

actly the same. The heterozygotes had phenotypic values of SKC intermediate between those of the two homozygotes in the ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 population that was constructed in this and previous studies, whereas they tended to have SKC levels similar to the plants homozygous for the ‘ZYQ8’ genotype in both *rss4* $\times$ ‘ZYQ8’ and *rss2* $\times$ ‘ZYQ8’ F_2 populations (Table 2 and data not shown). *rss4* was sensitive to salt stress and accumulated higher levels of Na^+ but lower levels of K^+ in shoots than the wild-type ‘Nipponbare’ under conditions of NaCl stress, which might be attributable to mutation of a single gene (*rss4*) located in the *qSNC-6* QTL on chromosome 6 (Deng *et al.* 2015b). Difference in salt tolerance between *rss4* and ‘Nipponbare’ results in different phenotypic values and frequency distribution pattern in terms of SNC and SKC in the *rss4* $\times$ ‘ZYQ8’ and ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 populations, and thus affects the phenotypic variability of SNC and SKC among the genotypes at the *qSKC-1* in the two populations. Therefore, the statistical difference in genetic effects of different genotypes at *qSKC-1* between the two F_2 populations might be partially conferred by the allele differences at the *rss4* locus between *rss4* and ‘Nipponbare’. Further study should determine the relationship between different alleles at the *qSKC-1* locus.

Besides *qSNC-1*, another QTL for SNC (*qSNC-2*) has been mapped to the long arm of chromosome 2 using the *rss4* $\times$ ‘ZYQ8’ F_2 populations (Deng *et al.* 2015b). This QTL explained 22.8% of the phenotypic variance, and the allele derived from ‘ZYQ8’ increased the SNC. In this study, a genetic map of chromosome 2 was also constructed using the ‘Nipponbare’ $\times$ ‘ZYQ8’ F_2 population, but no QTL for SNC or SKC was detected (data not shown), which was in accordance with the results for the QTL mapping using the *rss2* $\times$ ‘ZYQ8’ F_2 population (Zhou *et al.* 2013). These results suggest that *qSNC-2* is unstable across different genetic backgrounds and environments. An additional study should be performed to identify the effect of this QTL on salt tolerance in rice.

For the two QTLs *qSKC-1* and *qSNC-1*, the allele that increased SKC and decreased SNC was derived for ‘ZYQ8’ in all mapping populations (Deng *et al.* 2015b, Zhou *et al.* 2013). In previous studies, using a double haploid population derived from the cross between ‘ZYQ8’ and a *japonica* variety ‘Jingxi17’, Gong *et al.* (1999) identified a major QTL (*Std*) associated with the number of survival days of the seedlings (SDS) under salt stress on the short arm of chromosome 1. *Std* was located between the markers RG612 and C131, and the allele from ‘ZYQ8’ at this locus increased the SDS. In comparison, *qSKC-1* and *qSNC-1* are located adjacent to the chromosome region encompassing *Std*. In rice, many studies have suggested that SDS is negatively correlated with SNC but positively correlated with SKC in shoots (Cheng *et al.* 2012, Qiu *et al.* 2015, Sun *et al.* 2007, Zang *et al.* 2008). Based on these results, *qSKC-1*, *qSNC-1*, and *Std* may be the same locus associated with salt tolerance in ‘ZYQ8’.

In previous studies, several major QTLs associated with SNC, SKC and SNKR have been mapped to the short arm of rice chromosome 1. To compare the physical positions of these QTLs, we performed BLAST searches using the sequences of markers flanking each of these QTLs (Table 4). The QTL *SKC1* identified by Lin *et al.* (2004) has been cloned by a map-based cloning approach; *SKC1* encodes a HKT-type transporter (Os01g0307500) and is located at 11.46 Mb on chromosome 1. The *saltol* locus has been repeatedly identified and mapped to the chromosomal region between 10.71 and 15.12 Mb that encompasses the *SKC1* locus. The QTL clusters for the K⁺ and Na⁺ concentrations and Na⁺/K⁺ ratio identified by Haq *et al.* (2010) and Pandit *et al.* (2010) were located in the region corresponding to 11.08–14.63 Mb and 10.08–17.52 Mb on chromosome 1, respectively. In our previous studies, the *qSKC-1* locus was initially mapped between SSR markers RM283 (bp 4886915–4887070) and RM312 (bp 10063864–10063971) in two F₂ populations derived from crosses between *rss2* and ‘ZYQ8’ and between ‘Nipponbare’ and ‘ZYQ8’; it was then remapped to the interval flanked by RM283 and RM580 (bp 9606674–9606895) using a F₂ population derived from a cross between *rss4* and ‘ZYQ8’ (Supplemental Table 2; Deng *et al.* 2015b, Zhou *et al.* 2013). Based on these results, *qSKC-1* is located in the region corresponding to 4.89–10.06 Mb on chromosome 1. In this study, *qSKC-1* was mapped to the interval between IM7685 (bp 7685831–7685967) and IM9146 (bp 9146791–9146925) and was further delimited to a 445-kb region bracketed by the markers RM578 (bp 8411145–8411370) and IM8854 (bp 8855893–8856060), consistent with the results of our previous studies. In comparison, *qSKC-1* was located ~3.0 Mb upstream from the *SKC1* and *saltol* loci, as well as the QTL cluster identified by Haq *et al.* (2010). Thus, *qSKC-1* appears to be a distinct locus from the previously reported major QTLs for salt tolerance. Moreover, it is difficult to determine whether *qSKC-1* and the minor QTLs identified by Koyama

et al. (2001) and Zheng *et al.* (2015) are located at the same locus or different tightly linked loci, because they were mapped preliminarily, and the chromosomal regions encompassing them were greater than 10 Mb.

Recently, Negrão *et al.* (2011) constructed a list of rice genes shown to be involved in salt tolerance by semi-quantitative PCR, real-time PCR, or Northern blotting. Among these candidate genes related to salt tolerance, five are located on the short arm of chromosome 1: *OsDREB2A* (Os01g0165000), *OsLEA5* (Os01g0225600), *OsNTL3* (Os01g0261200), *SALT* (Os01g0348900), and Os01g0355100. Two of these genes [*OsLEA5* (bp 6922131–692278) and *OsNTL3* (bp 8792648–8797365)] are located in chromosomal regions encompassing the *qSKC-1* locus, based on the preliminary mapping, and *OsNTL3* is just within the 445-kb interval where *qSKC-1* was finally mapped. *OsNTL3* encodes a member of the NAM, ATAF, and CUC (NAC) transcription factors, referred to as *OsNAC8* or *ONAC074* (Kim *et al.* 2010). The NAC transcription factors have been found to be involved in responses to salinity in many plants. In rice, several NAC transcription factor family genes, such as *SNAC1*, *OsNAC5*, *OsNAC6* and *ONAC045*, are induced by salt stress, and overexpression of these genes enhances plant salt tolerance (Hu *et al.* 2006, Nakashima *et al.* 2007, Takasaki *et al.* 2010, Zheng *et al.* 2009). Several studies have found that the level of *OsNTL3* transcripts was induced significantly by multiple abiotic stresses, including high salinity, drought, and cold and heat, although the physiological functions and molecular mechanisms of this gene have not been reported (Fang *et al.* 2008, Kim *et al.* 2010). These results suggest that *OsNTL3* is an important candidate gene for *qSKC-1*. However, there are more than 60 other candidate genes annotated in the Rice Annotation Project Database (RAP-DB; <http://rapdb.dna.affrc.go.jp/>) within the 445-kb region encompassing the *qSKC-1* locus, and the accurate identification of this locus is difficult at present.

Additional experiments are needed to map the *qSKC-1*

Table 4. Previously reported quantitative trait loci (QTLs) for K⁺ and Na⁺ concentrations and Na⁺/K⁺ ratio on rice chromosome 1

QTL	Trait	Parentage	Marker flanking	Genomic position (bp) ^a	References
<i>SKC1</i>	SKC	Nona Bokra/Koshihikari	C1211–S2139 (Os01g0307500)	11458955–11463442	Lin <i>et al.</i> (2004), Ren <i>et al.</i> (2005)
<i>saltol</i>	SNC, SKC, SNKR	Pokkali/IR29	P3/M9-8–P1/M9-3 RM23–RM140 RM1287–RM10825 RM1287–RM7075	– 10705545–12302086 10839399–13323350 10839399–15119967	Gregorio (1997) Bonilla <i>et al.</i> (2002) Thomson <i>et al.</i> (2010) Alam <i>et al.</i> (2011)
–	leaf Na ⁺ concentration, leaf K ⁺ concentration, Leaf K ⁺ /Na ⁺ ratio	Co39/Moroberekan	RG811–RZ276	11078494–14627907	Haq <i>et al.</i> (2010)
<i>qNaSH-1.1</i> , <i>qKSH-1.1</i> , <i>qNa/KSH-1.1</i>	SNC, SKC, SNKR	CSR27/MI48	RM294–HvSSR01-46	10077939–17516026	Pandit <i>et al.</i> (2010)
–	Na ⁺ uptake, SKC, SNKR	IR4630/IR15324	–	–	Koyama <i>et al.</i> (2001)
<i>qSKC-1</i> , <i>qSNC-1</i>	SNC, SKC	Changbai10/Dongnong425	RM580–RM9	9606674–23326243	Zheng <i>et al.</i> (2015)

SKC, shoot K⁺ concentration; SNC, shoot Na⁺ concentration; SNKR: shoot Na⁺/K⁺ ratio.

^a The genomic position (bp) was inferred by NCBI blast analysis of the sequence of markers flanking QTLs (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

locus to a more confined chromosomal region using larger mapping populations. Thus, a more precise physical map covering the *qSKC-1* locus is currently being constructed using a larger 'Nipponbare' × 'ZYQ8' F₂ population. Moreover, we are currently developing a set of NILs by backcrossing the recombinants with 'Nipponbare' followed by selfing to eliminate non-target chromosome regions. The constructed NILs will be used not only for further fine mapping of *qSKC-1* but also to identify the relationship between *qSKC-1* and *qSNC-1*. In this and previous studies, *qSKC-1* and *qSNC-1* have always been mapped to the same chromosomal region, and the allele that enhances salt tolerance at the two loci is derived from 'ZYQ8' in all mapping populations, suggesting that *qSKC-1* and *qSNC-1* may be present within the same locus. Based on the results of progeny tests, however, not all the progenies from F₂ recombinants with low-extreme phenotypic values of SKC showed fixed, high levels of SNC, and not all those of with high-extreme phenotypic values of SKC showed fixed, low levels of SNC. One possibility is that there are more than one locus for SNC in the region encompassing *qSKC-1* and *qSNC-1*, and the recombination events among these loci resulted in the inconsistency between SNC and SKC in progenies from some groups (such as groups N1, N8, R1 and R6). Moreover, some additional undetected QTLs for salt tolerance located in other genomic regions may also be involved in the regulation of SNC in individuals of groups N1, N8, R1 and R6, and the combined action of them and *qSNC-1* are responsible for the moderate levels of SNC in these F₂ plants and their progenies. The F₂ individuals of groups N1, N8, R1 and R6 had extremely low or high phenotypic values in terms of SKC but not SNC, which were reliable for fine mapping of the *qSKC-1* locus, but cannot be used for recombinant screening and progeny tests in fine mapping of *qSNC-1*, considering that it is difficult to exclude the influence of other loci for SNC. As a next step, additional experiments are required to confirm the relationship between *qSKC-1* and *qSNC-1*, such as a comparison of Na⁺ and K⁺ concentrations in the NILs and functional analysis of the target *qSKC-1* gene after its isolation using a map-based cloning strategy.

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