

Phenotypic and Genotypic Screening of Rice Genotypes at Seedling Stage for Salt Tolerance

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Abstract: Selection for salinity tolerance genotypes of rice based on phenotypic performance alone is less reliable and will delay in progress in breeding. Recent advent of molecular markers, microsatellites or simple sequence repeats (SSRs) were used to find out salt tolerant rice genotypes. Three selected SSR markers viz., RM7075, RM336 and RM253 were used to evaluate rice genotypes for salt tolerance. Phenotypic and genotypic evaluation for salinity tolerance was done at the seedling stage. Phenotyping of 11 genotypes was done in hydroponic system using salinized (EC 12 dS/m) nutrient solution. IRR standard protocol was followed to evaluate salinity tolerance. Large variation in salinity tolerance among the rice germplasms was detected. Plant height and total dry matter of tolerant lines were reduced by 19.0% and 40.6%, respectively under salt stress (EC 12 dS/m), where as, those of susceptible lines were reduced by 46.0% and 73.5%, respectively. The markers showed polymorphism and were able to discriminate salt tolerant genotypes from susceptible. The genotypes having similar banding pattern with Pokkali were considered as salt tolerant. The SSR markers (RM7075, RM336 and RM253) identified 8, 9 and 7 salt tolerant genotypes, respectively. Through phenotypic and genotypic study, three genotypes viz., Pokkali, TNDB-100 and THDB were identified as salt tolerant rice cultivar. These SSR markers might have sequence homology with salt tolerant rice genotypes and consequently the markers could able to identify salt tolerant rice genotypes from susceptibles.

Key words: Rice, Salinity tolerance, SSR markers, Seedling stage

INTRODUCTION

Rice is the staple food of more than 50% of the world's population [1]. By the year 2025, 21% increase in rice production will be needed over that of year 2000 [2]. Salinity is one of the major obstacles in increasing production in rice growing areas worldwide, which is an ever-present threat to crop yield. Therefore, development of salt tolerant varieties has been considered as one of the strategies to increase rice production in saline prone coastal areas. The response of rice to salinity varies with growth stage. Several studies indicated that rice is tolerant during germination, becomes very sensitive during early seedling stage (2-3 leaf stage), gains tolerance during vegetative growth stage becomes sensitive during pollination and fertilization and then become increasingly more tolerant at maturity [3].

Screening of germplasms at seedling stage is readily acceptable as it is based on a simple criterion of selection; it provides rapid screening difficult at vegetative and reproductive stage [4]. Screening under controlled condition has the benefit of reduced environment effects and the hydroponic system is free difficulties associated with soil related stress factors. The conventional methods of plant selection for salt tolerance are not easy because of the large effects of the environment and low narrow sense heritability of salt tolerance [5]. This hinders the development of an accurate, rapid and reliable screening technique. However, DNA markers seem to be the best candidates for efficient evaluation and selection of plant material. Recent progress and technical advances in DNA marker technology permit reduction of time and accuracy of breeding where pronounced effects of environment lead to poor selection efficiency.

SSR or microsatellite markers are proved to be ideal for making genetic maps [6, 7], assisting selection [8] and studying genetic diversity in germplasms. SSR markers are playing important role to identify gene for salt tolerance that can be helpful for plant breeders to develop new cultivars. The aim of the present study was to screen rice germplasms under salinized and non-salinized conditions and to evaluate microsatellite markers for the identification of salt tolerant genotypes at the seedling stage.

MATERIALS AND METHODS

Plant Materials: Eleven rice germplasms with diversified genetic background were used in this study. Of which six were Bangladeshi landraces, four were BINA developed mutants and one salt tolerant Indian variety 'Pokkali' was used as check.

Phenotypic Study of Salinity Tolerance at Seedling Stage: The genotypes were screen for salt tolerance at seedling stage in hydroponic system using IRRI standard protocol [5]. Salinized and non-salinized setups with 3 replications were maintained. The evaluation was done using Yoshida *et al.* [9] nutrient solution at the glasshouse. The nutrient solution was salinized by adding crude salt to obtain desired EC (12 dS/m). The modified standard evaluation system (SES) was used in rating the visual symptoms of salt toxicity [10]. Visual rating of salinity tolerance was done according to Table 1. This scoring discriminated the susceptible from the tolerant and the moderately tolerant genotypes. Initial and final scoring was done at 13 days and 22 days after salinization. For phenotypic observation plant height and root length and total dry matter was recorded at salinized and non-salinized conditions.

CTAB Mini Preparation DNA Extraction: DNA isolation was done from fresh leaf tissues of 14-day old seedlings. DNA was extracted using the mini preparation CTAB method. First, grinding of leaf sample by extraction buffer and SDS. Then incubated the leaf sap at 65°C for 10 min. 100 µl NaCl and 100 µl CTAB were added sequentially and mixed well; and incubated again at 65°C for 10 minute. After that the suspensions were transferred to a new plate. 900 µl chloroform (Chloroform: Isoamyl: 24:1) was added and mixed well by a shaker. Then the sample was then centrifuged at 5700 rpm for 10 minutes. After that the supernatant were transferred into new eppendrof tubes. Then 600 µl ice-cold isopropanol was added into the new eppendrof tubes and shaken slowly and then centrifuged at 5700 rpm for 15 minutes. The supernatant was decanted

Table 1: Modified standard evaluation score (SES) of visual salt injury at seedling stage (Adapted from Gregorio *et al.*, [5])

Score	Observation	Tolerance
1	Normal growth on leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or 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 dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

and air dried for at least one hour. Pelletes were washed with 70% ethanol (200 µl), spinned for 15 minutes at 5700 rpm and then air-dried for 1/2-1hours. Then the ethanol was removed and air-dried. The pelletes were resuspended in 30.0 µl X TE buffer.

Amplification of Microsatellite Markers and Evaluation of Genotypes: Three selected primers were used for this study as those were used previously by Islam [6]; Bonilla *et al.* [11]; Niones [7] and Gregorio *et al.* [12] in recombinant inbred lines (RILs) of Pokkali X IR29 for tagging salt tolerance genes, where Pokkali was salt tolerant and IR29 was salt susceptible. Among them RM7075, RM336 and RM253 were polymorphic and showed clear bands; and used to reveal polymorphism. Each PCR reaction carried out with 15.0 µl reactions containing 1.5 µl 10 X buffer, 0.75 µl dNTPs, 1µl primer forward, 1µl primer reverse, 0.5 µl taq polymerase, 8.25 µl ddH₂O and 2.0 µl of each template DNA samples. PCR profile was maintained as initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 2 min; and final extension by 7 min at 72°C. Banding pattern of the genotypes was scored comparing the banding pattern of Pokkali. The germplasms showed similar banding pattern like Pokkali, were considered as tolerant and had different banding pattern were considered as susceptible.

RESULTS AND DISCUSSION

Screening of Genotypes for Salt Tolerance at Seedling Stage: All genotypes were grown robustly and showed uniform green colour and height in the non-salinized condition. In salinized condition, the genotypes showed wide variation in phenotypes ranging from score 1 (highly tolerant) and score 9 (highly susceptible) (Fig. 1). The most salinity tolerant germplasms were

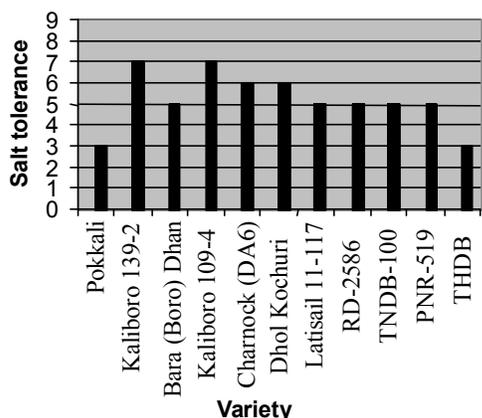


Fig. 1: Distribution of 11 genotypes under salt stress at seedling stage were grown in nutrient solution

Pokkali, THDB and TNDB-100. Four moderately salinity tolerant genotypes were identified as RD-2586, PNR-519, Dhol Kochuri and Bara Dhan. The most susceptible salt tolerant genotypes were Kaliboro 139-2 and Kaliboro 109-4. The modified standard evaluation system (SES) of IRRI [4] was used in rating the visual symptoms of salt injury.

Plant heights were shorter in salinized condition, compared to the plants grown in non-salinized conditions (Table 3). Plant height and total dry matter of susceptible genotypes showed higher percent reduction than tolerant genotypes. Lower percent reduction of plant height was recorded in genotypes Pokkali and THDB followed by genotypes TNDB-100, RD-2586, PNR-519 and Dhol kochuri. On the other hand, higher percent reduction of plant height was showed by genotypes, Kaliboro 139-2 and Kaliboro 109-4. The percent reduction of total dry

matter ranged from 40-75. Lower percent reduction of total dry matter was found in genotypes Pokkali, TNDB-100 and THDB. In contrast, Kaliboro 139-2 and Kaliboro 109-4 showed higher percent reduction of total dry matter. Tolerant cultivars showed less growth reduction than sensitive genotypes under salinized conditions [13].

Eleven genotypes showed wide variation in phenotypes. Salt tolerant seedlings were distinguished from the sensitive seedlings grown in salinized condition. Seedlings grown in salinized condition showed different visual symptoms of salt injury. The symptoms were prominent on the first and second leaves and were visualized by leaf rolling, formation of new leaf, brownish and whitish of leaf tip, drying of leaves and also reduction in root growth, stunted growth and stem thickness leading to complete cessation of growth and dying of seedlings occurred [4]. Salinity in rice was associated with Na^+ exclusion and increased absorption of K^+ to maintain a good Na^+/K^+ balance in the shoot under saline condition. It is considered that damage of leaves was attributed to accumulation of Na^+ from the root to the shoot in external high concentration [14]. In several species including rice, salt stress might increase or even include the expression of specific genes and repress or completely suppress the expression of others [15].

At the seedling stage, highly significant and positive correlations were found between plant height and total dry matter at salinized condition (Table 2). Whereas, at salinized condition correlations between salt tolerance and plant height, total dry matter and root length were inverse and significant. Which imply that salt tolerant genotypes (having lower salt tolerance score) exhibited

Table 2: Performance of plant height and total dry matter of 11 rice genotypes at seedling stage grown in hydroponic system

SL. No.	Variety	Plant height (cm)			Total dry matter (g)		
		Non-salinized	Salinized	% Red	Non-salinized	Salinized	% Red
1	Pokkali	61	50	18	9.12	5.48	40
2	Kaliboro 139-2	62	34	45	9.17	2.54	72
3	Bara (Boro) Dhan	55	39	29	7.7	3.61	53
4	Kaliboro 109-4	59	31	47	11.73	2.97	75
5	Charnock (DA6)	42	30	29	7.25	3.26	55
6	Dhol Kochuri	59	44	25	10.69	5.28	51
7	Latisail 11-117	55	40	27	8.44	3.75	56
8	RD-2586	44	34	23	6.7	3.85	43
9	TNDB-100	42	33	21	7.38	4.33	41
10	PNR-519	49	37	24	5.78	3.12	46
11	THDB	40	33	18	8.47	4.97	41
LSD _(0.05)		1.662	1.391		0.4415	0.2081	-

Table 3: Correlation of different traits at seedling stage under salinized condition

Parameter	Salt tolerance	Plant height	Total dry matter
Plant height	-0.406**		
Total dry matter	-0.74**	0.622**	
Root length	-0.278*	0.115	0.291*

* = Significant at 5% level of probability and ** = Significant at 1% level of probability

higher plant height and total dry matter. Peng *et al.* [16] reported that increasing plant height would allow greater biomass production. Zhang *et al.* [17] found similar result with their studied doubled haploid (DH) population consisting of 81 DH lines. They reported that increase of plant height was responsible for increase of biomass; so as to increase yield potential. It is crucial to note that Pokkali, THDB and TNDB-100 genotypes showed higher plant height and total dry matter and also performed as salt tolerant.

On the basis of SES score and phenotypic performance, three genotypes (Pokkali, THDB and TNDB-100) were identified as salt tolerant and RD 2586, Dhol Kochuri, PNR-519, Bara (Boro) Dhan, Latisail 11-117 and Charnock (DA6) were identified as moderately tolerant at seedling stage.

Screening of Salt Tolerance Through SSR Markers:

Three markers were used to evaluate germplasms for salinity tolerance. The markers were RM7075, RM336 and RM253. The bands obtained from other genotypes were compared to the band obtained from Pokkali. Pokkali was used as salt tolerant genotype in this study because it is known as salt tolerant genotype. The germplasms having

similar banding pattern to Pokkali were considered as tolerant and having different banding pattern to Pokkali were considered as susceptible. In case of RM253, Bara (Boro) Dhan, Latisail 11-117, RD-2586, TNDB-100, PNR-519 and THDB were found as tolerant. On the other hand, Kaliboro 139-2, Kaliboro 109-4, Charnock (DA6) and Dhol Kochuri were found as susceptible with RM253 (Fig. 2).

Considering the primer RM7075, genotypes Charnock (DA6), Dhol Kochuri, Latisail 11-117, RD-2586, TNDB-100, PNR-519 and THDB were found as tolerant. Whereas Kaliboro 139-2, Bara (Boro) Dhan and Kaliboro 109-4 were found as susceptible with RM7075. Bara (Boro) Dhan, Charnock (DA6), Dhol Kochuri, Latisail 11-117, RD-2586, TNDB-100, PNR-519 and THDB were identified as tolerant and Kaliboro 139-2 and Kaliboro 109-4 were identified as susceptible with RM336.

The marker RM7075 identified eight tolerant and three susceptible genotypes in comparison with Pokkali. Out of genotypically identified eight salt tolerant genotypes (identified genotypically), three were tolerant and five were performed as moderately tolerant based of phenotypic performance at the seedling stage. With regarding RM336, nine genotypes exhibited as salt tolerant and two genotypes were susceptible. Phenotypically three tolerant, six moderately tolerant at seedling stage were identified amongst the nine genotypically tolerant genotypes. Seven tolerant and four susceptible cultivars were found when eleven genotypes were tested with RM253. Considering these seven genotypically salt tolerant genotypes, phenotypically three tolerant and four moderately tolerant at seedling stage. Bhuiyan [8] identified 158

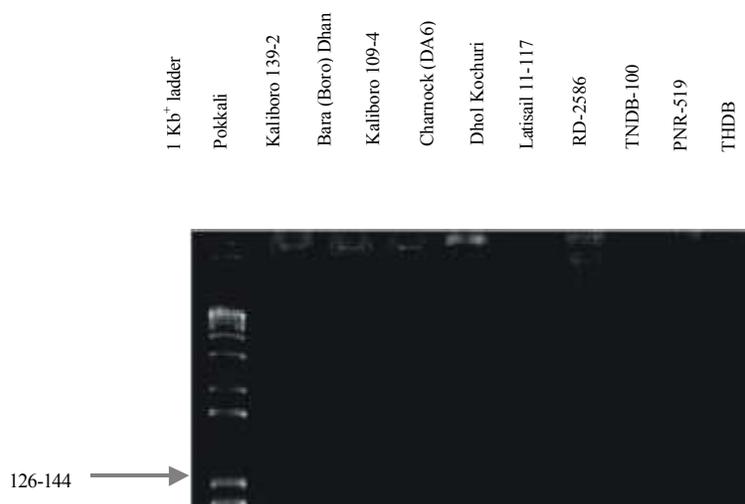


Fig. 2: Polymorphism exhibition of RM 253 among eleven genotypes

tolerant individuals of the F2 and F3 population of BRRI Dhan 28 X PSBRc88 with the marker RM493. Moreover, he observed 105 tolerant individuals phenotypically.

Considering phenotypic and genotypic observations, three genotypes TNDB-100, THDB and Pokkali were identified as salt tolerant. Four genotypes i.e. RD 2586, Dhol Kochuri, PNR-519 and Bara (Boro) Dhan were found as moderately tolerant. The selected markers (RM7075, RM336 and RM253) showed polymorphism in eleven rice cultivars. These markers were able to discriminate tolerant genotypes from susceptible. So these markers have relationship with salt tolerance alleles studied in rice genotypes. Molecular marker helps to identify alleles that are associated with key phenotypic traits [18]. Nguyen *et al.* [19] found that the marker RM315 had association with NaCl tolerant alleles at seedling population (IR64/ChengHui 448, IR64/OM1706 and IR64/FR13A) under EC 18 dS/m and salt stress genes were located at loci in chromosomes 1 and 8. Similar result was reported by Lang *et al.* [20]. They found that RM223 was closely linked to salt tolerance gene in chromosome 8. Since, the markers were used in this study showed polymorphism with the genotypes, these markers could be proficiently used in tagging salt tolerant genes, in marker-assisted selection and quantitative trait loci (QTL) mapping; and identified salt tolerant rice genotypes could be used in the improvement of salt tolerant rice genotypes.

REFERENCES

1. Aggarwal, R.K., V.V. Shenoy, J. Ramadevi, R. Rajkumar and L. Singh, 2002. Molecular characterization of some Indian Basmati and other elite rice genotypes using fluorescent – AFLP. *Theor. Appl. Genet.*, 105: 680-690.
2. Bhuiyan, N.I., D.N.R. Paul and M.A. Jabber, 2002. Feeding the extra millions by 2025: challenges for rice research and extension in Bangladesh. In: *Proceedings of the National Workshop on Rice Research and Extension*, Bangladesh Rice Research Institute, Gazipur, January 29–31, 2002.
3. IRRI (International Rice Research Institute). 1967. *Annual Report for 1967*. Los Baños, Laguna, Philippines. pp: 308.
4. Gregorio, G.B., D. Senadhira and R.D. Mendoza, 1997. Screening rice for salinity tolerance. IRRI Discussion Paper Series no. 22. Manila (Philippines): International Rice Research Institute. pp: 1-30.
5. Gregorio, G.B., 1997. Tagging Salinity Tolerant Genes in Rice Using Amplified Fragment Length Polymorphism (AFLP). Ph. D. dissertation. University of the Philippines Los Baños College, Laguna, Philippines, pp: 118.
6. Islam, M.M., 2004. Mapping salinity tolerance genes in rice (*Oryza sativa* L.) at reproductive stage. Ph.D. dissertation, University of the Philippines Los Baños College, Laguna, Philippines, pp: 1-149.
7. Niones, J.M., 2004. Fine mapping of the salinity tolerance gene on chromosome 1 of rice (*Oryza sativa* L.) using near-isogenic lines. MSc. Dissertation, University of the Philippines Los Baños College, Laguna, Philippines, pp: 78.
8. Bhuiyan, M.A.R., 2005. Efficiency in evaluating salt tolerance in rice using phenotypic and marker assisted selection. M.Sc. dissertation, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, Bangladesh. pp: 96.
9. Yoshida, S., D.A. Forno, J.H. Cook and K.A. Gomez, 1976. Laboratory manual for physiological studies of rice. International Rice Research Institute (IRRI), Los Baños, Laguna, Philippines, pp: 61-66.
10. IRRI (International Rice Research Institute). 1997. *Rice Almanac*. IRRI-WARDA-CIAT, Los Baños, Laguna, Philippines.
11. Bonilla, P.S., J. Dvorak, D. Mackill, K. Deal and G. Gregorio, 2002. RFLP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (*Oryza sativa* L.) using recombinant inbred lines. *The Philippines Agricultural Scientist*, 85: 64-74.
12. Gregorio, G.B., D. Senadhira, R.D. Mendoza, N.L. Manigbas, J.P. Roxas and C.Q. Guerta, 2002. Progress in breeding for salinity tolerance and associated abiotic stresses in rice. *Field Crops Research*, 76: 91-101.
13. Suplick-Ploense, M.R., Y.L. Qian and J.C. Read, 2002. Salinity tolerance of Texas bluegrass, Kentucky bluegrass and their hybrids. *Crop Sci.*, 42: 2025-2030.
14. Lin, H.X., M.Z. Zhu, M. Yano, J.P. Gao, Z.W. Liang, W.A. Su, X.H. Hu, Z.H. Ren and D.Y. Chao, 2004. QTLs for Na⁺ and K⁺ uptake of the shoots and roots controlling rice salt tolerance. *Theor. Appl. Genet.*, 108: 253-260.
15. Hasegawa, P.M., R.A. Bressan, J.K. Zhu and H.J. Bonhert, 2000. Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 51: 463-499.

16. Peng, S., K.G. Cassman, S.S. Virmani, J. Sheehy and G.S. Khush, 1999. Yield potential trends of tropical rice since the release of IR8 and the challenge of increasing rice yield potential. *Crop Sci.*, 39: 1552-1559.
17. Zhang, Z.H., P. Li, L.X. Wang, Z.L. Hu, L.H. Zhu and Y.G. Zhu, 2004. Genetic dissection of the relationships of biomass production and partitioning with yield and yield related traits in rice. *Plant Sci.*, 167: 1-8.
18. Xu, Y., H. Beachell and S.R. McCouch, 2004. Marker based approach to broadening the genetic base of rice in the USA. *Crop Sci.*, 44: 1947-1959.
19. Nguyen, T.L., S. Yanagihara and B.C. Buu, 2001. A microsatellite marker for a gene conferring salt tolerance on rice at the vegetative and reproductive stages. *SABRAO J. Breed. Genet.*, 33: 11-20.
20. Lang, N.T., S. Yanagihara and B.C. Buu, 2000. Quantitative trait loci for salt tolerance in rice via molecular markers. *Omon. Rice*, 8: 37-48.