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Marker-assisted introgression of *saltol* locus into genetic background of BRRI Dhan-49

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Abstract

The major purpose of this research was to introgress *Saltol* QTL into the genetic background of BRRI dhan49 through marker-assisted backcrossing. FL478 was used as a donor parent of *Saltol* QTL. Marker assisted backcrossing strategies were applied to develop BRRI dhan49-*Saltol* lines. A primer polymorphism survey was carried out between the two parental genotypes *viz.* BRRI dhan49 and FL478. A total of 363 SSR and *InDel* primers were surveyed and a total of 96 markers (27%) were found polymorphic. A cross was made between BRRI dhan49 and FL478 to produce F_1 seeds and F_1 was confirmed by RM493. Foreground selection was carried out using RM493 in all generations. Fifty six SSR markers were used for background selection. The Graphical Genotype (GGT) software was used to estimate the percentage recovery rate of the recurrent parent genome and to find out the genome ratio of the parents in the selected progenies of the backcross populations. From BC_1F_1 generation five best plants were selected based on background recovery of recurrent parent and backcrossed with recurrent parent. From BC_2F_1 generation, six plants were selected based on the highest background recovery of 80.7% to 89.5%. BC_2F_2 seeds were produced by selfing of selected BC_2F_1 individuals. The *Saltol* introgressed lines performed better in salt stress condition in hydroponic screening. The developed BRRI dhan49-*Saltol* lines would be useful for developing salt tolerant rice varieties or for using in other breeding programs.

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Introduction

Among the abiotic stresses, soil salinity is the second largest abiotic problem affecting rice, next to drought (Singh *et al.*, 2008). Rice production is severely affected by the deposition of soluble salts in the soils of arid and semi-arid tropics of the world (Ashraf *et al.*, 2008). Salinity is a serious problem in south Bangladesh, regularly affecting about one million hectares of rice lands in coastal areas (Karim *et al.*, 1990). There exist different degrees of salt tolerance within some rice cultivars, providing opportunities to develop salt-stress tolerant lines through genetic means. Previously, some attempts were taken to develop salt-tolerant genotypes by using highly tolerant traditional rice cultivars i.e., Pokkali and Nona-Bokra (Akbar *et al.*, 1985; Gregorio and Senadhira., 1993). A major QTL, *Saltol* for salinity tolerance at seedling stage on chromosome 1, provided the opportunity to apply marker-assisted backcrossing to introduce tolerance into popular, but salt-sensitive BRRI dhan49. *Saltol* is a major QTL for salinity tolerance at seedling stage on chromosome1 in rice. BRRI dhan49 is an early Transplanted Aman variety having 135 days growth duration and 4.5-5.0 t/ha yield. The variety does not possess the salt tolerant *Saltol* QTL. This study deals with converting the early T. Aman rice variety BRRI dhan49 into a salinity tolerant variety by incorporating *Saltol* using marker assisted backcrossing (MABC) approach. MABC works were done in BC_1F_1 and BC_2F_1 generations.

Materials and methods

The experiment was conducted at the experimental fields of Bangladesh Rice Research Institute (BRRI), Gazipur and Marker Assisted Selection Laboratory of Plant Breeding Division, BRRI, Gazipur.

Recurrent parent and Donor parent

BRRI dhan49 is an early maturing Transplanted Aman variety which was used as recurrent parent. This variety was derived from the cross BR4962-12-4-1/IR33380-7-2-1-3. The pedigree number of this variety is BR6592-4-6-4. The variety was released by BRRI in 2008. The average plant height is 100 cm. The growth duration is 135 days with a yield potential of 4.5-5 tha^{-1}. BRRI dhan49 is recommended for cultivation all over Bangladesh and the demand of this variety is increasing day by day which is a supplement to BR11 with Nizersail grain type. BRRI dhan49 is sensitive to salt stress.

FL478 (IR66946-3R-178-1-1) was used as a donor parent because of its high tolerance to salt stress at seedling stage. This is a recombinant inbred line developed from the cross of Pokkali, a salt tolerant landrace from India, with the lowland salt sensitive variety, IR29. The original population developed from Pokkali × IR29 cross was used for mapping and identification of *Saltol*, the major QTL on chromosome 1 which explained the 43.9% of the phenotypic variance for salinity tolerance (Bonilla *et al.*, 2002).

DNA extraction, PCR and gel electrophoresis

Leaf samples were collected from young leaves from the plants at 20-25 days after transplanting. DNA was extracted following modified Miniscale method (Zheng *et al.*, 1995). PCR was performed in 10 µl reactions containing around 25 ng of DNA template (3 µl DNA with 10X dilution factor), 1 µl 10X TB buffer (containing 200 mM Tris–HCl pH 8.3, 500 mM KCl), 1.35 µl 25 mM $MgCl_2$, 0.2 µl of 10 mM dNTP, 0.5 µl each of 10 µM forward and reverse primers and 0.1 µl of Taq DNA polymerase (5 U/µl) using thermal cycler (Chen *et al.*, 1997; Neeraja *et al.*, 2007). Twelve-channel pipette was used for transferring DNA from dilution plate to PCR plate. Ten micro liter of mineral oil was added in each well to prevent evaporation and the PCR plate was wrapped with adhesive film. After initial denaturation for 5 min at 94°C, each cycle comprises 45 sec denaturation at 94°C, 45 sec annealing at 55°C, and 2 min extension at 72°C with a final extension for 7 min at 72°C at the end of 35 cycles. 8% gel was used for PAGE. The reagents were added as Table 1.

After gel electrophoresis the acrylamide gel was removed carefully and transferred in the ethidium bromide staining solution (0.5 mg/ml) for 25 minutes

approximately.

The stained gels were put in the exposure cabinet of the gel documentation system (Alpha Imager EP, Alpha Innotech, CA, USA). The gel was exposed to UV light and the gel image was saved as a Jpeg file.

Allele scoring

The band having same level of BRRI dhan49 was scored as 'A' which indicated the homozygous allele of the recipient parent for the particular SSR marker. Again, the band having same level of FL478 was scored as 'B' which indicated the homozygous allele of the donor parent for the particular SSR marker. However, heterozygous alleles were scored as 'H' having both the bands of two parents. Importantly, heterozygous alleles always had the extra bands in most cases with bigger size. Unidentified or missing alleles were scored as 'U'.

Data analysis

The marker data was analyzed using computer software Graphical Genotyper (GGT 2.0) (VanBerloo, 2007). This software was freely available from: http://www.dpw.wau.nl/PV/. Analysis of variance and mean comparisons based on Least Significant Difference (LSD) for phenotyping at screenhouse used in this study were performed using SAS (Statistical Analysis System) (ver. 08) package.

Parental polymorphism survey

A primer survey was carried out between the two parental genotypes *viz.* BRRI dhan49, the recurrent or recipient parent and FL478, the donor parent of the MABC breeding program. A total of 363 microsatellite and *InDel* markers were used to screen the parents for identifying polymorphic markers.

Marker assisted backcrossing scheme

The marker-assisted backcrossing scheme used several cycles of crossing back to the recurrent parent as illustrated in Fig. 1. 56 SSR markers were used for background selection (Table 2) and 1SSR marker RM493 was used for foreground selection.

Phenotypic evaluation

Screening of selected *saltol* lines were done by hydrophonics system using Yoshida nutrient solution (Yoshida *et al.*, 1976). The experiment was set in the net house of Plant physiology division, BRRI, Gazipur during March-April, 2014. A randomized complete block design was used with three replications. In each tray, 6 BRRI dhan49-*Saltol* lines, the recurrent parent (BRRI dhan49), and the checks FL478 (tolerant, donor of *Saltol* QTL) and IR29 (sensitive) were included. Nine individual plants per line were evaluated in each replication. Seeds were kept for 5 days in an incubator set at 50°C to break their dormancy. Then the seeds rinsed with distilled water and placed in petri dishes lined with moistened filter paper and incubated at 32°C for 48 hours to germinate. One pre-germinated seed was sown per hole on the Styrofoam seedling float with a net bottom placed on a tray filled with distilled water. Distilled water was replaced with Yoshida nutrient solution after 3 days. After 14 days of seeding, salt stress was imposed by adding NaCl (Analytical grade) to the desired electrical conductivity (EC) of 12 dSm^{-1} in the Yoshida nutrient solution and maintained until final scoring. The pH of the solution was adjusted to 5.0 daily using either 1N NaOH or HCl. Visual symptoms of salt stress injury of the seedlings were evaluated based on the IRRI modified Standard Evaluation System (SES) scores (IRRI, 1996) with rating from 1 to 9 (Table 3).

The evaluation of visual injury symptoms was performed after two weeks of salinization. Root and shoot lengths were measured and washed with water. The samples were dried in an oven at 70°C for 5 days and then weighed to determine their biomass.

Results and discussion

Parental polymorphism survey

A total of 363 SSR and *InDel* primers were surveyed to find out polymorphic markers. A total of 96 markers (27%) were found polymorphic (Table 4), which is relatively lower because both parents are of indica origin, and the lower percentage polymorphism indicates the high degree of genetic

similarity between BRRI dhan49 and FL478. Out of these 96 markers, 74 exhibited distinct polymorphism and the rest 22 showed slightly polymorphic between two parents and evenly distributed in the genome. Fourteen polymorphic markers identified in chromosome 1 and 6, 13, 8, 8, 8, 7, 7, 8, 7, 5 and 5 markers were found polymorphic in chromosome 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, respectively. In total 96 primers were found as polymorphic, 246 markers were found as monomorphic and 21 markers did not amplify successfully (Table 4).

Table 1. Reagents for polyacrylamide gel.

Reagents	Final conc.	8% gel
Sterile nanopure H_2O		41.35 ml
10X TBE buffer	5X	6.0 ml
40% Acrylamide	8%	12 ml
10% APS	0.1%	600 µl
TEMED (Tetramethylethylene diamine)	1 µl/ml	50 µl
Total		60.0 ml

Confirmation and backcrossing

F_1s were confirmed using foreground marker RM493. The confirmed F_1 plants were backcrossed with recurrent parent BRRI dhan49 to produce BC_1F_1 seeds. The BC_1F_1 seeds were harvested and preserved for further backcrossing.

Foreground and background selection in BC_1F_1:

Fifty seven BC_1F_1 individuals were genotyped using foreground marker RM493, and 34 individuals were selected that were heterozygous for *Saltol* locus (Fig. 2). These 34 individuals were genotyped using 56 SSR background markers (Table2) that span the genome (Fig. 3). The recovery of the recurrent parental genome at the 56 markers varied from 27% to 63%. Five BC_1F_1 best plants were selected based on the highest recovery of the recurrent parent genome (Fig. 4). These selected BC_1F_1 plants were backcrossed with recurrent parent to produce BC_2F_1 seeds. The BC_2F_1 seeds were harvested and preserved for the next generation.

Table 2. List of background markers of all 12 chromosomes used in the background selection.

Chromosome 1	Chromosome 2	Chromosome 3	Chromosome 4	Chromosome 5	Chromosome 6
RM495	RM341	RM411	RM16301	RM153	RM314
RM283	RN110	RM520	RM470	RM413	RM136
RM272	RM324	RM293	RM280	RM289	RM275
RM490	RM525	RM468	RM567	RM440	RM340
RM212		RM565		RM421	
RM104		RM570		RM274	
		RM85		RM334	
Chromosome 7	Chromosome 8	Chromosome 9	Chromosome 10	Chromosome 11	Chromosome 12
RM432	RM337	RM296	RM216	RM202	RM12
RM346	RM407	RM23805	RM258	RM229	RM247
RM336	RM152	RM434	RM228		
RM428	RM5556	RM23958	RM333		
	RM72		RM590		
	RM458				
	RM447				

Table 3. Modified standard evaluation score (SES) of visual salt injury at seedling stage (IRRI, 1996).

Score	Observations	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: only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants are dead or dying	Highly susceptible

Foreground and background selection in BC_2F_1 generation

In BC_2F_1 generation, 364 individuals were genotyped using foreground markers RM493, and 92 individuals were selected that were heterozygous for *Saltol* locus (Fig. 5). These selected plants were then genotyped using 56 background markers that span the genome. In this generation, those background markers showed homozygous with recurrent parent in BC_1F_1 generation, were not used. The contribution of the recurrent parental genome in BC_2F_1 varied from 48.0% to 89.5%. From manual calculation, plant # 32-181 showed 89.5% background recovery. Six background markers and 1 foreground marker were used in chromosome 1 and out of them all background markers showed homozygous with recurrent parent. In chromosome 1, 2 and 4, 75% markers were homozygous with recurrent parent for this plant. In chromosome 3, 5, 6, 7, 9, 10 and 11 of this plant, the used markers showed 100% homozygosity with recurrent parent.

Table 4. Results of primer polymorphism survey between two parents of the MABC scheme.

Chromosomes	Polymorphic	Monomorphic	Non-amplification	Total number	% Polymorphism	Highest Rank	Lowest Rank
1	14	30	2	46	30.43		
2	6	18	3	27	22.22		2
3	13	23	3	39	33.33	3	
4	8	25	3	36	22.22		2
5	8	15	0	23	34.78	2	
6	8	20	1	29	27.59		
7	7	20	3	30	23.33		
8	7	21	1	29	24.14		
9	8	23	2	33	24.24		
10	7	11	0	18	38.89	1	
11	5	19	2	26	19.23		3
12	5	21	1	27	18.52		1
Total	96	246	21	363	Ave = 26.57		

Table 5. Performance of BRRI dhan49-*Saltol* lines and checks under salt stress of EC 12 dSm^{-1} at seedling stage in BC_2F_2 generation. Data are presented as the means of three replications.

Genotypes	SES	Root length (cm) (Saline)	Shoot length (cm)			Root biomass (g) (Saline)	Shoot biomass (g)			Total Biomass (g) (Saline)
			Control	Saline	% Reduction		Control	Saline	% Reduction	
15-42	5.3	18.8	34.1	25.5	25.07	0.30	2.11	1.60	24.42	1.90
15-64	4.9	18.5	33.1	25.3	23.36	0.34	2.24	1.66	26.01	1.99
15-66	4.8	17.9	33.8	23.7	29.71	0.30	2.14	1.61	24.65	1.91
32-172	5.5	16.5	31.9	24.8	22.13	0.26	1.51	1.38	8.68	1.64
32-181	5.2	16.4	34.8	25.9	25.47	0.26	1.74	1.48	14.92	1.74
48-348	5.6	17.5	31.3	24.7	21.21	0.28	2.28	1.56	31.75	1.84
BRRI dhan49	6.4	16.2	28.8	20.8	27.61	0.20	1.78	1.11	37.83	1.31
FL478	5.8	15.0	29.9	22.5	24.88	0.24	1.46	1.23	15.64	1.47
IR29	7.3	14.2	36.9	19.6	46.78	0.18	2.83	1.01	64.15	1.19
LSD(0.05)	0.49	1.6	-	0.86	2.71	0.04	-	0.21	11.14	0.23
%CV	5.00	5.5	-	2.10	5.73	9.22	-	8.71	23.35	8.26

Six plants, # 32-181, 32-172, 15-42, 15-64, 48-348, 15-66 were selected based on the highest recovery of the recurrent parent genome of 89.5%, 84.2%, 82.5%, 82.5%, 80.7% and 80.7%, respectively (Fig. 6). These six plants were selfed to produce BC_2F_2 BRRI dhan49-*Saltol* lines.

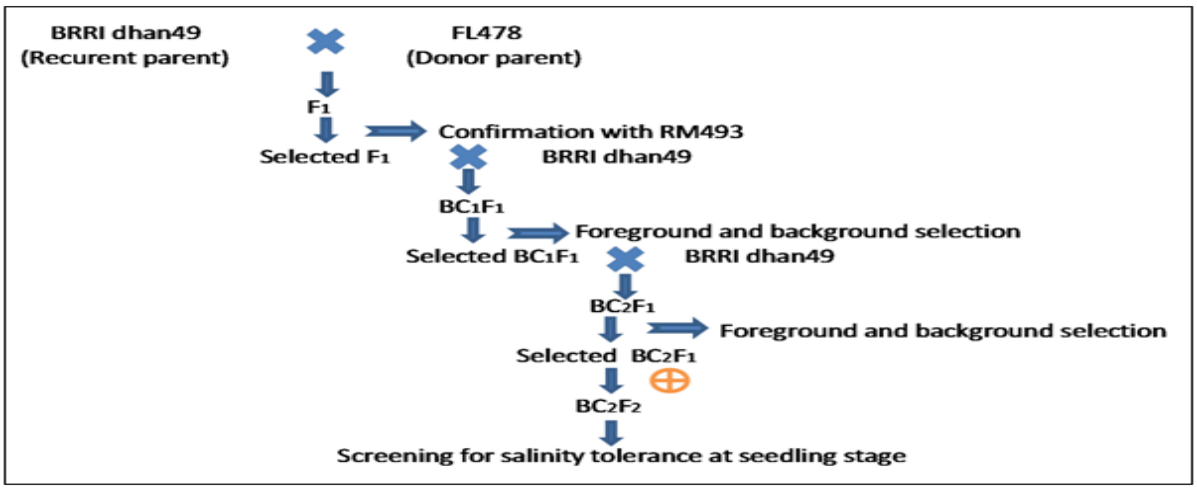

Fig. 1. Sequential order of events for introgression of *Saltol* locus into the genetic background of BRRI dhan49.

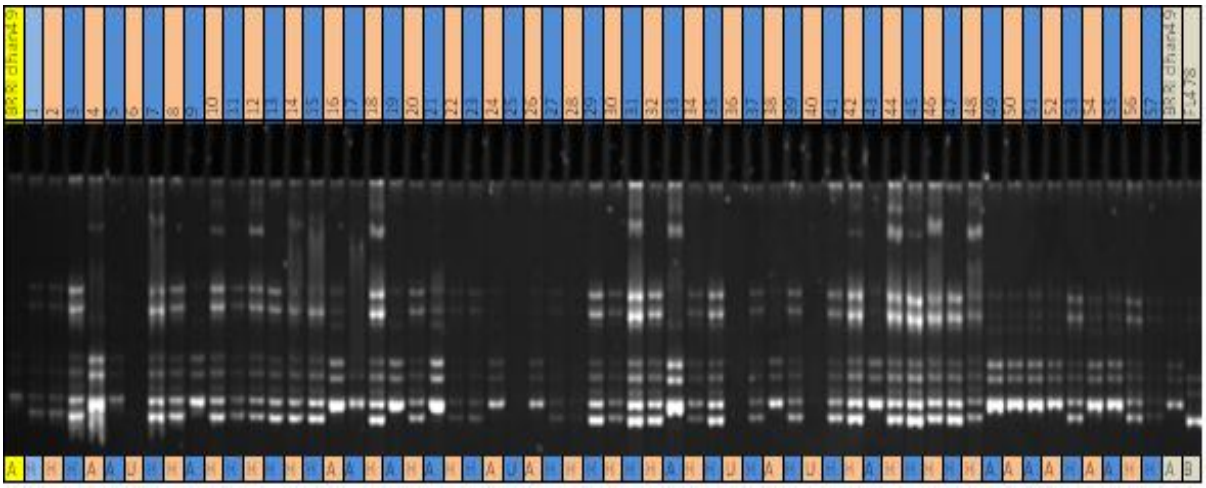

Fig. 2. Foreground selection with marker RM493 for detecting *Saltol* QTL in BC_1F_1 generation. A, B, H indicate recurrent parent, donor parent and heterozygous respectively.

Vu *et al.* (2012) used FL478 as donor parent of *Saltol* QTL and Bacthom7 as recurrent parent to develop a salt tolerant line. Two foreground markers RM3412 and RM493 were used for foreground selection to get heterozygous plants from BC_1F_1 population and a total of 80 SSR polymorphic markers were used for background selection. Eight best BC_1F_1 plants were selected that were heterozygous at target loci and 71% -76% homologous for the recurrent markers at other loci in all 12 chromosomes. These plants were used to backcross with Bacthom7 in order to develop the BC_2F_1 population for next step. For foreground selection in BC2F1 generation three polymorphic SSR markers (RM140, RM3412 and RM493) were used and 264 heterozygous plants from 852 plants of BC_2F_1 population were selected. After background selection, four plants were chosen based on conferred *Saltol* QTL region and background recovery with 88.5% - 95.5% homozygous ratio for the recurrent parent.

In this study, the *Saltol* QTL was introgressed in genetic background of BRRI dhan49. The background analysis in the introgression lines revealed the recovery up to 89.5% of recurrent parent alleles based on the screened background markers after two generations.

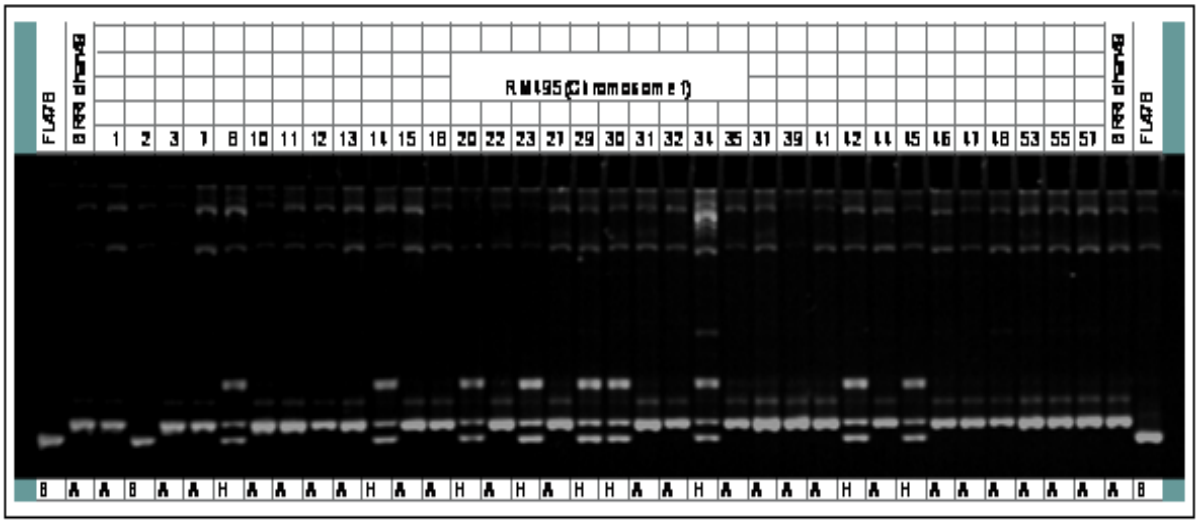

Fig. 3. Partial view of the gel pictures of the background selection in BC_1F_1 generation (Note: A = Recurrent parent: B = Donor parent; H = Heterozygous).

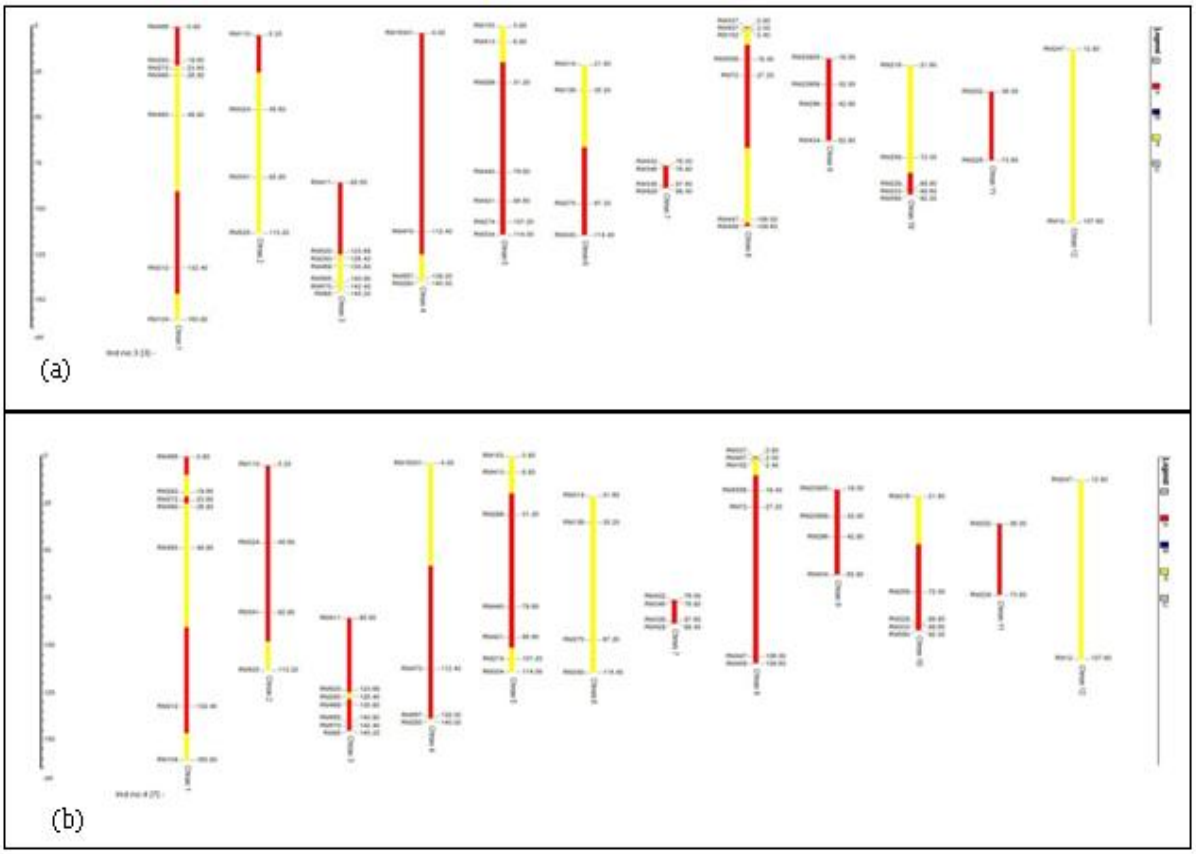

Fig. 4. Graphical genotyping of the plant # 3(a), plant # 7(b) in BC_1F_1 generation from the cross of BRRI dhan49*2/FL478. The red colored regions represent the homozygous regions of the recipient genome, whereas the yellow colored regions represent the heterozygous regions.

Phenotypic evaluation of BRRI dhan49-Saltol lines under salt stress at seedling stage

Phenotypic evaluation was carried out at seedling stage under salt stress of EC 12 dS m^{-1}. It was done in a replicated trial using hydroponic system with Yoshida nutrient solution. The six BRRI dhan49-*Saltol* lines were evaluated along with the recurrent parent (BRRI dhan49), the donor parent (FL478) and

a sensitive check, IR29. A randomized complete block design was used with three replications. Nine individual plants per line were evaluated in each replication. After 14 days of seeding, salt-stress was imposed. Two weeks after salinization the responses to salinity were assessed. All tested genotypes showed different levels of salt injury symptoms, such as: leaf rolling, delayed formation of new leaves, brownish and whitish coloration on the tips of leaves, stunting of growth and dying of seedlings. Significant differences were observed for all characteristics compared with the parental line, BRRI dhan49 and sensitive check, IR29 (Table 5). BRRI dhan49-*Saltol* lines had significantly lower SES scores (4.8 to 5.6) compared with the recurrent parent BRRI dhan49 (6.4) and the sensitive check IR29 (7.3), whereas the donor parent FL478 showed an average SES score of 5.8 (Fig. 7). All of the test entries had reduced shoot length and shoot biomass under saline condition. Shoot length of the *Saltol* lines was significantly higher than the recurrent parent, the sensitive check and donor parent. Shoot biomass of these lines was significantly higher than BRRI dhan49 and IR29. All the six BRRI dhan49-*Saltol* lines had relatively higher shoot biomass compared to FL478. Among the six BRRI dhan49-*Saltol* lines, five lines (15-42, 15-64, 15-66, 32-181, and 48-348) had significantly higher shoot biomass compared to FL478 in saline condition. All the six BRRI dhan49-*Saltol* lines had relatively higher total biomass and five lines (15-42, 15-64, 15-66, 32-181, and 48-348) had significantly higher total biomass compared to FL478 in saline condition. Salt stress severely affected growth related traits at seedling stage, which would negatively impact crop establishment in saline areas. That would cause, in its turn, major losses in stand and productivity of rice. By diluting Na^+ concentration in shoot tissues, vigorous seedling growth contributes to salt stress tolerance leading to better growth and survival rates on saline soils.

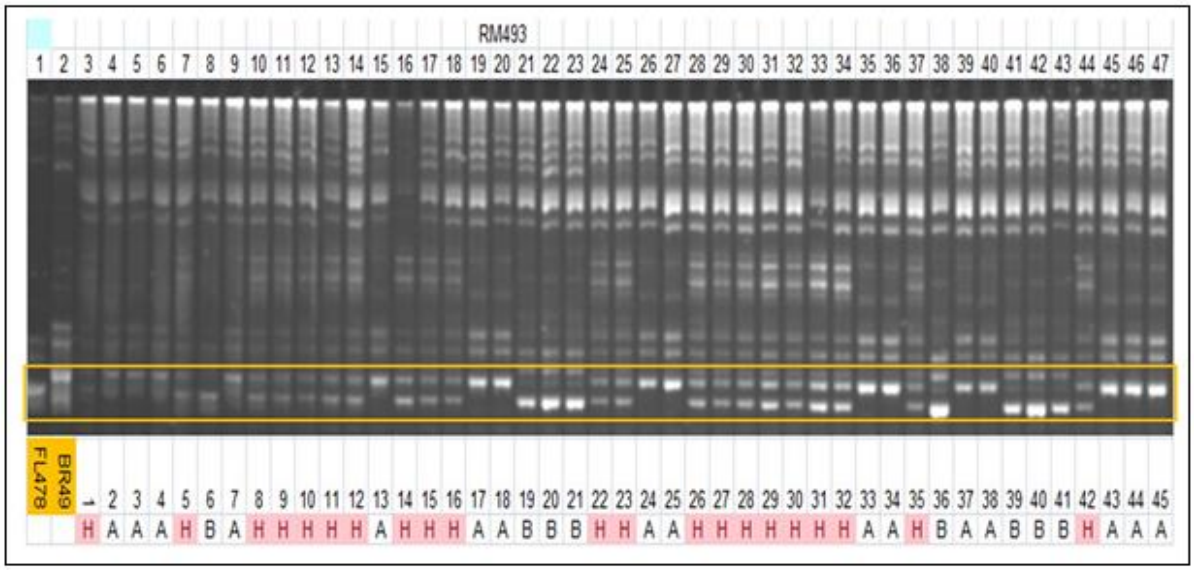

Fig. 5. Partial view of foreground selection with marker RM493 for detecting *Saltol* QTL in BC_2F_1 generation.

Sarker (2013) assessed performances of 10 BR29-*Saltol* lines in a replicated trail under salt stress of EC 12 dSm^{-1} at seedling stage. All BR29-*Saltol* lines showed significantly lower SES scores ranging from 5.5 to 5.8, compared to the recurrent parent BR29 (6.9), while the donor parent FL478 showed the lowest SES score of 4.7. In this study, plant # 15-64 showed SES score of 4.8 and all other lines had SES scores of less than 5.5 whereas BRRI dhan49 had higher SES score of 6.3 and susceptible check IR29 had 7.3. FL478, the donor parent of *Saltol* QTL had SES score of 5.8. The developed BRRI dhan49-*Saltol* lines showed lower SES scores compared with recurrent parent BRRI dhan49 which indicated that the developed lines were moderately salt tolerant at seedling stage.

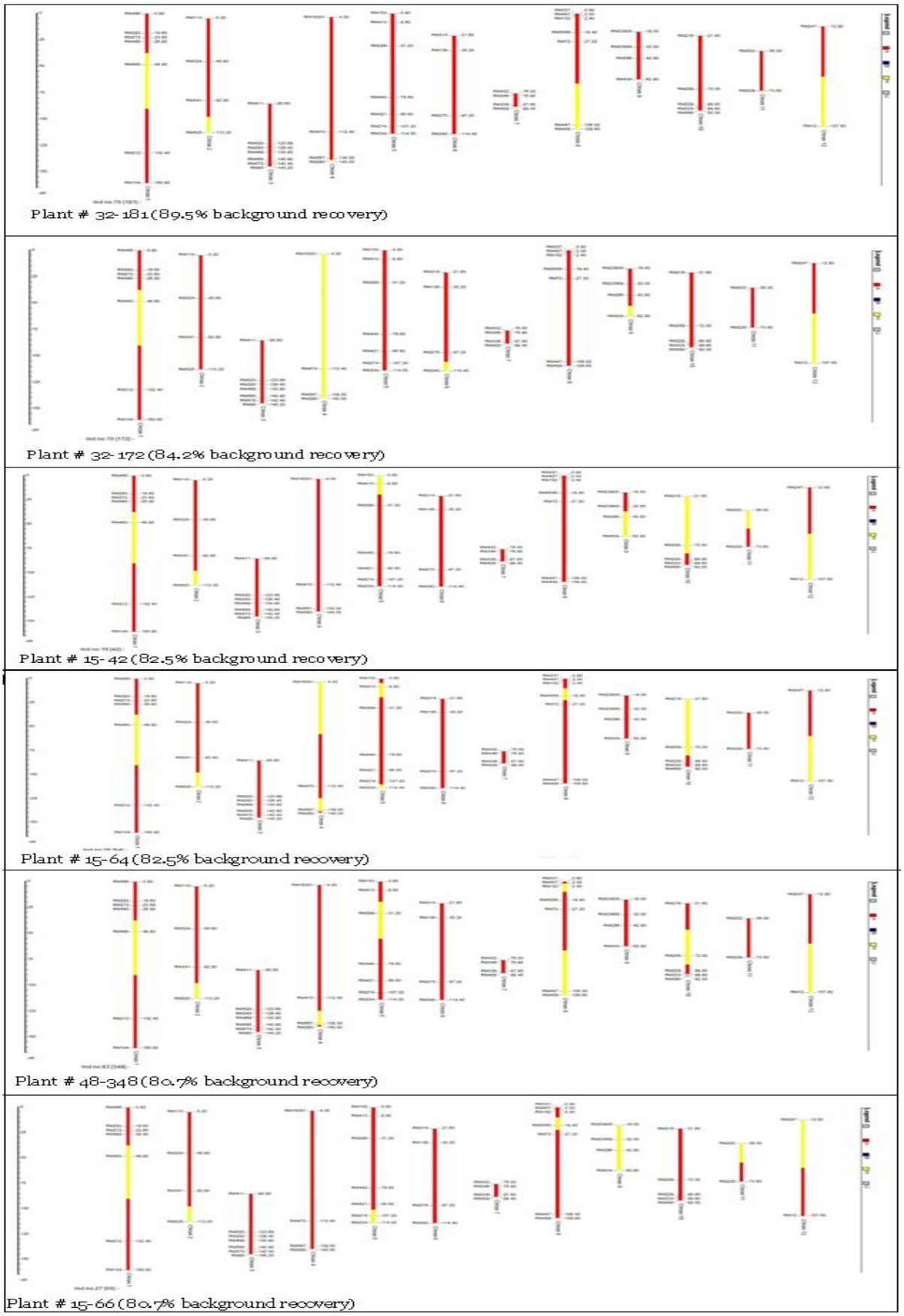

Fig. 6. Graphical genotyping of the plant # 32-181, 32-172, 15-42, 15-64, 48-348 and 15-66 in BC_2F_1 generation from the cross of BRRI dhan49*3/FL478. The red colored regions represent the homozygous regions of the recipient genome, whereas the yellow colored regions represent the heterozygous regions.

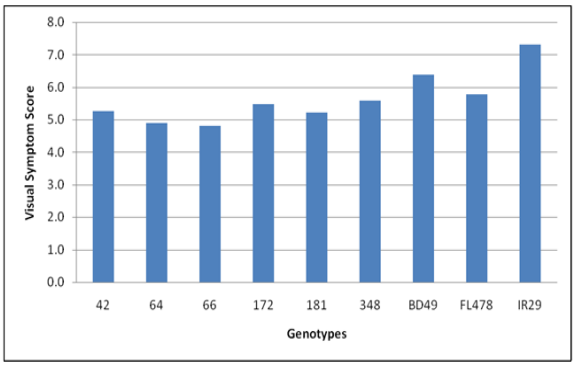

Fig. 7. Bar graph showing SES values for visual symptom score.

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