

Identifying QTLs Associated and Marker-Assisted Selection for Salinity Tolerance at the Seedling, Vegetative and Reproductive Stages in Rice (*Oryza Sativa* L.)

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Abstract— Salinity affects rice growth in all growth stages, with the seedling and reproductive stages being the most sensitive. Genetically improving salt tolerance of rice is an important objective of rice breeding programs. Hence, mapping quantitative trait loci (QTL) will be useful for marker-assisted selection in rice breeding programs. An advanced backcross population (BC₂F₂) was developed with the parents included OM5629 as a donor of salt tolerance and OM7347 as a recurrent parent with good quality traits and drought tolerance. Molecular markers associated with both qualitative and quantitative trait loci (QTL) salt tolerance were identified by using 416 polymorphic SSR markers. QTLs, associated with stress tolerance at EC = 15 dS/m at seedling stage, detected from the BC₂F₂ population of OM7347/OM5629, were located on chromosomes 1 and 3. Three QTLs were identified at the intervals of RM3252-S1-1 - RM10694, RM3740-RM5336 and RM11125-RM9 with genetic distance of 4.4, 4.5 and 18 cM on chromosome 1, respectively. Two QTLs at the intervals of RM3867-RM6959 and RM6876-RM4425 with genetic distance of 4.5 and 18.0 cM on chromosome 3, respectively. One QTL on chromosome 5 was detected at the interval of RM874 - RM10359, it was associated with salt stress tolerance under EC = 8dS/m at vegetative stage. Three QTLs at the regions of RM1324-RM2412, RM1185-RM24, and RM1282-RM2560 on chromosome 1, and one QTL of RM453-RM511 on chromosome 12, were related to salt tolerance under EC = 8dS/m at reproductive stage. Two tightly linked markers as RM3252-S1-1 and RM3867, were exhibited their effectiveness in identification of salt tolerance genotypes in BC₃F₆ population of OMCS2000/ Pokkali. The identification of new QTLs associated with salt tolerance will provide important information for the functional analysis of rice salinity stress.

Keywords— Seedling, reproductive stage, salinity, SSR, QTL, vegetative stage.

I. INTRODUCTION

Salinity is the second type of stress and is the most important loss rice yield production after drought [1]. Salty soil is one of the most common stress has a negative effect on crop production. Salty soil is the main limiting factor in the production of rice, a type of salt-sensitive plants, productivity is affected greatly by the ion toxicity [2]. The difference between plant species and on the tolerance to salinity [3]. Rice crops are relatively resistant to stress during germination, active and mature branches lying but very sensitive at the beginning stage of seedlings [4, 5]. Two stages of the rice plant sensitivity independent of each other and are controlled by genes, meaning, in the reproductive period the tree no resistant varieties in the stage of seedlings and vice versa. Moreover, salt tolerance at the reproductive stage is very important because the process of fertilization and seed formation occurs in this period and reproductive stages directly related to yield [6]. In rice, important traits such as salt-tolerance, yield and quality are controlled by polygenes that are described as quantitative trait loci (QTLs) [7]. QTL mapping related to environmental stresses, yield and quality are very important for the application of map-based cloning and marker-assisted selection (MAS) in rice breeding programs [8]. In rice, QTL analysis of salt tolerance has been conducted by several researchers [9, 10]. Lang *et al.*, [11] reported that salt tolerant genes tagging based on SSR markers with advanced backcross populations (BC₂F₂) of IR64/ChengHui 448, IR64/OM1706 and IR64/ FR13A derived alleles nearly located at on chromosome 1 while in the population of IR68552-55-3-2/OM1706, the alleles are linked with RM223 on chromosome 8. The major

gene for salinity tolerance (*Saltol*) was mapped on chromosome 1 and chromosome 8 [12, 11]. RM223 linked to salt tolerance gene at the distance of 6.3 cM on chromosome 8 at vegetative stage under EC = 10 dS.m⁻¹ from F₃ population of IR28/Doc Phung [12]. Tiwari *et al*, [13], reported a method for rapid identification of QTLs for reproductive stage salt tolerance in rice using bulked segregant analysis (BSA) of bi-parental recombinant inbred lines (RIL). The method was applied to CSR11/MI48 RILs segregating for reproductive stage salt tolerance. Genotyping of the parents and RIL bulks, made on the basis of salt sensitivity index for grain yield, revealed 6,068 polymorphic SNPs and 21 QTL regions showing homogeneity of contrasting alleles in the two bulks. BSA with 50K SNP chip revealed 5,021 polymorphic loci and 34 QTL regions.

In this study, we established QTL maps for the agronomical traits related to salinity tolerance at different stages to aim at selecting suitable genotypes adapted to both seedling and reproductive stages under Mekong Delta of Vietnam.

II. MATERIALS AND METHODS

1. Plant Materials

Two different populations were used to identify QTL analysis and marker-assisted selection in this study. Two hundred fifty-three elite lines were divided from the

BC₂F₂ population between OM7347, a good quality variety and drought tolerance and OM5629 which was considered as a donor of salt tolerance. Of 769 SSRs, 416 polymorphic markers related to salinity tolerance were applied to set up the QTL map. A second mapping population of 230 lines from the BC₃F₆ population of OMCS2000 / Pokkali was developed [14]. These plants were genotyped the Genetics and Plant Breeding Division of High Agricultural Technology Research Institute for Mekong Delta Vietnam and the Molecular Biology Lab of Biotechnology PCR Company, Cantho, Vietnam.

2. Phenotypic evaluation

This study carried out in two environments (EC of 15 dS/m and 8 dS/m) at seedling and reproductive stages, respectively. Pokkali is a tolerant variety and IR29 is a susceptible variety. The experiment consisted of in three replications, randomized complete design (RCD). Germinated seeds were put into the floating Styrofoam under sterilized water within 3 days. Then, the Yoshida solution was increased the EC up to 4dS/m and 8 dS/m, at pH = 5.0 – 5.5. After 21 days, the result was evaluated base on the survival day (SD) of the tolerant and susceptible genotypes based on the methods of Gregorio, [15] and IRR1 [16] (Table 1). The susceptible variety was almost dead completely.

Table.1: Modified IRR1 standard evaluation scoring (SES) system based on the visual symptoms of salt stress injury of rice.

SES	Description	Tolerance
1	Normal growth, only the old leaves show white tips while no symptoms on young leaves	Highly tolerant
3	Near normal growth, but only leaf tips burn, few older leaves become whitish partially	Tolerant
5	Growth severely retarded; most old leaves severely injured, few young leaves elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dried; only few young leaves still green	Susceptible
9	Almost all plants dead or dying	Highly susceptible

3. SSR markers genotyping

DNA was extracted using CTAB method which modified by Lang, [17]. Genomic DNA samples were loaded in 0.9% agarose gel in TAE 1X to check for its quality. PCR reactions were done in eight microliters of each reaction were run on the polyacrylamide gel with 769 SSR markers. Reactions were overlaid with mineral oil and processed in a programmable thermal controller set for 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, with a final extension at 75 °C for 5 min. After amplification, 10 µl of stop solution was added to the PCR product, which was then denatured at 94 °C for 2

min. Amplified genes from PCR were assessed on agarose gel 3% in TBE 1X.

4. Map construction and QTL analysis

The program MAPMAKER/EXP v.3.0 [18, 19] was employed to establish a genetic linkage map using the Kosambi mapping function [20]. Link-age groups were inferred based on the existing RFLP and micro-satellite maps of rice [21, 22, 23]. MAPMAKER/QTL version 1.1 was used to identify loci affecting quantitative traits on the basis of interval analysis [24, 19]. A LOD score of 3.0 was selected as the threshold for the presence of a QTL based on the total map distance, and the average distance

between markers [25]. With such a threshold, a false positive QTL would be detected anywhere in the genome with a probability of approximately 0.05 [24]. The independence test was performed where the initial scan suggested two or more QTLs located on the same chromosome as described by Paterson et al, [24]. and Lander and Bostein, [25]. The total phenotypic variation explained by all QTLs were estimated by fitting a multiple regression model in the MAPMAKER/QTL program. The interaction between all possible loci on the map was performed using QTLMapper version 1.0 [26]. (Wang et al., 1999).

Single-marker QTL analysis using linear regression was employed [27]. The marker alleles linked to salt stress were coded 1; and in contrast, a code 0 for conducting regression analysis. To analyze markers-QTLs association for each trait, single-point (single marker) analysis of QGene version. 4.0.2 [28] was performed. This analysis served as the primary method of detecting the association between markers and the target traits.

To determine the precise location of the putative QTLs, interval mapping of the MAPMAKER/EXP version 3.0 [24, 19] was conducted. Interval mapping analysis was also used to confirm the results of the single marker analysis.

LOD $\geq 3,0$ [with P -value $R^2 \geq 10\%$] was used as the threshold for detecting QTLs location. LOD peaks for significant QTLs were used to position the QTL on the linkage map and to identify significant markers linked to target genes.

GRAMENE database was used to widen putative gene regions and identify new markers for genetic diversity and shortening of target gene regions.

Combined data based on QGene, MapMarker and GGT (graphic genotyping) was used to analyze QTL maps. After that, only the most two specific traits were selected

to build QTL maps. QTL maps were analyzed by using the direction of phenotypic effect (DPE), additive value and probability with small errors [29].

III. RESULTS AND DISCUSSION

1. QTL map related to salinity tolerance in BC₂F₂ population of OM7347/ OM5629 at seedling stage

A total 253 individuals of the BC₂F₂ population of OM7347/OM5629 were used to set a QTL map on twelve chromosomes to identify target genes (Fig 1). Out of 769 SSRs, 416 SSRs were found polymorphism between drought tolerant gene of the recurrent parent (OM7347) and the donor (OM5629) in previous studies [30, 31]. PCR products of population OM7347/OM5629 based on 416 polymorphism markers showed in Table 1. Twelve linked groups on twelve chromosomes constructed were 4,447.5 cM in total length, with average distance at 10.69 cM among contiguous regions.

2. Analysis of QTL map

To elucidate the genetic basis and physiology of traits related to salt tolerance in rice, the overlapped QTLs, and beneficial genes were identified. Through CIM analysis, there were many significant QTLs related to salinity tolerance from BC₂F₂ with the average distance of 10.69 cM. The QTLs scattered on **chromosome 1** viz. RM3532S-RM10694; RM3740-RM5336 and RM11125-RM9RM explained 13.33%, 30.48% and 37.14% of phenotypic variance, respectively. QTLs located on chromosome 1 are major loci, which explained more than 50% of the phenotypic variance. The QTLs related to salinity tolerance under EC=18 dS/m at the seedling stage are located on chromosomes 1, 6, 8 and 9 [32, 30, 31]. Gong et al, [33], Bonilla et al, [34] and Lin et al, [35]. detected QTLs for salt tolerance on chromosome 1.

Table.1: Polymorphism of SSR markers on twelve rice chromosomes.

No.	Chromoeome	No. of linked markers	Length (cM)	Mean (cM)
1	1	72	872.3	12.11
2	2	48	518.4	10.80
3	3	53	549.7	10.37
4	4	31	254.6	8.21
5	5	33	414.5	12.56
6	6	27	411.0	15.22
7	7	21	213.3	10.14
8	8	21	153.3	7.30
9	9	45	385.7	8.57
10	10	19	198.2	10.43
11	11	23	232.0	14.04
12	12	23	244.7	10.63
Total		416	4447.5	10.69

In addition, Graphical representation of QTLs was located on linkage map is showed in Fig 2 using CIM analysis on chromosome1 and chromosome 3. Their LOD values, additive effects, and phenotypic variance are presented in Table 2. QTLs related to salinity tolerance at seedling stage on chromosome 1 (RM3532-S-RM10694; RM3740-RM5336 and RM11125-RM9) with genetic distance of 4.4 cM, 4.5 cM and 18 cM, respectively, and two QTLs revealed salinity tolerance at seedling stage with genetic distance of 4.5 and 18.0 cM on chromosome 3 (RM3867-RM6959 and RM6876-RM4425) in the BC₂F₂ population of OM7347/OM5629 under EC = 15 dS/m. [7]. reported that salt tolerance QTLs located on chromosomes 1 and chromosome 3 were QST01 and QST-3, respectively. Recently, Thomson *et al.* [37] reported that four QTLs related to salt tolerance are on chromosome 1 (1 QTL), chromosome 2 (1 QTL), chromosome 3 (1 QTL) and chromosome 12 (1 QTL). Markers linked the QTLs in MAS breeding permits to exactly identify the major and minor QTL regions. Results from QTL map analysis showed that marker RM3532S was tightly linked to *Saltol* locus (4.6 cM in genetic distance) on chromosome 1. QTLs for major traits detected on chromosome 1. The results are useful for MAS breeding as well as pyramiding breeding in the future.

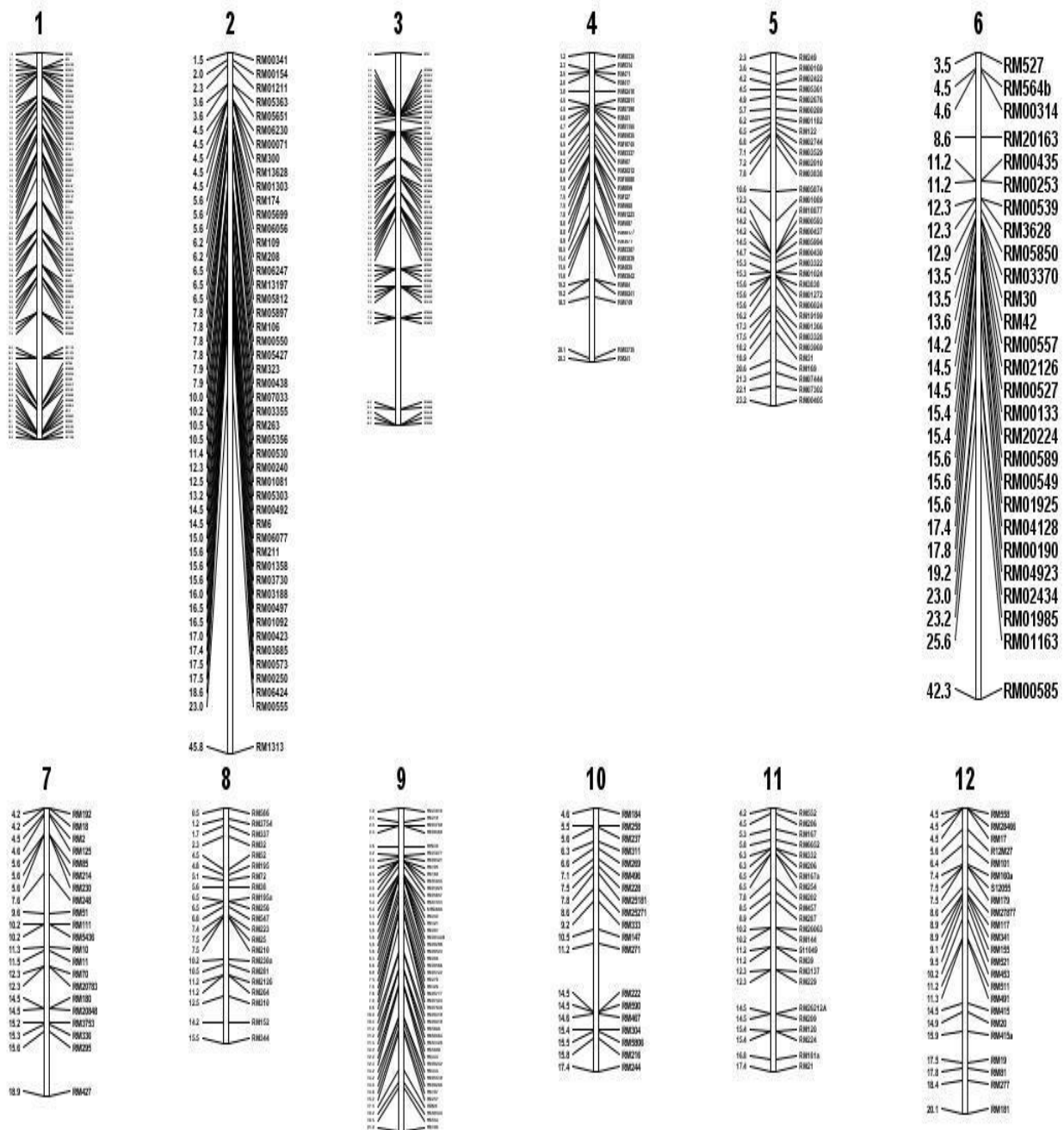


Fig. 1: QTL map for the traits related to salinity tolerance on twelve chromosomes in the BC₂F₂ population of OM7347/OM5629.

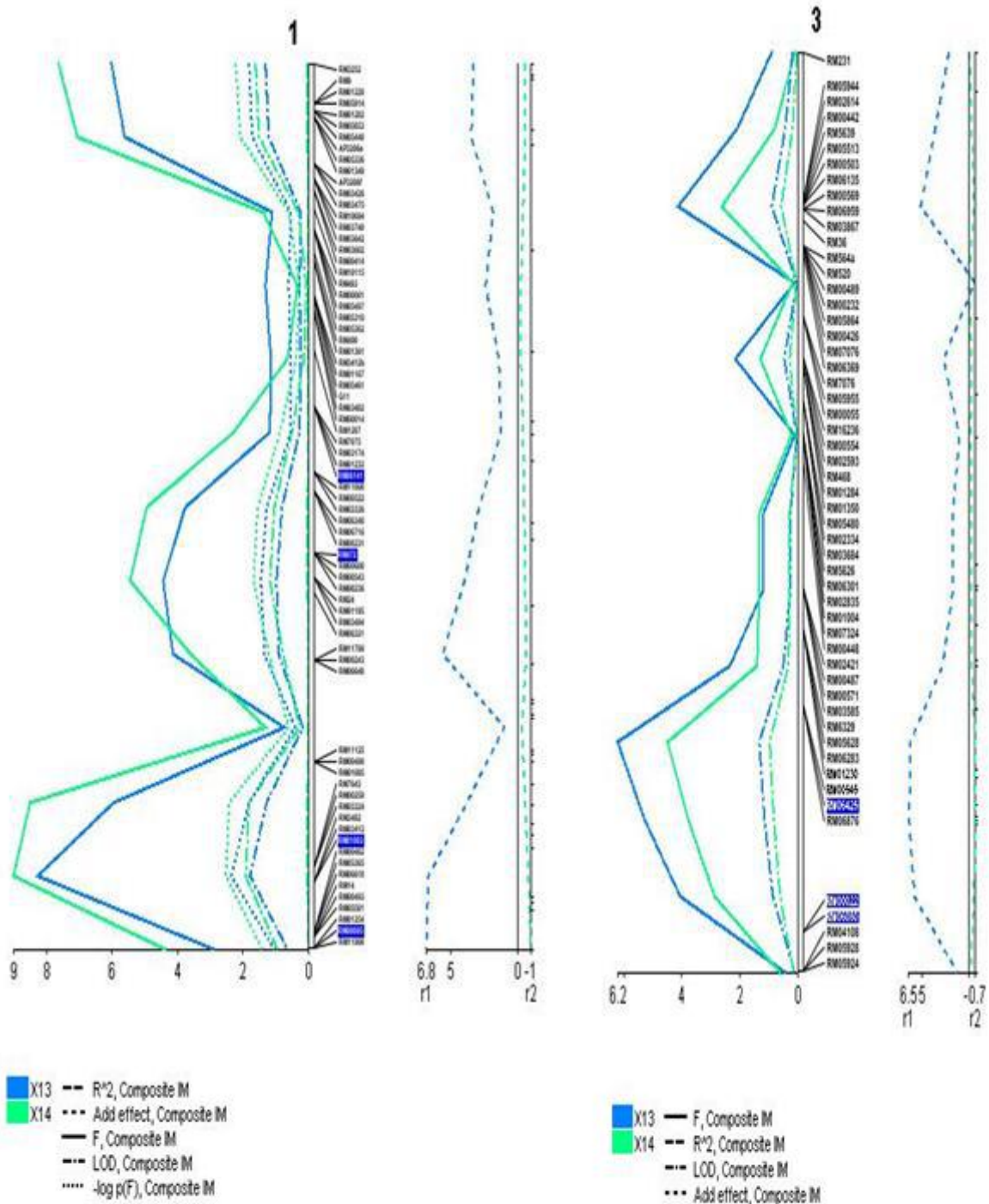


Fig. 2: LOD peaks for QTLs related to salinity tolerance at seedling stage on chromosome 1 and chromosome 3 in the BC₂F₂ population of OM7347/OM5629 under EC = 15 dS/m.

Table.2: QTLs related to salinity tolerance in the BC₂F₂ population of OM7347/ OM5629 at seedling stage.

Chr.	QTL	Location (cM)	CIM (Interval cM)	LOD	A	D	R ²
1	SD QTL-1	4.4	RM3252-S1-1 -RM10694	4.3	0.29	15.18	13.33
1	SD QTL-1	4.5	RM3740-RM5336	2.8	13.15	23.67	30.48
1	SEIQTL-1	18.0	RM11125-RM9	3.0	11.43	81.08	37.14
3	SD QTL-3	4.5	RM3867-RM6959	4.6	12.56	23.67	11.41
3	SEIQTL-3	18.0	RM6876-RM4425	17.1	4.85	24.50	17.40

P < 0.05; A: Additive, D: Dominant, R²: phenotypic variance explained, Chr: chromosome; SD: survival day; CIM: composite interval mapping

At vegetative stage

Similarly, the BC₂F₂ population of OM7347/OM5629 was assessed some key phenotypical traits at tillering stage. One QTL was detected at the region of RM874-RM10359 on chromosome 5 at LOD ≥ 3 (Fig 3).

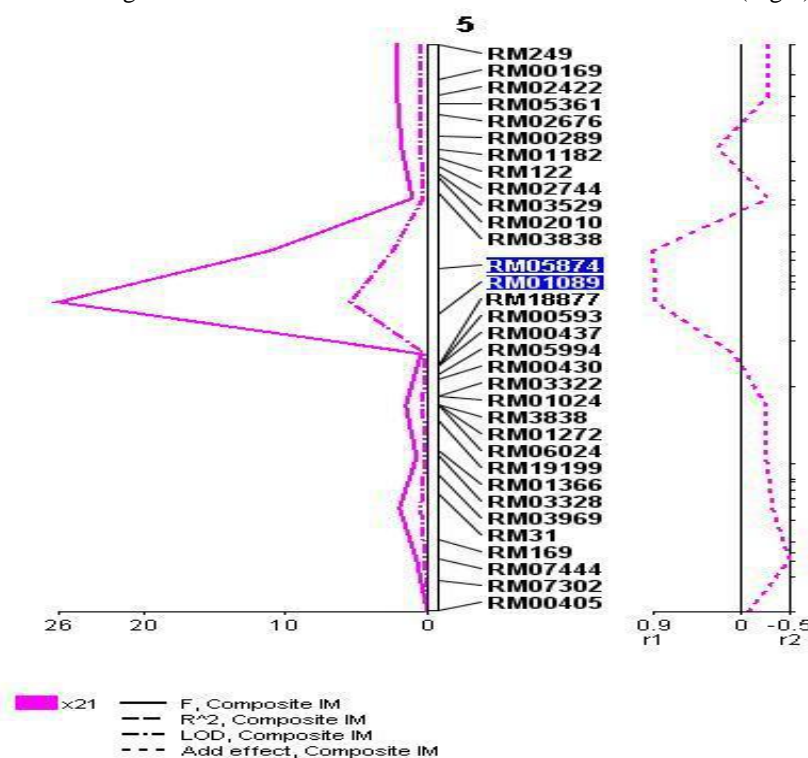


Fig. 3: LOD peaks for QTLs related to salinity tolerance at vegetative stage on chromosomes 5 in the BC₂F₂ population of OM7347 / OM5629 under EC = 8dS/m.

3. At reproductive stage

At reproductive stage, two hundred fifty-three individuals of BC₂F₂ population from OM7347/OM5629 were screened in Yoshida solution plus NaCl under salt stress of EC = 8dS/m. Yield components and grain yield were evaluated. The result showed that *Saltol* QTLs were mainly located on chromosomes 1 and 12. Three QTLs

located at the intervals of RM1324-RM2412, RM1185-RM24 and RM1282-RM2560 on chromosome 1 were corresponded to grain yield. One QTL at the region of RM453-RM511 on chromosome 12 corresponded to survival day (SD) and salinity tolerance score under EC = 8dS/m at reproductive stage (Fig 4, Table 3).

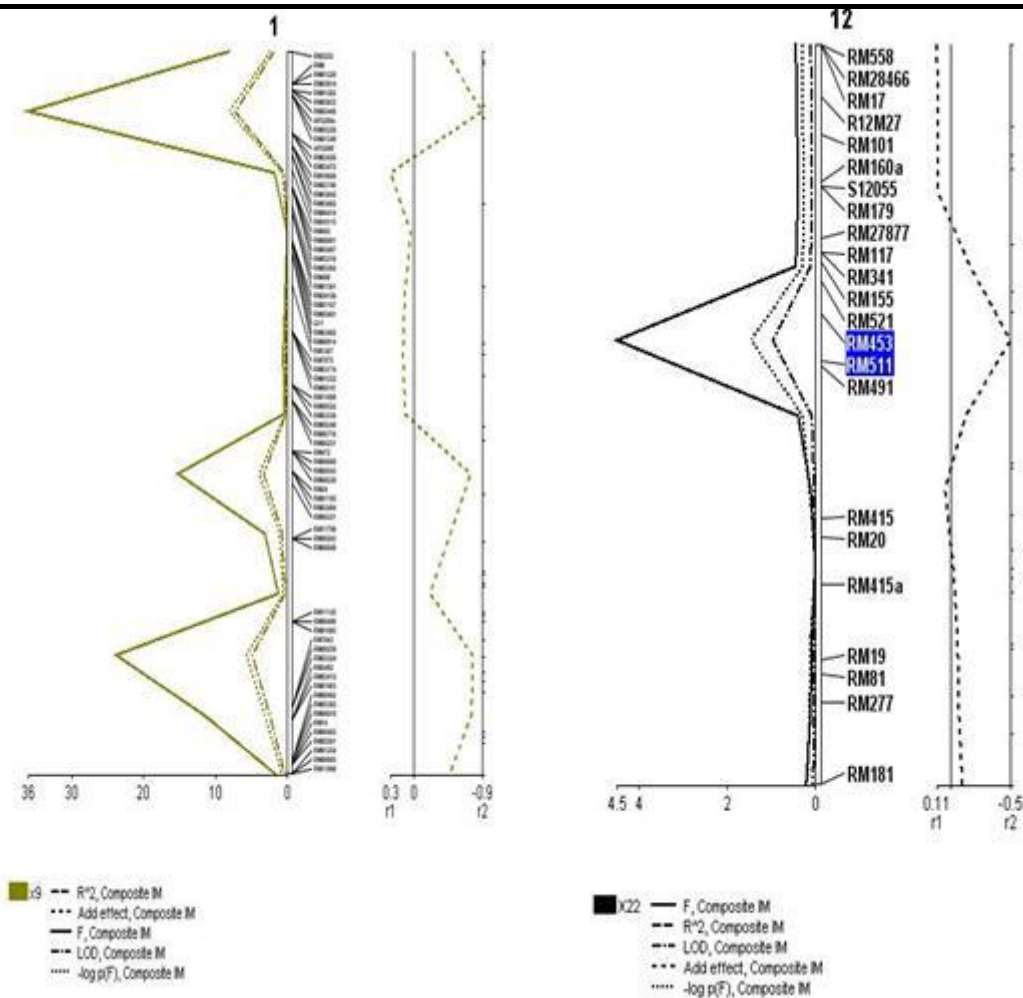


Fig. 4: LOD peaks for QTLs related to salinity tolerance at reproductive stage on chromosome 1 and chromosome 12 in the BC_2F_2 population of OM7347 / OM5629 under $EC = 8dS/m$.

Table.3: QTLs related to salt tolerance in the BC_2F_2 population of OM7347/OM5629 at reproductive stage.

Chr	QTL	Location (cM)	CIM (Interval cM)	LOD	A	D	R ²
1	SD QTL-1	6.8	RM1324- RM3412	7.1	1.61	7.19	12.15
1	SD QTL-1	11.6	RM1185-RM24	7.0	1.92	3.90	15.14
1	SD QTL-1	6.5	RM1282-RM2560	7.0	7.50	4.10	14.23
12	SD QTL-12	4.6	RM453-RM511	4.5	25.17	32.56	25.17

$P < 0.05$; A: Additive, D: Dominant, R²: phenotypic variance explained, Chr: chromosome, SD.

4. Marker-assisted selection in BC_3F_6 population of OMCS2000/Pokkali

Markers tightly linked to *Saltol* genes were RM3252-S1-1 and RM3867 on chromosome 1 and 3, respectively. They were used in marker-assisted selection in BC_3F_6 population.

One line namely BC_3F_6 -7 was exhibited at same band with Pokkali via RM3252-S1-1. Two lines as BC_3F_6 -17 and BC_3F_6 -22 expressed their heteromorphic bands. This

meant that RM3252-S1-1 was significantly used to select the elite rice lines, which tolerate salinity on chromosome 1 (Fig. 5).

In term of marker RM3867 on chromosome 3, PRC products of 24 selected lines were highly polymorphic. Two elite lines as BC_3F_6 -7 and BC_3F_6 -13 were exhibited at the same band of Pokkali on 3% agarose gel. No heteromorphism was recorded in case of RM3867 (Fig 5) accordingly.

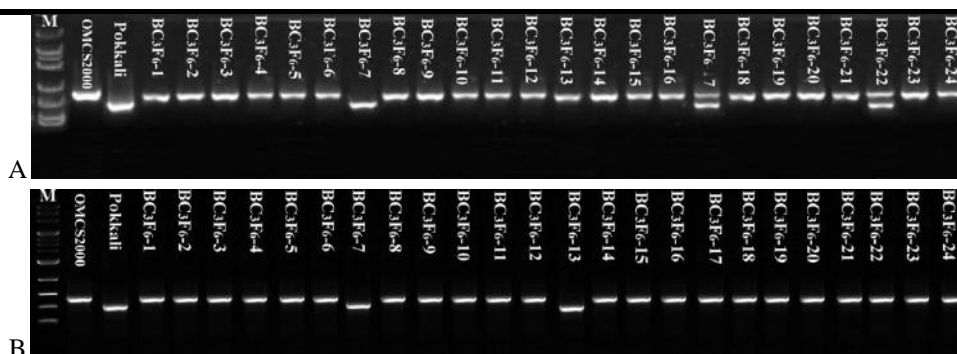


Fig. 5: PCR products of 24 lines of BC₃F₆ from OMCS2000/Pokkali on 3% agarose gel. (A): PCR products at locus RM3252-S1-1 on chr.1; (B): PCR products at locus RM3867 on chr. 3. M: Lambda leader (174/Hae III).

IV. CONCLUSION

In the backcross populations, parents were genotypically assessed on 12 chromosomes using SSR markers to identify the regions of rice genome where the contrary alleles (tolerant-susceptible alleles) showed “homogeneity” character of the polymorphic SSR markers. This method was applied at seedling stage. Success in QTLs mapping using polymorphic SSR markers in the BC population of OM7347/ OM5629 permits to identify new QTLs (located on chromosomes 1 and 3) beside previous QTLs reported. The candidate regions on chromosomes 1 and 3 from this study were then tested using the BC₃F₆ population of OMCS2000/Pokkali. The polymorphism in PCR products proved that two useful markers *viz.* RM3252-S1-1 and RM3867 were efficient.

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