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Research Article

Screening and marker trait association for salinity tolerance in rice (*Oryza sativa* L.)

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Abstract
The goal of this work was to find marker-trait association to assess ability of rice genotypes to withstand salinity, which is an important consideration in abiotic stress breeding. In order to breed rice genotypes with tolerance to salinity stress, it is necessary to identify highly tolerant germplasm sources. Eighty-six rice genotypes were evaluated under field conditions. In order to determine their relationship with salinity tolerance, 16 microsatellite markers were used concurrently. Microsoft Excel's regression-based Single Marker Analysis (SMA) was used to estimate this association. Nine of the 16 polymorphic markers RM3412, RM10843, RM562, RM10793, RM 10748, RM8094, RM10694, RM1287 and RM493 showed statistically significant marker-trait relationships, suggesting the presence of important putative genetic loci linked to chromosome 1 with ability to withstand salinity. The range of 1.0% to 24.9% for the percentage of total variance of phenotype explained by the relevant markers indicates the dependability of these genetic markers for enhancing breeding for salinity tolerance. The existence of these markers in the tolerant germplasm lines from the findings of the research could be utilized to salinity tolerance cultivars through marker-assisted breeding programs because they are associated with the *Saltol* gene/QTL, respectively.

Keywords: Single marker analysis, Screening, Salinity tolerance, Marker trait association

INTRODUCTION

Agricultural landscapes are changing due to abiotic stressors and climate change. Breeders can quickly adjust crops to these shifting conditions with the aid of molecular marker technology, which keeps agriculture productive and sustainable. Any plant breeding effort must include genetic variation. During the seedling and reproductive stages, rice is a crop that is vulnerable to salinity (Singh and Flowers, 2010; Munns and Tester, 2008; Hossain *et al*., 2015). However, rice has been shown to exhibit an enormous amount of genetic diversity in response to salinity, making it amenable for genetic modification for increased tolerance to salinity (Flowers and Yeo 1981; Akbar *et al*., 1972). Due to their greater capacity to absorb K^+, exclude Na^+, and maintain a low Na^+/K^+ ratio in shoots, indica genotypes are generally high tolerant to salinity than japonica cultivars (DeLeon *et al*., 2015;Gregorio and Senadhira 1993; Lee *et al*., 2003).

Plant phenotypic responses to salinity are highly impacted by the environment (Tack *et al*., 2015; Gregorio and Senadhira 1993; Krishnamurthy *et al*., 2015a, 2015b; Gregorio, 1997), and salinity is a complicated quantitative characteristic with low heritability (Yeo and Flowers 1986; Shannon, 1985). Due to the negative impacts of landraces, it becomes challenging to use them to introduce genes that are tolerant to salinity into conventional cultivars. To combat this, targeted breeding through molecular

methods is being used more frequently. This was made feasible with regard to salinity due to the identification of *Saltol*, a major gene/QTL linked to the seedling stage salinity tolerance and Na^+/K^+ ratio, which was found on chromosome 1 (Bonilla *et al*., 2002; Gregorio, 1997). Later, there was an abundance of SSR and RFLP markers in this area. Ever since, this area has been the most frequently used QTL for resistance to salinity at the seedling stage.

Around the world, numerous attempts have been made to introduce the *Saltol* gene/QTL into the locally preferred lines (Linh *et al*., 2012; Huyen *et al*., 2012, 2013; Singh *et al*., 2016; Usatov *et al*., 2015). A salt tolerant RIL, FL478 from the Pokkali × IR 29 cross, was the most widely used donor in these investigations. Numerous investigations were carried out to group the genotypes for salinity tolerance utilizing the markers linked to the *Saltol* gene/QTL and FL478 as tolerant checks (Davla *et al*., 2013; Islam *et al*., 2012; Babu *et al*., 2014; Ali *et al*., 2014; Krishnamurthy *et al*., 2014, 2015c; Chattopadhyay *et al*., 2014; Kordrostami *et al*., 2016; Dahanayaka *et al*., 2015). In order to determine the number and nature of genes or QTLs, it is very helpful to investigate the genetic makeup of a trait by using phenotypic and marker data to create marker-trait associations (MTAs).

Breeders can accurately target and select for desired characteristics via marker-trait associations. It takes years to create new crop kinds using traditional breeding techniques. By making it possible to identify traits that are wanted early on, molecular markers hasten the breeding process and cut down on the time needed to create stress-tolerant cultivars. This is essential for tackling the pressing issues of climate change and food security. Therefore, in the current investigation, single marker analysis was used to determine the association of reported *Saltol* related markers with the trait.

MATERIALS AND METHODS

Plant samples: In this research, 86 different rice genotypes including checks were used (**Table 1**). These genotypes came from Agricultural Research Station, Kampasagar, Nalgonda, Telangana. The experiment was carried out in 2020–2021, during the *Rabi* season. The FL 478 line was used to represent the tolerant check in this investigation, while Pusa44 was used to represent the susceptible check. These genotypes were evaluated under saline stress conditions.

Screening Methodology: A naturally existing plot subjected to stress from inland salinity was used as the field environment for screening the experimental materials. An electrical conductivity (E.C.) of 4.68 dSm^{-1}, pH of 9.30, and Exchangeable Sodium Percentage (ESP) of 88.0 were among the soil properties of the field. The Standard Evaluation Score (SES) was calculated in accordance with the IRRI Standard Evaluation System, 2013 standards during the reproductive stage (**Table 2**). Consistency was maintained by carefully implementing essential plant protection measures and suggested agricultural techniques for ensuring the crop's regular development in the main field. Data on ten different characteristics were obtained, namely mortality percentage (M%), days to 50% flowering (DFF), number of productive tillers per hill (NPT), plant height (PH), panicle length (PL), total number of grains per panicle (TNGP), number of sterile grains per panicle (UFG), sterility percentage (SP), 1000-grain weight (TW) and seed yield (SY).

Genotyping of lines using Saltol linked markers: *Saltol* QTL-associated SSR markers were used for DNA

Table 1. List of rice genotypes and checks utilized in the experiment

Sources	Germplasm lines
Germplasm collection	IR78222-20-7-148-2-B-B-B-B, IRRI 104, URAIBOOL IRGC 52785, Oryzica 1, CT118911-2-2-7-M, Kinandang patong, OM4900, IR6, Zanton:IRGC 31248-1, SUPA, M 202, MINGHU 163, ZHENSHAN 97B, IRRI 147, SANHUANGZHAN-NO2, IR10M 300, Manaw thukha, BR 28, Fedearroz 50, NSIC RC 240, GSRIR2-9-RI-Su3-Y2, IR13F 167, IR69726-116-1-1, IR77186-122-2-2-3, IRRI 154, IRBB 66, IR64-21, IR77298, N22 IRGC 19379-1, IR 93340, IR 93354, Khao Hlan on, IR 84984-83-15-481-B, IR10F 360, DJ123, TEQING, UPLR17:IRTP9897-C1, Jamir.
Varieties	MTU1010, Nanhi, Jasmine 85, Sambha mahsuri+sub1, Swarna, SADRI, TN1, Sahel 177.
Advanced breeding lines	KPS 13576, KPS 13577, KPS 13580, KPS 13584, KPS 10667, KPS 10672, KPS 13582, KPS 10628, KPS 10640, KPS 10656, KPS 10676, KPS 10683, KPS 10633, KPS 10658 KPS 10631, KPS 10642, KPS 10651, KPS 10654, KPS 10657, KPS 10661, KPS 10669, KPS 10316, KPS 10319, KPS 10321, KPS 10329, KPS 13575, KPS 13578, KPS 13579, KPS 13581, KPS 13583, KPS 13585, KPS 13586, KPS 13587, KPS 13588.
Checks	CSR 23 (Alkalinity and salinity tolerant check) CSR 36 (Alkalinity tolerant check) RNR 11718 (Local alkalinity and salinity check) FL 478 (Salinity tolerant check) KPS 2874 (Local yield check) PUSA 44 (Susceptible check)

Table 2. Scoring of damage for salt injury in field conditions in rice standard evaluation system scale (IRRI-SES 2013)

Score	Growth Scale	Salinity-induced reaction
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled.	Tolerant
5	Growth severely retarded, most leaves rolled, only a few are elongating	Moderately tolerant
7	Complete cessation of growth, most leaves dry, some plants drying	Susceptible
9	Almost all plants dead or drying	Highly susceptible

Table 3. List of SSR markers used in this study

S.No	Marker	Forward Sequence (5' —>3')	Reverse Sequence (3'—> 5')
1	RM3412	AAAGCAGGTTTTCCTCCTCC	CCCATGTGCAATGTGTCTTC
2	RM10793	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC
3	RM493	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
4	RM1287	GTGAAGAAAGCATGGTAAATG	CTCAGCTTGCTTGTGGTTAG
5	RM10764	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAGC
6	RM562	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC
7	RM10694	TTTCCCTGGTTTCAAGCTTACG	AGTACGGTACCTTGATGGTAGAAAGG
8	RM259	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT
9.	RM8094	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
10.	RM283	GTCTACATGTACCCTTGTTGGG	CGGCATGAGAGTCTGTGATG
11.	RM140	TGCCTCTTCCCTGGCTCCCCTG	GGCATGCCGAATGAAATGCATG
12.	RM10772	GCACACCATGCAAATCAATGC	CAGAAACCTCATCTCCACCTTCC
13.	RM10745	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
14.	RM10843	CACCTCTTCTGCCTCCTATCATGC	GTTTCTTCGCGAAATCGTGTGG
15.	RM10864	GAGGTGAGTGAGACTTGACAGTGC	GCTCATCATCCAACCACAGTCC
16.	RM10748	CATCGGTGACCACCTTCTCC	CCTGTCATCTATCTCCCTCAAGC

marker analysis. All 16 SSR markers associated with the *Saltol* gene/QTL on chromosome 1 (**Table 3**) were used to assess the genotypes' polymorphism, according to published literature (Nejad *et al*., 2008; Gregorio *et al*., 1997; Ganie *et al*., 2014; Islam *et al*., 2012). Using Murray and Thompson's (1980) CTAB method, DNA was extracted from the young lines' and succulent leaves. DNA quantification was performed using diluted uncut ladder DNA as a standard on a 0.8% agarose gel. Using the *Saltol* linked markers, 10µL reaction volumes were used for the PCR reactions. The reaction mixture included 4µl of TAKARA master mix, 2µl of double-distilled water, 0.5 µl of forward and reverse primers, and 2µl of template DNA. 35 cycles of 94°C for 60 seconds, 56°C for 45 seconds, and 72°C for 45 seconds were performed, with a final extension of 7 minutes at 72°C. For 5 minutes, the amplification profile was kept at 94°C. Using 1×TAE buffer, a 3% agarose gel was used to electrophoretically resolve the PCR generated products. The BIO-RAD Imaging gel documentation system was used to visualize DNA banding patterns.

Scoring: For both the presence and absence of the SSR bands, scores were assigned to each germplasm line. The presence or absence of clearly resolved, unambiguous bands with each *Saltol*-specific marker was visually scored. Band presence and absence in each germplasm line are indicated by the scores, which were obtained as a matrix with "1" and "0" respectively.

Data analysis and finding DNA markers linked to salt tolerance: Using the single factor standard analysis of variance (ANOVA) regression method, Single Marker Analysis (SMA) was used to estimate the marker-trait associations. Marker trait associations were taken to be significant when the P-value was below 0.05. The percentage R^2 value was used to estimate the percentage of the trait's phenotypic variance that can be explained by markers.

RESULTS AND DISCUSSION

Phenotypic screening for salinity tolerance: Screening is an important aspect of every breeding program. Although there are many rice genotypes that provide good yields in normal conditions, they do not perform well when exposed to salt stress conditions. The goal of the current study was to use field screening methods to evaluate how salt affected the 86 rice genotypes. One result, tolerance

to salinity, is produced by a collection of complex essential features. These SES scores assess the plant's overall vigor and/or survivability, making them useful predictors of how well the plant would respond under stress (Gregorio *et al*., 1997). During the reproductive stage, visual assessment of salt injury was recorded using the SES (Standard Evaluation Score). Based on the score, genotypes were categorized into different classes. Out of 86 genotypes, 8 genotypes were classified into tolerant, 13 genotypes as moderately tolerant, 21 genotypes as sensitive, 44 genotypes as highly sensitive and none of the genotypes showed highly tolerant reaction to salinity (**Table 4**). Sudharani *et al*. (2013), Raghavendra *et al*. (2020) and Bindu *et al*. (2022) also did a similar grouping of genotypes based on the salinity tolerance at reproductive stage.

Single marker analysis and salinity tolerance: At the molecular level, molecular markers can forecast a plant's ability to withstand stress, giving information about how well it might function in various environmental circumstances. This ability to predict is particularly useful in areas where abiotic stressors are unpredictable or changing. Ten of the sixteen SSR markers that were employed in association studies showed distinct banding patterns between resistant (FL478) and susceptible check (Pusa44), and they were associated to the *Saltol* gene/QTL on chromosome 1 and ranged in size from 10.4 to 15.3 MB. Ten SSR markers and eleven attributes were used to identify marker-trait relationships. A single-factor ANOVA was used, and Microsoft Excel was used for regression-based analysis (**Table 5**).

Based on the value $p<0.05$, 11 significant marker trait associations were found *viz,* RM3412 for TNGP and TW, RM10843 for M% and PL, RM1287 for TNGP, RM10694 for M%, RM8094 for TNGP, RM10748 for NPT, RM562 for TW, RM493 for M% and RM10793 for TW. Nine of the ten markers exhibited significant relationships with the characteristics, while two SSR markers (RM10843 and RM3412) demonstrated significant relationships with multiple traits. RM3412 for TNGP, RM8094 for TNGP, and RM10793 for TW had the highest significant association ($p<0.001$), while RM3412 for TW, RM10843 for M% and PL, RM1287 for TNGP, RM10694 for M%, RM10748 for NPT, RM562 for TW, and RM493 for M% had $p<0.05$. The associations between the remaining marker traits were non-significant, with p values ranging from 0.9987 for RM283 for SP to 0.0697 for RM140 for PH.

On the whole, the present investigation helped in the identification of 7 (IR78222-20-7-148-2-B-B-B-B, URAIBOOL IRGC 52785, Oryzica 1, CSR 23, CSR 36, RNR 11718 and CT118911-2-2-7-M) salinity tolerance donors which could be useful in breeding salinity tolerant rice varieties. In this study we confirmed the tolerant genotypes compared with known tolerant check FL478. Further investigations on presence of other mechanisms of tolerance need to be attempted to confirm the best germplasm lines as potential donors for salinity tolerance.

The R^2 value indicates that the proportional contributions of markers to the total variance of phenotype for salinity tolerance (Bearzoti and Vencovsky 1998) and it is very useful to understand the quantitative traits and marker

Table 4. Categorization of rice genotypes for salinity tolerance based on salt injury scores (IRRI-SES 2013) at the reproductive stage under saline field condition

S. No.	Salt injury score	Reaction to salinity	Number of genotypes	Genotypes
1.	1	Highly tolerant	0	-
2.	3	Tolerant	8	IR78222-20-7-148-2-B-B-B-B, URAIBOOL IRGC 52785, Oryzica 1, CSR 23, CSR 36, RNR 11718, FL 478, CT118911-2-2-7-M.
3.	5	Moderately tolerant	13	OM4900, IR6, Zanton:IRGC 31248-1, SUPA, KPS 2874, M 202, MINGHU 163, ZHENSHAN 97B, SADRI, IRRI 147, Sahel 177, IRRI 104, Kinandang patong.
4.	7	Sensitive	21	KPS 10628, KPS 10640, KPS 10656, KPS 10676, KPS 10683, SANHUANGZHAN-NO2, IR10M 300, MTU1010, Manaw thukha, BR 28, Fedearroz 50, KPS 13576, KPS 13577, KPS 13580, KPS 13584, KPS 10667, KPS 10672, KPS 13582, KPS 13586, KPS 13587, KPS 13588.
5.	9	Highly sensitive	44	NSIC RC 240, GSRIR2-9-RI-Su3-Y2, IR13F 167, KPS 10633, KPS 10658, Nanhi, IR69726-116-1-1, IR77186-122-2-2-3, IRRI 154, IRBB 66, Jasmine 85, KPS 10631, KPS 10642, KPS 10651, KPS 10654, KPS 10657, KPS 10661, KPS 10669, KPS 10316, KPS 10319, KPS 10321, KPS 10329, IR64-21, IR77298, Sambha mahsuri+sub1, N22 IRGC 19379-1, Swarna, IR 93340, IR 93354, Khao Hlan on, IR 84984-83-15-481-B, TN1, IR10F 360, DJ123, TEQING, UPLR17:IRTP9897-C1, Jamir, KPS 13575, KPS 13578, KPS 13579, KPS 13581, KPS 13583, KPS 13585, PUSA 44.

Table 5. Marker trait associations for SSR markers linked to *Saltol* QTL

S.No.	Marker	Trait	P-value	R^2
1	RM3412	SES	0.3124	0.0755
2	RM3412	M%	0.5614	0.0921
3	RM3412	DFF	0.2894	0.0452
4	RM3412	PH	0.9862	0.0014
5	RM3412	NPT	0.6325	0.0054
6	RM3412	PL	0.9888	0.0024
7	RM3412	TNGP	0.0011*	0.1204
8	RM3412	UFG	0.2586	0.0014
9	RM3412	SP%	0.3451	0.0562
10	RM3412	TW	0.0027*	0.2145
11	RM3412	SY	0.1452	0.0145
12	RM10843	SES	0.5874	0.054
13	RM10843	M%	0.0147*	0.0008
14	RM10843	DFF	0.9687	0.0258
15	RM10843	PH	0.8564	0.0897
16	RM10843	NPT	0.2654	0.0547
17	RM10843	PL	0.0247*	0.0025
18	RM10843	TNGP	0.8794	0.0258
19	RM10843	UFG	0.3547	0.0002
20	RM10843	SP%	0.4578	0.1479
21	RM10843	TW	0.1452	0.1532
22	RM10843	SY	0.3589	0.0486
23	RM1287	TNGP	0.0049*	0.0287
24	RM10694	M%	0.0031*	0.0963
25	RM8094	TNGP	0.0014*	0.0885
26	RM10748	NPT	0.0036*	0.0029
27	RM562	TW	0.0028*	0.0014
28	RM493	M%	0.0009*	0.0058
29	RM10793	TW	0.0012*	0.0459

assisted selection. Greater values of phenotypic variance suggest that they regulate a significant portion of genetic variation and may serve as trustworthy genetic markers for future advancement. Out of the 11 significant marker-trait relationships, RM3412 for TW had the highest R^2 value (0.2145), followed by RM10843 for TW (0.1532), while RM10843 for UFG had the lowest contribution to the overall phenotypic variance (0.0002). Up to 24% of the observed phenotypic variance was explained by the significant marker-trait relationships (R^2). Higher phenotypic variance values indicated that a considerable amount of genetic variability in salinity response was controlled by markers. They may also serve as trustworthy genetic markers for enhancing breeding for salinity tolerance.

Banumathy *et al*. (2018) screened sixty eight BC_3F_3 lines using foreground marker RM 3412. Among the 68 lines tested, 32 BC_3F_3lines showed the presence of RM 3412 marker. The selected marker RM 3412 was able to discriminate tolerant lines from susceptible. They identified linked marker RM 3412 can be used in marker-assisted selection programme in identifying tolerant lines and also gene pyramiding of rice salinity breeding.

Senguttuvel *et al*. (2010) found similar substantial marker-trait relationships in the instance of RM493 for the Na^+/K^+ ratio when examining 25 different genotypes and phenotyping using yoshida nutrient solution under salinity conditions. In addition to RM493, they found that RM8053 and RM23 were trustworthy markers for marker-assisted selection to find salinity tolerance in rice. Based on sequence homology with previously identified salt-tolerant rice genotypes, these markers are used for screening large collection of germplasm to find and distinguish salt tolerant rice genotypes from susceptible ones.

Using 300 F_2 segregating plants, Islam *et al*. (2012) conducted QTL mapping for salinity tolerance in rice at

the seedling stage. They discovered that RM8094 was significantly linked with salt tolerance with a P < 0.001 based on a single marker analysis in chromosome 1. There was a substantial correlation (P<0.01) between the salinity tolerance of seven markers (RM3412, RM493, RM10665, RM1287, RM10825, RM11008 and CP03970). These findings demonstrated the presence of significant gene/QTL for tolerance to salinity in chromosome 1 segment.

According to Sellammal *et al.* (2013) marker - trait association was assessed by single marker analysis utilizing 121 markers. They reported out of that eighteen markers showed putative association with at least one of the investigated trait. They concluded RM 256 in chromosome 8, RM 245 in chromosome 9 were linked with yield under moderate stress and these markers were also linked with other associated traits viz, harvest index, leaf senescence and leaf rolling and these might be useful for marker assisted selection for rainfed rice improvement.

According to association analysis, RM3412, RM8094, RM1287, RM493, AP3206, RM140, RM5, RM490, and RM10793 were shown to be linked with morphological features under stress circumstances by Kordrostami *et al*. (2016) while assessing the salinity tolerance of forty-four rice varieties from iran.

We may conclude from the results that the Saltol QTL, which imparts resistance to salt, is associated with the markers RM493, RM562, RM10793, RM3412, RM10843, and RM10694. In order to develop salinity-tolerant cultivars, these markers may be used in marker-assisted and breeding programs. The study's most significant markers, RM10843 for M%, PL and RM3412 for TNGP and TW, showed a substantially correlated with more than two attributes. This would improve the efficacy and precision of stress-resistant breeding programs.

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