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Validation of a major QTL for salinity tolerance on chromosome 1 of rice in three different breeding populations

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INTRODUCTION. – Rice (*Oryza sativa* L) is one of the most important food crops of the world. It is the staple food of South and Southeast Asia and widely consumed in South America and Africa. Eighty five percent of rice production is devoted for human consumption (IRRI, 1997). It occupies almost one-fifth of the total world area. The world population grows over 80 million per year and is predicted to reach 10 billion within 2050 (KURODA *et al*, 1997). For these increasing people now need to increase the rice production for fulfill their demands.

Salinity is the most common abiotic problem in rice growing areas of the world (SENADHIRA, 1987). Millions of hectares in the tropics, arid and semi-arid region are technically suitable for rice cultivation but they are left idle or cultivated with low yielding varieties due to the lack of suitable tolerant high yielding varieties. About 400-950 million hectares land of the world is affected by different levels of salinity (LIN *et al*, 1998). More than 54 million hectares of rice land in Asia are affected by salinity; another 9.5 million hectares of saline soils can be managed by large-scale irrigation and drainage schemes and by chemical treatment of soil, but the scale of problem makes these solutions too costly (GREGORIO *et al*, 2002). The rice plant is one of the most suitable crops for saline soils, although it is considered moderately sensitive to salinity (MORI and KINOSHITA, 1987).

Salt tolerance is a complex, quantitative, genetic character controlled by many genes (SHANON, 1985; YEO and FLOWERS, 1986). Using conventional breeding methods, plant selection for salt tolerance is not easy because of the large environmental effects and low heritability of salt toler-

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ance (GREGORIO and SENADHIRA, 1993; GREGORIO, 1997). DNA/molecular markers are widely accepted as potentially valuable tools for crop improvement of rice, especially abiotic stresses (MACKILL *et al* 1999).

Despite the importance of developing rice varieties with salinity tolerance, only a small number of quantitative trait loci (QTL) mapping experiments have been conducted. QTLs for salinity tolerance in rice have been mapped using Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), and microsatellite or simple sequence repeat (SSR) markers in different populations (GREGORIO, 1997; LANG *et al*, 2000; TUAN *et al*, 2000; BONILLA *et al*, 2002, NIONES, 2004). Microsatellite markers have been useful for tagging and mapping of genes/QTLs associated with salinity tolerance (LANG *et al*, 2001). A major QTL for salt tolerance was mapped at chromosome 1 by using F₈ recombinant inbred line (RIL) of Pokkali/ IR29 cross (GREGORIO, 1997). This QTL was designated as the *SalTol* QTL. The segment of chromosome 1 contained a QTL that controlled the Na⁺ - K⁺ absorption ratio and accounted for 64.3 to 80.2% of the phenotypic variation in salt tolerance with LOD > 14.5. This chromosome 1 segment was further saturated in RFLP and SSR markers using the RIL population by BONILLA *et al*. (2002). The identified Na⁺, K⁺ and the Na⁺ - K⁺ absorption ratio QTLs accounted for 39.2, 43.9 and 43.2% of the phenotypic variation with LOD > 6.7 (BONILLA *et al*, 2002). This segment of the chromosome 1 was confirmed by fine mapped by using near isogenic lines (NILs) of the backcross of Pokkali/ IR29. Using interval mapping analysis, two LOD peaks were detected: one peak is associated with four microsatellite markers within 4.3 cM distance; CP03970, RM8094, RM493 and CP6224 and another one is with RM140 (NIONES, 2004).

Prior to marker-assisted selection (MAS), QTLs and tightly-linked markers should be validated by testing their effectiveness in determining the target phenotype in independent populations and different genetic backgrounds (CAKIR *et al.*, 2003; COLLINS *et al.*, 2003). In this study, the *SalTol* QTL on chromosome 1 was validated in three different F₂ populations to test if this QTL was present in different genetic backgrounds by using microsatellite markers. QTL results were then further confirmed using F₃ derived population using the same parental genotypes.

MATERIALS AND METHODS. – *Plant materials.* – The three F₂ populations were: (1) ‘BRR1 dhan40’/ ‘IR61920-3B-22-2-1’ (population 1) where ‘BRR1 dhan40’ was a popular variety cultivated in Bangladesh and susceptible at seedling stage and IR line was highly tolerant to salinity and released as salinity tolerance variety in the Philippines;

(2) 'BRR1 dhan28'/'IR50184-3B-18-2B-1' (population 2) where 'BRR1 dhan28' was a very popular variety for irrigated ecosystem cultivated in Bangladesh highly susceptible and IR line was moderately tolerant to salinity and (3) 'Kajalshail'/'IR52713-2B-8-2B-1-2' (population 3) where both of the parents were tolerant to salinity. Significant marker-QTL associations detected in the F_2 populations were subsequently confirmed using F_3 derived population.

Screening for salt tolerance. – Screening of 300 F_2 and 600 F_3 plants of each cross was done under controlled environment conditions following the method described by GREGORIO *et al.* (1997). A nutrient solution was used as described by YOSHIDA *et al.* (1976) for growth media. The salinity of EC 12 dSm⁻¹ was applied. The screening test was conducted in IIRI Phytotron Glass house maintained at 29°C/ 21°C day night temperature, a relative humidity of 70% during the day and natural daylight.

A modified standard evaluation score (Tab. 1) was used in rating the symptoms of salt damage. Score 1 is representing normal growth and no leaf symptoms and considered as highly tolerant; similarly score 3 for nearly normal growth, but leaf tips or few leaves whitish and rolled and considered as tolerant; score 5 for growth severely retarded; most leaves rolled; only a few are elongating and considered as moderately tolerant; score 7 for complete cessation of growth, most leaves dry, some plants dying and known as susceptible and finally score 9 was revealed for almost all plants dead or dying and known as highly susceptible. In the scoring, odd number from 1-9 was used and no even number was used to discriminate clearly among the classification. Scoring was done three weeks after salinization or after the death of the susceptible check (IR29).

Molecular marker analysis. – Twenty SSR and two EST markers from 49.6 to 87.1 cM position of chromosome 1 segment (according to Gramene database (www.gramene.org) (WARE *et al.*, 2002) including the markers were used for fine mapping by NIONES (2004) were tested for polymorphism. Genomic DNA was extracted using the CTAB method described by ZHENG *et al.* (1995). Microsatellite analysis was performed using the methods described by TEMNYKH *et al.* (2000). PCR was carrying out in a PTC-100 dyad thermocycler machine (MJ Research), and was placed the 384-well plate. Amplification products (2-3 µl) were resolved by polyacrylamide gel electrophoresis (8%, 10% or 12% gels). The gel was run for 2-5 hrs at 100 volts. The gels were stained with ethidium bromide staining solution and visualized under UV light.

Linkage and QTL Analysis. – Linkage analysis was performed using the Map Manager/QTXT computer program (MANLY *et al.*, (2001) using the Kosambi function using a linkage evaluation of $P = 0.001$. The ripple command was used to verify the marker order. The newly constructed linkage maps were compared with the existing maps.

QTL analysis was performed using Windows QTL Cartographer version 2.0 (BASTEN *et al.*, 2001). For interval mapping analysis (IM), a LOD threshold score of 2.5 was selected. The proportion of the total phenotypic variation explained by each QTL was calculated as R^2 value ($R^2 =$ ratio of the sum of squares explained by the QTL to the total sum of squares). For more accurately determining QTL positions, composite interval mapping (CIM) was performed with default parameters.

RESULTS. – *Phenotypic Scoring.* – Among the 288 plants in population 1, around 150 plants were scored as moderately tolerant (Fig. 1(a)). 125 were classified as tolerant to highly tolerant and more

than 15 plants were scored as susceptible to highly susceptible to salinity. In Population 2, among the 281 plants, 185 plants were scored as susceptible to highly susceptible to salinity tolerance and around 80 plants were scored as moderately tolerant and only few were scored as tolerant (Fig. 1(b)). Population 3 showed that among 292 plants more than 230 were scored 3 and around 20 plants scored 1, which indicated that most of the plants of this cross were highly tolerant to salinity (Fig. 1(c)). Around 40 plants were scored as moderately tolerant and there was no susceptible.

Construction of linkage map. – In population 1, seven PCR based markers were used, among them six markers found in one linkage group to saturate the target region of the chromosome 1 where the major salinity tolerance gene was located (Fig. 2). RM6681 marker was unlinked with others. Figure 2 shows the linkage map of different markers and their interval distances for population 1, population 2, population 3 and the previous fine map. In population 2, seven markers were used for constructing linkage map but only four markers could be scored. The four markers gave one linkage group and constructed SSR map. In population 3, five markers were used and found all the five markers in one linkage group and construct SSR map.

Quantitative trait loci (QTL) analysis. – The results using single marker analysis are summarized for all the three populations in Table 2. In population 1, RM8094 was found to be strongly associated with salinity tolerance with significant on $P < 0.001$. Other four markers RM1287, RM3412, RM493 and CP03970 also gave significantly association with salinity tolerance ($P < 0.05$).

The graph derived from IM and CIM are shown in Fig. 3. The QTL was tightly linked with the marker RM8094 with the LOD score of 2.81 and R^2 value was 0.0535. A LOD peak was found from IM and CIM line graph which was located near RM8094, between the flanking markers RM8094 and RM3412. In populations 2 and 3, none of the four microsatellite markers was significant in single marker analysis (Tab. 2).

Reconfirmation in the F_3 population. – Among the 575 plants that were tested, total 320 plants were scored as 3 and only 4 scored 1, these were considered as tolerant and highly tolerant to salinity, respectively (Fig. 4). 188 plants were scored as moderately tolerant to salinity and 63 plants were scored as susceptible to highly susceptible to salinity. The frequency distribution of the salinity reaction was continuous and slightly skewed towards tolerance.

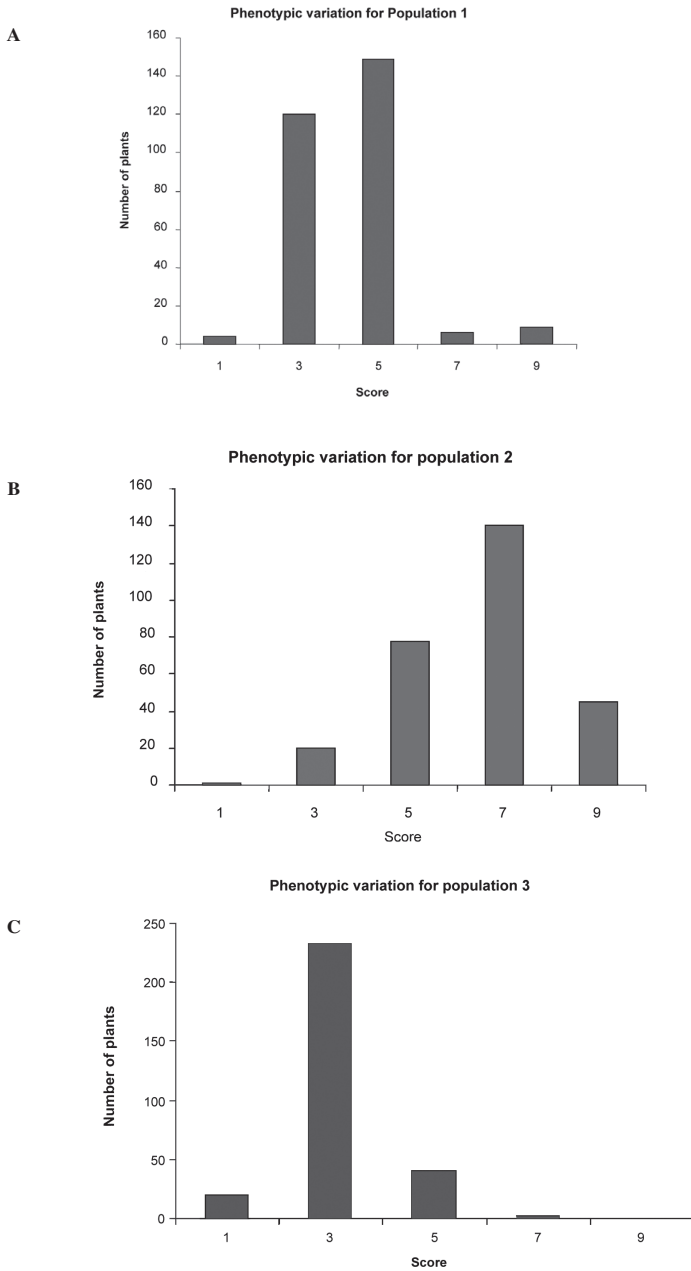


FIG. 1. – Phenotype variation of salinity tolerance for three F₂ populations.

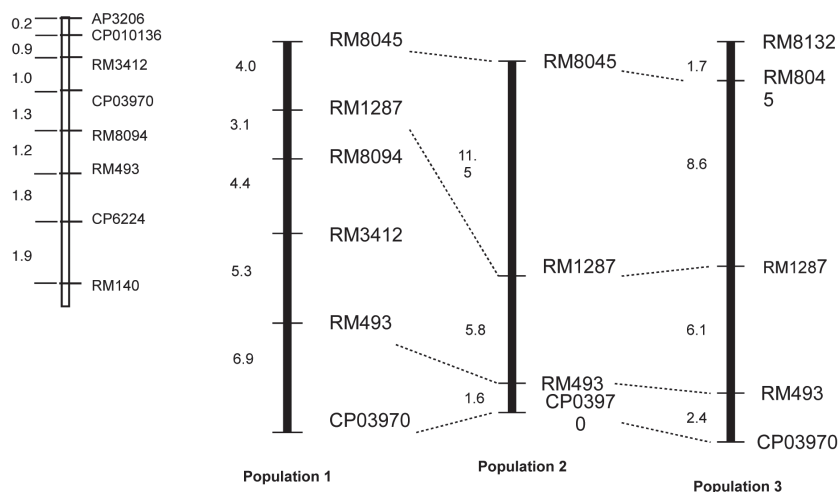


FIG. 2. – Results of Linkage Map analysis for three F_2 populations with previous fine map.

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

Score	Observation	Classification
1	Normal growth, 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

All of the markers were found in one linkage group flanking the *SalTol* region on chromosome 1. The markers sequence of this linkage map and linkage map from F_2 population of the same cross was similar but interval distances slightly changed. RM8094 was found to be strongly associated with salinity tolerance with significant ($P < 0.0001$) using single marker analysis (Tab. 3). The four markers RM1287, RM3412, RM493 and CP03970 were also found to be significantly associated with salinity tolerance ($P < 0.01$).

IM and CIM analysis showed that there was one QTL associated in this segment of chromosome 1 associated with salinity tolerance. The RM8094 marker was tightly linked with the QTL with the LOD score of

TABLE 2. – Single marker analysis for three F₂ populations.

Markers	Population I		Population II		Population III	
	F-value	P- value	F-value	P- value	F-value	P- value
RM8132	--	--	--	--	0.23	0.636
RM8045	3.42	0.065	0.04	0.834	0.20	0.653
RM1287	6.22	0.013*	2.69	0.102	2.92	0.089
RM8094	13.34	0.000***	--	--	--	--
RM3412	4.64	0.033*	--	--	--	--
RM493	5.81	0.017*	2.42	0.121	2.72	0.100
CP3970	5.74	0.018*	2.79	0.096	2.27	0.133
RM6681 ^a	0.24	0.658	--	--	--	--

-- Marker gave monomorphism

* Significance at 5% level

** Significance at 1 % level

^a Unlinked Marker

Note: Parents gave polymorphism in population II but F₂ progenies gave like susceptible parent (BR28) for RM8132, RM3412 and RM6681

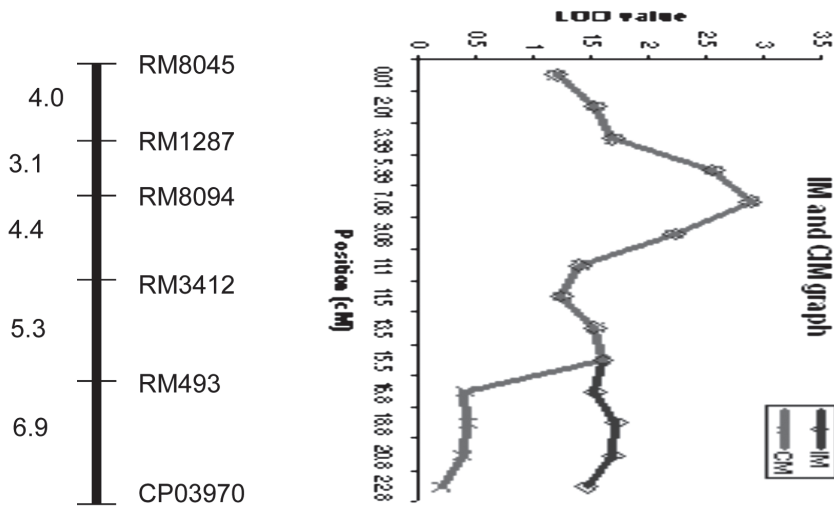


Fig. 3. – Interval Mapping (IM) and Composite Interval Mapping (CIM) curve for Population I of F₂ population.

3.7086 and its R² value was 0.0222 (Fig. 5). The LOD peak was found from IM and CIM line graph which was located near RM8094 between flanking markers RM8094 and RM3412. The IM and CIM results were identical.

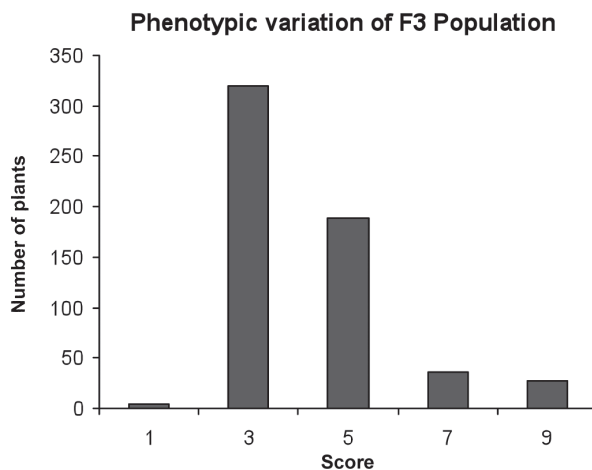


Fig. 4. – Phenotypic variation of salinity tolerance for F₃ Population.

TABLE 3. – Single marker analysis for F₃ population.

Markers	F ₃ population		
		F-value	P- value
RM8045		3.47	0.630
RM1287		9.85	0.002**
RM8094		17.66	0.000****
RM3412		9.03	0.003**
RM493		8.17	0.004**
CP3970		7.74	0.006**
Fine Map (IR29/Pokkali)	Population 1 (IR61920/BRRI dhan40)	Population 2 (IR50184/BRRI dhan28)	Population 3 (IR52713/Kajalsail)

DISCUSSION. – *Phenotypic Scoring*. – The phenotypic or trait data were taken on the basis of leaf injury symptoms. Leaf injury is highly correlated with salt effect to the plants reactions as tolerant or susceptible. The established classification of susceptible, moderate and tolerant based on field, laboratory and Phytotron glass house was clearly related to visual injury symptoms rating and Na⁺/ K⁺ absorption ratio (GREGORIO, 1997).

In population 1, 288 seedlings were tested. After three weeks of salinization (or death of the susceptible check, ‘IR29’) phenotypic scoring was done for salinity tolerance or susceptibility. In population 1, the frequency distribution of the tested plants was continuous while the frequency distribution of population 2 showed continuous and skewed

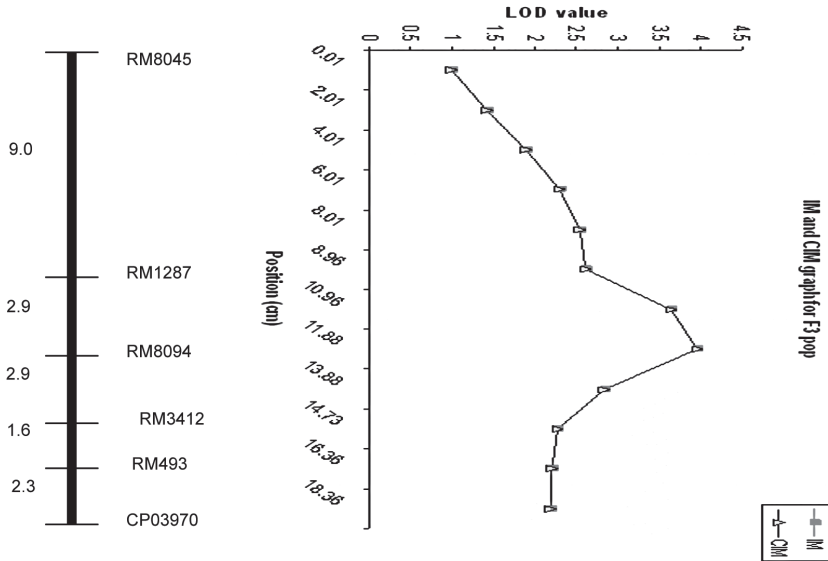


Fig. 5. – IM and CIM curve for F3 population.

towards the right (susceptibility). In population 3 the frequency distribution of the tested plants showed continuous and skewed towards left (tolerance). This was consistent with the phenotypes of both parents (i.e. tolerant to salinity).

Molecular and QTL analysis. – Linkage analysis showed that all of the markers formed a single linkage group except RM6681, which was unlinked. This map covered only from 52.4 to 66.5 cM of chromosome 1 segment according to Gramene data base. Although there are some differences of marker positions from the previous map, the F₂ maps were very similar to the previous fine-map by NIONES (2004) except for slight difference in distances between markers.

The three methods single marker analysis, interval mapping (IM) and composite interval mapping (CIM) for each of the three F₂ populations were used to find the more accurate association and position of the QTL. Three methods of QTL analysis (single marker analysis, IM and CIM) were used in order to confirm QTL results and because some differences in results are sometimes obtained using different methods. From single marker analysis in population 1, RM8094 was found to be strongly associated with salinity tolerance with significant on P<0.001. Four markers RM1287, RM3412, RM493 and CP03970 were also

significantly associated with salinity tolerance ($P < 0.05$). These results revealed that there was important QTL for salinity tolerance in this region of the chromosome 1 segment.

To more precisely determine the location of the identified QTL for salt tolerance by single marker analysis, IM and CIM analysis was performed. The LOD plot of IM and CIM was very similar. A LOD peak was found from IM and CIM line graph which was located at RM8094 and flanking between RM1287 and RM3412. From this result it was assumed that a QTL was tightly linked with the marker RM8094 with the LOD score of 2.81 and R^2 value was 0.0535. NIONES (2004) also reported that a QTL for salinity tolerance was present in this region of chromosome 1 segment and the position of QTL was between the marker loci CP6224 and RM8094 (1.5 cM) by using NILs (BC_3F_4) of the cross of Pokkali/ IR29. BONILLA *et al* (2002) saturated this segment of chromosome 1 with RFLP and microsatellite markers using the RIL population and reported that two microsatellite markers, RM23 and RM140 flanked the *SalTol* QTL with 16.4 and 10.1 cM distance, respectively. The source of salinity tolerance in population 1 was from IR61920-3B-22-2-1 (NSIC106) for this study which was donated by the ancient parent of this variety TKM6, Kitcheli Chamba or Vallaikar. The source of salinity tolerance of the NILs and RIL populations was used by NIONES (2004) and BONILLA *et al* (2002) was Pokkali, which has been the most commonly-used source of salinity tolerance in rice to date. Interestingly, a QTL was detected in the same region of chromosome 1.

Using the F_3 population derived from population 1, RM8094 was found to be strongly associated with salinity tolerance ($P < 0.0001$). Four other markers RM1287, RM3412, RM493 and CP03970 were also found to be significantly associated with salinity tolerance ($P < 0.01$). These results confirmed our previous result in the F_2 population that there was a QTL for salinity tolerance in this region of the chromosome 1 segment. IM and CIM were identical. The maximum LOD score value of 3.7086 and its R^2 value was 0.0222 which was slightly higher compared to the maximum LOD score value in the F_2 .

In populations 2 and 3, none of the markers were significant using single marker analysis. However, the most tightly-linked markers associated with the *SalTol* QTL were not polymorphic in both of these populations. The failure to detect a QTL in population 2 was unexpected because of the difference in phenotype between both parents and that one parent (IR50184) was derived from Pokkali. In population 3 it was found that

most of the plants were tolerant; this result was obtained may be due to the effect of both parents, because both of the parents were salinity tolerant. This population was included for validation in this study because previous IIRI data indicated that the Kajalshail parent was susceptible. However, in this study, this variety was clearly tolerant. These results revealed that the population of those crosses of 'BRRI dhan28'/'IR50184-3B-18-2B-1' and 'Kajalshail'/'IR52713-2B-8-2B-1-2' may not be suitable for the introgression of the *SalTol* QTL using marker assisted selection.

The QTL analysis results confirmed the presence of a QTL at the *SalTol* locus in an independent population. This was interesting because the pedigrees of the tolerant parents used were different. The results in this study also indicated the importance of verifying QTLs in three different populations, even if the large QTL effects were previously reported. For a marker assisted breeding program for introgressing the *SalTol* QTL, parents that differ widely in phenotype should be selected (i.e. highly tolerant and highly susceptible or susceptible genotypes to salinity).

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SUMMARY. – The effect of a major quantitative trait locus (QTL) for salinity tolerance in rice, designated as *SalTol* in a previous study, was tested using three F₂ breeding populations. The populations were derived from the following F₁ hybrids: ‘BRRI dhan40’ (susceptible)/ ‘IR61920-3B-22-2-1’ (highly tolerant); ‘BRRI dhan28’ (highly susceptible)/ ‘IR50184-3B-18-2B-1’ (moderately tolerant); and ‘Kajalsail’ (tolerant)/ ‘IR52713-2B-8-2B-1-2’ (tolerant). Targeted mapping of the chromosome region containing *SalTol* (49.6 to 87.1 cM) on chromosome 1 was conducted using 20 SSR and two EST markers. Comparisons of linkage maps of the three populations were very similar to the previous QTL map that identified *SalTol*. A QTL was only detected for ‘BRRI dhan40’/ ‘IR61920-3B-22-2-1’ population. The SSR marker RM8094 was the most tightly-linked marker ($P < 0.001$); four other markers, RM1287, RM3412, RM493 and CP03970, were also significantly associated with salinity tolerance ($P < 0.05$). An F₃ population of the cross ‘BRRI dhan40’/ ‘IR61920’ was used to reconfirm this result. This was interesting because the tolerant parent in this population was not related to the tolerant parent used for the original mapping population. QTLs were not detected at the *SalTol* locus for either of the other two populations. This was consistent with the phenotypes of the parents used to construct these populations, and indicates that the *SalTol* QTL may only be effective in specific populations.

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