Genetic and Phenotypic Characterization of Rice Backcrossed Inbred Sister Lines of Saltol in Temperate Saline Reclaimed Area

Jae-Hyuk Han, Na-Hyun Shin, Je-Hoon Moon, Changhwan Yi, Soo-Cheul Yoo, Joong Hyoun Chin

ABSTRACT Saltol is one of the most well-known quantitative loci (QTLs) for salinity tolerance in rice. It has been used to develop highly tolerant rice varieties in saline and coastal areas in Southeast Asia, South Asia, and Africa. However, the functional activity of Saltol is not well known, and the molecular marker application of readily developed linked markers in Saltol has not always been successful in the rice breeding programs for salinity tolerance improvement. Interestingly, two BC2F9 sister backcrossed inbred lines (BILs), which have been developed by marker-assisted backcrossing utilized the linked markers of Saltol to improve the salinity tolerance of MS11 (a temperate japonica growing in tropical condition). The BILs showed very different phenotypic and stress tolerance, although both contained the Saltol QTL. The genomic similarity of the two BILs was 73%, and we have identified the genomic sites of different genic constitutions between the lines utilizing background genotyping. The stress response of the two BILs showed difference in survival rate, grain yield under highly saline field condition, and SPAD, SES in hydroponic conditions. MS11-SaltolA showed salinity tolerance through Na+/K+ homeostasis with relatively high K+ ion uptake and low Na+ ion uptake in the seedling stage. Further genomic analyses with whole genome resequencing is ongoing to study on gene interactions. The developed highly tolerant MS11-SaltolA can be used as an improved donor in rice molecular breeding for high salinity tolerance.

Keywords Rice, Salinity, Saltol, Backcross inbred lines, SNP markers, Molecular breeding

INTRODUCTION

Climate change has added a new dimension of uncertainty to world food production. Global food requirements are expected to increase by 90% by 2050, and soil salinity in agricultural areas is increasing worldwide due to irrigation with salty water and seawater encroachment on low-lying coastal regions (Glauber 2018). Its impact on crop production is further growing as the global demand for food means agriculture extending into naturally salt-affected lands. Higher salt concentration in soil would increasingly reduce growth. Nevertheless, the extent of yield reduction is hard to predict as saline soils are never uniformly saline across a given area and at depth (Munns et al. 2020).

As land devastation and urban proliferation continues, it is necessary to bring the benefits of agricultural productivity on marginal land, including saline soils (Nutan et al. 2020). Salinity in soil affects almost all aspects of plant development, including germination, vegetative growth, and the reproductive stage. To overcome these problems, the genetics regarding salinity tolerance in rice has been studied for many years. Several QTLs and genes have been reported on morphological and physiological characteristics associated with salinity tolerance. However, the application of QTLs and molecular markers for the development of salt-tolerant rice varieties is still tricky and slow.
The majority of QTLs detected in various mapping populations have been small effect QTLs that have not been validated or used to improve salinity tolerance in breeding programs (De Leon et al. 2017).

Stress tolerance is a matter of high survival rates in the early-stage and high yielding in the maturity of rice. One source for the tolerance in rice, Pokkali showed high tolerance in the reproductive stage. However, the developed breeding lines using it, FL478, had a different allele tolerance in the reproductive stage. However, the developed sources for the tolerance in rice, Pokkali showed high early-stage and high yielding in the maturity of rice. One programs (De Leon et al. 2010). OsHKT1;5 transporter gene associated with salinity in early vegetative growth, which is also known as SKC1, is selected as a major gene of Saltol (Kobayashi et al. 2017). The gene is associated with Na+/K+ homeostasis function and is activated under salinity stress. However, the utilization of Saltol is limitedly useful in breeding programs, as shown in the study of De Leon et al. (2017). A salinity tolerance of Saltol containing isogenic lines (ILs) was not different from non-Saltol lines. Instead, the other QTLs on chromosome 1, such as qSIS1.39 for salt injury score (10.154%) and qKI.3863 for K+ content (10.66%) linked with RM3810 (39.49 Mb on chromosome 1) containing Pokkali alleles, were effective.

MS11 is a good yielding temperate japonica variety in tropical conditions (Ha et al. 2011). However, it shows intolerance against salinity, therefore tolerance improvement utilizing salinity tolerant lines are economically essential to be grown under tropical conditions. Furthermore, well-known and widely used generous donors, Pokkali and Nona Bokra, are low yield and have many undesirable agricultural properties that complicate the breeding process. They are tall, susceptible to lodging, sensitive to photoperiod, and the grains are awned with red pericarp (De Leon et al. 2017).

Recently, various genes were reported by a genome-wide association study (GWAS) using japonica rice panels (Batayeva et al. 2018). In this study, some additional QTLs complementary to Saltol were reported (De Leon et al. 2016, 2017). In our study, the Korean reclaimed area saline field area, the parents, MS11 and MS11-SaltolB1, a sister line, were not tolerant as MS11-SaltolA. Moreover, there might be more QTLs/genes interacting with Saltol and some genes located outside Saltol in MS11. Enhancing salinity tolerance in japonica rice could consider both points in our study. We have listed the genes/QTLs linked to the differential SNP markers among the lines for the following studies.

**MATERIALS AND METHODS**

**Plant materials**

MS11, a japonica rice variety adaptable to tropical regions, was released from the cross between Jinnibyeyo and Cheolweon 46 through the Germplasm Utilization for Value Added (GUVA) breeding program of the Rural Development Administration (RDA) with International Rice Research Institute (IRRI), INGER and PhilRice as collaborating partners (Ha et al. 2011). IR64-Saltol (FL478 allele) is the donor parent for the Saltol QTL. The MS11-Saltol backcross breeding lines (BILs) have been developed by marker-assisted backcrossing utilizing IR64-Saltol (FL478 allele), which was imported from the IRRI through a material transfer agreement.

**Material development and genotyping**

In the BC1F1 generation, Saltol markers were applied to confirm the heterozygosity of Saltol QTL and homozygosity in BC2F2 (Neeraja et al. 2007). In the early generation of backcrossing, background selection was conducted using indica-japonica-specific STS markers (Chin et al. 2007). At BC2F2, a total 192 SNP markers utilizing Fluidigm genotyping system, BioMark™ HD system (Fluidigm, San Francisco, CA, U.S.A.) and 96.96 Dynamic Array IFCs (Fluidigm, San Francisco, CA, U.S.A.), were used for background genotyping. Two 96-plex indica/japonica SNP sets were developed based on polymorphism between indica and japonica in the Crop Molecular Breeding Lab., Seoul National University (Seo et al. 2019). To ensure maximum similarity, phenotypic and agronomic comparisons at all stages of selection after BC2F2 generations have been conducted. A combination of genotypic and phenotypic selection was performed to select the most desirable homozygote plants containing Saltol. At the BC2F2 generation, two sister BILs were selected and the propagated plants of the BILs were further evaluated under hydroponic, and
normal/reclaimed area (Fig. 1).

Total genomic DNA from the leaf tissues were extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thomson 1980). Foreground selection method has been conducted by DNA amplification using the microsatellite markers, RM3412b and RM3206f (Thomson et al. 2016). Polymerase chain reaction (PCR) was conducted in a PCR thermocycler (SimpliAmp, Thermo Scientific, UK). The 20 µL of the reaction mix (CellSafe, Korea) constituted with 30 ng of template DNA, and 10 pmol each of both forward and reverse primers (Bioneer, Korea). The PCR represented an initial 5 minutes denaturation at 95°C, followed by 38 cycles of 30 second denaturation at 95°C, 30 seconds annealing at 55°C and 1 minute extension at 72°C. After 38 cycles, the final extension was done for 7 minutes at 72°C, and the products cooled to 4°C. The amplicons were size separated through electrophoresis in 3.5% agarose gel (Inclone, Korea).

**Screening for salinity tolerance at the seedling stage in the hydroponic system**

Rice seeds were sterilized in 2% (v/v) sodium hypochlorite for 10 minutes and pre-germinated for three days at 30°C in a paper towel under dark condition. The pre-germinated seeds were transferred to styrofoam trays and were grown for three weeks in a hydroponic nutrient solution containing 1 g/L of Jack’s Professional fertilizer 20-20-20 (J.R. Peters, Inc., U.S.A.) supplemented with 300 mg/L ferrous sulfate (De Leon et al. 2015). After 21 days of transplanting, 120 mM NaCl (= 12 dS/m NaCl) was added to the nutrient solution with the pH maintained between 5.0 and 5.1. Control plants were grown simultaneously in a nutrient solution without NaCl. All experiments were conducted in a greenhouse with temperatures set between 25 and 35°C. The entire experiment was performed with a random block design and repeated three times.

Phenotype data was collected from five uniformed seedlings for all replications. Chlorophyll content was measured using a SPAD-502 meter (Spectrum Technologies, Inc., U.S.A.). Stress tolerance of each plant was measured following the Standard Evaluation System for rice (SES) at IRRI with a modification of susceptibility range: 1 (tolerance)-10 (susceptible).

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**Fig. 1.** Development of MS11-SaltoA and B. (A) Breeding scheme of backcross inbred lines (BILs) and foreground genotyping using Saltol QTL linked markers. (B) Background genotyping of BILs in BC_{2}F_{7}.
Field trials

Two BILs with Saltol were named MS11-SaltoA and MS11-SaltoB. The BILs and their parents, MS11 and IR64-Saltol, were sown in the greenhouse on April 18, 2019. The forty-day-old seedlings were transplanted to one seedling per hill at a paddy field (Jangdoek-ri, Namyang-eup, Hwaseong-si, Gyeonggi-do, South Korea), due to cold stress delaying seedling growth. The plants were grown with 30 × 15 cm intervals under normal irrigation condition. Fertilizers (21-11-21 kg/10 a, N-P-K) were applied on the day of transplanting and pesticide was applied conventionally. The same plant materials were sown in the same greenhouse on April 27, 2019. One-month-old seedlings were transplanted to one seedling per hill at a reclaimed area (Chiljeon-ri, Buseok-myeon, Seosan-si, Chungcheongnam-do, South Korea) with a planting interval of 30 × 15 cm in irrigation plot. The plants were cultivated with high-N fertilizer conditions (30-7-9 kg/10 a, N-P-K). Due to the severe salinity in soil, active water circulation was applied. The water salinity, EC and pH were measured using a water quality meter (WM-32EP, TOADKK, Japan).

For, Na\(^+\)-K\(^+\) analysis, the concentration of sodium and potassium in the root and shoot was analyzed for each genotype grown in saline conditions at 4-weeks old plants. Five plants per genotype were rinsed with distilled water and then dried for two days at 65\(^\circ\)C. Each dried tissue was ground by mortar and pestle. A 100-mg sample was digested with 5 mL of nitric acid and 3 mL of hydrogen peroxide at 152-155\(^\circ\)C for three hours in a hood. The digested tissue was diluted into a final volume of 12.5 mL, and the concentration of sodium and potassium were quantified using a flame photometer (PFP7, Jenway, UK). The estimated concentration was calculated from a standard curve. The absolute concentration was computed based on the dilution factor of the sample.

Co-located QTLs and genes for salinity in additional background identified in previous studies

The genes and QTLs located within +/-10kb regions linked to the differential markers in this study between MS11-SaltoA and MS11-SaltoB were listed. They are located within the additional background outside Saltol with non-MS11 alleles. In this study, firstly targeting the readily known function, the genes/QTLs reported previously for salinity tolerance were solely selected (Kumar et al. 2015; De Leon et al. 2017; Bataeyea et al. 2018; Frouin et al. 2018; Naveed et al. 2018; Li et al. 2019). We also included co-located salinity QTLs from the QTL databases, such as TropGENE (https://tropgenedb.cirad.fr/tropgene/JSP/index.jsp) and QTARO (http://qtaro.abr.affrc.go.jp/).

RESULTS

Development of Saltol breeding lines by marker-assisted backcrossing (MABC)

For the development of the Saltol near-isogenic lines, we have followed a MABC approach that was applied for the development of salinity tolerant rice varieties (Fig. 1A). The F\(_1\) plants obtained from the cross of MS11 (used as the recurrent parent) × IR64-Saltol were crossed with the MS11 parent to get more than 200 BC\(_1\)F\(_1\) seeds. In the BC\(_1\)F\(_1\) generation, individual plant heterozygous at the Saltol locus were identified using markers specific for Saltol (RM3412b), followed by background genotyping. The individuals with the highest recurrent parent background were crossed with the recurrent parent to obtain 24 BC\(_2\)F\(_1\) lines. The selected BC\(_2\)F\(_1\) plants were self-pollinated to obtain twelve BC\(_3\)F\(_2\) lines. In BC\(_3\)F\(_2\), the plants were genotyped with the Fluidigm genotyping system (Seo et al. 2019) and the two BILs were selected based on the genotyping results with minimum background. The background recovery with MS11 alleles in MS11-SaltoA and MS11-SaltoB were 73% and 74%, respectively by MABC (Fig. 1B). Except for chromosome 6 and 11, chromosome segments originating from IR64-Saltol were detected. There were some chromosomal differences detected on chromosomes 1, 3, 4, 7, 8, and 9 between the BILs (Fig. 1B).

Screening for seedling stage salt tolerance

Saltol introgression lines were screened in reclaimed area and in hydroponic systems along with the parents, MS11. In the reclaimed area, salt concentration was over 1.2% at early growth stage stage (4-6 weeks after trans-
planting) (Fig. 2C). MS11-SaltolA showed a highly tolerant phenotype compared to MS11-SaltolB (Fig. 2A). The survival rate of MS11-SaltolA was the highest, 91.0% ± 4.4% (total 182 out of 200 plants in all reps). MS11-SaltolB (50 plants, 25.0% ± 10.6%) was even lower than MS11 (91 plants, 45.8% ± 26.4%) (Fig. 2B). Among the reps, MS11-SaltolA showed a smaller variation in survival. In the hydroponics system, the leaves of MS11 became decolorized and dried after treatment (SES = 8.5) (Fig. 3A, D). On the contrary, the average SES score of MS11-SaltolA was 2.9 ± 0.2, which is similar to that of the original salinity tolerance donor, FL478 (Fig. 3A, D). The SPAD values of MS11-SaltolA and FL478 were higher than MS11 and MS11-SaltolB, implying that MS11-SaltolA contains genetic components associated with higher chlorophyll content under salinity stress more than MS11-SaltolB (Fig. 3C). On the other hand, MS11-SaltolA and B both were taller than MS11, implying the basic growth potential before the stress treatment was not different from each other (Fig. 3B).

Field testing

All the plants were transplanted in six rows (around 100 plants per rep) with three reps in normal (Hwaseong) and saline reclaimed areas (Seosan). Under the saline reclaimed area at Seosan, MS11-SaltolA performed 15.26 g plant⁻¹, which is similar to that of MS11 survivors (Table 1). However, due to the high survival rate of MS11-SaltolA, when estimated to 10 a yielding capacity, its potential yield was estimated to 305 kg/10 a, which is equivalent to 87.4% at normal condition. On the contrary, those of MS11 and

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Fig. 2. Salinity screening of BILs (BC₂F₉) in reclaimed land under salinity stress conditions at seedlings stage. (A) 60 Days after transplanting (DAT) of MS11-SaltolA and -SaltolB. (B) Survival rate of MS11 and Saltol QTL introgression lines. (C) Salinity in growth duration. Values within different letters show significantly different within each other at $P < 0.05$ by Duncan’s multiple range test. All values are mean ± standard errors (SE).
MS11-SaltolB yielded 241 kg/10 a (59.8%) and 222 kg/10 a (55.4%), respectively. As shown in Fig 2B, the survival rate of MS11 and MS11-SaltolB were far lower than MS11-SaltolA. However, the performance of the survivors was not poor. The yield per plant of MS11-SaltolB was 40.72 g which was not lower than in normal condition (one could argue that it was even better, because of the higher panicle numbers due to the larger space allowed for them).

In 1,000-grain-weight, MS11-SaltolA in the reclaimed area was 23.3 g (73.0% compared to normal), which is not different from those of MS11 and MS11-SaltolB. Thus, the major genetic factor conferring tolerance in the reclaimed area was tolerance in the early stages associated with seedling survival.

On the other hand, the yield of MS11-SaltolA under normal condition was lower than MS11 and MS11-SaltolB, although the maturity of fertile grains was good according to 1000-grain-weight. The major cause of low yield under irrigation condition was due to smaller panicle numbers per plant than those of MS11 and MS11-SaltolB.

**Estimation of ion concentration in shoot and root**

The plants grown in the reclaimed area during the seedling stage under stress were used to measure the ion concentration of Na\(^+\) and K\(^+\) (Table 2). In shoot, MS11-SaltolA had higher K\(^+\) concentration (16.3 g/kg) and lower
Na\(^+\) concentration (23.9 g/kg) than MS11 and MS11-SaltolB. The Na\(^+\) to K\(^+\) ratio of MS11-SaltolA was significantly lower than the others. However, not like shown in the shoot parts, both Na\(^+\) and K\(^+\) concentrations in roots were higher in MS11-SaltolA than the others. MS11-SaltolB had the highest shoot/root ratio Na\(^+\) concentration. MS11 showed the highest K\(^+\) concentration in the shoot/root ratio. In brief, higher partitioning of Na\(^+\) concentration in the root of MS11-SaltolA was observed.

Reported genes in the differentially present genome blocks of MS11-SaltolA from those of MS11-SaltolB

We have listed the genes/QTLs altering protein sequences linked to differential SNP markers among the lines for the following studies (Table 3). Because the average non-TE gene size of rice is 2,853 bp (Kawahara et al. 2013), around six genes in the blocks have been checked. In all the detected differential genome blocks, genes associated with saline stress-related responses were reported.

DISCUSSION

The Saltol QTL on the short arm of chromosome 1 in rice was identified from a recombinant inbred line population developed between Pokkali and IR29 (Thomson et al. 2010). Saltol and its linked genes have been focused in various researches targeting the improvement of salinity tolerance in rice. In this study, we have developed backcross inbred lines (BILs) containing Saltol locus. In BC\(_2\)F\(_7\), two BILs (MS11-SaltolA and MS11-SaltolB) were selected by foreground and background genotyping. These two BILs and their parents were tested under salinity conditions.

Although these BILs have Saltol, only MS11-SaltolA showed salinity tolerance at seedling stages under reclaimed area and hydroponic conditions (Fig 2, 3). Notably, in the reclaimed area, MS11-SaltolA showed over 90% survival rate despite over 1.2% salinity concentration in the seedling stage (Fig 2B, C). In Table 2, MS11-SaltolA showed salinity tolerance through Na\(^+\)/K\(^+\) homeostasis, with a relatively high K\(^+\) ion uptake and low Na\(^+\) ion uptake in seedling stage. On the other hand, MS11-SaltolB was
Table 2. Na$^+$ and K$^+$ ion concentration at seedling stages grown in reclaimed area.

<table>
<thead>
<tr>
<th>Traits</th>
<th>Ion concentration (g/kg)</th>
<th>MS11</th>
<th>MS11-SaltolA</th>
<th>MS11-SaltolB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>Na$^+$</td>
<td>30.6 ± 0.4a</td>
<td>23.9 ± 1.6b</td>
<td>32.1 ± 2.5a</td>
</tr>
<tr>
<td></td>
<td>K$^+$</td>
<td>11.6 ± 0.7b</td>
<td>16.3 ± 0.3a</td>
<td>8.7 ± 1.1c</td>
</tr>
<tr>
<td></td>
<td>Na$^+$/K$^+$</td>
<td>2.7 ± 0.1b</td>
<td>1.5 ± 0.1c</td>
<td>3.3 ± 0.1a</td>
</tr>
<tr>
<td>Root</td>
<td>Na$^+$</td>
<td>0.9 ± 0.1b</td>
<td>1.3 ± 0.0a</td>
<td>0.4 ± 0.1c</td>
</tr>
<tr>
<td></td>
<td>K$^+$</td>
<td>0.7 ± 0.0c</td>
<td>2.9 ± 0.6a</td>
<td>2.4 ± 0.4ab</td>
</tr>
<tr>
<td></td>
<td>Na$^+$/K$^+$</td>
<td>1.1 ± 0.2a</td>
<td>0.9 ± 0.4a</td>
<td>0.3 ± 0.1c</td>
</tr>
<tr>
<td>Shoot/Root</td>
<td>Na$^+$</td>
<td>32.9 ± 1.4b</td>
<td>17.6 ± 1.8c</td>
<td>64.3 ± 12.3a</td>
</tr>
<tr>
<td></td>
<td>K$^+$</td>
<td>17.3 ± 0.6a</td>
<td>6.1 ± 1.4b</td>
<td>4.3 ± 0.6bc</td>
</tr>
</tbody>
</table>

Values within different letters show significantly different within each other at $P < 0.05$ by Duncan’s multiple range test. All values are mean ± SE.

Table 3. List of genes/QTLs co-located with the linked markers within the background introgressions, which are differently present between MS11-SaltolA and MS11-SaltolB.

<table>
<thead>
<tr>
<th>Chr.</th>
<th>Marker name</th>
<th>QTL</th>
<th>Gene</th>
<th>Trait</th>
<th>Position (Mb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>id1009557</td>
<td>-</td>
<td>-</td>
<td>Na$^+$ concentration in shoot</td>
<td>11.0-14.6</td>
<td>Haq et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>Na$^+$/K$^+$ ratio</td>
<td>11.0-14.6</td>
<td>Haq et al. (2010)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- LOC_Os01g25460</td>
<td>Na$^+$/K$^+$ ratio Stress</td>
<td>14.4</td>
<td>Kumar et al. (2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>id1022407</td>
<td>-</td>
<td>-</td>
<td>Shoot length</td>
<td>33.9-37.7</td>
<td>Takehisa et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>qSKD_1.1</td>
<td>-</td>
<td>SK'D</td>
<td>Shoot dry weight</td>
<td>32.9-38.6</td>
<td>Batayeva et al. (2018)</td>
</tr>
<tr>
<td>4</td>
<td>id4001096</td>
<td>-</td>
<td>K$^+$ uptake</td>
<td>1.0-6.6</td>
<td>Koyama et al. (2001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- LOC_Os01g25460</td>
<td>Na$^+$/K$^+$ ratio Stress</td>
<td>14.4</td>
<td>Kumar et al. (2015)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>cmb0703.2</td>
<td>-</td>
<td>-</td>
<td>Shoot length</td>
<td>2.6-3.3</td>
<td>Takehisa et al. (2004)</td>
</tr>
<tr>
<td>7</td>
<td>cmb0718.0</td>
<td>-</td>
<td>-</td>
<td>Biomass</td>
<td>16.8-17.5</td>
<td>MacMillan et al. (2006)</td>
</tr>
<tr>
<td>7</td>
<td>id7003072</td>
<td>LOC_Os07g32880</td>
<td>Na$^+$/K$^+$ ratio Stress</td>
<td>18.2-21.8</td>
<td>Kumar et al. (2015)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>GW8-AG</td>
<td>-</td>
<td>-</td>
<td>Biomass</td>
<td>24.1-27.7</td>
<td>MacMillan et al. (2006)</td>
</tr>
<tr>
<td>9</td>
<td>TAC1-CT</td>
<td>-</td>
<td>LOC_Os09g35970</td>
<td>Score for salt toxicity symptoms</td>
<td>20.6-20.8</td>
<td>Naveed et al. (2018)</td>
</tr>
</tbody>
</table>

$^a$Chr.: chromosome.  
$^b$QTL: quantitative trait locus.  
$^c$Predicted physical position based on IRGSP 1.0 Nipponbare pseudomolecule (http://rice.plantbiology.msu.edu/).

susceptible to saline damage due to the higher absorption of Na$^+$ ions in shoots than in roots, as shown by the shoot/root ratio Na$^+$ concentrations. MS11-SaltolA showed a high survival rate in seedlings, resulting in salinity tolerance due to the lowest decrease in yield per unit area during the harvest stage (Table 2, Fig. 4). The FL478 allele of Saltol was reported as a tolerance QTL in the seedling stage, which was associated with low Na$^+$/K$^+$ ratio (Thomson et al. 2010). It was confirmed in our study using MS11-SaltolA, however in addition, the phenotypes of MS11-SaltolB implied the presence of the additional genetic components to Saltol even in seedling stage.

Many QTLs and genes associated with salinity have been identified in rice. A major QTL qSKC1, was identified for shoot K$^+$ concentration on chromosome 1 (Thomson et al. 2010). Fine mapping of qSKC1 led to the cloning of the HKT1;5 gene located at the 11.46 Mb region. The gene was implicated in regulating Na$^+$/K$^+$ homeostasis by unloading Na$^+$ ions from xylem for salinity tolerance (Ren et al. 2005). Platten et al. (2013) reported the OsHKT1;5 allele...
for several mechanisms that affect leaf Na\(^+\) concentrations and rare cases of accessions displaying different mechanisms to occur. A transcription factor OsGATA8 within Saltol was reported to increase seed size and tolerance to abiotic stresses in both Arabidopsis and rice. However, as shown in our study, transferring Saltol locus to elite varieties using molecular markers showed a limitation to develop salinity tolerant varieties. The additional QTLs/gens are needed to explain the complete salinity tolerance with Saltol.

Previous studies of De Leon et al. (2016, 2017) reported additional QTLs for salinity. In the study, Saltol and non-Saltol QTLs showed the same level of seedling salinity tolerance. Evaluation of near-isogenic lines containing Saltol locus in the field under salt stress did not show higher yield performance than the susceptible IR29. Thus, we regarded that the possibility of epistatic QTLs in salinity tolerance. Therefore, we compared the differences between MS11-SaltolA and MS11-SaltolB in the background genotyping results. There are some chromosomal differences detected on chromosomes 1, 3, 4, 7, 8, and 9 between the BILs (Fig. 1B). Therefore, we have listed the genes/QTLs altering protein sequences linked to differential SNP markers among the lines for the following studies (Table 3). Most of the genes identified in the differential blocks are related with Na\(^+\)/K\(^+\) homeostasis functions and shoot biomass. It is not well known that the effect of salinity stress to roots in those studies. Thus, the more focus to the functional characters of roots in our future studies. Furthermore, the candidate genes in the differential genome blocks can be listed through resequencing of the lines. Then, the functional assessment of the genes should be studied more accurately. Meanwhile, new recombinants generated from MS11-SaltolA and SaltolB will be useful resources to detect the additional genes.

Regardless of more than the seven years’ efforts, marker-assisted backcrossing seems to develop only simple trait pyramiding. To identify gene interactions, MS11-SaltolA \(\times\) MS11-SaltolB crossing population is being studied. The molecular breeding programs enhancing salinity tolerance should employ more genes outside Saltol to develop the forage rice adoptable for saline areas in temperate regions. The developed highly tolerant MS11-SaltolA can be used as an improved donor in rice molecular breeding for high salinity tolerance in salinity area.

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