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Association Mapping of Salinity Tolerance in Rice Using Molecular Markers

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Abstract

A study was conducted to determine the association between simple sequence repeat (SSR) markers and salt tolerance QTLs qSKC-1 and qSNC-7 in rice. Genetic variability for salt tolerance among 172 rice accessions was assessed using two physiological indices viz., shoot potassium concentration (SKC) and shoot sodium concentration (SNC). A wide range of variance for the two indices indicated the existence of substantial genetic diversity for salt tolerance among the germplasm studied. Eight SSR markers near SKC QTL (qSKC-1) locus on chromosome 1 and six SSR markers near SNC QTL (qSNC-7) locus on chromosome 7 were selected. Dendrogram constructed based on physiological traits and SSR marker data grouped the rice accessions into salt tolerant, moderately tolerant and susceptible lines. Marker-trait associations were studied for the two physiological traits using a set of 40 polymorphic SSR loci, 26 polymorphic loci for SKC QTL and 14 polymorphic loci for SNC QTL. Association mapping strategy identified four loci significantly associated with qSKC-1 QTL and two loci associated with qSNC-7 QTL by simple linear as well as multiple regression analyses. Marker loci identified in the present study may be useful for MAS, map based cloning of salt tolerance genes and functional genomics studies.

1. Introduction

Soil salinity is a major problem affecting agricultural production both in irrigated and rainfed ecosystems. Salinity adversely affects quantity and quality of crop produce (Gepstein et al., 2006; Blumwald and Grover, 2006). About 26% of the world's cultivated land is affected by salinity. In Asia alone, 21.5 million hectares of land area is affected by salinity. In India, 63.23 lakh hectares are affected by salinity and about 0.45 million hectares of culturable Command Area (CCA) in Tamil Nadu State is affected by salinity (Ramasamy et al., 2005). The problem of soil salinity is further increasing due to the use of poor quality water for irrigation and poor drainage. Salt stress is also a major problem for rainfed agriculture in coastal areas. Overcrowding, food shortages and land scarcity are compelling the developing countries to bring lands affected by salinity under cultivation of crops. Breeding crop varieties for increased salt tolerance is now considered as a more promising and economical approach than major irrigational management techniques and soil amelioration practices which are beyond the limits of marginal farmers.

Rice continues to hold the key for sustained food security in Asia. Soil salinity is the most widespread soil toxicity problem facing the rice production (Hood-Nowotony and Shaheen, 2005). About 30% of the world's rice growing land is affected by salinity (Prasad et al., 2000). Rice is a moderately salt sensitive crop species. In Asia, the human population is growing at the rate of 2.7%. In order to feed the growing population, genetic improvement of salt tolerance in rice appears to be the most feasible strategy.

Salt tolerant lines were identified in certain traditional *indica* varieties (Yeo et al., 1990), but not in japonica rice (Lee, 1995). In traditional salt tolerant *indica* rice varieties viz., Pokkali, Nona Bokra and Kararata, the salt tolerance is governed by polygenes. Detection of marker-trait association through association mapping (AM) (or linkage dis-equilibrium mapping, LD) in germplasm has potential advantages over classical linkage analysis and QTL mapping (Jannink and Walsh, 2002). AM is a higher resolution mapping of the QTL. It is used to dissect a complex trait in natural populations. Hence, there is no need to develop large segregating generations. AM allows to detect

tight linkage between a marker and a trait. It complements the previously detected QTL region and refines it. The molecular markers associated with a quantitative trait may be used as a reliable marker for MAS. AM studies using natural populations were reported in wheat (Breseghello and Sorrells, 2006), rice (Agrama et al., 2007) and sorghum (Swetha, 2006). In the present study, an attempt was made to identify SSR markers associated with salt tolerance in rice through AM.

2. Materials and Methods

2.1. Materials

A total of 172 rice genotypes consisting of 164 accessions, five varieties and three checks were used in the study**.** Seeds of rice accessions and varieties were obtained from Directorate of Rice Research (DRR), Hyderabad, India. Seeds of the local checks were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamilnadu state, India.

A total of fourteen SSR markers in the shoot potassium concentration (SKC) and shoot sodium concentration (SNC) QTL regions, respectively on rice chromosome 1 and 7 were selected for genotyping (Lin et al., 2004). The rice physical map developed by McCouch et al. (2002) was used as a bridge in selecting the SSR markers, since the SKC and SNC QTLs were originally mapped using RFLP markers by Lin et al. (2004). Eight markers from SKC QTL region and six from the SNC QTL region were selected for marker-trait association

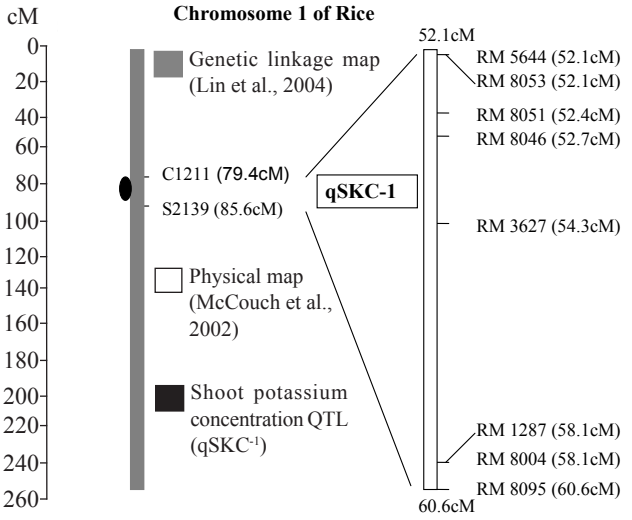

Figure 1: The genetic linkage map showing the location of qSKC-1 QTL under salt stress (140 µM NaCl) in the Nona Bokra/Koshihikari F_2 population (Lin et al., 2004) and physical map of rice showing the region in between the RFLP markers flanking the qSKC-1 QTL along with the SSR markers in that region (McCouch et al., 2002) (Distances in Kosambi centi Morgan (cM))

(Figure 1 and Figure 2). The SSR primer pairs were synthesized from Sigma- Genosys, USA.

2.2. Methods

2.2.1. Screening rice accessions for salt tolerance

The set-up included thermo-cole floats (each measuring 54.5 cm x 35 cm) with 180 circular holes (each 1.75 cm diameter) placed on a rectangular 18 L plastic tray. The seeds were incubated at 45 ºC for a week to break dormancy, and then germinated at 35 ºC for 48 hrs. Uniform pre-germinated seeds were positioned in the holes of the thermo-cole floats, which were placed on trays. For initial three days, the trays were filled with distilled water and later with Yoshida's nutrient solution (Yoshida et al., 1976). Eight days after sowing, the trays were filled with nutrient solution containing 140 µM of sodium chloride (NaCl). Ten days after treatment with NaCl solution, the shoots were harvested and rinsed using distilled water. The shoots were dried at 105 ºC for 2 hrs and dry biomass was taken in an electronic balance. Dried and powdered shoots were then extracted in triple acid (nitric, sulphuric and perchloric acids in the ratio of 9:2:1, respectively) digest. The shoot sodium and potassium concentrations were determined using a Flame Photometer (ELICO^{TM}, CL 361).

2.2.1. Statistical analysis

The descriptive statistics and correlation coefficients were computed for both the physiological traits viz., shoot potassium concentration (SKC) and shoot sodium concentration (SNC). In addition, shoot Na^+/K^+ ratio was also calculated for classifying the rice accessions and varieties into salt tolerant, moderately salt tolerant and salt sensitive lines. Principal component analysis of the traits was employed to examine the

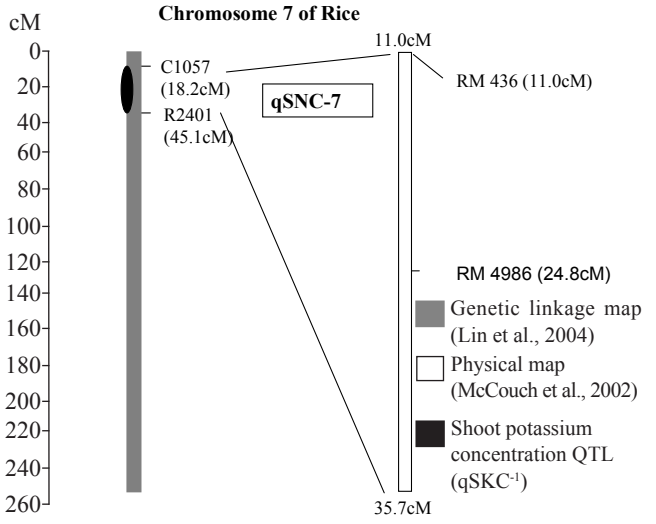

Figure 2: Chromosome 7 of rice showing the SSR markers associated to the qSNC-7 QTL region (Distances in Kosambi centi Morgan (cM))

percentage contribution of each trait to total genetic variation. The principal component analysis was done using the software MINITAB version 15.0. Cluster analysis using the physiological data was done based on dissimilarity matrix obtained using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973). Cluster analysis was carried out using NTSYS-pc version 2.20L software (Rholf, 2005).

2.3. Molecular marker assay

2.3.1. Isolation of genomic DNA

Leaf samples were collected from 15 day old seedlings grown in the transgenic green house, Centre for Plant Molecular Biology, TNAU, Coimbatore. The leaves were cut and kept immediately in -70°C freezer till DNA was extracted. A healthy leaf blade (about 2 cm long) was used to extract DNA following the method described by Kangel et al. (1995) with slight modification. The leaf tissue was cut into small pieces (about half cm long) and was taken in 1.5 mL eppendorf tube. The tube was capped and placed on ice. Four hundred µL of extraction buffer was added. The tissue was ground using glass rod as pestle and again 100 µL of extraction buffer was added. Then 400 µL of chloroform was added and mixed well, spun for 20 minutes at 10,000 rpm. The supernatant was transferred to another 1.5 mL eppendorf tube. To which 400 µL of ice cold iso-propanol was added and mixed gently. Again it was spun for 20 minutes at 10,000 rpm. The supernatant was decanted and the pellet was washed with 70% ethanol and air dried. Finally DNA was suspended in 100 µL of TE and then stored at -20ºC for genotyping. DNA was checked for its purity and intactness by running on 0.8% agarose gel following the protocol of Sambrook et al. (1989) and was visualized in a gel documentation system (Alpha Imager™ 2200, Alpha Innotech Corp., CA, USA). DNA quantity was assessed following the method of Brunk et al. (1979).

2.3.2. PCR assay and agarose gel electrophoresis

After quantification, both visually on agarose gel and flourometrically, the DNA was diluted to a final concentration of 25 ng μl^{-1} for SSR analysis. PCR amplification reaction was done in a total volume 15µl containing, template DNA 2 µL, dNTPs (5 µM) (Sigma- Genosys, USA) 0.70 µL, Primer (Sigma- Genosys, USA) (10µM), 0.8 µL, 10X assay buffer 1.5 µL, *Taq* polymerase (3 units μl^{-1}) (Sigma- Genosys, USA) 0.2 µL.
The reaction mixture was given a short spin for thorough mixing of the cocktail components. Then the 0.2 mL PCR tubes were loaded on to a thermal cycler (PTC-100™ MJ Research Inc., USA). The thermal cyclers were programmed as follows:
The thermal cycler was set at 94°C for 5 min for initial denaturation step. This was followed by 32 cycles of 94°C for 45 seconds for denaturation, 53°C-60°C for 1 min for annealing and 72°C for 1 min for primer extension. Finally 1 cycle of 5 min at 72°C was used for final extension. After PCR, agarose (3.5%) gel electrophoresis was done to separate the amplified products.

2.3.3. Statistical analysis of SSR data

The genotypes were scored for the presence or absence of the SSR primer bands and the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The genetic associations between varieties were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the SSR primers (Jaccard, 1908). Similarity matrix was generated and employed to construct dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to infer genetic relationships and phylogeny (Mathew et al., 2000). Polymorphism information content (PIC) was calculated for the SSR primer pairs based on the formula $PIC=\Sigma pi^2$, where *pi* is the frequency of the i^{th} allele (Smith et al., 1997). In addition, Principal Component Analysis (PCA) based clustering was done using software MINITAB version 15.0, in order to visualize the difference between the individuals graphically in 2D image (Rohlf, 2005).

2.3.4. Marker-trait association

Simple and multiple linear regression analyses were done to detect marker-trait association with traits as dependent, while SSR marker data as independent variables. For simple linear regression, data on individual physiological trait were regressed on whole marker data using MS- Excel program. Multiple regression analysis was performed using a software MINITAB version 15.0 for the subset of markers that had significant correlation on an individual basis with the trait.

3. Results and Discussion

Linkage disequilibrium or association mapping is a novel approach, providing opportunities to explore the genetic variation in natural populations for high-resolution mapping of simple as well as complex traits in crop plants (Skøt et al., 2005). In the present study, an attempt has been made to determine the association between SSR markers and salt tolerance QTLs viz., qSKC-1 and qSNC-7 detected by Lin et al. (2004). Among these QTLs, the shoot potassium concentration QTL (qSKC-1) and shoot sodium concentration QTL (qSNC-7) explained relatively large phenotypic variation, 48.5 and 40.1%, respectively. Thus SSR markers in these QTL regions were utilized in the present study. Shoot potassium concentration (SKC) and shoot sodium concentration (SNC) were determined to assess the genetic variability present among the 172 rice accessions (Table 1). Sodium concentration was low in the salt tolerant accessions and high among the susceptible lines. Likewise the

Table 1: Descriptive statistics of SNC and SKC for 172 rice genotypes under salinity stress

Trait	Mean ± SE	Variance	Co-efficient of variance	Range
Shoot sodium concentration (SNC) (%)	4.449± 0.016	0.044	4.72	4.00-5.18
Shoot potassium concentration (SKC) (%)	4.693± 0.018	0.057	5.10	3.83-5.10

shoot potassium concentration was lower in susceptible lines and higher among tolerant accessions. The shoot Na^+/K^+ ratio was lower in salt tolerant accessions and higher in susceptible lines. Similar findings were reported earlier in rice (Koyoma et al., 2001). Low Na^+/K^+ ratio was mainly due to Na^+ exclusion (Ren et al., 2005).

The descriptive statistics of the two physiological traits were of the greater order and indicated the existence of genetic diversity among the rice accessions, thus providing scope for the improvement of salt tolerance. The principal component analysis of the two physiological traits showed that the first component, SKC contributed up to 55% of the total variance (Table 2). The second component, SNC contributed to 45% of the variance. This analysis showed that SKC explained more phenotypic variance than SNC as reported earlier (Lin et al., 2004).

Dendrogram obtained using physiological indices grouped the 172 rice genotypes into two major clusters (data not shown). The cluster A consisted of salt sensitive rice varieties, Mandya, Vijaya and Rasi. The cluster B had all other rice accessions and varieties. Rice accessions were classified into salt tolerant, moderately tolerant and susceptible genotypes based on their degree of tolerance to salt stress. The sub clusters B3, B4 and B7 had all the salt susceptible accessions. The sub cluster B5 had Pokkali, a salt tolerant landrace from Kerala, India. The sub cluster B8 comprised of both salt susceptible and moderately salt tolerant rice genotypes.

Fourteen SSR markers were used to assess the genetic divergence for salt tolerance among rice accessions (Table 3). The PIC value was higher for RM1287. Hence, RM1287 might be useful to determine genetic variation for salt tolerance among rice germplasm. The mean polymorphism was 97.6% as also reported by Chakravarthi and Naravaneni (2006). The number of alleles produced by the SSR primers ranged from 2-4. Most of the primers produced two alleles. Similar banding was reported earlier (Akagi et al., 1997).

The Jaccard's similarity coefficient for the SSR data set varied from 0.316 to 1.000. The SSR marker profile also resulted in

Table 2: Principal component analysis showing the contribution of SNC and SKC among the 172 rice genotypes subjected to salinity stress

Variable	PC1	PC2
SNC	0.742	-0.671
SKC	0.742	0.671
EIGEN value	1.099	0.900
% Variance	0.550	0.450
Cumulative % variance	0.55	1.00

SNC: Shoot sodium concentration; SKC: Shoot potassium concentration; PC: Principal component

Table 3: SSR marker profile across rice accessions and varieties

Shoot potassium concentration (SKC) QTL / Chromosome 1					
1.	RM 1287	4	4	100	0.742
2.	RM 3627	4	4	100	0.697
3.	RM 5644	3	2	66.66	0.486
4.	RM 8004	5	5	100	0.726
5.	RM 8046	3	3	100	0.614
6.	RM 8051	2	2	100	0.531
7.	RM 8053	2	2	100	0.500
8.	RM 8095	2	2	100	0.498
Shoot sodium concentration (SNC) QTL / Chromosome 7					
9.	RM 427	2	2	100	0.169
10.	RM 436	2	2	100	0.499
11.	RM 2006	2	2	100	0.500
12.	RM 4986	3	3	100	0.674
13.	RM 8007	2	2	100	0.500
14.	RM 8263	3	3	100	0.519

two main clusters, A and B (not shown), in agreement with the cluster analysis of physiological data. The main clusters in turn were divided into sub, sub-sub clusters and so on. Even though, they were being sub clustered, the main clusters clearly exhibited divergence among rice accessions. The cluster A consisted of salt susceptible accessions and cluster B with moderately salt tolerant accessions and varieties Salt tolerant Pokkali was in a separate sub cluster. The rice genotypes at the extreme clusters may be useful in breeding to generate higher variability. The SSR variation in relation to salt tolerance was found to be more among the rice accessions and varieties. To confirm the clustering pattern, the principal component analysis was followed. PCA was employed to exploit the resolving power of ordination. The two-dimensional ordination of the rice accessions and varieties confirmed the clustering pattern obtained by the cluster analysis. Clear distinction of the salt

tolerant and susceptible rice accessions and varieties were evident in two-dimensional ordination.

The association mapping analysis has been performed for the physiological traits, SNC and SKC with SSR markers following simple linear regression and multiple regressions. The regression analysis was capable of identifying those markers, which showed a strong association with QTLs. Eight markers selected for SKC QTL generated 26 polymorphic loci and the six markers for SNC QTL generated 14 polymorphic loci. SKC was regressed with each of the 26 polymorphic loci and SNC was regressed with each of the 14 polymorphic loci. Simple linear regression showed that eight loci were associated with SKC and five loci with SNC. The eight loci significantly associated with SKC by simple linear regression were regressed as a whole against SKC. Likewise, five loci significantly associated with SNC were also regressed as a whole against SNC. The multiple regression analysis revealed that four loci were significantly associated with SKC QTL and two loci with SNC QTL (Table 4).

Table 4: Descriptive statistics of SNC and SKC for 172 rice genotypes under salinity stress

Quantitative trait locus	Associated SSR marker[y]
Shoot potassium concentration QTL (qSKC-1)	$RM8046_{140bp}$, $RM1287_{154bp}$, $RM8053_{209bp}$ and $RM8053_{195bp}$
Shoot sodium concentration QTL (qSNC-7)	$RM436_{81bp}$, $RM4986_{147bp}$

[y]Numbers in lowercase (next to the SSR marker), indicate band size of the associated loci

4. Conclusion

These associated SSR markers may be potential candidates for marker-assisted selection to improve salinity tolerance in rice. These markers may also be used to screen large germplasm to identify additional donors to breed for salt tolerant rice.

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