



Evaluation of salinity tolerance in rice genotypes

G. Mohammadi-Nejad^a, R.K. Singh^b, A. Arzani^c, A.M. Rezaie^c,
H. Sabouri^{d,*}, G.B. Gregorio^b

^aAssist. Prof. Department of Agronomy and Plant Breeding, College of Agriculture, Shahid Bahonar University of Kerman, P.O.Box 76169-133 Kerman-Iran.

^bProf. International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines.

^cProf. Department of Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, Iran.

^dAssist. Prof. Department of Plant Production, College of Agriculture Science and Natural Resource, Gonbad High Education Center.

*Corresponding author. E-mail: Hos.Sabouri@gmail.com

Received 21 August 2009; Accepted after revision 9 May 2010; Published online 14 June 2010

Abstract

Salinity is considered as one of important physical factors influencing rice (*Oryza sativa* L.) production. Knowledge of salinity effects on rice seedling growth and yield components would improve management practices in fields and increase our understanding of salt tolerance mechanisms in rice. This study was designed to assess the role of *Saltol* QTL in regards to effects of salinity on plant growth and yield components of different genotypes of rice at different growth stages. A greenhouse study was conducted to evaluate the response of 30 rice genotypes to three levels of salt stresses (0, 60, 100 mM NaCl) at reproductive stage. The seedling stage response of these genotypes to salinity with electrical conductivity at 12 dSm⁻¹ also investigated. Pollen viability, number of unfilled and filled grain and grain yield per plant were evaluated. The rice genotypes differed significantly for salt tolerance at seedling stage. The genotypes were also significantly varied for the traits measured at the reproductive stage. The interactions of genotypes × salinity treatments were significant for pollen viability, number of unfilled grain and grain yield. Grain yield reduction due to salinity was more severe for control to 60mM than for 60mM to 100mM. Pollen viability was found to be a robust criterion to screen the genotypes for salt tolerance at the reproductive stage. Pokkali cultivar possessing a major quantitative trait locus (QTL) for salt tolerance at seedling stage (*Saltol*) mapped on chromosome 1 was used as reference for haplotyping. Thirty rice genotypes divided into 16 different haplotypes based on *Saltol* QTL. RM8094 and RM10745 microsatellite markers found to be the most effective markers for discriminating the salinity tolerant genotypes.

Keywords: Haplotype diversity; Rice; Salinity tolerance; *Saltol* QTL.

Introduction

Rice is one of the most important world food crops, serving as the staple food for over one-third of the world's population (Khush, 1997). It is one of the most widely grown crops

in coastal areas frequently inundated with saline sea water during high tidal period (Akbar et al., 1972; Maas and Hoffman, 1977; Mori and Kinoshita, 1987). Salinity is considered as one of important physical factors influencing rice production. At the present, salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious constraint to increased rice production worldwide (Gregorio, 1997). There exists tremendous variation for salt tolerance within species in rice (Sabouri et al., 2009b), providing opportunities to improve crop salt-stress tolerance through genetic means. Some attempts to develop salt-tolerant genotypes were based on highly tolerant traditional rice cultivars i.e. Pokkali and Nona-Bokra (Akbar et al., 1985; Gregorio and Senadhira, 1993). Despite of much of Research conducted for the salinity tolerance at the seedling stage (Sabouri et al., 2009a; Mohammadi-Nejad et al., 2008) very little attention has been devoted to the reproductive stage (Sabouri and Biabani, 2009b).

Assessment of genetic diversity is very important in plant breeding as it provides the basis for selection. Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level (Weir, 1990).

A major QTL located on chromosome 1 was identified for salt tolerance using F_8 recombinant inbred lines (RILs) of Pokkali/IR29 cross (Gregorio, 1997). This QTL governed the Na^+ / K^+ uptake ratio and accounted for 64.3 to 80.2% of the phenotypic variation in salt tolerance. This segment of chromosome 1 was further saturated by RFLP and SSR markers using the RILs (Bonilla et al., 2002). The identified QTLs for Na^+ , K^+ and Na^+ / K^+ uptake ratio accounted for 39.2, 43.9 and 43.2% of the phenotypic variation, respectively. This segment of the chromosome 1 was further fine mapped using microsatellite markers in a near isogenic lines (NILs) population of IR29 in which Pokkali used as the donor (Niones, 2004). It should be mentioned that Pokkali has been the most widely used salt tolerant parent by rice breeders. A newly developed line FL478 derived from a cross between IR29 and Pokkali was used as a novel source of salinity tolerance at seedling stage (Walia et al., 2005). Eight QTLs were found responsible for the variation in their K^+ and Na^+ content, among which SKC1 distinguished as a major QTL for the K^+ and Na^+ shoot content and was mapped on chromosome 1, using F_2 populations derived from a cross between Niponbare and Koshihikari cultivars (Ren et al., 2005).

The objectives of this study were (i) to evaluate the response of rice genotypes to salinity at the seedling and reproductive stages (ii) to assess the most discriminating microsatellite (SSR) markers of salinity tolerant haplotypes using Pokkali cultivar which contains salinity tolerance QTL (*Saltol*), (iii) to identify salinity tolerance rice germplasm possessing different QTLs compared with *Saltol*.

Materials and Methods

This experiment was carried out in International Rice Research Institute, (IRRI). Thirty rice (*Oryza sativa* L.) genotypes including land races, pure lines and improved cultivars obtained from Plant Breeding, Genetics, and Biotechnology Division (PBGB) of IRRI, were used (Table 1). Rapid screening method for evaluation of responses of genotypes to salinity stress at seedling stage as described by Gregorio et al. (1997) was used in a salinized culture solution with electrical conductivity 12 dSm^{-1} for 21 days under controlled

conditions in the IIRRI phytotron at 34 °C/ 29 °C day and night temperatures with relative humidity of 70%. The genotypes were grouped under 1, 3, 5, 7 and 9 score categories for salt tolerance on the basis of relative seedling shoot and root growth 21 days after salinity treatment (Gregorio et al., 1997).

The response of genotypes to salt stress at seedling stage was evaluated under controlled greenhouse condition at IIRRI using salt concentrations of 0, 60, and 100 mM NaCl at the reproductive stage in completely randomized blocks design (RCBD) with three replications. Four pre-germinated seeds were sown in 1-liter capacity perforated plastic pots with nylon mesh lining and filled with pre-fertilized soil (N: P: K 50:25:25 mg kg⁻¹). The pots were kept in concrete tanks with non-saline water. Water level was maintained at the same level as of the soil. Two weeks after seeding, seedlings were thinned to three per pot and water level was raised about 1-2 cm above the soil level. Saline water solution of 60 and 100 mM was prepared by dissolving calculated amount of NaCl in water in separate tanks. As the genotypes had different flowering periods, for imposing the stress very specific only to the reproductive stage and to eliminate the effect of salinity before flowering, the pots were immediately transferred to salinized tanks when the tip of the first flag leaf of any tiller appeared. Water level and salinity were maintained regularly. Salinity stress was relieved after two weeks from complete flowering as the seed ripening is relatively tolerant stage (Makihara et al., 1999; Singh et al., 2004).

In reproductive stage, pollen viability, number of unfilled grains, number of filled grains and grain yield were recorded under both normal (control) and salinized conditions according to the standard procedure as mentioned by Gregorio (2002).

Six to seven unopened spikelets collected randomly during heading from all the replications in 3 treatments and stored in refrigerator in 70% ethanol until analyzed. While analyzing, anthers from the stored spikelets were removed and crushed thoroughly so as to release all the pollens. Pollens were stained with a drop of 1% potassium iodide solution. The samples were covered with the cover-slip and viewed under light microscope. Fertile and unfertile pollens were counted at three different areas per slide. Pollen viability was calculated by dividing number of fertile pollen grains to total number of pollen grains and denoted in percentage. The analysis of variance (ANOVA) was conducted using the SAS version 6.12 (SAS, 1996).

Microsatellite (SSR) analysis

Genomic DNA was extracted from young leaf tissue according to Delaporta et al. (1983). Eight polymorphic SSR markers including: RM140, RM493, RM1287, RM3412, RM8094, RM10745, RM10764, RM10772, located on chromosomes 1 were used. SSR primers were obtained from Invitrogen Corp., Carlsbad, California, USA. The PCR reaction was performed in a 25 µl volume using a PTC-200 MJ thermocycler (MJ Research, Inc., Waltham, MA). The reaction mixture contained 1x PCR buffer, 1.5 mM of MgCl₂, 1.0 µl dNTPs, 0.5 µM of each primer, 1 U of *Taq* polymerase and 30ng of template DNA. After 5 min of denaturation at 94 °C, 35 cycles were performed with 1 min at 94 °C, 45 seconds at 55 °C, 1 min at 72 °C, and a final extension step of 7 min at 72 °C. PCR amplified products were separated in a 8% polyacrylamide gel at 100 V for 2.5 h in 1 x TBE buffer and stained

with SYBR Safe (from Invitrogen Corp., Carlsbad, California, USA). DNA banding patterns were visualized using Alpha Imager (Alpha Innotech Corporation, San Leandro, CA).

Molecular weight for each band was measured by using Alfa Imager software version 5.5. Polymorphism information content (PIC) values were calculated with the following formula (Anderson et al., 1993).

$$PIC_i = 1 - \sum_{j=1}^n p_{ij}^2$$

Where n is the number of marker alleles for marker i and P_{ij} is the frequency of the j th allele for marker i . PIC value and other statistics for SSR marker alleles were analyzed using Power Marker version 3.25 (Liu and Muse, 2005). For the comparison and validity of the dendrograms, separate trees were constructed based on the morphological and molecular data and the comparison between two was done using Maximum Agreement Sub-Tree (MAST) module of the DARwin software (Perrier et al., 2003). Software analyze the results based on the number of the units conserved with respect to the order (o) of the MAST that can be considered as the measure of the resemblance between trees. The maximum order is 'n' which is obtained for the identical trees while the minimum value is 3 for commonality of the tree. So, the distance between two trees (d) = $(n-o)/(n-3)$, where $0 \leq d \leq 1$. Haplotype diversity was prepared according to McCartney et al. (2004) and Liu and Anderson (2003).

Results and Discussion

The rice genotypes differed significantly for salt tolerance at seedling stage. The genotypes were classified into five groups from highly tolerant (score 1) to highly sensitive (score 9) as 14 (highly tolerant), 4 (tolerant), 7 (moderate tolerant), 5 (sensitive) and 6 (highly sensitive) (Table 1). Most of the tested IRRI elite lines were ranked as highly tolerant to salt stress at the seedling stage. Rice cultivars showed different reaction to salinity tolerance at reproductive stage viz Pokkali, Kala-rata were ranked as highly tolerant genotypes while Karuna and Baothai ranked as highly sensitive genotypes. Rice genotypes differed significantly for all the studied traits as revealed from partitioning of variances. Salinity as a stress factor also exerted the significant differences in the performance of all the traits (Table 1 and 2). However, a significant genotype \times salt treatment interaction component of variation revealed the differential effect of stress on genotypic component of variation for pollen viability, unfilled grains and grain yield per plant, not for the filled grains (Table 2). The degree of discrimination among the genotypes for their performance varied more under saline conditions in comparison to non-stress conditions (Table 1). In general, genotypes showed the higher reduction in performance when the stress was increased from control to the 60 mM rather than the increase from 60mM to 100 mM as evident from the yield per plant, pollen viability and number of filled grains. Obviously the number of unfilled grains increased at 60 and 100mM in comparison to 0 mM due to salinity stress. The imposition of stress at the reproductive stage affected the performance of studied traits differentially.

Table 1. Means of studied traits of 30 rice genotypes under control (C) and salinity treatments (S).

Cultivar/line	Score at seedling stage	No. unfilled grain		No. filled grain		Grain yield per plant (g)		Pollen viability (%)	
		C	S	C	S	C	S	C	S
IR65209-3B-6-3-1	1	29.4	62.9	47.1	27.3	1.4	1.0	46.7	14.6
IR65858-4B-11-1-2	1	27.9	48.8	82.6	28.4	4.4	0.8	48.1	48.0
IR69588-4R-P-11-3	1	46.2	48.7	27.0	9.7	2.0	0.5	36.6	18.8
IR72046-B-R-7-3-1-2	1	21.2	34.8	18.7	20.0	1.3	1.1	49.4	24.7
IR71832-3R-2-2-1	1	37.2	35.7	34.0	9.5	1.7	0.4	19.5	12.3
IR71899-2-1-1	1	23.3	43.3	62.9	19.5	2.6	1.1	34.0	14.9
IR71991-3R-2-6-1	1	55.9	65.6	58.7	30.3	3.6	1.4	27.8	22.1
IR71995-3R-1-2-2	1	44.2	44.8	49.8	49.0	3.8	2.6	38.2	15.7
IR74099-3R-3-3	1	47.2	54.0	13.2	10.6	1.9	0.8	52.3	19.7
IR74105-3R-2-1	1	24.0	28.0	13.0	4.0	0.6	0.2	54.9	19.5
IR70023-4B-R-12-3-1	1	64.4	69.4	40.8	39.9	2.7	1.9	81.2	46.2
Cheriviruppu	3	44.1	47.2	83.1	72.7	6.8	4.2	59.4	20.8
Kala Rata 1-24	1	17.8	26.3	25.1	24.1	3.1	1.5	69.0	48.6
Bhirpala	5	44.9	50.6	51.3	30.3	2.1	1.4	45.1	26.2
IR4630-22-2-5-1-3	5	30.2	32.2	23.8	18.4	1.0	0.7	60.1	45.6
Pokkali (Ac.108921)	1	68.4	67.7	91.0	79.8	5.5	5.2	67.7	29.6
IR66946-3R-178-1-1	1	88.3	90.0	37.6	5.7	2.7	0.5	73.0	47.4
IR64	3	18.4	39.7	8.4	8.1	0.9	0.9	46.6	8.2
IR65185-3B-8-3-2	5	64.4	64.7	36.7	16.2	2.7	0.6	16.0	19.7
IR72046-B-R-4-3-2-1-2B-1	3	28.3	50.3	57.0	22.3	2.2	1.0	48.8	18.2
IR72043-B-R-6-3-3-3	5	16.0	18.7	35.4	13.0	1.5	0.3	39.3	15.4
IR72046-B-R-8-3-1-3	5	21.3	29.8	60.7	22.7	3.8	0.8	47.4	17.2
IR75000-69-2-1	5	30.8	45.5	65.1	40.3	1.8	1.6	50.6	26.4
IR29	9	9.1	19.0	6.1	5.4	0.2	0.2	24.7	18.3
Mojang Kor	7	38.2	47.1	23.9	6.3	1.0	0.5	57.9	27.6
Bao thai	9	23.9	26.8	10.1	9.2	0.3	0.3	24.9	13.8
CN499-160-13-6	9	41.2	52.2	89.2	74.5	3.9	2.0	42.2	26.9
Karuna	9	26.8	28.5	27.1	8.1	0.6	0.2	36.0	33.4
TCA4	7	63.8	74.6	38.0	19.4	2.1	0.6	29.8	16.5
Kinandang Patong	7	49.3	87.4	30.6	10.8	1.2	0.5	56.4	27.4
Mean Value		38.2	47.8	41.6	24.5	2.3	1.2	46.1	24.8

Table 2. Analysis of variances of the studied traits in 30 rice genotypes.

Source of variation	dF	Mean of Square			
		Pollen viability (%)	No of unfilled grains	No of filled grains	Grain yield (g)
Replication	2	114.16	481.46	80.46	0.79
Stress levels (S)	2	15182.15**	1939.2**	8862.34**	42.51**
Genotypes (G)	29	1270.7**	2896.5**	3665.50**	12.50**
G × S interaction	58	214.71**	443.32*	285.95 ^{ns}	1.191**
Error	178	83.18	330.03	219.36	0.55

*, **; significant at P= 0.05 and P =0.01 level, respectively.

^{ns}: Non significant at P=0.05.

Salinity stress caused the reduction in overall vigor especially in the number of filled grains and grain yield per plant. This might be probably due to consequent high reduction in pollen viability under stress. This is more pronounced in sensitive genotypes rather than traditional tolerant rice varieties like Pokkali, Cheriviruppu, Kalarata 1-24 and Bhirpala etc. Moradi et al. (2003) hypothesized that slow growth of plants due to osmotic stress forced by a high concentration of salts in the root zone significantly decreased the grain yield.

Based on grain yield reduction and also pollen viability at 100 mM salinity, Cheriviruppu, Pokkali were ranked as the tolerant ones, whereas IR29, Mojang and Karuna were the most sensitive genotypes (Table 1). Based on Spearman correlation, the rank of all genotypes showed the reduction from control to the 60mM for grain yield ($r=0.64^{**}$), also it had significant relation to the reduction from control to the 100 mM ($r=0.77^{**}$). This indicates that salinity stress as low as 60 mM affects the plant vigor. However, there was non-significant correlation between reduction of grain yield at 60 and 100 mM ($r=0.42^{ns}$). This shows that the genotypes with changing degree of tolerance behaves differently at different stress levels and this discriminatory performance is much more pronounced at higher degree of stress as the tolerant genotypes resist better to further reduction in the mean performance from 60 to 100 mM in comparison to sensitive genotypes.

Reproductive stage is one of the most sensitive growth stages under the saline conditions (Makihara et al., 1999; Singh et al., 2004). This is the most important stage as far as grain yield is concerned because the successful fertilization at this stage is ultimately translated to grain yield. Since the rice genotypes in this experiment were grown under saline condition during the boot stage, their pollen viability was drastically affected. This resulted into poor fertilization and consequent poor seed setting. Almost all the varieties reduced their pollen viability under stress but those which reduce the pollen viability drastically in conjunction with very high reduction in grain yield were considered as the sensitive genotypes for reproductive stage (Khatun and Flowers, 1995). The reduction alone either in the pollen viability or grain yield is not considered as the criterion for salinity tolerance. Few traditional land races such as Cheriviruppu, Kalarata, Bhirpala and Pokkali were found relatively tolerant at reproductive stage due high pollen viability (21 to 49%) under stress and also less grain yield reduction (up to 50% with exception for very poor yielders). Fortunately some of the improved genotypes were also identified as tolerant at reproductive stage based on similar criteria i.e. IR72046-B-R-7-3-1-2, IR4630-22-2-5-1-3 and CN499-160-13-6. There are two varieties viz. IR65858-4B-11-1-2 and IR66946-3R-178-1-1, which showed the pollen viability as high as 47-48% but the grain yield reduction over normal was as high as 82 percent with very poor yield, hence considered as sensitive for the reproductive stage. This might be due to the some sampling bias while collecting the florets or due to the adverse effects of salinity on stigma receptivity / abortive ovule rather than on pollen viability due to salinity stress resulting into poor grain yield. This is also considered as sensitivity at the reproductive stage. Seedling and reproductive stage tolerance are independent to each other (Moradi et al., 2003; Singh et al., 2004), and could be confirmed in this study as shown from performance of one of the genotypes i.e. CN499-160-13-6 which is a proven sensitive genotype for seedling stage salinity tolerance but behaved as tolerant one for the reproductive stage. This supports that separate sets of genes are responsible for seedling and reproductive stage tolerance.

Sodium distribution within the plant is not homogenous (Yamanouchi et al., 1987). Composition and development of the panicle and yield components such as tillering, spikelet number, sterility and grain weight may depend upon sodium concentration in the panicle (Khatun and Flowers, 1995). The changes in grain weight and sterility were significantly correlated with changes in the panicle sodium concentration at several panicle development stages (Folkard et al., 1999). Germination, active tillering and maturity are considered to be less sensitive to salinity than the seedling stage, early reproductive stage, pollination and seed formation (Singh et al., 2004; Babu, 1985). In accordance with these studies, pollen viability, a very important trait that is greatly influenced by the ionic toxicity

under salinity, was found to be a governing trait for the ultimate grain yield, thus grouped separately in cluster analysis. Better partitioning mechanism for the excess influx of sodium or other harmful ions into the older tissues like leaf sheaths or older leaves leads to better tolerance in genotypes.

Eight tightly linked SSR markers to the *Saltol* positioning at 10.8 to 12.28 Mb (www.gramine.org) were used for haplotyping. All the eight SSR markers amplified polymorphic bands using 30 rice genotypes (Figure 1 and Table 4). The lowest amplicon size belonged to RM10796 (131 bp) and the highest amplicon size belonged to RM10772 (386 bp). The number of microsatellite alleles of used markers ranged from 5 to 14 of which RM8094 produced the highest numbers of alleles (14) while RM140 produced the lowest (5) (Table 4). Polymorphic information content (PIC) value varied from 0.56 to 0.88, the highest value belonged to RM8094, while RM8095 showed the lowest PIC value (PIC=0.56). The SSR marker RM8094 was found to be superior for analysis of genetic diversity among the markers in this region (Table 4).

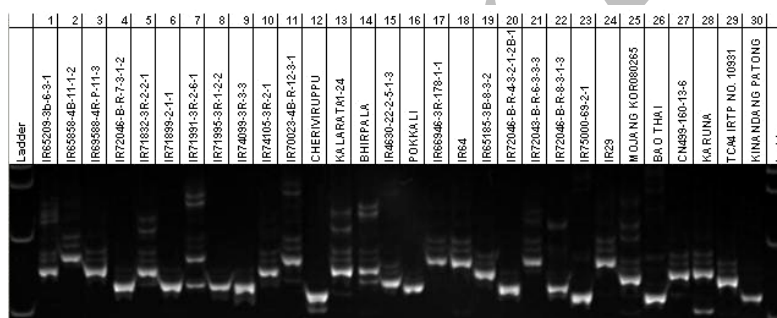


Figure 1. DNA bands amplified from leaves of 30 rice genotypes using microsatellite RM8094 marker and electrophoresed in a 8% polyacrylamide gel. Ladder = 100 bp ladder.

Table 3. Means of studied traits at different salinity stress levels; 0-60 mM: percent reduction at 60mM over control; 0-100 mM: percent reduction at 100 mM over control; and 60-100 mM: percent reduction at 100 mM over 60 mM.

Traits	Salinity stress			Percent reduction		
	Control	60mM	100mM	0-60 mM	0-100mM	60-100mM
Pollen viability (%)	46.11	29.11	20.49	36.9	55.6	29.6
No of unfilled grain	38.22	47.3	45.58	-23.8	-19.3	3.6
No of filled grain	41.6	28.97	21.4	30.4	48.6	26.1
Grain yield /plant (g)	2.32	1.39	0.93	40.1	59.9	33.1

Table 4. Number of alleles and polymorphism information content (PIC) value of SSR markers for 30 rice genotypes.

Marker	Frequency of major allele	No. of allele	Locus heterozygosity	PIC value	Amplicon size range (bp)
RM140	0.58	5	0.60	0.56	248-264
RM493	0.42	9	0.78	0.76	193-253
RM1287	0.31	11	0.84	0.82	147-192
RM3412	0.36	11	0.82	0.81	225-260
RM8094	0.22	14	0.88	0.89	166-220
RM10745	0.47	6	0.71	0.68	182-201
RM10764	0.25	8	0.81	0.78	131-171
RM10772	0.17	12	0.90	0.88	321-386

Sixteen haplotypes were identified among the 30 rice genotypes (Table 5). Ten genotypes allocated to 9 single haplotypes. None of the 29 genotypes produced similar haplotype as Pokkali. In the other words, none of the used genotypes possess all of the microsatellite markers belonging to Pokkali. Twenty-three genotypes had different combinations of some Pokkali's alleles, while six genotypes did not have any common marker alleles with the Pokkali haplotype (haplotype number 16). From comparison of the haplotypes with Pokkali haplotype, it can be assumed that the rice genotypes possessing the Pokkali band type for locus RM8094 marker were either highly tolerant or tolerant to salinity stress at the seedling stage (Table 5). IR70023, IR65858, IR69588, IR74105, IR71832 and IR74099, Cheriviruppo and IR66946-3R-178-1-1 which is known as FL478 had the Pokkali marker allele for the RM8094 marker and all were found to be either highly tolerant or tolerant to salinity stress (Table 5). Therefore, this marker appears to have a strong and positively associated with seedling salt tolerance in rice. Some of salt-tolerant genotypes had the Pokkali marker allele for RM10745. The genotypes that had the same marker allele as Pokkali for the RM3412 and RM1287 markers showed different reaction to salinity while Pokkali's markers RM8094 and RM10745 consistently discriminated the salt tolerant genotypes (Table 5). Rice haplotype which did not contained any common marker alleles with Pokkali haplotype responded either sensitive or highly sensitive to salinity stress (Table 5).

Table 5. Sixteen rice haplotypes produced by SSR markers located on *Saltol* QTL region on chromosome 1 with reference to Pokkali*.

RM1287																
RM8094																
RM3412																
RM10745																
RM10764																
RM10772																
RM493																
RM140																
Haplotype No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

*1: Pokkali, 2: FL478, 3: IR75000, Mojangkor, Baothai, 4: IR64, Kala-Rata, IR71991, IR72043, 5: IR29, 6: SAL411, 7: Cheriviruppo, 8: IR65858, IR69588, IR74105, IR70023, 9: IR74099, 10: IR71899, IR65209, IR4630, K.Patong, 11: Bhirpala, 12: IR71832, 13: SAL518, 14: SAL764, 15: IR71995, 16: TCA4, IR65185, CN499, Karina.

These results suggested that it may be other QTLs rather than *Saltol* affecting reproductive stage in these genotypes. CN499-160-13-6 line proved as a sensitive genotype for seedling stage while behaves as tolerant for the reproductive stage. This line did not possess any common allelic loci (markers) with Pokkali. Therefore, these genotypes may represent novel sources of salinity tolerance, and could be potentially exploited for the development of new breeding lines with high levels of salinity tolerance at reproductive stage. The results of present study further verified that RM8094 and RM10745 are useful for marker assisted selection of *Saltol* QTL.

Acknowledgements

The authors wish to thank D.L. Adorada and A.G. Sajise, for their supervision in the lab and Phytotron, N.A. Vispo and R. Mogul for their technical help and Ms. R.J. Melgar for her valuable help. The authors are also grateful to IRRI administration for financial and technical supports.

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Article reference # ijpp09-222; Editorial responsibility: A. Soltani