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Phenotypic screening and Single marker analysis for salinity resistance in rice (*Oryza sativa* L.)

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ABSTRACT: This study focuses on the identification of marker-trait associations for salinity tolerance in rice, a critical factor in abiotic stress breeding. The research employed both in vitro and field screening techniques to assess 36 test entries for their responses to salinity stress. Leveraging 15 SSR markers linked to the *Saltol* QTL on chromosome 1, this study identified eight markers with distinctive banding patterns between resistant and susceptible rice varieties, further used to establish marker-trait associations. Single-factor ANOVA and regression-based analyses were conducted, resulting in the identification of nine significant marker-trait associations, contributing valuable insights into the genetic basis of salinity tolerance in rice. Notably, RM3412 emerged as a marker with a highly significant association, highlighting its potential as a robust tool for marker-assisted breeding programs. These findings provide a foundation for marker-assisted screening and breeding programs aimed at developing salinity-tolerant rice varieties. Through the use of molecular markers, the study demonstrates an efficient and cost-effective alternative to extensive field trials in identifying stress-tolerant rice genotypes, ultimately contributing to enhanced food security and economic sustainability in regions prone to salinity stress. The significant marker-trait associations identified here, particularly the strong correlation with RM3412, RM562, and RM10843, offer promising prospects for advancing rice breeding efforts, ensuring crop resilience to abiotic stress factors, and supporting sustainable agriculture in the face of climate change.

Keywords: Rice, Salinity, Single marker analysis, Microsatellite markers, Marker-trait associations.

INTRODUCTION

Climate change and abiotic stresses are causing shifts in agricultural landscapes. Molecular marker technology allows breeders to rapidly adapt crops to these changing conditions, ensuring that agriculture remains sustainable and productive. Genetic variation is a pre-requisite for any plant breeding programme. Rice is a salt-sensitive crop, at seedling and reproductive stages (Munns and Tester, 2008; Singh and Flowers 2010; Hossain *et al*., 2015). But a vast genetic variability was reported in rice in response to salinity which makes it acquiescent to genetic manipulation for enhanced salinity tolerance (Akbar *et al*., 1972; Flowers and Yeo 1981). Overall, the indica genotypes are more tolerant to salinity than japonica cultivars because of their superior ability of excluding Na^+, absorbing K^+, and maintaining a low Na^+/K^+ ratio in shoot (Gregorio and Senadhira 1993; Lee *et al*., 2003; DeLeon *et al*., 2015). Salinity is a complex quantitative trait with low heritability (Shannon, 1985; Yeo and Flowers 1986) and phenotypic responses of plants to salinity are greatly influenced by environment (Gregorio and Senadhira 1993; Gregorio, 1997; Krishnamurthy *et al*., 2015a, 2015b; Tack *et al*., 2015) and use of landraces for transferring salt tolerant genes into traditional varieties becomes difficult because of the side effects of using landraces. To overcome this there is an increased exploitation of targeted breeding using molecular methods which was made possible in the context of salinity because of the discovery of a major QTL associated with Na^+/K^+ ratio and seedling stage salinity tolerance, named *Saltol*, was located on chromosome 1 (Gregorio, 1997; Bonilla *et al*., 2002). Later, this region was saturated with RFLP and SSR markers. Since, then this region was the most exploited QTL for seedling stage salinity tolerance. Many attempts were made across the globe to introgress *Saltol* QTL into the locally popular varieties (Huyen *et al*., 2012, 2013;

Linh *et al.*, 2012; Usatov *et al.*, 2015; Singh *et al.*, 2016). The popular donor among these studies was FL478, a salt tolerant RIL from Pokkali × IR 29 cross. Using the markers associated with *Saltol* QTL and FL478 as tolerant check, many studies were conducted to group the genotypes for salinity tolerance (Islam *et al.*, 2012; Davla *et al.*, 2013; Ali *et al.*, 2014; Babu *et al.*, 2014; Chattopadhyay *et al.*, 2014; Krishnamurthy *et al.*, 2014, 2015c; Dahanayaka *et al.*, 2015; Kordrostami *et al.*, 2016). Establishing marker-trait associations (MTAs) using phenotypic and marker data is highly useful in investigating the genetic nature of a trait that can aid in the identification of the number and nature of genes/QTLs.

Marker-trait associations allow breeders to precisely target and select for desired traits. Traditional breeding methods are time-consuming, taking several years to develop new crop varieties. Molecular markers expedite the breeding process by enabling the early identification of desirable traits, reducing the time required to develop stress-tolerant varieties. This is crucial in addressing urgent food security and climate change challenges. Thus, in the present study, marker analysis was done with the reported *Saltol* linked markers to identify their linkage to the trait using single marker analysis.

MATERIAL AND METHODS

A. Plant Material

A total of 36 rice genotypes, including reference checks, were employed in this research (refer to Table 1 for details). These genotypes were sourced from the Agricultural Research Station in Kampasagar, Nalgonda, within the state of Telangana. The experimentation was conducted during the Rabi season of 2020-2021. The resistant check for this study was represented by the FL 478 line, while the susceptible check was designated as Pusa44. The evaluation of these genotypes took place under prevailing saline stress conditions, both within naturally occurring infested plots and in controlled *in-vitro* environments.

Table 1: List of the genotypes studied in the experiment.

Sr. No.	Genotype Name	Remarks
1.	IR 69726	Germplasm Collection
2.	IR 77186	Germplasm Collection
3.	NSICRC 240	Germplasm Collection
4.	IRRI 154	Germplasm Collection
5.	GSRIR 2	Germplasm Collection
6.	CT 11891	Germplasm Collection
7.	IR 13F 167	Germplasm Collection
8.	Sahel 177	Germplasm Collection
9.	Jasmine 85	Germplasm Collection
10.	M 202	Germplasm Collection
11.	KPS 10628	Advanced Breeding Line
12.	KPS 10631	Advanced Breeding Line
13.	KPS 10633	Advanced Breeding Line
14.	KPS 10640	Advanced Breeding Line
15.	KPS 10642	Advanced Breeding Line
16.	KPS 10651	Advanced Breeding Line
17.	KPS 10654	Advanced Breeding Line
18.	KPS 10656	Advanced Breeding Line
19.	KPS 10657	Advanced Breeding Line
20.	KPS 10658	Advanced Breeding Line
21.	KPS 10661	Advanced Breeding Line
22.	KPS 10667	Advanced Breeding Line
23.	KPS 10669	Advanced Breeding Line
24.	KPS 10672	Advanced Breeding Line
25.	KPS 10676	Advanced Breeding Line
26.	KPS 10683	Advanced Breeding Line
27.	KPS 10316	Advanced Breeding Line
28.	KPS 10319	Advanced Breeding Line
29.	KPS 10321	Advanced Breeding Line
30.	KPS 10329	Advanced Breeding Line
31.	FL 478	Salinity tolerant check
32.	Pusa 44	Susceptible check
33.	CSR 23	Alkalinity and salinity tolerant check
34.	CSR 36	Alkalinity tolerant check
35.	RNR 11718	Local alkalinity and salinity check
36.	KPS 2874	Local check

B. Screening Methodology

The experimental materials were subjected to screening in a field environment, specifically within a naturally occurring plot exposed to inland salinity stress. The field's soil characteristics included a pH level of 9.30, an electrical conductivity (E.C) of 4.68 dSm^{-1}, and an Exchangeable Sodium Percentage (ESP) value of 88.0. Additionally, all experimental materials underwent screening under controlled in-vitro conditions. In the in-vitro setup, the Standard Evaluation Score (SES) was determined following the guidelines of the IRRI Standard Evaluation System, 2013 (Table 2), and this assessment was performed after subjecting the materials to treatment for 16 days.

To maintain consistency, recommended agricultural practices and essential plant protection measures were diligently implemented to ensure the normal development of the crop in the primary field. Data on ten distinct traits were collected, namely seedling mortality (SM), days to 50% flowering (DFF), plant height (PH), panicle length (PL), number of productive tillers per hill (NPT), number of grains per panicle (NGP), number of filled grains per panicle (NFG), sterility percentage (SP), 1000-grain weight (TW), and yield (in kilograms per hectare, kg ha^{-1}). Notably, data concerning days to 50% flowering and yield (kg ha^{-1}) were recorded at the plot level.

Table 2: Standard Evaluation System scale (IRRI-SES 2013).

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of few leaves	Tolerant
5	Growth severely retarded, most leaves rolled, only a	Moderately tolerant
7	Complete cessation of growth, most leaves dry, some	Susceptible
9	Almost all plants dead or drying	Highly susceptible

C. Genotyping of lines using Saltol linked markers

DNA marker analysis was carried out by using SSR markers linked to *Saltol* QTL. Based on published literature (Gregorio *et al.,* 1997; Nejad *et al.,* 2008; Islam *et al.,* 2012; Ganie *et al.,* 2014) a total of 15 SSR markers linked with *Saltol* QTL on chromosome 1, were used to study the polymorphism among the genotypes (Table 3). Genomic DNA was extracted from young and succulent leaves of the lines using the CTAB method suggested by Murray and Thompson (1980). The quantification of DNA was carried out on 0.8 per cent agarose gel with diluted uncut ladder DNA as standard. The PCR reactions were performed in 10µL reaction volumes using the *Saltol* linked markers. The reaction mixture contained 2µl of template DNA, each 0.5 µl of forward and reverse primers, 4µl TAKARA master mix and 2µl of double distilled water. The amplification profile was maintained at 94°C for 5 min followed by 35 cycles of 94°C for 60 sec, 56°C for 45 sec and 72°C for 45 sec with a final extension of 7 min at 72°C. The amplified PCR products were electrophoretically resolved on a 3% agarose gel using 1×TAE buffer. DNA banding patterns were visualized using BIO-RAD Imaging gel documentation system. The list of the markers used is presented here under. The well-separated and consistently reproducible, amplified DNA fragments were scored as being present (1) or absent (0) for each allele of the SSR markers using a 100 base pair ladder (Takara).

D. Single Marker Analysis

The marker-trait associations were estimated by Single Marker Analysis (SMA) with regression method using single factor standard analysis of variance (ANOVA). The marker trait associations with P-value < 0.05 were identified as significant. The proportion of phenotypic variance of the trait that is accounted by markers was estimated in per cent R^2 value.

Table 3: Details of SSR markers used in this study.

Sr. No.	Marker	Forward Sequence (5' —>3')	Reverse Sequence (3'—> 5')
1	RM8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
2	RM3412	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
3	RM10793	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC
4	RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
5	RM1287	GTGAAGAAAGCATGGTAAATG	CTCAGCTTGCTTGTGGTTAG
6	RM10764	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAGC
7	RM562	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC
8	RM10694	TTTCCCTGGTTTCAAGCTTACG	AGTACGGTACCTTGATGGTAGAAAGG
9	RM140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG
10	RM10772	GCACACCATGCAAATCAATGC	CAGAAACCTCATCTCCACCTTCC
11	RM10745	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
12	RM10843	CACCTCTTCTGCCTCCTATCATGC	GTTTCTTCGCGAAATCGTGTGG
13	RM10864	GAGGTGAGTGAGACTTGACAGTGC	GCTCATCATCCAACCACAGTCC
14	RM10748	CATCGGTGACCACCTTCTCC	CCTGTCATCTATCTCCCTCAAGC
15	RM7075	TATGGACTGGAGCAAACCTC	GGCACAGCACCAATGTCTC

RESULTS AND DISCUSSIONS

Molecular markers can predict a plant's stress tolerance at the molecular level, providing insights into how well it may perform under different environmental conditions. This predictive ability is especially valuable in regions prone to unpredictable or variable abiotic stress factors. In the present study, an attempt was made to assess the effect of salinity on the 36 test entries using invitro and field screening techniques. A set of complex key traits give a single outcome i.e., tolerance to salinity. These SES scores measure overall survival and/or vigour of the plant and therefore are good indicators of performance of the plant under stress (Gregorio *et al*., 1997). Fifteen SSR markers linked to *Saltol* QTL on chromosome 1 spanning from 10.4–15.3 MB were used of which 8 markers showing differential banding patterns between resistant (FL478) and susceptible check (Pusa44) and were further used in association studies. Marker trait associations using 8 SSR markers and 11 traits were identified by single-factor ANOVA and Regression-based analysis were done using Microsoft Excel (Table 4). There were 9 significant marker-trait associations identified based on the value $p<0.05$ viz, RM562 for NGP, RM493 for TW, RM3412 for NGP, and NFP, RM10793 for SM (%), RM10694 for TW, RM10843 for NGP, NFP, and SP (%). Out of eight, six markers showed significant associations with the traits and two SSR markers (RM3412 and RM10843) showed significant associations with more than one trait. Highest significant association i.e. $p<0.001$ was observed in case of marker RM3412 for NFP (0.00126) and $p<0.05$ was observed in case of RM3412 for NGP (0.00384), RM562 for NGP (0.00750), RM10694 for TW (0.01207), RM10793 for SM (%) (0.02584), RM10843 for NGP (0.02709) and RM10843 for NFP (0.03866), RM493 for TW (0.04246) and the least significant association was observed in case of RM10843 for SP (%) (0.04270) and the remaining marker trait associations were non-significant and for these non-significant associations 'p' values ranged from 0.9690 in case of RM7075 for ISES to 0.05946 in case of RM10843 for DFF.

The relative contribution of these markers to the total phenotypic variance for salinity tolerance is given by the R^2 value. This estimate is quite useful because it plays a quite important role in understanding quantitative traits and marker-assisted selection. Higher phenotypic variance values indicate that they control a considerable amount of genetic variation and could be reliable genetic markers for further improvement. For the 9 significant marker-trait associations, the highest R^2 value was observed in the case of RM3412 for NFP (0.26682) followed by RM562 for NGP (0.22684) and the least contribution to the total phenotypic variance with RM10793 for SM (%) 0.00042.

Similar studies of significant marker-trait associations were observed by Senguttuvel *et al.* (2010) in the case of RM493 for Na^+/K^+ ratio while studying 25 diverse genotypes and phenotyping with Yoshida solution under salinity stress. They concluded that along with RM 493, RM 23 and RM 8053 are the most reliable markers for marker assisted selection to identify salinity tolerant in rice and these markers can be used to screen a large set of germplasm collection to identify and discriminate more salt tolerant rice genotypes from susceptible ones based on sequence homology with already identified salt tolerant rice genotypes. Mohammadi-Nejad *et al.* (2010) using 30 rice varieties introduced 16 different haplotypes based on *Saltol* QTL. As in this study, RM8094 and RM10745 microsatellite markers were found to be the most effective markers for discriminating the salinity-tolerant varieties. Aliyu *et al.* (2011) in case of RM493 and RM3412 for leaf diameter with a P-value of (0.025 and 0.045 respectively) and concluded in their study that the RM 10793 was best in marker assisted selection followed by RM 3412 then RM 493.

Islam *et al*. (2011) while doing QTL mapping for salinity tolerance at seedling stage in rice using 300 F2 segregating plants found out that from single marker analysis in chromosome 1, RM8094 was found to be strongly associated with salinity tolerance with significant on $P < 0.001$. Other three markers RM3412, RM493 and CP03970 found significantly associated with salinity tolerance ($P < 0.01$) and other four markers RM10665, RM1287, RM10825 and RM11008 were also significantly associated ($P<0.05$). These results revealed that there was important QTL for salinity tolerance in this region of the chromosome 1 segment.

Table 4*:* Marker trait associations for SSR markers linked to *Saltol* QTL on chromosome no. 1.

Sr.	Marker	Trait	P value	R^2 value	Sr.	Marker	Trait	P value	R^2 value
1	RM562	ISES	0.23303	0.01456	45	RM3412	ISES	0.20880	0.04605
2	RM562	SM (%)	0.86998	0.00437	46	RM3412	SM (%)	0.31148	0.03011
3	RM562	DFF	0.1149	0.00381	47	RM3412	DFF	0.59478	0.00841
4	RM562	PH	0.20677	0.01831	48	RM3412	PH	0.89687	0.00050
5	RM562	PL	0.27494	0.01790	49	RM3412	PL	0.91241	0.00036
6	RM562	NPT	0.65770	0.02350	50	RM3412	NPT	0.69725	0.00451
7	RM562	NGP	0.0075**	0.22684	51	RM3412	NGP	0.00384*	0.22073
8	RM562	NFP	0.18198	0.01407	52	RM3412	NFP	0.00126*	0.26682
9	RM562	SP	0.10001	0.08685	53	RM3412	SP	0.45369	0.01662
10	RM562	TW	0.16683	0.10045	54	RM3412	TW	0.37322	0.02339
11	RM562	Yield	0.48251	0.00396	55	RM3412	Yield	0.12031	0.06949
12	RM7075	ISES	0.96650	0.00029	56	RM10793	ISES	0.19930	0.03134
13	RM7075	SM (%)	0.60813	0.00012	57	RM10793	SM (%)	0.02584*	0.00042
14	RM7075	DFF	0.30382	0.002859	58	RM10793	DFF	0.15890	0.04285
15	RM7075	PH	0.91064	0.01148	59	RM10793	PH	0.34105	0.03775
16	RM7075	PL	0.82596	0.00240	60	RM10793	PL	0.92630	0.00001
17	RM7075	NPT	0.16916	0.00731	61	RM10793	NPT	0.52963	0.05185
18	RM7075	NGP	0.29860	0.09212	62	RM10793	NGP	0.83242	0.00072
19	RM7075	NFP	0.85598	0.01068	63	RM10793	NFP	0.22910	0.04677

Kordrostami *et al.* (2016) while evaluating salinity tolerance of Iranian 44 rice varieties found that according to association analysis, RM1287, RM8094, RM3412, RM493, RM140, RM5, RM10793, AP3206 and RM490 were detected to be associated with morphological traits under stress conditions.

Based on the results we can say that these markers RM562, RM493, RM3412, RM10793, RM10694, and RM10843 are linked to *Saltol* QTL which confers tolerance to salinity. Hence these markers have the potential for use in marker-assisted screening and marker-assisted breeding programs for the development of salinity tolerant varieties and these would increase the efficiency and accuracy of stress resistance breeding program.

CONCLUSIONS

Abiotic stress breeding often involves the screening of a large number of plants to identify stress-tolerant individuals. Molecular markers offer a cost-effective alternative to extensive field trials, conserving resources, and reducing the expenses associated with maintaining and evaluating large breeding populations. Developing crop varieties that are resilient to abiotic stress can enhance farmers' economic sustainability by reducing yield losses due to adverse environmental conditions. Farmers can achieve better returns on their investments, enhancing overall food security. In this study, association analyses of SSR markers linked to *Saltol* QTL revealed that the markers in this region were significantly associated with the related traits and are capable of properly explaining the phenotypic variance of the mentioned traits. The most important markers in this study were RM3412 and RM10843 which revealed a significant association even with more than two traits.

FUTURE SCOPE

Identifying marker-trait associations through molecular work in agriculture, particularly in abiotic stress breeding, offers a pathway to more efficient, precise, and sustainable crop improvement. It empowers breeders to develop crop varieties that can withstand harsh environmental conditions, ultimately contributing to global food security and agricultural resilience in the face of climate change.

Conflict of Interest. Authors have declared that no competing interests exist.

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