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Marker Assisted Introgression of *Saltol* QTL into "APMS 6B" to Enhance Salt Tolerance at Seedling Stage of Rice (*Oryza sativa L.)*

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Authors' contributions

This work was carried out in collaboration among all authors. 'All authors read and approved the final manuscript.

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ABSTRACT

APMS 6B which is highly sensitive to salinity was used as a recurrent parent to introgress *Saltol* QTL from FL-478. Two gene linked markers *viz.,* RM8094 and RM10793 were used for used to confirm backcross generations for *saltol QTL.* Two successive backcrosses were attempted to transfer target alleles of *Saltol* from FL478 into APMS-6B. After backcross progrmme, the BC_2F_1 plants subjected to foreground selection by using gene specific markers, positive plants for target gene were selected and selfed to raise BC_2F_2 populations. Twenty BC_2F_2 plants were identified to be homozygous dominant for *Saltol* QTL and these BC_2F_2 lines were also screened in plastic trays using Yoshida solution at the seedling stage. A total of BC_2F_2 lines were confirmed for the target gene and displayed a high level of salinity tolerance without any symptoms on their leaves, with a score of '0'.

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1. INTRODUCTION

Rice is that the prime food crop, it forms the staple food of over three billion people which accounts for more than 21% of the calorific need of the world's population and upto 76% of the calorific intake of the population of southeast Asia [1] and [2]. Rice yields about one-third of the entire carbohydrate source and it provides a considerable amount of Zinc and Niacin [3]. Rice protein is biologically abundant as its digestibility is incredibly high (88%). It is the second most significant crop which covers almost 90% of the area across Asia alone after wheat. World rice production is around 471.83 million tons grown over 161 million hectares with a productivity of 4.41 tonnes per hectare. India has an area of 43.66 million hectares under rice cultivation along with a production of 105.31 million tonnes, with productivity of 2393 kg/ha. India ranks first in area and second in production following China, the largest producer of rice. However with the expanding population, the increase in production of the crop is the critical need in order to keep national food and livelihood security system.

Among the abiotic stress factors, salinity is the second type of stress and is the most predominant hindrance to rice production after drought. In India, nearly 8.50 M ha land is salt affected and the yield reduction is estimated to tune of 30 -50 % cent. In the state of Telangana alone, more than 50% of the area is covered by rice where more than 20% of agricultural land is salinity affected. Rice is susceptible to salinity, specifically, at the early vegetative and later reproductive stages [4]. Rice genotypes show wide variations in salinity tolerance due to additive gene [5]. Rice plants are highly sensitive to salinity at seedling [6], panicle initiation and pollination stages [7] [8], resulting in poor crop establishment. Salinity affects yield components such as panicle length, spikelet number per panicle, grain yield and also delays panicle emergence and flowering [8].

The huge amount of genetic variability present in rice in response to salinity makes it amenable to genetic manipulation to increase its salinity tolerance [9] [10]. There are some landraces and traditional cultivars like Nona Bokra and Pokkali which are naturally tolerant to salt stress due to their adaptation to thrive on salt affected land for generations. However, negative agronomic characteristics such as tall plant stature, poor grain quality, low yield, and photosensitivity [11] [12] and polygenic nature have presented challenges for traditional breeding to make significant advancement and has led to increased interest in molecular breeding methods.

Mapping of quantitative trait loci (QTLs) can open up the possibility of future efforts to develop salinity tolerance varieties by precisely transferring QTLs into popular. Recently, in pokkali remarkable effort has been made for the identification of candidate genes localized within the Saltol QTL by genome wide transcriptome analysis [13]. Several salt tolerant rice lines has been developed by precisely transferring QTLs into popular varieties and pyramiding multiple relevant QTLs for a particular stress-prone environment.

The objectives of the current study were to introgress *Saltol* Qtl from FL 478 to APMS-6B and resulting improvement in salinity tolerance at seedling stage of the APMS-6B BC_2F_2 lines.

2. MATERIALS AND METHODS

The plant materials employed in the experiments were selected from the crop improvement section, Indian Institute of Rice Research, Hyderabad. The parental lines which were employed in crossing programme selected based upon the results of screening for salt tolerance at seedling stage in plastic trays using IRRI standard protocol. Salinized and non-salinized setups with two replications were maintained. The modified standard evaluation system (SES) was employed in rating the visual symptoms of salt toxicity. This SES scoring discriminated the susceptible from the tolerant and also the moderately tolerant genotypes. Final scoring was done at 22 days after salinization.

The variety APMS 6B was selected as recipient parent and it was developed by ANGRAU, Hyderabad. APMS 6B is an improved, high yielding, fine grain variety but susceptible to salinity. The variety FL-478 rice genotype was selected as a donor parent (Pokkali x IR-29) to introgress *Saltol* QTL into the genetic background of APMS 6B. It was developed by TNAU, Tamilnadu.

Sequences of PCR based SSR markers were selected from the Gramene database (www.gramene.org) and selected gene specific

markers were employed in the Marker Assisted Selection programme. Totally 200 markers were used for the polymorphism study between the parents. The markers with peak and clearly differentiating between the parents were employed for the confirmation and foreground selection of the target genes. Out of 200 markers, thirty six markers showed polymorphism between the parents. Among the thirty six markers, two peak markers were selected for foreground selection which were given in Table 2.

2.1 Introgression of *Saltol QTL* into the Genetic Background of APMS-6B

The recipient parent APMS-6B was crossed with FL-478 and the F_1s derived from this cross were confirmed for their hybridity using the SSR markers RM 10793 and RM 8094, which were polymorphic between donor and recipient combinations. The confirmed F_1s (positive for *Saltol* QTL) were crossed with recurrent parent, FL-478 to generate BC_1F_1 plants. These were then subjected to foreground selection with the gene linked markers RM 10793 and RM 8094 and the plants with target genes in heterozygous condition were identified. The identified positive plants of BC_1F_1 were again backcrossed with recurrent parent to generate BC_2F_1s and foreground selection was employed. Positive BC_2F_1 plants for target gene were selected and selfed to raise BC_2F_2 population. Phenotyping of BC_2F_2 was done under salinity condition as per IRRI Standards and screening also done with the gene specific markers for confirming the *Saltol* QTL. . The various steps involved in Introgression of *Saltol* QTL are depicted in Fig. 3.

2.2 Screening of the Marker Assisted Breeding Derived Lines for Salinity Tolerance at IIRR, Hyderabad

Identified, homozygous dominant backcross derived BC_2F_2 lines with *Saltol* QTL in APMS-6B background were evaluated for salinity tolerance at Hybrid Rice, IIRR, Hyderabad during *Kharif, 2016.* Sowing of seeds of introgressed lines along with susceptible parent, APMS-6B and donor parent, FL-478 (positive check) was carried out in the plastic nursery trays. The test material was surrounded by both parents in plastic nursery trays. The salinity solution at the concentration of 90 mM was imposed at the fourth-leaf stage. The salinity condition was maintained up to 30 days after sowing. Imposed seedlings were monitored for the development of salinity symptoms. The stress reaction on each line was recorded after 15 days of imposition, following standard 0-9 scale.

3. RESULTS AND DISCUSSION

Advancement in rice breeding for salt tolerance has accelerated since the identification of major loci conferring salt tolerance especially at the seedling stage. Microsatellite markers have been used to map QTLs associated with salt tolerance [14] [15] [16] [17]. A major QTL for salt tolerance at the seedling stage, *Saltol*, was identified using F8 recombinant inbred lines (RILs) of Pokkali/IR29 [18]. The basis of a marker-assisted backcrossing (MAB) strategy is to transfer a specic allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genome. The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increases the speed of the selection process, thus increasing genetic gain per unit time. In this context, in the present study, APMS 6B popular maintainer line of DRRH-3 hybrid has been introgressed with *Saltol Qtl* of FL-478through marker-assisted backcross breeding. The donor and recipient parents, both were selected from salinity screening at seedling stage (Fig. 1). The results of the study are described and discussed below.

Table 1. Modified standard evaluation score (SES) of visual salt injury at seedling stage (Adapted from Gregorio *et al.*, [18])

Score	Observation	Tolerance
1	Normal growth on 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

3.1 Parental Polymorphism Study

Two hundred gene specific SSR markers were used for surveying the parental polymorphism between APMS-6B and FL-478 at molecular level. Out 200 markers used,36 markers for *Saltol Qtl* were showed polymorphism between two parents. between the recipient and donor parents. Among them, two markers *viz.,*RM8094 and RM10793 which were clearly differentiated at the selected genomic regions (Fig. 2). These reported gene specific markers, showing polymorphism between the parents for the target genes in APMS-6B genetic background, were employed for the confirmation and selection during introgression programme.

Table 2. Details of primers and Primer sequence

Primer Name		Primer sequence	Product size	AT^{0}C
RM8094	Forward	AAGTTTGTACACATCGTATACA	209	55
	Reverse	CGCGACCAGTACTACTACTA		
RM10793	Forward	GACTTGCCAACTCCTTCAATTCG	123	55
	Reverse	TCGTCGAGTAGCTTCCCTCTCTACC		

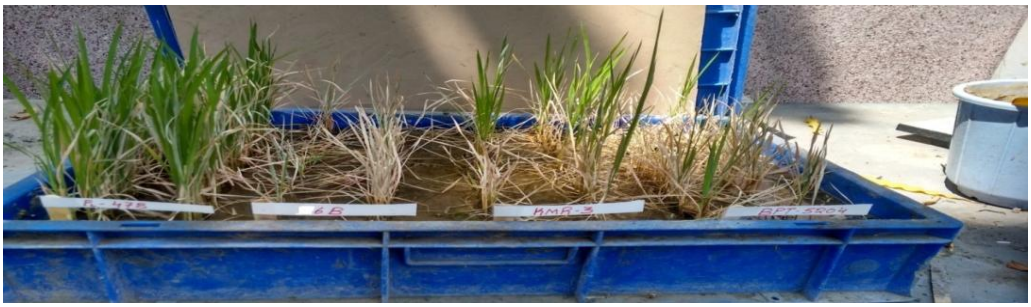

Fig. 1. Screening of parental lines for salinity tolerance at seedling stage

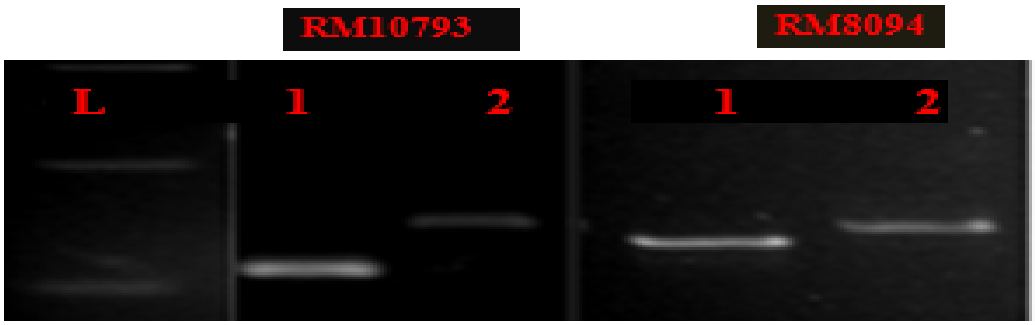

Fig. 2A. Garose gel showing polymorphism between the parents by SSR markers 1-FL-478 and 2-APMS-6B

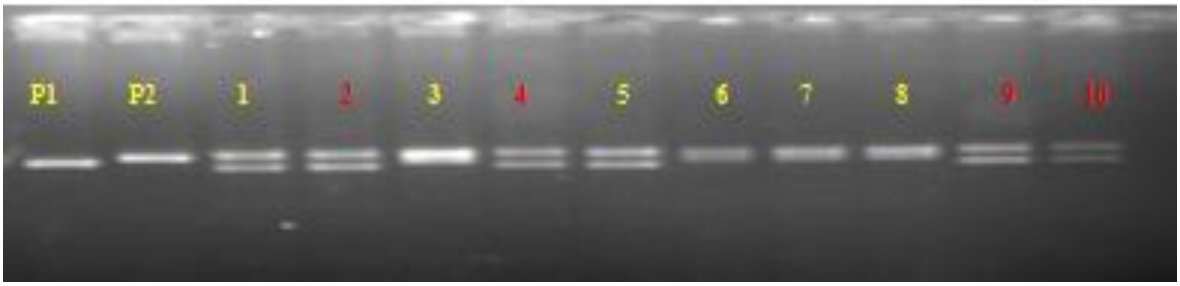

Fig. 3. Hybridity confirmation study for the APMS-6B x FL-478by using RM8094 (P_1:FL-478; P_2:APMS-6B; 1-10: F_1s)

The polymorphic SSR markers selected (RM8094 and RM10793 for *Saltol Qtl*) are differentiating the parents with the expected resistant amplicon product sizes of 209 and 123 bp respectively (www.gramene.org). So the choice of the markers for the selection/screening of the generated material is appropriate.

3.2 Introgression of *saltol* Gene in Rice Variety APMS-6B

The genotypes, FL-478 (donor for salinity tolerance *Saltol Qtl*) were used to generate salt tolerant lines. The recipient parent APMS-6B was crossed with FL-478 and the F_1s derived from this cross were confirmed for their hybridity using the SSR markers RM 10793 and RM8094, which are polymorphic between donor and recipient combinations. For the introgression of *saltol* gene in rice variety APMS-6B panicles of APMS-6B were hand emasculated and pollinated with the pollens of FL-478. A total of 25 crosses were made which produced 20 F1 seeds. Out of these, only 10 seeds were germinated when sown in pots. DNA from individual plant was isolated and foreground selection with SSR markers RM-10793 and RM-8094 were done (Figs. 3 & 4). It identified four true hybrids with both markers and identified true hybrids were similar with APMS-6B type.

3.3 Generation of BC_1F_1 Plants and their Foreground Selection using the Gene Linked Markers

The identified positive F_1s possessing *Saltol Qtl* were back crossed with recurrent parent, APMS-6B and BC_1F_1lines derivedfrom these crosses were checked for the presence of *Saltol Qtl* using the linked molecular markers RM8094 and RM10793 respectively (Figs 5 & 6). Among the 50 BC_1F_1 plants derived from true F_1s and recurrent parent APMS-6B cross, screened with gene linked markers, 24 plants were identified positive for *Saltol Qtl* using RM10793 and 27 plants were identified for *Saltol Qtl* using RM10793 and 15 plants were identified positive for both markers RM10793 and RM8094.

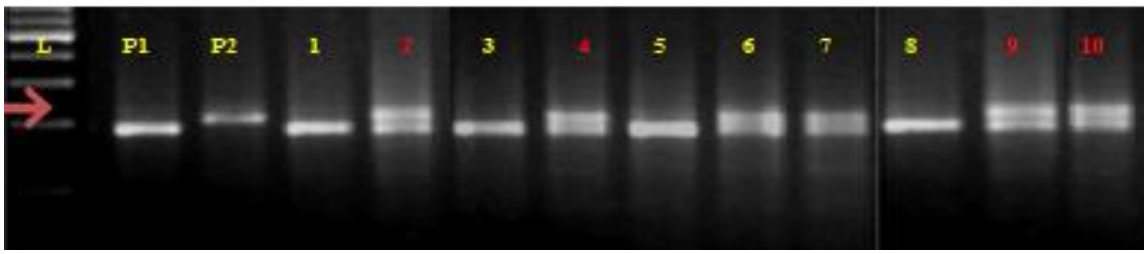

Fig. 4. Hybridity confirmation study for the APMS-6B x FL-478by using RM10793 (P_1:FL-478; P_2:APMS-6B; 1-10: F_1s)

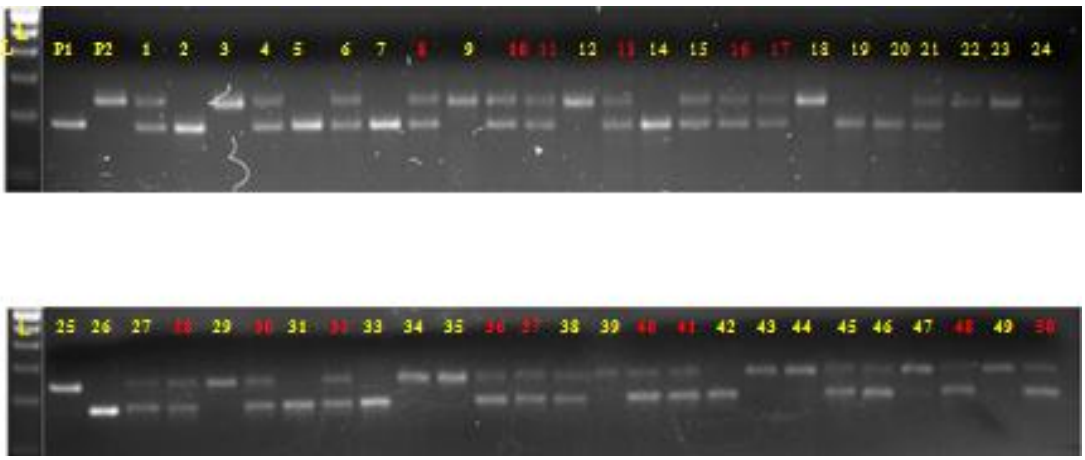

Fig. 5. Screening of the BC_1F_1plants for *SaltolQtl*by using RM10793 marker (P_1: FL-478; P_2: APMS-6B 1-50: BC_1F_1s)

3.4 Development of BC_2F_1 Lines from the Selected BC_1F_1 Plants and Theirmarker-Assisted Selection

Strict phenotypic selection was carried out among the 15 positive BC_1F_1 plants, one plants possessing similar plant type characters to recurrent parent were selected and once again backcrossed with recurrent parental line. A total 60 BC_2F_1 seeds were produced from the cross between BC_1F_1 and recurrent parent APMS-6B. Out of 60 BC_2F_1 seeds, only 42 seeds were germinated when sown in pots. Foreground selection was practiced in BC_2F_1 to identify the plants possessing the resistance genes *SaltolQtl* using the linked molecular markers RM10793 and RM8094 respectively (Figs 7 and 8). A total eleven BC_2F_1 plants (*viz.,* P-3, P-11, P-14, P-15, P-18, P-22, P-27, P-31, P-33, P-39 and P-40) derived from BC_1F_1 and recurrent parent APMS-6B, were identified to be positive for *SaltolQtl.* The selected BC_2F_1 plant had a very high similarity to the recurrent parents for most of the plant type characters. Even though there is no apparent reason to explain this higher percentage of recurrent parent genome recovery, it can be assumed that this could be because of some unknown mechanisms which might have resulted in transfer of some chromosomal segments from recurrent parent genome to be transmitted as such transmitted to the progenies in the second cycle of backcrossing.

Fig. 6. Screening of the BC_1F_1 plants for *SaltolQtl* by using RM8094 marker (P_1: FL-478; P_2: APMS-6B 1-50: BC_1F_1s)

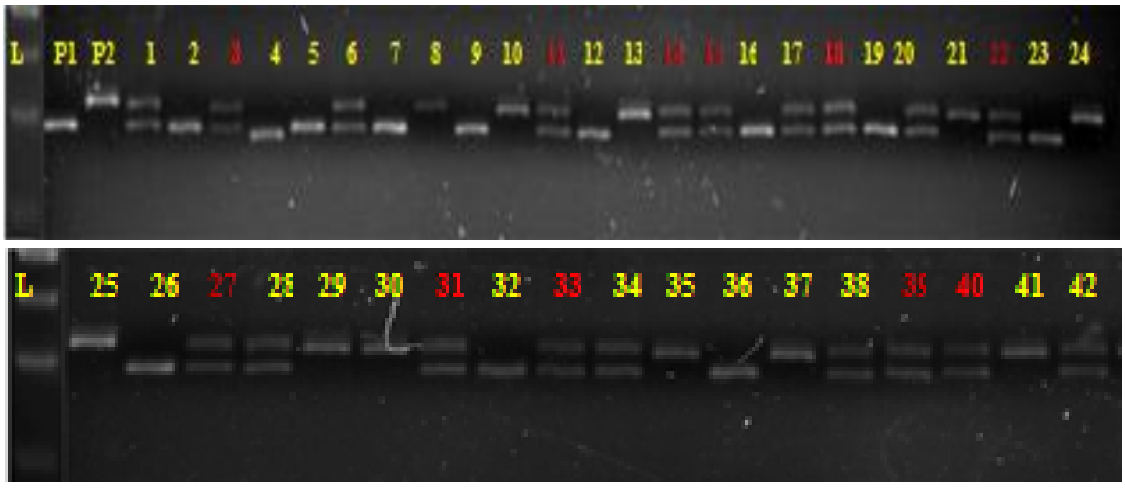

Fig. 7. Screening of the BC_2F_1 plants for *SaltolQtl* by using RM10793 marker (P_1: FL-478; P_2: APMS-6B 1-50: BC_2F_1s)

3.5 Identification of BC_2F_2 Plants Homozygous for *Saltol Qtl* using the Gene Linked Markers

Among the 11 BC_2F_1s, a single best positive plant number P-15 which was resembling (showing maximum similarity) to recurrent parent for most of the plant type characters was selected and selfed to generate BC_2F_2 population. A total of 200 seeds were produced from P-15 selfing. Genotyping of 200 BC_2F_2s with gene linked markers was done to identify those BC_2F_2 plants possessing *Saltol Qtl* in a homozygous condition. Out of these 200 plants, 20 plants (i.e. P-15-6, P-15-12, P-15-16, P-15-17, P-15-25, P-15-31, P-15-41, P-15-51, P-15-63, P-15-77, P-15-81, P-15-101, P-15-105, P-15-109, P-15-119, P-15-154, P-15-167, P-15-177, P-15-180 and P-15-195) were identified to be homozygous dominant for *Saltol Qtl* (Fig. 9 and Fig. 10). Identification of homozygous BC_2F_2 lines is very important because if the selected BC_2F_2lines contains one or more of the target genes in heterozygous condition, they will segregate in next generation. In the present study, only homozygous lines with the desirable target gene were selected for further advancement and evaluation, thus ensuring homozygosity of the material with respect to salinity tolerance. The use of gene specific markers *viz.*, RM10793 and RM8094 will be of prime importance for the selection and tagging of *Saltol Qtl* genes associated with salinity tolerance.

3.6 Screening of BC_2F_2 Lines for Salinity Tolerance at Seedling Stage

BC_2F_2 lines obtained from the cross between BC_2F_1 andrecurrent parent APMS-6B were screened for salinity tolerance as described in materials andmethods. The donor parent FL-478 showed high level of tolerance for salinity with '0' score and recurrent parent, APMS-6B showed presence of salinity symptoms in more than 75% leaf area with scoring scale '9'. A total of 13 improved lines (i.e. homozygous BC_2F_2 plants) developed through marker assisted backcross breeding displayed a high level of salinity tolerance without any symptoms on their leaves with a score of '0' and four breeding lines has nearly normal growth butleaf tips or few leaves whitish and rolled score of '3' (Fig. 1).

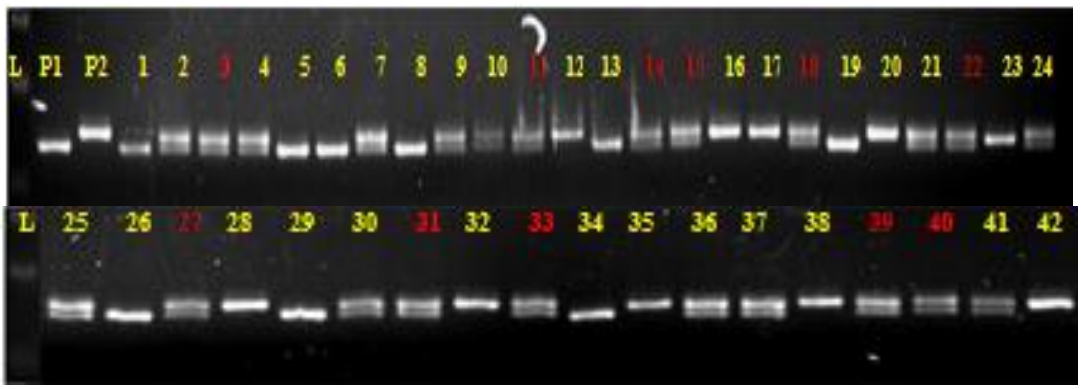

Fig. 8. Screening of the BC_2F_1plants for *SaltolQtl*by using RM8094 marker (P_1: FL-478; P_2: APMS-6B 1-50: BC_2F_1s)

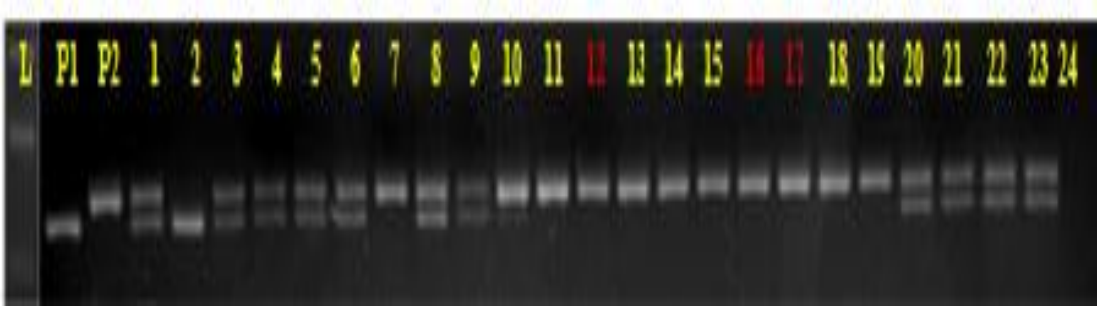

Fig. 9. Screening of the BC_2F_2plants for *Saltol Qtl* by using RM8094 marker (P_1: FL-478; P_2: APMS-6B 1-24: BC_2F_2s)

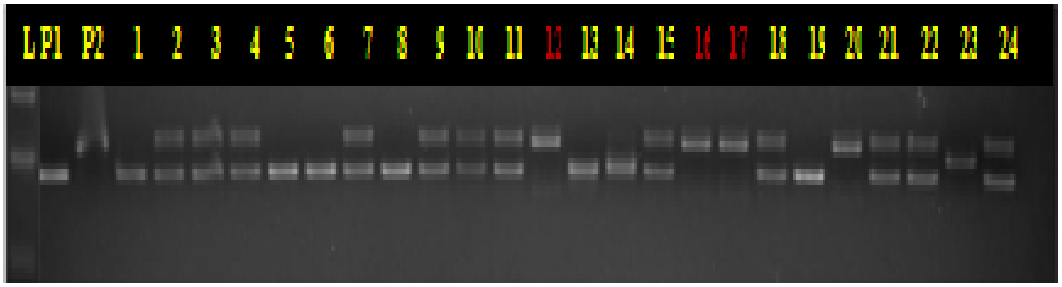

Fig. 10. Screening of the BC_2F_2 plants for *Saltol Qtl* by using RM10793 marker (P_1: FL-478; P_2: APMS-6B 1-24: BC_2F_2s)

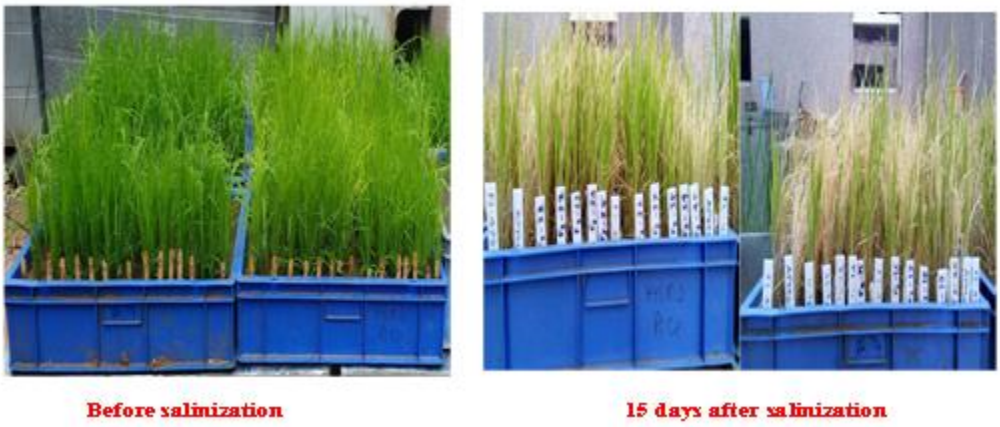

Fig. 11. Screening of BC_2F_2 lines for salinity tolerance at seedling stage

The results obtained from the introgression of *Saltol Qtl* into the genetic background of APMS-6B is in conformity with the outcomes of Hoque *et al.,* [19] where '*Saltol'* QTL was introgressed into the genetic background of BRRI dhan49 through marker-assisted backcrossing from FL478 [20].

4. CONCLUSION

In the present experiment, introgression of the Saltol QTL enhance of seedling stage salinity tolerance in APMS-6B and these improved lines showed marked enhancement of salt tolerance at seedling stage.. A total of 13 improved BC_2F_2 lines displayed a high level of salinity tolerance may be evaluated for their suitability in breeding programmes for improving their reproductive stage salt tolerance. Additionally, a comprehensive evaluation of the improved BC_2F_2 under salt-affected soil will reveal, other than agronomic performance, physiological improvements such as photosynthetic efficiency gained by incorporation of salt tolerance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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