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Short Communication

Identification of SSR Markers for Salt-tolerance in Rice Variety CSR10 by Selective Genotyping

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A population of 171 F_3 genotypes derived from a cross between CSR10 (salt tolerant, *indica*) and Taraori Basmati (HBC19) was evaluated for various salt-tolerance attributes at vegetative stage using a hydroponic culture system. Substantial variation was observed in F_3 population for relative growth rate (range 0.065-0.187), Na-K ratio (0.023-0.376) and visual injury symptoms (score 1-9). The mean individual score of CSR10 x HBC19 F_3 plants ranged from 1.7 to 9.0 with mean value of 5.07. Seven of the F_3 plants showed transgressive segregation for salt tolerance. F_3 individuals at both extremes of the response distribution were selected and genotyped using 30 SSR markers displaying polymorphism between the two parental genotypes. As many as 18/30 SSR markers showed distorted segregation ratios among the 30 selected salt-tolerant and salt-sensitive CSR10 x HBC19 F_3 plants. Linear regression analysis showed significant association of three markers (RM162 mapped on chromosome 6, and RM209 and RM287 on chromosome 11) with relative growth rate and two markers (RM212 on chromosome 1 and RM206 on chromosome 11) with Na-K ratio explaining 31.3% and 25.6% of phenotypic variation, respectively.

***Key words*:** Basmati, *Oryza sativa*, rice, SSR, salt-tolerance.

Soil salinity limits rice yield and productivity and prevents its cultivation over large areas around the world (1). Often flooded paddy raises local ground water level, bringing salts to the surface and such seasonal increase in top-soil salinity can hardly be avoided. In Basmati rice belt (North-Western regions of India), the problem of salinity and water logging have reached serious proportions and is increasing further (2). The gene pools from wild progenitor species/ landraces have been exploited for breeding for salinity tolerance in rice and a few salt-tolerant rice varieties have been developed and released for commercial cultivation (1). However, the Basmati rice breeding has been difficult because of poor combining ability, incompatibility with *indica*/*japonica* cultivars and high inter-group hybrid sterility (3). Introgression of salt tolerance trait into Basmati rice is further complicated because of the need to keep all the Basmati rice grain and cooking quality traits intact during the selection process. The efficacy and the precision of Basmati rice breeding programs can be greatly enhanced with the aid of genetic maps, gene/QTL (quantitative trait loci) analysis and marker assisted selection (4). In this paper, we report the association of SSR markers with QTLs contributing to salt tolerance at vegetative stage using a 'selective genotyping' approach (5) in an interspecific F_3 population of a cross between salt tolerant *indica* (CSR10) and salt sensitive Basmati (HBC19) rice varieties. Use of already-mapped SSR markers for linkage mapping has an edge over the other marker types, as it directly provides information about the putative chromosomal segments carrying the genes/QTLs for a specific trait (6, 7).

A population of 171 CSR10 x HBC19 F_3 seedlings/ plants was used for salinity tolerance analysis. CSR10 (selection from CSR1/Jaya) developed at CSSRI, Karnal (India) has been recommended for cultivation in saline soils. HBC19 (a pure line selection from Taraori Basmati) is a commercially important premium traditional Basmati rice variety. Evaluation of salt tolerance was carried out in a net-house during rice growing season using a hydroponic culture system as described earlier (8). The de-husked seed of 171 F_2 and parental rice genotypes were allowed to germinate directly onto nylon mesh supported on floating thermocol sheets (specially-designed sheets having 1 cm diameter holes with attached fine nylon mesh on the lower surface) in Yoshida nutrient solution (pH 5.0; electrical conductivity 0.7 dS/m) (9). After 14 days, the nutrient solution was replaced with the solution containing 30 mM NaCl (EC 4.8 dS/m, pH 5.0). Solutions were renewed after every three days and water level and pH were adjusted on alternate days. After 3 weeks of salinity treatment, 171 CSR10 x HBC19 F_3 plants were individually evaluated for salt tolerance by measuring relative growth rate (RGR),

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Na^+ and K^+ contents and visual salt-injury symptoms. RGR is measured using the following equation given by Salim and Pitman (10): $RGR = \ln(sl_2\text{-}sl_1)/\Delta t$; where sl_2 and sl_1 are the shoot lengths at time t_2 and t_1 respectively, whereas Δt is the change in time ($t_2 - t_1$). Na^+ and K^+ were estimated in µmol/g tissue dry weight units using the Flame Photometer (Systronics 128, India). Data was recorded on visual effects of salinity including shoot tip burning, yellowing of leaves, leaf curling and physical appearance of the plants as per IRRI standard evaluation system (11). Every F_3 plant was scored for each trait on a 1-9 scale (lower score state for salt tolerance). The scores were averaged and used for the selection of 15 most salt-tolerant and 15 salt-sensitive (surviving) plants from the population of 171 CSR10 x HBC19 F_3 plants. The population of 171 F_3 plants was grouped under 1, 3, 5, 7 and 9 score categories and mean was calculated using Snedecor and Cochran (12) method. The F_3 population was tested for normal distribution using 'Z' statistics.

Genomic DNA was extracted from young leaf samples of parental rice genotypes and selected F_3 plants using the modified CTAB method (13). DNA was purified and checked for its quality and quantity by 1% agarose gel electrophoresis using a standard containing 100 ng per µl genomic λDNA. A total of 30 SSR markers (RM152, 162, 180, 201, 206, 208, 209, 210, 212, 220, 223, 234, 235, 240, 241, 242, 247, 250, 251, 252, 255, 258, 260, 264, 287, 310, 312, 324, 335 and 339) showing polymorphism between CSR10 and HBC19 were used for molecular analysis of 30 selected CSR10 x HBC19 F_3 plants. The map position, original source and repeat motifs for these markers can be found in RiceGenes database (http://www.gramene.org/microsat/RM_primers.html). PCR amplification, denaturing polyacrylamide gel electrophoresis and silver staining were carried out as described earlier (14). The genetic associations among 30 CSR30 x HBC19 F_3 and parental genotypes were evaluated by calculating the 'Jaccard' similarity coefficient for pair-wise comparisons based on the proportion of shared bands (alleles) and two-dimensional PCA (Principal Component Analysis) (7). The Mendelian segregation ratio of 30 SSR markers was tested by chi square analysis ($P<0.05$) and the markers were scored as 0 (homozygous type of susceptible parental line), 2 (homozygous type of tolerant parental line) and 1 (heterozygous type). Association between molecular markers and salt tolerance were detected by linear regression analysis where markers were considered as independent variables. The associations were considered significant at $P<0.01$, in which the coefficient of determination (R^2) was used to estimate the amount of phenotype variation explained by the markers.

At 30 mM NaCl, substantial variation was observed for relative growth rate (RGR), Na-K ratio and visual salt-injury symptoms in the population of 171 CSR10 x HBC19 F_3 plant saplings and parental rice varieties (Fig. 1). CSR10 showed a relative growth rate (RGR) of 0.168 compared to 0.080 in HBC19, while RGR ranged between 0.065-0.187 in CSR10 x HBC19 F_3 plants with a mean value of 0.138±0.002. Na-K ratio was significantly low in CSR10 (0.086) in comparison to HBC19 (0.289); the ratio varied between 0.023-0.376 in F_3 plants with an average of 0.132±0.004. Data on salt stress injuries including shoot tip burning, yellowing of leaves and leaf curling showed CSR10 as highly tolerant with a score of 1.0 and HBC19 as highly sensitive with a score of 9.0; the score ranged between 1 and 9 in F_3 plants with a mean value of 4.310±0.153. The two parental rice varieties, CSR10 and

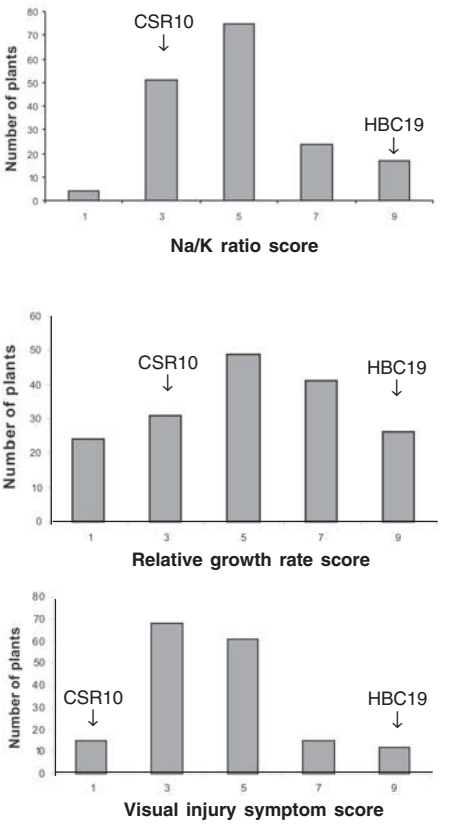

Fig. 1. Frequency distribution of relative growth rate, Na/K ratio and visual salt injury symptoms in 171 CSR10 x HBC19 F_3 plants.

HBC19 had overall mean scores of 2.3 and 8.3, respectively. The mean salinity score of the CSR10 x HBC19 F_3 plants ranged from 1.7 to 9.0 with a mean value of 5.076. Z statistics data of population suggested a good fit (ψ^2=9.09, *p*=0.01) to normal distribution (data not shown). Maximum number of F_3 plants (95 plants) was in score 5.0 category (Fig. 1). Four per cent (7 plants) of F_3 plants displayed even greater salt tolerance than CSR10 and 3.5% (6 plants) were as sensitive as HBC 19. A significant positive rank correlation (P<0.01) was observed between RGR and visual salt injury symptoms (0.340), RGR and Na-K ratio (0.259) and Na-K ratio and visual salt injury symptoms (0.239). Na-K ratio was found to be more variable with a covariance of 49.8 compared to relative growth rate, which had a covariance of 18.2. F_3 individuals at both extremes of the response distribution (salt tolerant and salt-susceptible genotypes) were selected on the basis of mean score. Selected F_3 plants did show variability for score for three parameters but within moderate to tolerant/sensitive range (data not shown).

The salt injury starts with reduction in effective leaf area. The oldest leaves start to roll, and turn yellowish and/or whitish then die followed by next older leaves. These internal salt injuries is due to the accumulation of salt in the transpiring leaves to excessive levels, causing premature senescence and reducing the photosynthetic capacity of the plant to a level that can not sustain further growth. The salt sensitive HBC19 and F_3 plants may be lacking an effective mechanism for regulating the Na^+ across the membranes. A positive correlation between RGR, visual salt-injury symptoms and Na-K ratio observed in CSR10 x HBC19 F_3 population is in agreement with the notion that excess Na^+ may be the primary cause of salt sensitivity in rice genotypes (15). Notably, some of the F_3 plants showed transgressive segregation for salt tolerance, which may have resulted due to accumulation and/or differential combination of genes/QTLs conferring salt tolerance (16).

Selected F_3 plants had either one or both of the parental alleles (Fig. 2). Eleven of 30 F_3 plants had new (rare) alleles at 1-3 of the 30 marker loci with a parental allele or in the homozygous state, except a salt tolerant F_3 plant (plant # 12; Fig. 2) that had rare alleles at 11/30 loci. The origin of these rare alleles may be another interesting area to work on. Theoretically, these new alleles could arise due to recombination (17) and/or unequal crossing over/

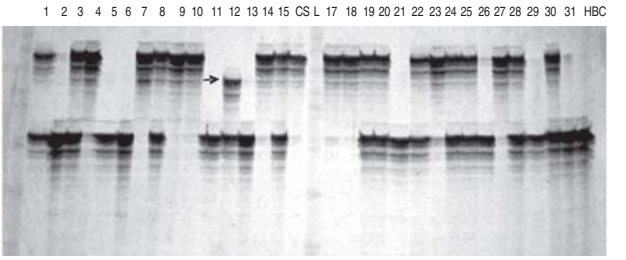

Fig. 2. A silver stained gel showing allelic polymorphism among the selected salt tolerant (1-15) and salt-sensitive (17-31) F_3 plants at RM335 locus. CS, CSR10 (144 bp); HBC, HBC19 (126 bp); L, 10 bp ladder (L). Arrow indicates the rare allele (138 bp) in line 12.

replication slippage (18) events at two cycles of meiosis in F_1 and F_2 generations.

SSR allelic database for 30 CSR10 x HBC19 F_3 plants and two parental genotypes was used for generating similarity matrices data and two dimensional PCA (data not shown). The similarity coefficient between CSR10 and HBC19 was 0.231. Selected salt tolerant F_3 plants showed an average similarity of 0.609 and 0.563 with CSR10 and HBC19, respectively. Two dimensional PCA scaling showed scattered clustering of 30 F_3 plants between the two parental rice varieties. A number of salt tolerant F_3 plants (plant nos. 5, 6 and 13) with a mean score of 1.7 and ability to maintain a low Na-K ratio under salt stress were clustered close to salt-sensitive parent, HBC19. Similarly, a few salt sensitive F_3 plants (plant nos. 20 and 29) were close to salt tolerant parent, CSR10.

Goodness of fit to the expected segregation ratio revealed that a significant number of markers (18 in numbers, data not shown) were distorted from expected segregation ratio using chi square analysis. In order to identify the markers associated to salt tolerance, the markers which showed distorted segregation were subjected to linear regression analysis and single marker analysis using one-way ANOVA. The analysis led to the identification of five SSR markers, RM162 (chromosome 6), RM206 (chromosome 11), RM209 (chromosome 11), RM212 (chromosome 1), RM287 (chromosome 11), which were significantly associated with the salt tolerance trait (Table 1). Regression analysis identified three markers significantly associated with relative growth rate and two markers associated with Na-K ratio explaining 31.3% and 25.6% of phenotypic variation, respectively.

Table 1. Markers associated with salt tolerance trait in rice as detected by linear regression and one factor ANOVA analyses. The coefficient of determination (R^2) represents the proportion of phenotype variation explained by each marker

Markers	Chromosome location	F value* (calculated)	RGR		Na-K ratio	
			R^2 (%)	p value	R^2 (%)	p value
RM162	6	5.301	8.26	0.005	-	-
RM209	11	7.389	10.78	0.0033	-	-
RM287	11	5.624	12.25	0.0008	-	-
RM212	1	4.711	-	-	16.82	0.0001
RM206	11	6.712	-	-	8.73	0.0021
Total			31.29		25.55	

*Significant at $p < 0.05$ level, calculated using one factor ANOVA.

Selection for a trait is expected to change allele frequency of genes affecting that trait (5). In this case, it means increasing the frequency of favorable alleles in the salt tolerant and of un-favourable alleles in salt sensitive F_3 individuals. For monogenic traits, the change in allele frequency can be easily monitored in subsequent generations of selection. But, same is not true in case of a quantitative trait like salt tolerance. However, if some marker loci are associated with segregating QTLs (either directly due to pleiotropic effect or, more likely, due to linkage) for a trait, the marker allele frequencies will also change in response to selection. Thus, any significant change in marker allele frequencies due to selection can be attributed to the linkage of marker loci with QTL(s) affecting the trait under selection (5).

There have been several reports on QTL analysis for salt-tolerance in rice (4). In this study, selective genotyping of the salt-tolerant and salt sensitive CSR10 x HBC19 F_3 plants led to the identification of four genomic regions, one each on chromosome 1 and 6 and two on chromosome 11, which showed significant association with QTLs contributing for salt tolerance. Whether each of these regions contains only a single QTL or a cluster of linked QTLs could not be ascertained in this study. Further studies are required to define the role of these chromosomal segments in salt tolerance, which is being pursued using the CSR10 x HBC19 RILs (recombinant inbred lines) recently developed by us. A major QTL 'Saltol' controlling Na-K ratio has also been reported earlier on chromosome 1 in rice (19). Position of RM212 (chromosome 1) is in close vicinity of a QTL for root Na^+ quantity identified by Lin *et al* (20). The chromosomal position of RM162 putatively linked with QTL for relative growth rate is similar to that of the QTL for survival days of seedling under salt stress reported by Lin *et al* (20) and in close vicinity of a QTL for root length identified by Prasad *et al* (21). Microsatellite marker, RM209 associated with relative growth rate has earlier been reported to be associated with shoot fresh weight QTL present on chromosome 11 (16).

It is interesting to note that a few salt tolerant CSR10 x HBC19 F_3 plants (plant nos. 5, 6 and 13) with the ability to maintain low Na-K ratio (<0.90; comparable to CSR10) were in fact genetically closer to the salt sensitive parent, HBC19. Of the various Basmati grain quality components, seed harvested from these plants had length-breadth ratio and aroma comparable to HBC19 (unpublished data). The results indicate that lines with intact salt tolerance and Basmati rice attributes can be obtained from such a cross combination. Both, physiological components and molecular marker analysis can be meticulously combined and used to improve the efficacy in the Basmati rice breeding for stress tolerance. However, further work is required to find marker closely associated with this trait.

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