Title:

Identification of quantitative trait loci associated with shoot sodium accumulation under low potassium conditions in rice plants

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Na accumulation in rice plants under low K supply

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Abstract

Sodium application has marked beneficial effects on plant growth when the potassium supply is low. Under low K supply, three *japonica* rice (*Oryza sativa* L.) cultivars, Koshihikari, Nipponbare, and Sasanishiki, accumulated more sodium than three *indica* cultivars, IR36, IR64, and Kasalath, and the effect of sodium application on growth was greater in *japonica* Koshihikari plants than in *indica* IR64 plants. A quantitative trait locus analysis using a population of backcross inbred lines derived from *japonica* Koshihikari and *indica* Kasalath identified two significant loci associated with shoot sodium concentration on chromosomes 3 and 6. The quantitative trait locus for shoot sodium accumulation on chromosome 6 was confirmed in a population of chromosome segment substitution lines. The major QTL detected in this study could be useful for increasing crop productivity under low K input.

Key words: *Oryza sativa*, potassium, QTL, sodium accumulation, substitution effect

INTRODUCTION

Potassium (K) is a basic component of crop fertilizers besides nitrogen and phosphorus, and the world demand for K fertilizer is increasing because of population growth and the expansion of intensive agriculture. The annual growth rate of the global demand for K fertilizer was estimated as 3.0% for the period 2007–2011 (FAO 2008) and 3.8% for the period 2011–2014 (Heffer and Prud'homme 2010). Although there is no urgent concern regarding reduced world K reserves, the cost of K fertilizer is increasing because the reserves are concentrated in a few countries (USDA 2011, USGS 2012). Furthermore, excessive application of fertilizers causes environmental pollution. Therefore, it is important to decrease K fertilizer use for both economic and ecological reasons; to minimize K fertilizer use without decreasing crop yields it is important to improve the efficiency with which crops use K.

Sodium (Na) is not an essential element for higher plants; however, application of Na salts is beneficial when plants are grown under low K conditions (Lehr 1953, Flowers and Läuchli 1983, Wakeel et al. 2011). The extent of the beneficial effect of Na salts differs from species to species (Lehr 1953). Takahashi and Maejima (1998) demonstrated that Na application did not counteract the growth reduction in K-deficient maize (*Zea mays* L.) and kidney bean (*Phaseolus vulgaris* L.) plants, but the growth of K-deficient barley (*Hordeum vulgare* L.) plants recovered to up to 78% of that of K-sufficient plants. Cultivar differences with respect to the effect of Na application under low K supply was also found for tomato (*Solanum lycopersicum* L.; Figdore et al. 1987). The extent of growth stimulation by Na salts in different species and cultivars was correlated positively with Na concentration in the shoots (Takahashi and Maejima 1998, Figdore et al. 1987); therefore, it is likely that the ability to accumulate Na is important for the utilization of Na under low K conditions. As Na is ubiquitous and its concentration in the soil solution is

generally higher than that of K (Flowers and Läuchli 1983), enhancement of the ability of crops to take up soil Na will enhance crop productivity.

Rice (*Oryza sativa* L.), one of the most important crops in humid Asia, consumes 13% of world K fertilizer supplies (Heffer 2009), and the grain yield of rice decreased by 10% when K fertilizer was not applied in a long-term field experiment (Momii and Izawa 2007). Studies on the substitution of K by Na in rice plants showed that rice plants took up more Na under low K conditions than under adequate K conditions (Hasegawa et al. 1987, Takahashi and Maejima 1998, Akai et al. 2012), and supplying NaCl made the flaccid rice leaf erect and increased plant dry weight under K-deficient conditions (Yoshida and Castaneda 1969). Takahashi and Maejima (1998) estimated that supplementation with NaCl could reduce the growth reduction caused by K deficiency to 52%. Horie et al. (2007) indicated that the Na transporter OsHKT2;1 contributed to Na uptake under K-deficient conditions.

The final goal of our study is to breed new rice that can take up more soil Na and produce a high yield without K fertilizer application. In this report, we first reveal cultivar differences in shoot Na accumulation in rice under low K conditions and investigate the relation between Na accumulation ability and growth response under low K conditions. We then performed a quantitative trait locus (QTL) analysis to identify the chromosomal regions responsible for the difference in shoot Na accumulation, which could provide a foundation for breeding rice with high Na accumulation ability and therefore high yield under low K fertilizer input.

MATERIALS AND METHODS

Plant materials

Three *japonica* cultivars of rice (*Oryza sativa* L.), Koshihikari, Nipponbare, and Sasanishiki, and

three *indica* cultivars, IR36, IR64, and Kasalath, were used in this study.

Two hybrid populations, a backcross inbred line (BIL) population (Ma et al. 2002) and a chromosome segment substitution line (CSSL) (Ebitani et al. 2005), both derived from a cross between the *japonica* Koshihikari and the *indica* Kasalath, were obtained from the Rice Genome Resource Center (RGRC), Tsukuba, Japan. The BIL population consisted of 182 lines, of which 132 randomly selected lines were used in this study. The CSSLs consisted of 39 lines carrying Kasalath chromosome segments on a genetic background of Koshihikari. The substituted chromosome segments covered most of the 12 chromosomes by 39 lines.

The genotype data of BILs and CSSLs were downloaded from the RGRC website (http://www.rgrc.dna.affrc.go.jp/). The physical position of the marker probes was based on the Os-Nipponbare-Reference-IRGSP-1.0 in the rice annotation project database (Rice Annotation Project 2008).

Culture conditions

Seeds were soaked for 3 days at 30°C in distilled water supplemented with a fungicide (3% w/v, TORIFUMIN; Nippon Soda Co., Ltd., Tokyo, Japan). Ten seeds were sown on a nylon mesh (18 mesh, 24 × 36 mm) stretched on a plastic frame then floated on a culture solution. The plants on the mesh were raised in a growth chamber (NS-280 FHW, Takayama Seisakusyo, Kyoto, Japan) under the following conditions: temperature 30°C, relative humidity 80%, photoperiod 12 h, and light intensity 350 μmol m^{-2} s^{-1}.

The culture solution was prepared in distilled water. The control culture solution contained 0.75 mol m^{-3} $(NH_4)_2SO_4$, 0.25 mol m^{-3} $(NH_4)_2HPO_4$, 0.75 mol m^{-3} KCl, 0.50 mol m^{-3} $CaCl_2$, and 0.50 mol m^{-3} $MgCl_2$. Iron was supplied at 5.0 g Fe m^{-3} as Fe-citrate for the comparison

among rice cultivars and the growth experiment, or FeNa-EDTA for the experiment using BIL and CSSL populations. When FeNa-EDTA was used, the culture solution basically contained Na at 0.09 mol m^{-3}. Micronutrients were supplied according to Arnon's formula (Hewitt 1966). The pH was adjusted to 6.0 with HCl. For the low K treatment, the KCl concentration was decreased. Supplemental Na was added as NaCl.

Comparison of shoot Na and K concentrations among rice cultivars

To evaluate the cultivar difference in shoot Na and K concentration, six cultivars were grown in 2 L of culture solution containing 0.08 or 0.75 mol m^{-3} KCl. NaCl concentration in the culture solution was 0.38 mol m^{-3}. Plants were harvested 10 days after sowing. Plants on a mesh were divided into three groups and subjected to analysis.

Growth experiment

Seedlings of Koshihikari and IR64 were grown on the control culture solution as described above. Seven days after sowing, plants with uniform size were selected and two plants each were transferred to a 40-mL glass vial to start the low K treatment. The plants were fixed in the vial with a piece of sponge. The KCl concentration in culture solution was 0.08 mol m^{-3} and the NaCl concentration was 0 or 0.38 mol m^{-3}. At 3, 7, 10, and 14 days after the beginning of the low K treatment, the roots were gently blotted and the fresh weights of the plants were determined. After fresh weight determination, plants were returned to the freshly prepared culture solution. Plants were harvested 18 days after the beginning of the low K treatment, and shoot dry weights and shoot Na and K concentrations were determined. The experiment was performed with three replications.

Low K treatments for BIL and CSSL populations

For QTL analysis using the BIL population, KCl and NaCl concentrations in the culture solution were 0.08 and 0.38 mol m^{-3}, respectively. Thirty-three lines of BILs with the parent cultivars Koshihikari and Kasalath were grown in a 10-L plastic container and harvested 9 days after sowing. All plants on a mesh were bulked and subjected to analysis.

CSSLs were grown in culture solution containing KCl and NaCl, both at 0.38 mol m^{-3}. Each of the six lines and Koshihikari and Kasalath were grown in a 2-L plastic container and harvested 10 days after sowing. The plants on a mesh were divided into three groups and subjected to analysis.

Na and K determination

At harvest, the plants were separated into shoots and roots, and the shoots were rinsed with distilled water and blotted dry. The shoots were dried in an oven at 70°C for 2 days and weighed. The shoots in a test tube were digested with HNO_3-H_2SO_4 and the digest solution was brought to 20 mL with 100 mol m^{-3} HCl. Concentrations of Na and K were determined by flame photometry (AA-6200, Shimazu Seisakusyo, Kyoto, Japan).

QTL analysis

QTL analyses were performed using QGene software (Nelson 1997). The simple interval mapping method was used and the log likelihood (LOD) score was used to estimate the QTL for traits. Based on permutation tests with 1000 permutations, a threshold of 2.9 ($P < 0.05$) was applied to QTL detection.

RESULTS

Cultivar differences in shoot Na concentration under low K condition

Shoot K and Na concentrations of the six rice cultivars are shown in Fig. 1. For both K and Na concentration, two-way ANOVA revealed significant differences between treatments and among cultivars, and their interaction was also significant ($P < 0.01$). The shoot K concentration was decreased at the lower K concentration in the medium (Fig. 1). The Na concentration in the culture solution was uniform under both treatments, but there was a significant difference between treatments in shoot Na concentration (Fig. 1). The shoot Na concentration was low in the control (0.75 mol K m^{-3}) condition, but increased at the low medium K concentration. The shoot Na concentration under the low K condition was higher in the *japonica* cultivars (Koshihikari, Nipponbare, and Sasanishiki) compared with the *indica* cultivars (IR36, IR64, and Kasalath).

Contribution of Na accumulation ability to growth

To clarify the significance of Na accumulating ability for better growth under the low K condition, the effect of Na application on the growth rate of Koshihikari, a cultivar that accumulates high levels of Na, and IR64, a cultivar that accumulates low Na levels, was compared. Growth rate was similar in both cultivars when NaCl was not applied (Fig. 2); however, NaCl application significantly increased the fresh weight of Koshihikari plants ($P < 0.05$). The fresh weight of IR64 plants was slightly increased by NaCl application, but the difference between the treatments was not significant. Also, shoot dry weights of 25-day-old plants were not different between cultivars when NaCl was not applied, but significantly higher

in Koshihikari plants than IR64 plants under NaCl application (Table 1). The shoot NaCl concentration of Koshihikari plants was significantly higher than that of IR64 plants (Table 1).

QTL for shoot Na concentration

A total of 132 BILs and their parent cultivars, Koshihikari and Kasalath, were grown using four containers, each with 10-L capacity. The parent cultivars were included in each container as the reference. The mean shoot K concentration was 292 ± 37.8 μmol g^{-1} DW for Koshihikari and 324 ± 56.4 μmol g^{-1} DW for Kasalath. The mean shoot Na concentration was 333 ± 77.5 μmol g^{-1} DW for Koshihikari and 85.5 ± 19.7 μmol g^{-1} DW for Kasalath. The values varied among containers, however; the ratio of the values of Kasalath to Koshihikari were similar in all the containers: 1.04, 1.20, 1.06, and 1.14 for K, and 0.27, 0.25, 0.25, and 0.25 for Na. To normalize the differences among containers, the BIL/Koshihikari concentration ratios in the same container were used for analysis.

The relative shoot K concentrations of BILs ranged from 0.61 to 1.41. The distribution of relative shoot Na concentrations was broader than that of K, and ranged from 0.24 to 1.67 (Fig. 3).

QTL analysis was performed using 161 markers. A QTL peak was detected for shoot K concentration close to marker C25 on chromosome 3 (LOD = 4.0, Table 2, Fig. 4). At the C25 locus, the Koshihikari allele increased shoot K concentration, and genotype explained 12% of the phenotypic variation. For the shoot Na concentration, two QTL peaks were detected. One was close to marker C721 on chromosome 3 (LOD = 3.0), and the other was close to marker R1167 on chromosome 6 (LOD = 17.1, Table 2, Fig. 4). The Kasalath allele increased shoot Na concentration at the C721 locus, and the Koshihikari allele increased shoot Na concentration at

the R1167 locus. Genotype at the C721 and R1167 loci explained 8% and 74% of the phenotypic variation, respectively.

Sodium accumulation in CSSLs

Figure 5 indicates the relative shoot Na concentrations of CSSLs. Shoot Na concentration was similar to that of the corresponding genetic background, Koshihikari, in most of the CSSLs; however, shoot Na concentrations of SL215 and SL218 were low, and the lower Na concentrations were reproduced in several independent experiments. Both SL215 and SL218 carry the Kasalath-derived allele on chromosome 6; moreover, the shoot Na concentrations of SL216 and SL217, which also carry the Kasalath derived allele on chromosome 6, were similar to that of Koshihikari (Fig. 5). It was therefore considered that the gene responsible for Na accumulation was in the Kasalath-derived chromosome region common to SL215 and SL218, but excluding the Kasalath-derived region in SL216 and SL217. The candidate region was delimited between marker R2549 and the end of the long arm (Fig. 6), and corresponded to the QTL detected in the BIL population. The physical length of the candidate region was approximately 6.4 Mb.

Some lines, such as SL208, SL209, SL210, SL212, SL238, and SL239, showed higher shoot Na concentrations compared with Koshihikari, but the data were not reproducible in parallel experiments (data not shown).

DISCUSSION

Efficient K use by crops is an integral phenomenon of K uptake ability by the root and internal K use efficiency. Internal K use efficiency is determined by several factors, such as translocation

capacity, ability to maintain cytosolic K, and ability to substitute K by Na (Rengel and Damon 2008). Cultivar or ecotypical differences in K use efficiency have been shown in several plant species, such as barley (Pettersson and Jensen 1983), wheat (*Triticum aestivum* L.) (Woodend and Glass 1993), *Brassica oleracea* L. (White et al. 2010), tomato (Figdore et al. 1987), rice (Yang et al. 2003), and *Arabidopsis thaliana* (Harada and Leigh 2006), using dry weight under low-K stress, K concentration in plants, or dry weight production per unit of K as the index.

In this study, we examined the Na accumulation ability of rice plants under low K supply as an index of K use efficiency. Shoot Na concentrations in rice plants were low under the control condition, but were higher at the low K concentration in the culture solution (Fig. 1), consistent with previous findings (Hasegawa et al. 1987, Takahashi and Maejima 1998). Three *japonica* cultivars, however, accumulated more Na compared with three *indica* cultivars (Fig. 1). Though the rice cultivars used in this experiment also differed in their shoot K concentrations under low K supply, the variation was much smaller than that in the shoot Na concentrations (Fig. 1). Such variation among cultivars in Na accumulation has not been described previously, and this result suggests that the Na accumulation ability of *indica* cultivars could be enhanced through crossing with *japonica* cultivars.

Rice cultivars with different Na-accumulating ability showed different growth responses to Na application under the low K supply. The growth rate of Koshihikari, a high Na-accumulating cultivar, was significantly increased by the NaCl application, whereas the effect of NaCl application on IR64, a low Na-accumulating cultivar, was negligible (Fig. 2, Table 1). This result indicates the contribution of Na accumulation ability to improved growth of rice under low K conditions and the possibility that the enhancement of Na-accumulating ability results in a better yield under low K input farming.

The QTL analysis revealed two significant loci responsible for the cultivar difference in shoot Na concentration (Table 2), and a significant QTL for shoot K concentration was also detected despite similar shoot K concentrations in the parent cultivars (Table 2). Wu et al. (1998) performed QTL analyses for growth parameters and K uptake of rice under low K stress and found several QTLs on chromosomes 2, 3, 4, 5, 7, and 8, but none of these correspond to the QTLs detected in this study. This might be because of the difference in the combination of parent cultivars or the different culture conditions.

One of the QTLs for Na uptake was detected near marker C721 on chromosome 3, and the Kasalath allele increased shoot Na concentration at this QTL. Although the shoot Na concentration of a CSSL line SL208, which may carry the Kasalath-derived QTL allele, was higher than that of Koshihikari in the confirmation experiment (Fig. 5), the higher shoot Na concentration was not detected in a similar trial. More intensive examination is necessary in order to validate the QTL.

The other QTL for Na uptake, which was located at the end of chromosome 6, strongly contributed to the phenotypic variation (Table 2, Fig. 4), and this QTL was confirmed in the CSSLs (Fig. 5). In previous studies, several QTLs for Na uptake in rice under NaCl salinity were detected (Koyama et al. 2001, Lin et al. 2004), one of which was located at the end of chromosome 6 (Koyama et al. 2001). However, it is uncertain whether these two QTL genes are identical, because the Na uptake of SL215 and SL218, which are CSSLs with the Kasalath-derived allele at the end of chromosome 6, under 100 mol m^{-3} NaCl salinity was not different from that of Koshihikari in a preliminary experiment (data not shown).

A database search showed the occurrence of a transporter gene, *OsHKT2;1*, in the 6.4 Mb candidate region on chromosome 6. OsHKT2;1 is a Na transporter, which has been characterized

in the context of salt injury in rice (Horie et al. 2001, Golldack et al. 2002). Horie et al. (2007) later showed that OsHKT2;1 was responsible for Na uptake under K-deficient conditions, on the basis of the result that *Tos17* insertion mutants, in which the *OsHKT2;1* gene is disrupted, accumulated less Na. They also showed that *OsHKT2;1* expression was induced in the K-deficient condition. Because of its position on the chromosome and its function, it is likely that *OsHKT2;1* is responsible for the cultivar difference in Na accumulation under low K conditions. However, we observed that the predicted amino acid sequences of OsHKT2;1 were identical in Koshihikari and IR64 (data not shown). Furthermore, Oomen et al. (2012) reported that the main functional properties of OsHKT2;1 were conserved in the six kinds of protein variants that were identified in a survey of 49 diverse rice cultivars. Therefore, it might be the level of expression rather than the functional difference that correlates with the cultivar difference in Na accumulation found in this study. We are examining the expression level of *OsHKT2;1* to verify this possibility. In addition, we are trying to narrow the candidate region using the map-based cloning technique, because many other genes are annotated in the candidate region.

The results reported here indicate that the Na uptake of *indica* cultivars under low K input conditions could be increased, resulting in better performance under low K input. Marker-assisted breeding of near-isogenic lines of the popular *indica* cultivar IR64, in which the QTL region on chromosome 6 is substituted by the allele from Koshihikari, is now in progress. To enhance the Na uptake ability of *japonica* rice further, it is necessary to screen more diverse cultivars and determine high-Na uptake cultivars as a gene source.

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Table 1 Shoot dry weight, shoot K concentration, and shoot Na concentration of 25-day-old rice plants grown under low K condition. Plants were grown and exposed to low K as indicated in the legend to Fig. 2, and harvested 18 days after the beginning of low K treatment.

	Shoot dry weight (mg)	Shoot K conc. (μmol g^{-1} dw)	Shoot Na conc. (μmol g^{-1} dw)
-NaCl			
Koshihikari	122^{b} ±10.3	175^{a} ±10.5	1.13^{c}± 0.74
IR64	114^{b} ±10.5	165^{a} ± 7.18	0.91^{c}± 0.26
+NaCl			
Koshihikari	159^{a} ± 0.81	133^{b} ± 4.62	170^{a} ±18.7
IR64	123^{b} ± 3.00	148^{b} ± 5.90	87.8^{b} ±12.6

Values are means and SD (n=3). Values in a column followed by the same letter are not significantly different ($P < 0.05$, Holm-adjusted t-test).

Table 2 QTLs for shoot K and shoot Na concentrations in rice under low K condition.

Traits	Chr.	Nearest marker	LOD	Additive effect†	Contribution‡ (%)
Shoot K conc.	3	C25	4.0	0.056	12
Shoot Na conc.	3	C721	3.0	-0.13	8
	6	R1167	17.1	0.26	74

†Positive values indicate the allele from Koshihikari increases the phenotypic value.

‡Values indicate the phenotypic variance explained by each QTL

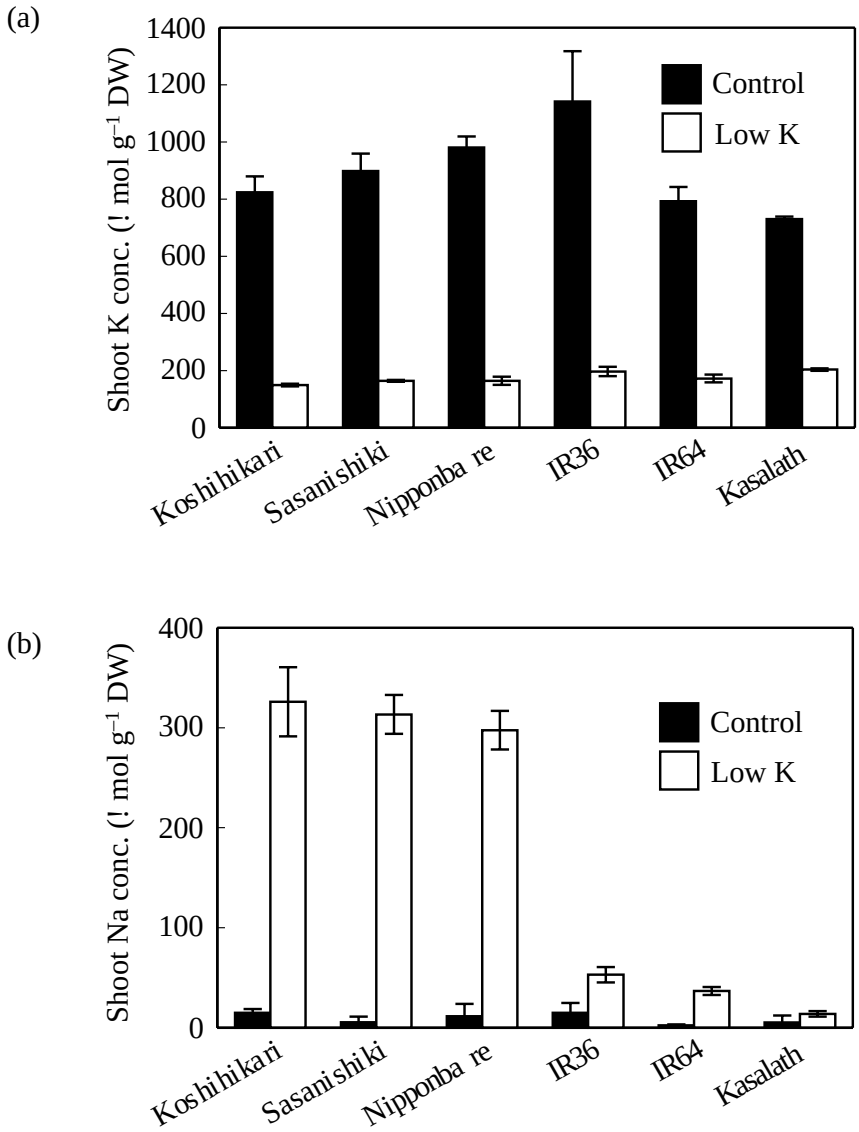

Figure 1 Shoot K and Na concentrations in 10-day-old seedlings of six rice cultivars. (a) Shoot K concentration, (b) shoot Na concentration. Plants were grown in a culture solution containing KCl at 0.75 mol m^{-3} (Control) or 0.08 mol m^{-3} (Low K). The culture solution was supplemented with 0.38 mol m^{-3} NaCl for both K treatment conditions. Values are means with SD (n = 3). For both the K and the Na data, there were significant differences between the K treatment and among cultivars, and the interaction was also significant in two-way ANOVA ($P < 0.01$).

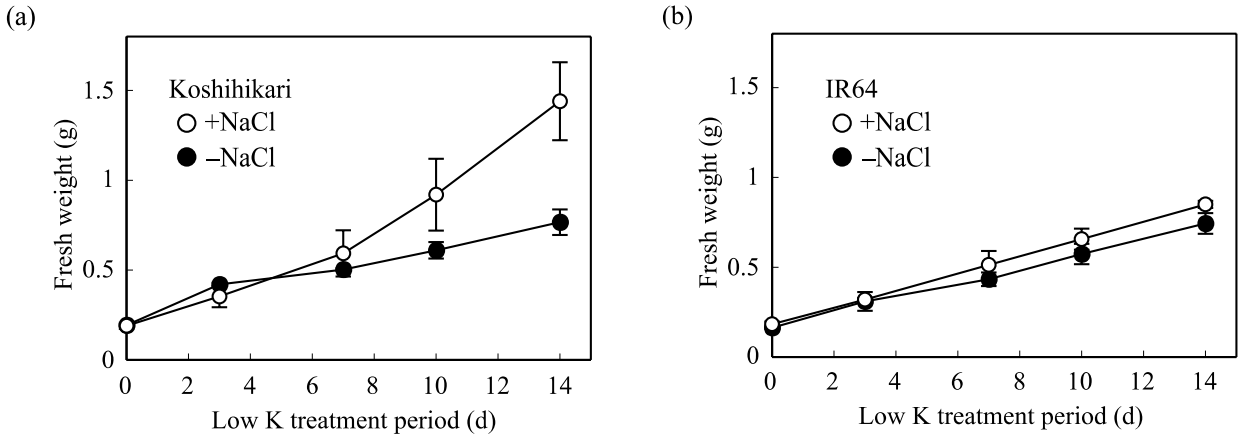

Figure 2 Changes in fresh weight of rice plants under the low K condition. (a) Koshihikari, (b) IR64. Seven-day-old seedlings raised in a K-sufficient condition were transferred to low K culture solution. The K concentration in the culture solution for the low K treatment was 0.08 mol m^{-3} and the NaCl concentrations were 0 (black circle) or 0.38 (white circle) mol m^{-3}. Values are means with SD (n = 3). Significance of the factors are as follows. Koshihikari: NaCl*, period**, NaCl × period**, IR64: $NaCl^{ns}$, period**, NaCl × $period^{ns}$ (ns, not significant; *P < 0.05; **P < 0.01, two-way repeated measures ANOVA).

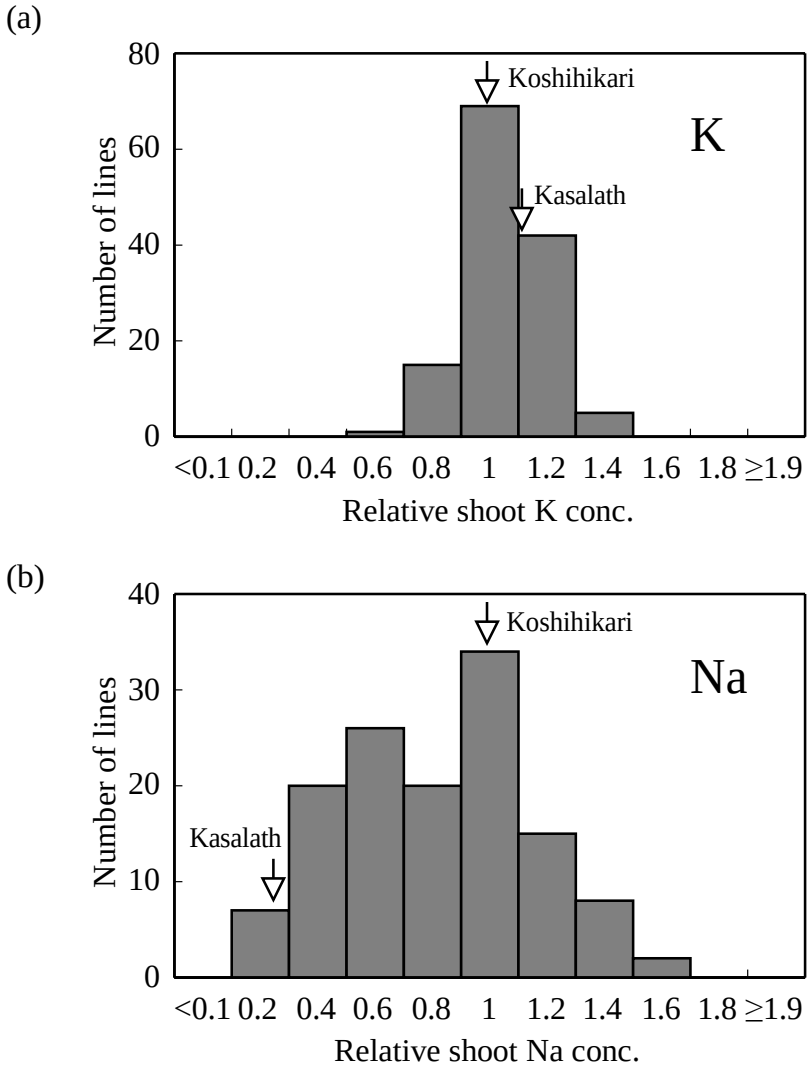

Figure 3 Frequency distributions of shoot K and Na concentrations of 10-day-old seedlings of BIL populations derived from Koshihikari and Kasalath. (a) Shoot K concentration, (b) shoot Na concentration. Arrows indicate Na and K concentrations of the parent cultivars. Plants were grown in a culture solution containing 0.08 mol m^{-3} KCl and 0.38 mol m^{-3} NaCl. K and Na concentrations are shown as the ratio with respect to Koshihikari. Analysis of elements was performed for 10 bulked plants for each line or cultivar.

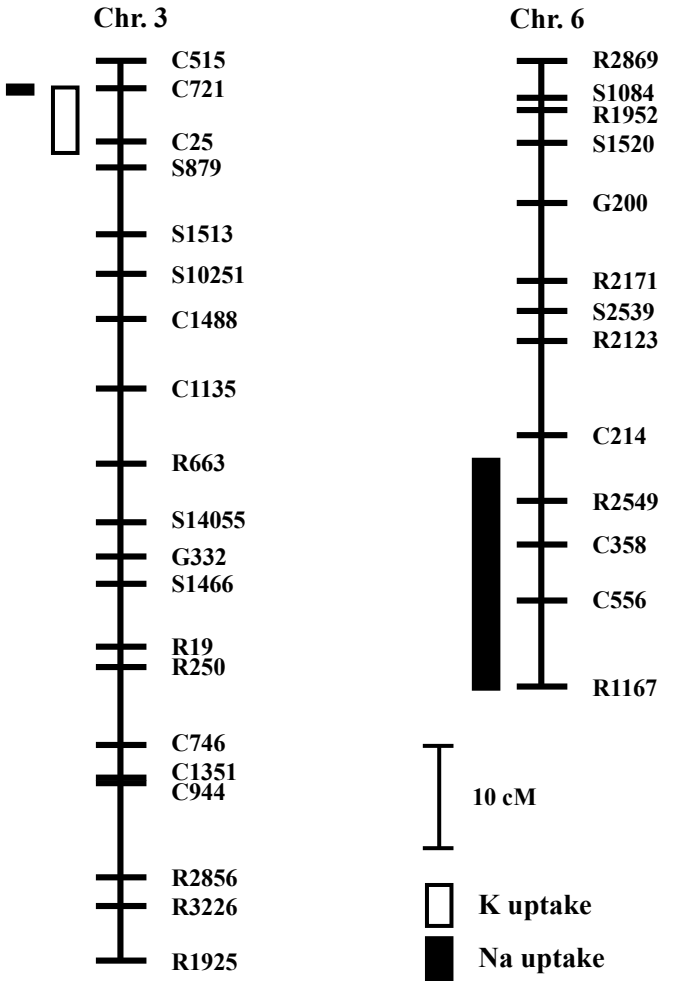

Figure 4 QTLs for shoot K and shoot Na concentrations in BILs derived from Koshihikari and Kasalath. Data for the linkage map were obtained from Rice Genome Resource Center (http://www.rgr.dna.affrc.go.jp/jp/index.html). Marker names are indicated on the right of each linkage map. Bars on the left of the linkage maps indicate chromosome regions in which LOD values exceeded the threshold determined by the 1000 permutation tests.

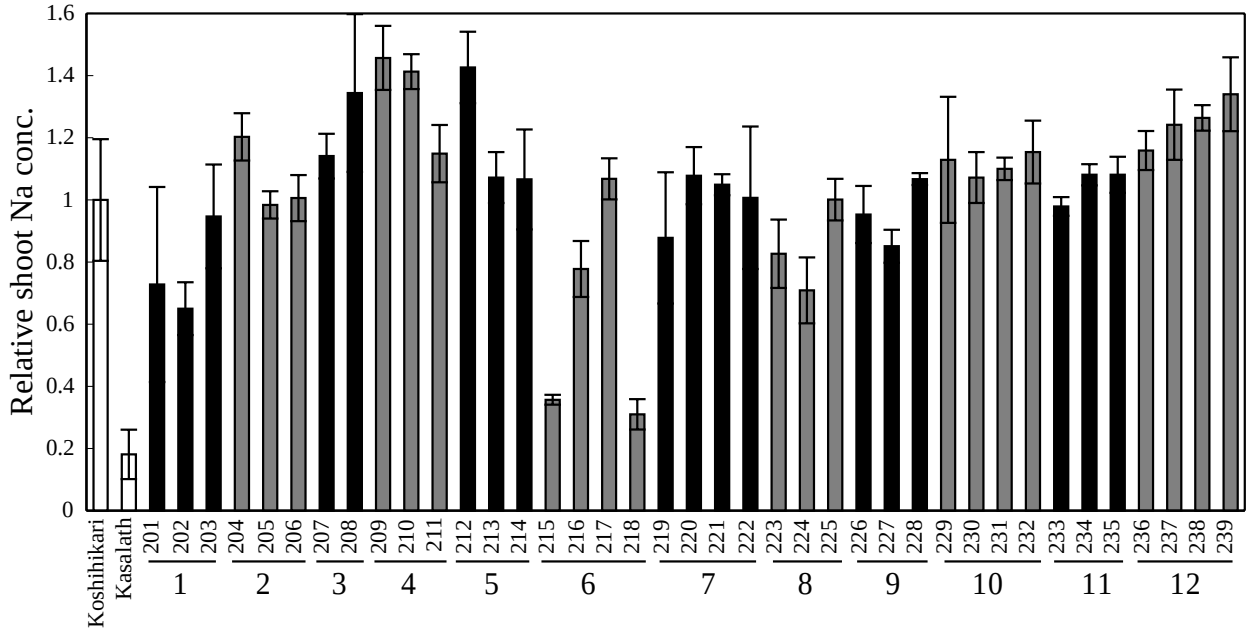

Figure 5 Relative shoot Na concentration of 9-day-old CSSLs (201–239) and their parent cultivars. Plants were grown in culture solution containing KCl at 0.38 mol m^{-3} and NaCl at 0.38 mol m^{-3}. Values are means with SD (n = 3 for CSSLs and n = 21 for Koshihikari and Kasalath). CSSLs carrying a segment from Kasalath on the same chromosome are distinguished by different colors. Numbers under line numbers indicate the chromosome in which the substitution occurred.

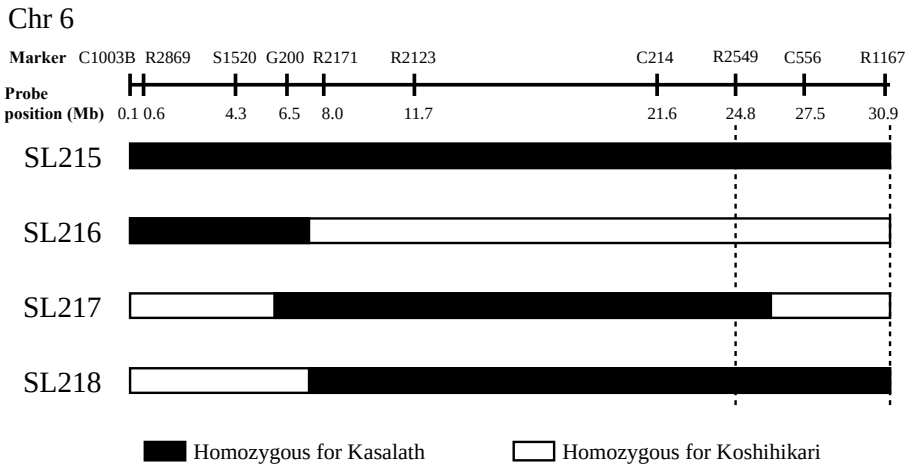

Figure 6 Graphical genotypes of SL215, SL216, SL217, and SL218. Genotype data were obtained from the Rice Genome Resource Center (http://www.rgr.dna.affrc.go.jp/jp/index.html). The physical position of the probes is based on the Os-Nipponbare-Reference-IRGSP-1.0.