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APPLICATION OF SSR TECHNIQUE FOR THE IDENTIFICATION OF MARKERS LINKED TO SALINITY TOLERANCE IN RICE

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ABSTRACT

A cross was made between high yielding salt susceptible BINA variety (Binadhan-5) with salt tolerant rice landrace (Harkuch) to identify salt tolerant rice lines. Thirty six F_3 rice lines of Binadhan-5 x Harkuch were tested for salinity tolerance at the seedling stage in hydroponic system using nutrient solution. In F_3 population, six lines were found as salt tolerant and 10 lines were moderately tolerant based on phenotypic screening at the seedling stage. Twelve SSR markers were used for parental survey and among them three polymorphic SSR markers viz., OSR34, RM443 and RM169 were selected to evaluate 26 F_3 rice lines for salt tolerance. With respect to marker OSR34, 15 lines were identified as salt tolerant, 9 lines were susceptible and 2 lines were heterozygous. While RM443 identified 3 tolerant, 14 susceptible and 9 heterozygous rice lines. Eight tolerant, 11 susceptible and 7 heterozygous lines were identified with the marker RM169. Thus the tested markers could be efficiently used for tagging salt tolerant genes in marker-assisted breeding programme.

Key words : Microsatellite markers, rice and salt tolerance

INTRODUCTION

Salt stress is a major constraint to cereal production worldwide. The total saline area forms a third of the 9 million hectares of total cultivated area in Bangladesh (ABSPII, 2006). Agriculture is a major sector of Bangladesh's economy and the coastal area of Bangladesh is very fertile for growing rice. Increase in salinity intrusion and increase in soil salinity will have serious negative impacts on agriculture. Conventional breeding programs for salinity tolerance include the development of rice varieties tolerant to salt and efforts to incorporate salt tolerance to rice from wild related species. To improve the salt tolerance of crops through conventional breeding programmes have met with very limited success, due to complexity of the traits: salt tolerance is complex genetically and physiologically (Flowers, 2004). A number of genomic tools, such as molecular markers and gene profiling methods, can greatly improve the efficiency of breeding programs, and should be fully exploited for conventional breeding initiatives. Microsatellite markers have been used effectively to map QTLs associated with salt tolerance (Singh *et al*., 2007). An important region containing the salinity tolerance QTLs on rice chromosome 1 was identified by QTL analysis in Pokkali x IR29 cross using microsattelite markers (Islam,

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2004). SSR markers were used to sorting salt tolerant progeny from segregating generations (Bhuiyan, 2005) and microsatellite marker analysis is attractive for developing marker-assisted selection programs (Gregorio *et al*., 2002). The present work has aimed to identify markers linked to salinity tolerance in rice by SSR technique and to select the salt tolerant F_3 rice lines through phenotypically and genotypically.

MATERIALS AND METHODS

Thirty six F_3 rice lines were selected based on better agronomic performances from Binadhan-5 x Harkuch. These rice lines were screened for salinity tolerance at the seedling stage. Out of 36 F_3 rice lines, 26 F_3 rice lines were selected for genotyping based on the greenhouse evaluation of salt tolerance through marker analysis (using SSR markers). Hydroponic system with IRRI standard protocol (Gregorio *et al*., 1997) was used at the glasshouse to evaluate for salt tolerance in F_3 rice lines using nutrient solution (Yoshida *et al*., 1976). Two pregerminated seeds were sown per hole on the styrofoam seedling float with tap water. The seedlings were allowed to grow for 3 days; tap water was replaced with nutrient solution. The nutrient solution was salinized at EC 12 dS/m by adding crude salt (non-refiend). Culture solution was replaced at every 8-day and the pH was maintained at 5.25 daily. The modified standard evaluation score (SES) of IRRI was used to assess the visual symptoms of salt toxicity (Table 1). Initial and final scoring was done at 15-day and 21-day after salinization.

Table 1. Modified standard evaluation score (SES) of visual salt injury at seedling stage of rice

Score	Growth condition	Level of tolerance
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 dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

The modified Cetyl Trimethyl Ammonium Bromide (CTAB) mini-prep method was used to extract DNA from healthy leaf samples of 25-day old seedlings. Three polymorphic SSR markers viz., OSR34, RM443 and RM169 were selected to evaluate the 26 F_3 rice lines for salt tolerance (Table 2). Each PCR reaction carried out with 15.0 µl reactions containing 1.5 µl 10X buffer, 0.75 µl dNTPs (100 mM), 1 µl primer forward (5 µM), 1 µl primer reverse (5 µM), 0.5 µl taq polymerase (3 u/µl), 8.25 µl ddH_2O and 2.0 µl of each template DNA samples. The PCR reaction were: initial denaturation at 94°C for 5 min., denaturation at 94°C for 1 min., primer annealing at 55°C for 1 min., primer extension at 72°C for 2 min., cycle to step 2 for 34 more times and incubation at 72°C for 7 min. Then electrophoresis was done in 1.5% agarose gel and soaked in ethidium bromide (10 mg/ml) solution for

20-25 min. The gels were viewed by the Gel Documentation system. The segregating population having similar banding pattern to Harkuch were considered as tolerant, banding pattern having similar to Binadhan-5 were considered as salt susceptible and having one allele from Binadhan-5 and one allele from Harkuch was considered as heterozygous.

Table 2. The sequence and size of the microsatellite markers used for screening salt tolerant rice lines

Primer	PCR product size (bp)	Sequence		Annealing temperature (°C)
		Forward	Reverse	
OSR34	n.a	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	55
RM443	118-124	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGCTTCG	55
RM169	164-194	TGGCTGGCTCCGTGGGTAGCTG	TGGCTGGCTCCGTGGGTAGCTG	67

RESULTS AND DISCUSSION

In phenotypic screening, F_3 rice lines under salt stress, expressed wide variation with scoring 3 (tolerant), 5 (moderately tolerant), 7 (susceptible) and 9 (highly susceptible). Under salinity stress at 12 dS/m, growth of seedlings were suppressed and salt injury induced severe symptoms on sensitive lines leading to complete cessation of their growth and consequently seedlings died early. Six lines, SAL-05-7, SAL-05-13, SAL-05-18, SAL-05-20, SAL-05-25 and SAL-05-26 were identified as salt tolerant (Table 3). The moderately salinity tolerant lines were SAL-05-5, SAL-05-9, SAL-05-10, SAL-05-11, SAL-05-16, SAL-05-19, SAL-05-27, SAL-05-29, SAL-05-39 and SAL-05-42. Fifteen lines were selected as susceptible and rests of the lines were found as highly susceptible. About 16.6% of the F_3 populations were found as tolerant as Harkuch, 27.7% were found as moderately tolerant, 41.6% as susceptible and 13.8% as highly susceptible. The sodium concentration within the leaves of rice exposed to salinity shows a wide distribution between individuals and transgressive segregation, features of a multigenic character. Some plants in this population were more tolerant or susceptible than their parents, suggesting that loci other than the major one are involved in the determination of salinity tolerance. Breeding for salt tolerance using traditional screening and selection methods have been limited by the complex and polygenic nature of salt tolerance traits. Because of environmental effects on the expression of the tolerance, recombinant inbred lines could be used to improve salt tolerant rice variety. Sohrawardy *et al*. (2008) investigated 26 F_3 rice lines of PNR-519 x IR29 to evaluate salt tolerance at the seedling stage using hydroponic system following IRRI standard protocol and reported that lines 05-29, 05-33 and 05-47 were tolerant, 4 lines were moderately tolerant and 11 lines were susceptible. In F_3 population, Kaushik *et al*. (2003) reported that salt tolerance score ranged from 1.72 to 8.45 with a mean value of 5.308 and they found some plants in this population were even more tolerant or susceptible (transgressive segregants) than their parents. Islam *et al*. (2007) also used modified SES of IRRI standard protocol and they found the strains Pokkali, Jamainaru, Harkuch, RC-STL-33, RD-2586, and RC-STL-1 to be salt tolerant and

RC-STL-2, Bakibalam, Rajasail, BR10, BR23, Atomita 4, Y-1281 and TNDB100 to be moderately salt tolerant. In one study of Sheetal *et al*. (2008) found that traditional Basmati rice varieties (Basmati 370 and HBC19) were more sensitive than the salt sensitive control variety, MI-48 when assessed for salinity tolerance on 1–9 scale using seedling growth parameters, visual salt injuries and Na-K ratio.

Table 3. Performance of F_3 rice lines under salinized condition (EC 12 dS/m) grown in hydroponic system at the seedling stage

Parents/ lines no.	SES score	Tolerance level	Parents/ lines no.	SES score	Tolerance level
SAL-05-1	9	HS	SAL-05-19	5	MT
SAL-05-2	7	S	SAL-05-20	3	T
SAL-05-3	7	S	SAL-05-21	7	S
SAL-05-4	7	S	SAL-05-22	7	S
SAL-05-5	5	MT	SAL-05-23	9	HS
SAL-05-6	7	S	SAL-05-24	9	HS
SAL-05-7	3	T	SAL-05-25	3	T
SAL-05-8	7	S	SAL-05-26	3	T
SAL-05-9	5	MT	SAL-05-27	5	MT
SAL-05-10	5	MT	SAL-05-28	7	S
SAL-05-11	5	MT	SAL-05-29	5	MT
SAL-05-12	7	S	SAL-05-30	7	S
SAL-05-13	3	T	SAL-05-31	9	HS
SAL-05-14	7	S	SAL-05-37	7	S
SAL-05-15	9	HS	SAL-05-39	5	MT
SAL-05-16	5	MT	SAL-05-41	7	S
SAL-05-17	7	S	SAL-05-42	5	MT
SAL-05-18	3	T	SAL-05-44	7	S
Binadhan-5(P_1)	7	S	Harkuch(P_2)	1	HT

P = Parent, 1-9 Scale, where 1 = highly tolerant (HT), 3 = tolerant (T), 5 = moderately tolerant (MT), 7 = susceptible (S), and 9 = highly susceptible (HS)

In genotyping salinity tolerance, identification of molecular markers tightly linked to salt tolerant genes can serve as land marks for the physical localization of such genes facilitating marker assisted selection (MAS). In respect of the marker OSR34, 15 lines were identified as salt tolerant, 9 lines were susceptible and 2 lines were heterozygous in F_3 population (Fig. 1, Table 4). Three tolerant, 14 susceptible and 9 heterozygous lines were identified by RM443 (Fig. 2). Eight tolerant, 11 susceptible and 7 heterozygous lines were identified when 26 F_3 lines were genotyped with marker RM169 (Fig. 3). Line SAL-05-12,

SAL-05-13, SAL-05-19 and SAL-05-27 were tolerant using OSR34 and RM169 markers; line SAL-05-26 was tolerant by RM443 and RM169 markers. Line SAL-05-4 was found susceptible using OSR34, RM443 and RM169 markers (Table 4). Considering the marker OSR34, out of 15 genotypically tolerant rice lines, phenotypically two rice lines were identified as tolerant and 4 lines were moderately tolerant. With respect to marker RM443, out of 3 genotypically tolerant rice lines, phenotypically 2 tolerant lines were tagged. Regarding marker RM169, out of 8 tolerant lines, 4 tolerant and 2 moderately tolerant lines were identified by phenotypically at the seedling stage. On the basis of phenotypic and genotypic performances, line SAL-05-7, SAL-05-13, SAL-05-18, SAL-05-20, SAL-05-25 and SAL-05-26 were selected as tolerant and SAL-05-11, SAL-05-16, SAL-05-19 and SAL-05-27 were tagged as moderately tolerant. Several SSR markers were used by Sohrawardy *et al*. (2008) for the identification of salt tolerant rice lines of PNR-519 x IR29 in F_3 population. They reported that line 05-28 and 05-33 were found as salt tolerant. Islam *et al*. (2008) selected different SSR markers to evaluate F_2/F_3 rice lines for salt tolerance and identified 15 rice lines as salt tolerant using RM231 and RM24 markers. Six tolerant rice lines were identified by Paul *et al*. (2008) when marker RM152 and RM334 were used in F_2 progenies of Binadhan-5 x Bawoi Jhak. Bhuiyan (2005) also identified 158 tolerant individuals of F_2 and F_3 population of BRRI Dhan 28 x PSBRc88 with the marker RM493.

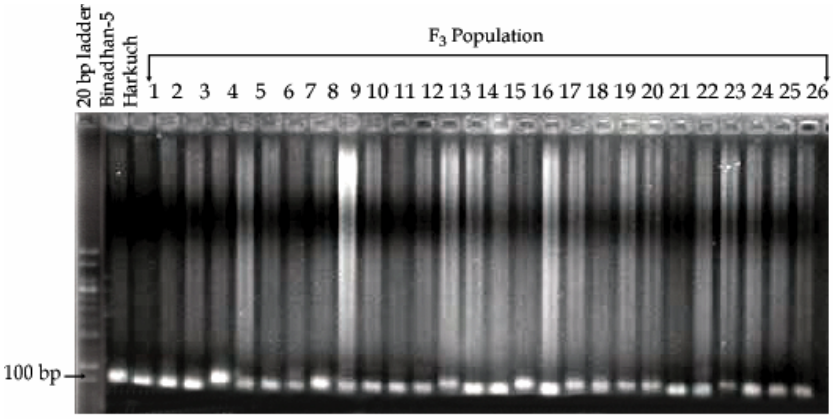

Fig. 1. A gel image of banding profiles of two parents and 26 F3 population derived from the cross Binadhan-5 x Harkuch using primer OSR34

To examine the accuracy of the OSR34 genetic marker for identifying tolerant and susceptible homozygotes, genomic DNAs from 26 F_3 individuals were amplified and the genotypes were identified. Among these rice lines, 6 were tolerant, 5 moderately tolerant, 12 susceptible and 3 highly susceptible to salinity at the seedling stage. Of the 11 tolerant and moderately tolerant lines, 6 lines were homozygous for the Harkuch allele and 4 lines were homozygous for the Binadhan-5 allele and 1 was heterozygous. This gives an accuracy of prediction of salt tolerance based on the marker of 54.5%. Of the 15 rice lines

that were scored as susceptible and highly susceptible, 5 rice lines were homozygous for the Binadhan-5 allele, giving an accuracy of prediction of 33.3% (Table 5).

Regarding the marker RM443, of the 11 tolerant and moderately tolerant lines, 2 lines were homozygous for the Harkuch allele, giving an accuracy of prediction of 18.8%. Of the 15 susceptible and highly susceptible lines, 8 lines were homozygous for the Binadhan-5 allele, giving an accuracy of prediction of 53.3% (Table 6).

Table 4. Genotypic performances of F_3 segregating population using SSR markers

Lines no.	Salt tolerance with SSR markers			Lines no.	Salt tolerance with SSR markers		
	OSR34	RM443	RM169		OSR34	RM443	RM169
SAL-05-2	T	S	S	SAL-05-17	T	H	S
SAL-05-3	T	S	S	SAL-05-18	S	H	T
SAL-05-4	S	S	S	SAL-05-19	T	S	T
SAL-05-6	T	H	S	SAL-05-20	S	T	H
SAL-05-7	T	S	S	SAL-05-21	S	S	H
SAL-05-8	H	H	T	SAL-05-22	S	H	S
SAL-05-9	S	H	H	SAL-05-23	S	H	H
SAL-05-11	T	S	H	SAL-05-24	T	S	H
SAL-05-12	T	S	T	SAL-05-25	H	H	T
SAL-05-13	T	S	T	SAL-05-26	S	T	T
SAL-05-14	T	H	H	SAL-05-27	T	S	T
SAL-05-15	S	T	S	SAL-05-28	T	S	S
SAL-05-16	T	S	S	SAL-05-30	T	S	S

T = Tolerant, S = Susceptible and H = Heterozygous

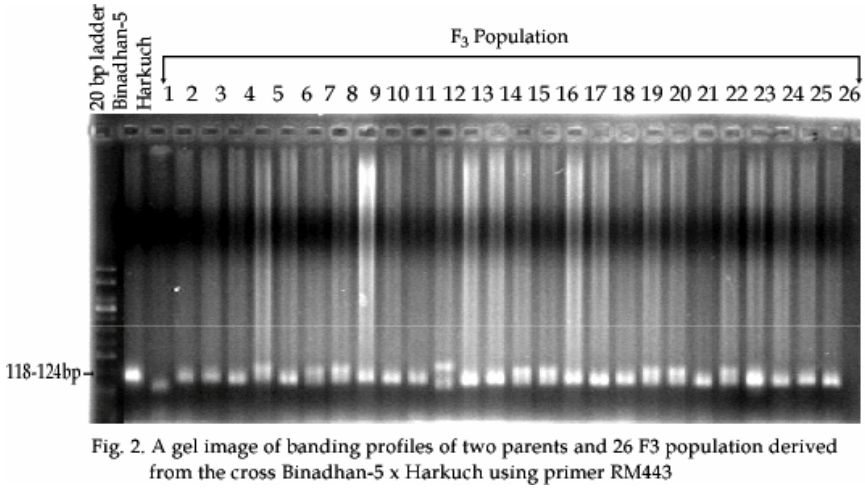

Fig. 2. A gel image of banding profiles of two parents and 26 F3 population derived from the cross Binadhan-5 x Harkuch using primer RM443

Considering the marker RM169, of the 11 tolerant and moderately tolerant lines, 6 lines were homozygous for the Harkuch allele, giving an accuracy of prediction 54.5%. Of the 15 susceptible and highly susceptible lines, 9 lines were homozygous for the Binadhan-5 allele, giving an accuracy of prediction of 60% (Table 7).

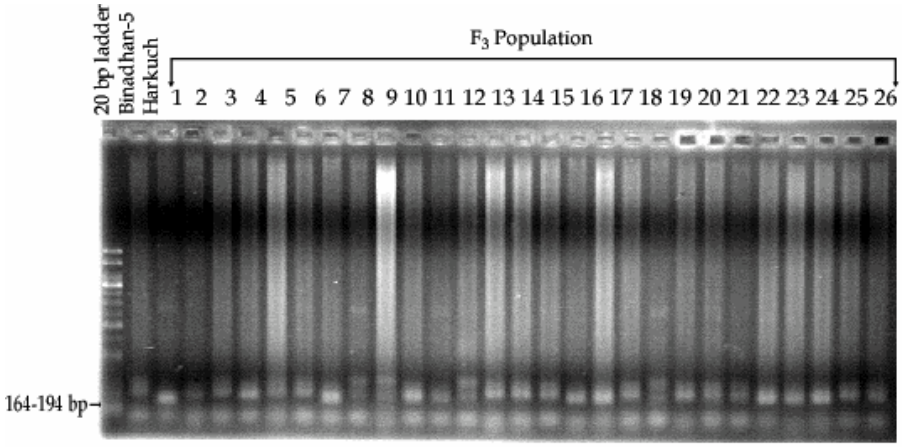

Fig. 3. A gel image of banding profiles of two parents and 26 F3 population derived from the cross Binadhan-5 x Harkuch using primer RM169

Table 5. F_3 lines with respect to the alleles amplified by the microsatellite primer OSR34 in the DNA of the tolerant parent Harkuch (allele T) or of the sensitive parent (allele S) in the lines that were rated as tolerant or susceptible to salt at the seedling growth stage

Salinity rating of F_3 lines (seedling stage)	No. of F_3 lines	F_3 genotypes at the OSR34 locus			Accuracy of classification by marker (%)
		TT	TS	SS	
Tolerant and moderately tolerant	11	6	1	4	54.5
Susceptible and highly susceptible	15	9	1	5	33.3

Table 6. F_3 lines with respect to the alleles amplified by the microsatellite primer RM443 in the DNA of the tolerant parent Harkuch (allele T) or of the sensitive parent (allele S) in the lines that were rated as tolerant or susceptible to salt at the seedling growth stage

Salinity rating of F_3 lines (seedling stage)	No. of F_3 lines	F_3 genotypes at the RM443 locus			Accuracy of classification by marker (%)
		TT	TS	SS	
Tolerant and moderately tolerant	11	2	3	6	18.8
Susceptible and highly susceptible	15	1	6	8	53.3

Table 7. F_3 lines with respect to the alleles amplified by the microsatellite primer RM169 in the DNA of the tolerant parent Harkuch (allele T) or of the sensitive parent (allele S) in the lines that were rated as tolerant or susceptible to salt at the seedling growth stage

Salinity rating of F_3 lines(seedling stage)	No. of F_3 lines	F_3 genotypes at the RM169 locus			Accuracy of classification by marker (%)
		TT	TS	SS	
Tolerant and moderately tolerant	11	6	3	2	54.5
Susceptible and highly susceptible	15	2	4	9	60

Evaluating and selecting salt tolerance among rice lines are not easy tasks because measurements of physiological and morphological phenotypes are highly affected by environmental factors. However, larger number of samples and adequate number of primers would be necessary to generate and construct an appropriate genetic relationship, for identification of samples with salt tolerant genes and to get more reliable markers. Molecular markers that are linked to genes controlling salinity tolerance could facilitate selection and improve rice varieties with salinity tolerance having high heritability and expressivity, which in turn increase rice production in saline environments. Thus, this study implies that SSR technique in tagging salt tolerant gene is simple, rapid and efficient and these markers (OSR34, RM443 and RM169) could be efficiently used in tagging salt tolerant genes, in marker-assisted selection and quantitative trait loci QTL) mapping. This will be useful for developing salinity tolerant variety through Marker Assisted Selection.

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