Diversity and validation of microsatellite markers in Saltol QTL region in contrasting rice genotypes for salt tolerance at the early vegetative stage

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Abstract

The diversity in microsatellite markers in the Saltol-QTL region among 30 accessions from saline tracts was examined and validated by using 37 breeding lines that were salt tolerant at the seedling stage. The diversity was assessed in terms of morpho-physiological traits related to salt stress (at 12 dS m⁻¹) and polymorphism of molecular marker alleles in the Saltol QTL region. Principal component analysis of the data on microsatellite markers showed that all moderately tolerant accessions collected from coastal areas in two Indian states, West Bengal and Odisha, were distant from a Saltol-introggressed line, namely FL478. That polymorphism of molecular markers in the Saltol QTL region did not generate clusters based on salt tolerance suggests the involvement of other QTLs. The specific marker alleles RM3412 and RM10745 were also found in ‘Pokkali’ and ‘Chettivirippu’ and FL478-specific marker alleles for 11 polymorphic primers in the Saltol region (10.8–12.7 Mb) were found in F₂ lines derived from the cross Annapurna x FL478. The most tightly associated marker, RM10772, was found in a 12.1 Mb region. However, the absence of marker alleles specific to FL478 in the Saltol region of three of the phenotypically tolerant lines clearly suggests that we should look, through whole-genome graphical genotyping, for QTLs other than Saltol for markedly higher salt tolerance at the seedling stage.

Keywords: coastal regions; Oryza sativa; principal component analysis; rice breeding; seedling stage.

Introduction

Although rice is sensitive to salt stress at the seedling stage, it is a preferred crop in salt-affected coastal areas. Rice can withstand water-logging, and standing water helps in diluting and leaching salts from surface soil (Ismail et al., 2008). Therefore, despite their low yields, many landraces are still preferred by farmers in coastal areas. The degree of salt tolerance varies widely in these landraces, and the variability offers an opportunity for varietal improvement for salt tolerance. The Pokkali cultivars have been recognized since long as highly potential salt-tolerant donors and extensively exploited in genetic as well as physiological studies because the cultivars are more salt tolerant than other cultivars. Pokkali refers to a system of rice cultivation under saline conditions in Kerala, a state in southern India along its western coast. These tracts are characterized by prolonged partial flooding, and farmers alternate rice cultivation with shrimp farming (Shylaraj and Sasidharan, 2005). Limitations of conventional breeding have prompted many mapping studies to identify QTLs associated with salt tolerance, a highly polygenic trait in rice (Ammar et al., 2009; Haq et al., 2010; Singh and Flower, 2010). Salt tolerance is governed by several physiological traits, and ion homeostasis which ensures a low Na⁺:K⁺ ratio, is one of the major mechanisms for salinity tolerance at the seedling stage (Munns and Tester, 2008). A major QTL for salt tolerance at the seedling stage, namely Saltol, was mapped on the short arm of chromosome 1 in between RM23 and RM140 (10.7–12.2 Mb). The QTL explains 43% of the variability in shoot Na⁺:K⁺ ratio (Bonilla et al., 2002). Thomson et al. (2010) reported the presence of different ‘Pokkali’ alleles in the Saltol region between 11.0 Mb and 12.2 Mb and suggested that Saltol is controlled by the same gene that controls the SKCI QTL located at 11.46 Mb and first detected in Nona Bokra (Ren et al., 2005). However, lack of tightly linked markers in this region may be the reason why marker-assisted selection based on this QTL has not been widely successful. Many other salt-tolerant varieties besides Pokkali are cultivated along India’s eastern coast, especially in Odisha and West Bengal. The coastal saline tract of Odisha lies along the state’s 480 km coastline, extending 10–20 km inland. In West Bengal, on the other hand, Sundarbans (22° N, 89° E; 0–10 m above the mean sea level), covering parts of India and Bangladesh, is the largest single block of tidal halophytic mangrove forests in the world, a site declared by the UNESCO as a world heritage site. People in Sundarbans depend mostly on rain-fed paddy cultivation and they continue to grow traditional rice varieties because of their stable yields and preferred grain quality. However, coastal areas are increasingly vulnerable to frequent cyclones, high tidal waves, and erratic rainfall. The super cyclone (wind
speeds greater than 220 km h−1) that hit coastal Odisha and
cyclone Aila that hit Sundarbans led to the failure of high-yielding
rice varieties sensitive to salt stress, resulting in a
sharp increase in the extent of uncultivable land (Bhushan, 2012) and, in turn, prompting rice growers and researchers to
cultivate and conserve indigenous salt-tolerant lines. New salt-
tolerant donors from the rice germplasm available in
coastal eastern India are yet to be systematically evaluated
based on their molecular and physiological traits (Mahata et
al., 2010). As part of this effort, it is necessary to estimate

genetic distances in the Saltol QTL region of these landraces
using landraces from the well-characterized ‘Pokkali’ and its
tolerant derivative, FL478, used frequently (Vu et al., 2012)
in the marker-assisted breeding programme to introgress this
QTL into a high-yielding background. Therefore, the present
investigation sought to (a) assess allelic diversity in the Saltol
region for using potential donors that have unique marker
alleles in breeding for salt tolerance at the seedling stage and
(b) validate the Saltol QTL with the associated molecular
markers in salt-tolerant introgressed lines.

Results and discussion

Phenotypic diversity in levels of salt tolerance

Analysis of variance showed significant genotypic
differences (p < 0.01) for shoot dry weight (g), K⁺ and Na⁺
concentration (µg mg⁻¹) in shoot, shoot length (cm), root
length (cm), increase in shoot length (%), Na⁺-K⁺ ratio in
shoots, and SES score for salinity tolerance (Supplementary
Table 1). The standard evaluation system (SES) score was
negatively correlated with shoot dry weight (r = −0.89), K⁺
concentration (r = −0.94), shoot length (r = −0.88), increase
in shoot length (r = −0.94), and root length (r = −0.47) but
positively correlated with Na⁺ concentration (r = 0.88) and
Na⁺-K⁺ ratio (r = 0.94) in shoots (p < 0.01). Some of the
observations were in agreement with those reported earlier,
recommending low Na⁺-K⁺ ratio and Na⁺ concentration as
reliable indicators of tolerance (Lee et al., 2003; Lisa et al.,
2004). The tolerant phenotype was associated with nearly
normal growth, low Na⁺ concentration, high K⁺
concentration, and low Na⁺-K⁺ ratio in shoots. Therefore,
based on these phenotypic data, genotypes were grouped by
the degree of their salt tolerance. A wide variation in
Euclidian distance coefficient values was observed in the
matrix derived from these morpho-physiological traits
(Supplementary Table 2). The UPGMA dendrogram derived from
the coefficient values of all the pairs showed five
distinct clusters (Fig 1). The clustering was governed largely
by Na⁺-K⁺ ratio (Supplementary Table 1). Cluster II and
Cluster IV contained only tolerant and moderately tolerant
genotypes. FL478 was in Cluster II whereas all Pokkali and
Chettivirippu accessions were in Cluster IV. The average
Na⁺-K⁺ ratio of moderately tolerant genotypes in Cluster I
was considerably higher than that of tolerant and moderately
tolerant genotypes in Cluster II and Cluster IV. Cluster V
contained all the susceptible lines. Thus, the clustering was
clearly in accordance with the degree of salt tolerance at the
seedling stage and corroborated the results of an earlier
investigation (Theerawitaya et al., 2011).

Marker-allele diversity in tolerant and susceptible lines

Primers were selected based on their capability to deliver a
clear, positive, reproducible, and polymorphic banding
pattern in all the genotypes. Although all the five primers
designed from the gene and EST clone sequences resulted in
clear and reproducible bands, none was found to be
polymorphic in agarose gel. A total 284 bands distributed in
32 different marker alleles in 30 rice genotypes were
identified using selected polymorphic primers
(Supplementary Table 3) located in the Saltol QTL region
(10.8–12.5 Mb) on the short arm of chromosome 1. Primers
RM10682, RM10719, RM10745, and RM3412 proved more
informative, as shown by their higher PIC and MI values
(Supplementary Table 3) as well as their capability to
produce greater numbers of polymorphic bands. The FL478
haplotype for six important Saltol marker loci, namely
RM493, RM10772, RM10720, RM10745, RM8094, and
RM3412, was detected only in Pokkali (AC39416); that for
RM3412 marker allele at 190 bp was detected only in the
tolerant and moderately tolerant genotypes such as Pokkali
(AC41585, AC39416), Chettivirippu (AC39388, AC39389)
and Murishal; and that for RM10745 marker at 200 bp was
detected only in the tolerant and moderately tolerant

genotypes Pokkali (AC39416), Chettivirippu (AC39388), and
Hasawi. Therefore, the two most important Saltol primers for the FL478 haplotype, namely RM3412 and RM10745, were found in Pokkali (AC39416) and Chettivirippu (AC39388). Earlier reports also indicate that these two primers were among the most potential markers that had been used in marker-assisted selection (Mohammadi-Nejad et al., 2010; Thomson et al., 2010). RM10745 produced another marker allele, at 205 bp, which was present only in highly tolerant genotypes such as Pokkali (AC41585) and Chettivirippu (AC39389). A unique band at 415 bp for locus RM10772 was identified in Chettivirippu (AC39416). All these observations point to a remarkable variability in marker alleles in the Saltol QTL region even within Pokkali and Chettivirippu accessions. A low average value (0.30) of pairwise Jaccard’s similarity coefficients generated from SSR profiling is another manifestation of the wide variability for this region among the genotypes (Supplementary Table 4). Although FL478 and Pokkali (AC39416) were not phenotypically similar (the Euclidian coefficient was 3.5), we observed the closest similarity in the Saltol QTL region of these two genotypes. Single-feature polymorphism in the Saltol region showed that FL478 contained a 0.9 Mb fragment from a Pokkali accession at 10.6–11.5 Mb on chromosome 1, flanked by an IR 29 allele (Kim et al., 2009). Therefore, the allelic similarity between them in the present study is probably due to their possessing a similar chromosomal fragment responsible for tolerance. Low allelic similarities in the Saltol region were seen in the tolerant genotype pairs Pokkali (AC41585) – Talmugur and Nona Bokra – Chettivirippu (AC39389) (Supplementary Table 4). Such genotypes should be tested further to examine the differences in their genomic region responsible for salt tolerance with correspondingly different mechanisms of tolerance. IR 29, a highly susceptible variety, was 62% similar in the Saltol QTL region to the tolerant cultivars SR 26B and Rahnpunjur. The similarities of these tolerant lines with their susceptible counterparts are commensurate with their distances from Pokkali (similarity coefficients of 0.18–0.25) in the Saltol QTL region. Therefore, some other genes or QTLs or their epistatic interaction may explain the tolerance reaction under salt stress. For this reason, the UPGMA dendrogram (Fig 2), which was based only on pairwise similarity coefficient values on marker data, failed to match the earlier dendrogram (Fig 1) based on the data for phenotypic traits under salt stress. As a whole, the genotypes fell broadly into nine clusters in the dendrogram (Figure 2). FL478 shared Cluster V with two accessions, a Pokkali (AC39416) and a Chettivirippu (AC39388), and Cluster VII contained only two highly tolerant accessions, namely Pokkali (AC41585) and Chettivirippu (AC39389), whereas the rest of the clusters comprised genotypes with varying levels of tolerance, from tolerant to highly susceptible. Similar observations on the presence of genotypes with varying levels of tolerance in a single cluster based on Saltol markers has been frequently noted before (Mohammadi-Nejad et al., 2008; Gregorio et al., 2010), which suggests that the trait is polygenic and also strongly supports the probability that QTLs or genes other than Saltol can explain a substantial portion of the phenotypic tolerance or salinity tolerance found among the genotypes tested in this study.

Assessment of molecular and phenotypic diversity in indigenous salt-tolerant germplasm

The genetic diversity in the germplasm was presented visually, in the form of a 2-D plot, by principal component analysis on the basis of phenotypic traits. The first two principal components explained 52% of the genetic variation. Two main groups, comprising tolerant to moderately tolerant genotypes, were identified based on positional proximity in the 2-D biplot (Fig 3). Accessions of Pokkali and Chettivirippu along with a few other tolerant and moderately tolerant lines such as Nona Bokra, Rahnpunjur, and Talmugur were located close to one another whereas FL478, FL496, SR 26B, Kamini, Pattnai, and Hasawi occupied a different location on the 2-D plot. Hosseini et al. (2012) also reported that PCA could group genotypes by their salt tolerance. On the other hand, the first two principal components, which accounted for 65% of the total genetic variability based on SSR marker polymorphism in the Saltol QTL region, were the two axes of the 2-D plot (Fig 4). In contrast to the 2-D plot based on phenotypic diversity, in that based on marker-allele polymorphism at the Saltol QTL region FL478 was located close to Pokkali (AC39416). Principal component analysis also showed the highly salt-tolerant genotypes Pokkali (AC41585) and Chettivirippu (AC39389) close to

![Fig 2. UPGMA dendrogram using Jaccard’s similarity coefficients among 30 rice genotypes based on eight microsatellite markers in the Saltol QTL region of chromosome 1.](image-url)
FL478 in this 2-D plot. The results of PCA were broadly congruent with the grouping observed in the UPGMA dendrogram.

Many genotypes from Sundarbans turned out to be not particularly tolerant; indeed, some of them were highly sensitive to salt stress at the seedling stage. And yet, these genotypes are being cultivated in this region as salt-tolerant cultivars. This anomaly could be explained by their origin and seasonal effects. In Sundarbans, salinity during the wet season occurs initially in patches; at later stages, crop growth is affected mainly by excess water and not as much by salt stress. Therefore, even salt-sensitive genotypes can escape salt stress and yield reasonably well—so long as they can tolerate other forms of abiotic stress such as excess water. The present investigation showed that even the salt-tolerant genotypes were generally only moderately so. Initial growth of Talmugur, Nona Bokra, Rahspunjur, and Patnai under stress was comparable with that of Pokkali and FL478. Similarly, Na⁺ uptake and ion homeostasis, manifested as low Na⁺/K⁺ ratio in shoots in some of the germplasm derived from coastal Sundarbans and Odisha, indicated a physiological similarity in the nature of tolerance although none of these moderately tolerant genotypes was genetically closer to FL478 in the Saltol QTL region. Such allelic diversity has been studied frequently (Mohammad-Nejad et al., 2008; Thomson et al., 2010). Talmugur showed the highest allelic dissimilarity with FL478, indicating its allelic mismatch with Pokkali in the Saltol QTL region. On the other hand, the UPGMA dendrogram and the 2-D plot based on PCA showed all the moderately tolerant genotypes, such as Patnai, Rupshal, Matla, Talmugur, and Kamini from Sundarbans, to be genetically closer to one another. These observations suggest that such genotypes can be used for identifying additional QTLs with similar or dissimilar physiological mechanisms that can complement Saltol in achieving higher levels of salt tolerance at the early vegetative stage.

**Marker validation for Saltol QTL region**

Thirty-seven tolerant and moderately tolerant (SES 3–5) F₁ lines derived from the cross Annapurna × FL478 were subjected to SSR analysis for validating the microsatellite markers in the Saltol QTL region. The Saltol region on chromosome 1 of all the 37 lines was examined (Fig 5). Marker alleles specific to FL478 at different loci in the 10.8–12.7 Mb region on chromosome 1 were found in all the lines except three, and were either homozygous or heterozygous (Supplementary Table 5). Salt tolerance is reported to be dominant over susceptibility (Lang et al., 2010). The 34 tolerant and moderately tolerant lines sharing a common segment from the donor FL478 probably carry the Saltol QTL in homozygous or heterozygous form in this region (Fig 5). However, the absence of marker alleles specific to FL478 in the Saltol region of three of the phenotypically tolerant lines clearly suggests that the Saltol region did not contribute to salt tolerance in these lines. Similarly, in earlier studies, tolerant lines derived from FL478 had no FL478 alleles in the Saltol region (Alam et al., 2010; Thomson et al., 2010; Islam et al., 2012). Their salt tolerance in the absence of the Saltol QTL was believed to be due to some other QTLs inherited from Pokkali. However, whole-genome graphical genotyping of FL478 and its tolerant derivatives is required for gaining further insights into different regions inherited from Pokkali and probably responsible for salt tolerance. All the lines developed in this study (Supplementary Table 6), with or without Pokkali and FL478 alleles in the Saltol QTL region, showed better crop establishment under salt stress and were found suitable for cultivation in the dry season for ensuring higher productivity and profitability in coastal West Bengal and Odisha. Marker-assisted selection has been similarly used in the past to generate breeding lines with Saltol allele from FL478 in the 11.4–12.5 Mb region on chromosome 1 in the background of different popular varieties (Huyen et al., 2012; Vu et al., 2012).

**Materials and methods**

**Plant materials**

Thirty accessions were studied, comprising a few germplasm accessions from Sundarbans, a few from coastal Odisha, and the rest from coastal Kerala, along with the salt-susceptible check IR29 and the salt-tolerant check FL478 (Supplementary Table 1). In addition, 37 advanced breeding lines derived from the cross Annapurna × FL478 by the
pedigree method of selection were also employed to validate the Saltol QTL. These lines were tolerant to moderately tolerant to salinity stress at the seedling stage and known to be high yielding (3–5 t ha\(^{-1}\)) under coastal saline conditions in Odisha during the dry season.

**Experimental details**

The experiment was conducted under a net house at the Central Rice Research Institute (CRRI), Cuttack, India (20°30'N, 85°40' E) in July–August, 2011. The average maximum and minimum temperatures during the study period were 36.5 °C and 24 °C; relative humidity was moderate to high (72%–94%); and the average sunshine hours were low (4.0). The same experiment was repeated with the same set of materials in 2012. Pre-germinated seeds were placed on styrofoam seedling floats resting on plastic trays filled with Yoshida nutrient solution (Yoshida et al., 1976) containing 40 ppm N, 10 ppm P, 40 ppm Ca, 40 ppm Mg, 0.5 ppm Mn, 0.05 ppm Mo, 0.2 ppm B, 0.01 ppm Zn, 0.01 ppm Cu, and 2 ppm Fe.

After three days of seedling growth, NaCl was added to maintain EC of 6 d m\(^{-1}\)lor acclimation and, after another three days, to raise the EC of the nutrient solution to 12 dS m\(^{-1}\). When the susceptible check (IR 29) showed severe symptoms of salt stress, all the genotypes were scored visually on a scale from 1 to 9 (highly tolerant to highly susceptible) based on a modified SES (Gregorio et al., 1997). Mean shoot length of 5 randomly selected plants from each row was recorded when salt stress was introduced (after 6 days of seedling growth as mentioned above) and at the end of the experiment. Plant growth was expressed as the increase in shoot length (as a percentage of the initial length). Shoot samples were dried, powdered, and analysed for sodium and potassium concentration by flame photometry after 48 h of extraction with IN HCl, following the procedure described by Yoshida et al. (1976).

**DNA isolation and amplification**

Fresh leaves (0.5–1.0 g), from a pooled sample of five seedlings from each genotype, were cut into small pieces, crushed in liquid nitrogen, and their DNA isolated using the DNeasy Plant Mini kit (Quagen, Valencia, California). Twelve SSR primers in the Saltol QTL region (McCouch et al., 2002; IRGSP, 2005) and five primers (SAL-1-5) capable of amplifying intron sequences, designed for the present study using Primer3 (http://www.genome.wiunut.edu/cgi/bin/primer/primer3-www.cgi) from Os01g0756700 and OSISAP1 genes, SKC1 mRNA, and two plasma membrane H\(^{+}\) ATPase mRNA EST clone sequences derived from the NCBI database were tested for polymorphism (Supplementary Table 3). Further, polymorphism in Annapurna and FL478 was studied using 61 primers from the Saltol region and locations adjacent to this region on chromosome 1 (Supplementary Table 7). The polymerase chain reaction was conducted in a solution (25 µl) containing 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% gelatine, 200 µM dNTPs, 100 ng µL\(^{-1}\) primer, 1 unit Taq DNA Polymerase (Promega), and 100 ng of the template DNA. The amplification reaction consisted of preheating for 5 min at 94 °C and 35 cycles of 1 min at 94 °C (denaturation), 1 min at 55–61 °C (annealing) and 3 min at 72 °C (elongation), followed by 7 min at 72 °C (extension) in a PCR system (Bio-Rad). The amplified products were separated in 1.5% agarose gel (Promega) containing 0.5 ng mL\(^{-1}\) of EtBr (ethidium bromide). The separated PCR products were made visible under UV light and photographed using a Kodak Electrophoresis Documentation & Analysis System.

**Data analysis**

For microsatellite DNA fingerprinting of the rice genotypes, polymorphism was scored for the presence (1) or absence (0) of bands on agarose gel. Polymorphic information content (PIC) was calculated based on allelic patterns of all the genotypes, and marker index (MI) was calculated by using the formula MI = PIC × proportion of polymorphic bands × average number of loci per assay unit (Powell et al., 1996). This is also called the resolving power of molecular markers that indicates their ability to detect polymorphism for differentiating genotypes. The average proportion of alleles (bands on gels) shared between any two accessions was used for deriving Jaccard’s similarity coefficients matrix, which was then deployed for cluster analysis by UPGMA. A taxonomic distance matrix on standardized morphophysiological dataset was computed using the SIMNIT function and Euclidian distance coefficients. A dendrogram
was generated using UPGMA, based on the genetic distance matrix. Principal component analysis (Jolliffe, 2002) was carried out using eigen vectors and projected genotypes on 2-D scatterplots based on the SSR and morpho-physiological datasets. These analyses were carried out using a software package, namely NTSYS-PC ver. 2.11f (Rohlf, 2000). On the other hand, analysis of variances of all the phenotypic data and correlation coefficients were calculated by standard procedures (Chowdhury et al., 1982). The physical locations of validated molecular markers on the short arm of chromosome 1 in introgression lines from the cross Annapurna × FL478 were marked by using a software package, namely Graphical GenoTyping (GGT 2.0) (Van Berloo, 2008).

**Conclusion**

Diversity analysis based on marker alleles in the *Saltol* QTL region grouped the salt-tolerant genotypes in a pattern that broadly corresponded with their geographical areas. The observed diversity in landraces from Sundarbans (West Bengal) and Odisha in the *Saltol* QTL region can be used effectively by including a genetically diverse group of salt-tolerant donors in the breeding programme to broaden the genetic base for salt tolerance at the vegetative stage. In the present investigation, salt tolerance in the derived lines was associated with the introgression of a fragment with the *Saltol* QTL region from FL478. The absence of FL478 alleles in the *Saltol* region in the derived lines points to the need for genome-wide mapping to identify additional QTLs even from Pokkali. One of the Pokkali accessions (AC41585) was reported to show relatively high salt tolerance at the reproductive stage (Chattopadhyay et al., 2013), which is desirable, along with tolerance at the vegetative stage, in rice varieties to ensure stable yields. The molecular markers used for validating the *Saltol* QTL region in the lines derived from the cross Annapurna × FL478 can be useful in marker-assisted backcross breeding from this particular cross. The diversity of marker alleles in this region opens up further lines of investigation that can lead to fine-mapping this region, which will certainly be useful in marker-assisted backcross breeding across the parental combination.

**Acknowledgements**

The authors are grateful to the National Initiative on Climate Resilient Agriculture (NICRA) Project, ICAR, New Delhi, India, for funding and acknowledge the help received from In-Charge, KVK, Nimpith, West Bengal, India, in acquiring seed material from Sundarban.

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