Phenotypic and genotypic screening of rice genotypes at seedling stage for salt tolerance

Selección fenotípica y genotípica de genotipos de arroz para tolerancia a la salinidad en la etapa de plántulas

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ABSTRACT

Selection for salinity tolerant 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), have been useful in finding salt tolerant rice genotypes. Three selected SSR markers already known to be polymorphic, *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 was done in hydroponic system using salinized (EC 12 dS/m) nutrient solution following IRRI standard protocol. Large variation in salinity tolerance among the rice germplasms was detected. Salt stress (EC 12 dS/m) reduced seedling height by 19.0% and total dry matter of tolerant lines by 40.6%, whereas, total dry matter of susceptible lines were reduced by 46.0-73.5%. All the tested markers were polymorphic and were able to discriminate salt tolerant genotypes from susceptible. The genotypes having similar banding pattern with Pokkali were considered as salt tolerant. Markers RM7075, RM336 and RM253 identified eight, nine and seven salt tolerant genotypes, respectively. Through phenotypic and genotypic study, three genotypes *viz*., Pokkali, TNDB-100 and THDB were identified as salt tolerant rice genotypes. 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 susceptible ones.

Key words: rice, salinity tolerance, SSR markers, seedling stage.

RESUMEN

La selección para resistencia a la salinidad de genotipos de arroz, basada solamente en el comportamiento fenotípico, es menos confiable y retarda el avance en el mejoramiento. Se han utilizado avances recientes en marcadores moleculares, microsatélites o repeticiones de secuencias simples (SSR por sus siglas en inglés) para determinar genotipos de arroz tolerantes a la salinidad. Se utilizaron tres marcadores SSR *viz*., RM7075, RM336 y RM253 para evaluar genotipos de arroz para tolerancia a la salinidad. La evaluación fenotípica y genotípica para la tolerancia a la salinidad se realizó en la etapa de plántula. La fenotipificación de once genotipos se realizó en un sistema hidropónico utilizando solución nutritiva salinizada (CE 12 dS/m). Se siguió el protocolo estandarizado del IRRI para evaluar la tolerancia a la salinidad. Se detectó una gran variación en la tolerancia a la salinidad entre el germoplasma de arroz. La altura de las plántulas y la materia seca total de las líneas tolerantes se redujeron en un 19,0 y 40,6%, respectivamente, bajo estrés salino (CE 12 dS/m), en tanto que las de las líneas susceptibles se redujeron en un 46,0% y 73,5%, respectivamente. Los marcadores mostraron polimorfismo y fueron capaces de discriminar los genotipos tolerantes a la salinidad de aquellos susceptibles. Los genotipos con un patrón similar de bandas a Pokkali se consideraron como tolerantes a la salinidad. Los marcadores SSR (RM7075, RM336 y RM253) identificaron ocho, nueve y siete genotipos tolerantes a la salinidad, respectivamente. A través del estudio fenotípico y genotípico, tres genotipos *viz*., Pokkali, TNDB-100 y THDB se identificaron como cultivares de arroz tolerantes a la salinidad. Estos marcadores SSR podrían tener homología de secuencias con genotipos de arroz tolerantes a la salinidad y por consiguiente, los marcadores podrían ser capaces de identificar genotipos de arroz tolerantes a la salinidad de aquellos susceptibles.

Palabras clave: Arroz, tolerancia a la salinidad, marcadores SSR, etapa de plántulas.

INTRODUCTION

Rice is the staple food of more than 50% of the world's population (Aggarwal *et al*., 2002). By the year 2025, 21% increase in rice production will be needed over the production of 2000 (Bhuiyan *et al*., (2002). Salinity is one of the major constraints to productivity 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 salinity-prone coastal areas. The response of rice to salinity varies with growth stage. Several studies have indicated that rice at early seedling stage (2-3 leaf stage) and during pollination and fertilization is more sensitive to salinity than during, germination or vegetative growth stage or late reproductive stages (IRRI, 1967).

Screening of germplasm at seedling stage is readily acceptable as it is based on a simple criterion of selection; whereas rapid screening becomes difficult at vegetative and reproductive stages (Gregorio *et al*., 1997). Screening under controlled condition has the benefit of reduced environment effects and the hydroponic system is free from complex variations 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 (Gregorio, 1997). 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 permits rapid and improved accuracy in breeding for traits prone to pronounced environmental effects leading to poor selection efficiency.

SSR or microsatellite markers have been proved to be ideal for making genetic maps (Islam, 2004; Niones, 2004), assisting selection (Bhuiyan, 2005) and studying genetic diversity in germplasms. SSR markers play an important role while identifying gene for salt tolerance or in introgressing the genes to develop new cultivars. The aim of the present study was to screen rice germplasm for salinity response and to evaluate microsatellite markers for the identification of salt tolerant genotypes at the seedling stage.

MATERIALS AND METHODS

Plant materials

Eleven rice germplasm accessions, with diverse genetic background, were used in this study. Of which six were Bangladeshi landraces, four were Bangladesh Institute of Nuclear Agriculture (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 screened for salt tolerance at seedling stage in hydroponic system using IRRI standard protocol (Gregorio, 1997). Salinized and non-salinized setups with three replications were maintained. The evaluation was done using Yoshida *et al.* (1976) 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 was used in rating the visual symptoms of salt toxicity (IRRI, 1997). 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. Other observations are seedling height, root length and total dry matter recorded both at salinized and non-salinized conditions.

Table 1. Modified standard evaluation score of visual salt injury at seedling stage (Method adapted from Gregorio *et al.*, (1997)).

Score	Observation	Response category
1	Normal growth with no 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

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. Grinding of leaf sample with extraction buffer and SDS was followed by incubating 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 minutes. After that the suspensions were transferred to a new plate. 900 µl chloroform : isoamyl (24:1) was added and mixed well by a shaker. The sample was then centrifuged at 5700 rpm for 10 minutes. After that the supernatant were transferred into new eppendorf tubes. Then 600 µl ice-cold isopropanol was added into the new eppendorf tubes and shaken slowly and then centrifuged at 5700 rpm for 15 minutes. The supernatant was decanted 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 1/2-1 hours. 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 (2004); Bonilla *et al.* (2002); Niones (2004) and Gregorio *et al.* (2002) 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 Each PCR reaction was carried out with 15.0µl reaction mixtures 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 ddH_2O 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 germplasm lines that showed similar banding pattern like Pokkali, were considered as tolerant and that with different banding pattern were considered as susceptible.

Data analysis

Data obtained were subjected to one way analysis of variance for completely randomized design. Treatment means were compared using Least Significant Difference. Correlation coefficients of different traits at seedling stage under salinized condition were also calculated.

RESULTS AND DISCUSSION

Screening of genotypes for salt tolerance at seedling stage

All genotypes grew robust and were uniform in colour and height in the non-salinized condition. In salinized condition, the genotypes showed wide ranging variation in phenotypes from score 1 (highly tolerant) to 9 (highly susceptible) (Figure 1). The most salinity tolerant germplasm were 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 of IRRI (Gregorio *et al.*, 1997) was used in rating the visual symptoms of salt injury.

Seedling height was shorter in salinized condition, compared to the seedlings grown in non-salinized conditions (Table 2). Seedling height and total dry matter of susceptible genotypes showed higher percent reduction than tolerant genotypes.

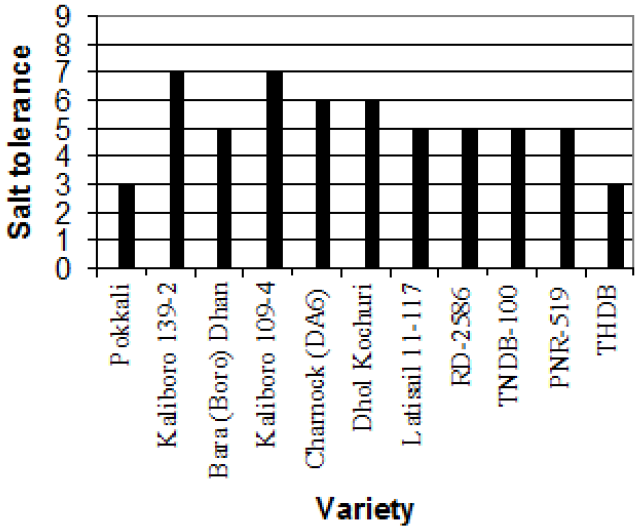

Figure 1. Visual symptoms score distribution in rice genotypes (n = 11) under salt stress at seedling stage when grown in hydroponics with nutrient solution.

Lower percent reduction of seedling 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 seedling 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 (Suplick-Ploense *et al*., 2002).

All the eleven genotypes showed a wide variation in phenotypes. Salt tolerant seedlings were distinct 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 shoot growth with thickened stem leading to a complete cessation of growth and dying of seedlings (Gregorio *et al.*, 1997). 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 (Lin *et al*., 2004). 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 (Hasegawa *et al*., 2000).

At the seedling stage, highly significant and positive correlations were found between plant height and total dry matter at salinized condition and correlations between salt tolerance and plant height, total dry matter and root length were inverse and significant (Table 3). This implies that salt tolerant genotypes (with lower salt tolerance score) exhibited higher plant height and total dry matter. Peng *et al*. (1999) reported that increasing plant height would allow greater biomass production. Zhang *et al*. (2004) found similar result in their study with doubled haploid (DH) population consisting of 81 lines. They reported that increase of plant height was responsible for increase in 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.

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

Traits	Salt tolerance	Seedling height	Total dry matter
Seedling height	-0.406 **		
Total dry matter	-0.740 **	0.622 **	
Root length	-0.278 *	0.115 ns	0.291 *

* = Significant at 5% level of probability
** = Significant at 1% level of probability
ns = Non significant at > 5% level of probability

Table 2. Seedling height and total dry matter in rice genotypes (n = 11) under both non-saline and saline conditions at seedling stage when grown in hydroponics with nutrient solution.

Serial No.	Genotypes	Seedling height (cm)			Total dry matter (g)		
		Non-salinized	Salinized	% reduction	Non-salinized	Salinized	% reduction
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
$LSD_{(0.05)}$		1.7	1.4	-	0.442	0.208	-

On the basis of standard evaluation system 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 SSR markers, RM7075, RM336 and RM253, were used to evaluate germplasms for salinity tolerance. 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 while others 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 (Figure 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. 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.

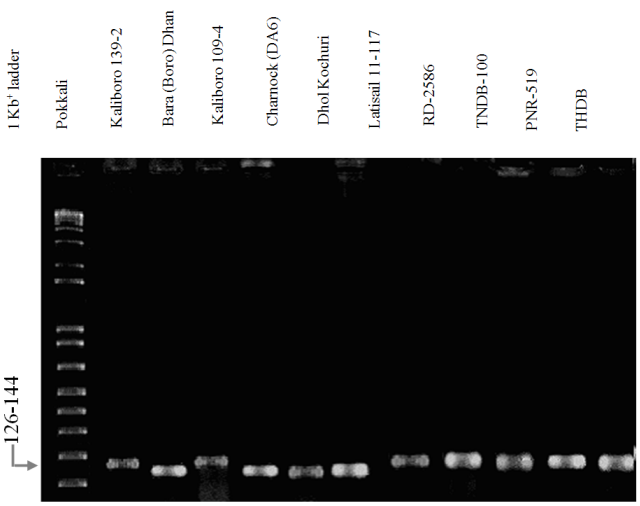

Figure 2. Polymorphism exhibition of RM 253 among eleven rice genotypes

The marker RM7075 identified eight tolerant and three susceptible genotypes in comparison with Pokkali. Out of genotypically identified eight salt tolerant genotypes, three were tolerant and five were moderately tolerant based of phenotypic performance. With marker 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 (2005) identified 158 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 both 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 characterised as moderately tolerant. The selected markers (RM7075, RM336 and RM253) showed good level of polymorphism with the eleven rice genotypes. These SSR markers were able to discriminate well tolerant genotypes from susceptible. Thus these markers have a clear relationship with salt tolerance alleles studied in rice genotypes. Molecular marker helps to identify alleles that are associated with key phenotypic traits (Xu *et al*., 2004). Nguyen *et al*., (2001) 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*., (2000). They found that RM223 was closely linked to salt tolerance gene in chromosome 8. Since, the markers that were used in this study showed polymorphism, 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.

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