

Marker Assisted Introgression of *Saltol* QTL into “APMS 6B” To Enhance Salt Tolerance at Seedling Stage of rice (*Oryza sativa* L.)

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 programme, the BC₂F₁ plants subjected to foreground selection by using gene specific markers, positive plants for target gene were selected and selfed to raise BC₂F₂ populations. Twenty BC₂F₂ plants were identified to be homozygous dominant for *Saltol* QTL and these BC₂F₂ lines were also screened in plastic trays using Yoshida solution at the seedling stage. A total of BC₂F₂ 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’.

Keywords: Salinity, *Saltol*, QTL, introgression, MABB

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 (Ma *et al.*, 2007 and Melissa *et al.*, 2009). Rice yields about one-third of the entire carbohydrate source and it provides a considerable amount of Zinc and Niacin (Gopalan *et al.* 2007). 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 (Shannon, 1998). Rice genotypes show wide variations in salinity tolerance due to additive gene (Sahiet *al.*, 2006). Rice plants are highly sensitive to salinity at seedling (Khan *et al.*, 1997), panicle initiation and pollination stages (Khatun and Flowers, 1995; Zeng *et al.*, 2001), 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 [Zeng *et al.*, 2001].

The huge amount of genetic variability present in rice in response to salinity makes it amenable to genetic manipulation to increase its salinity tolerance (Akbar *et al.* 1972; Flowers and Yeo 1981). 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 [Ismail *et al.* 2007 and Thomson *et al.* 2010] 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 [Soda *et al.*, 2013]. 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 BC₂F₂ lines.

MATERIAL 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.

Table 1: Modified standard evaluation score (SES) of visual salt injury at seedling stage (Adapted from Gregorio *et al.*, 1997)

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

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.

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		

Introgression of *Saltol* QTL into the genetic background of APMS-6B

The recipient parent APMS-6B was crossed with FL-478 and the F₁s 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 F₁s (positive for *Saltol*QTL) were crossed with recurrent parent, FL-478 to generate BC₁F₁ 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 BC₁F₁ were again backcrossed with recurrent parent to generate BC₂F₁s and foreground selection was employed. Positive BC₂F₁ plants for target gene were selected and selfed to raise BC₂F₂ population. Phenotyping of BC₂F₂ 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 Figure 3.

Screening of the marker assisted breeding derived lines for salinity tolerance at IIRR, Hyderabad

Identified, homozygous dominant backcross derived BC₂F₂ 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.

RESULT 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 (Lang *et al*, 2000, 2001; Singh *et al*, 2007; Mohammadi-Nejad *et al*, 2008; Hossain *et al*, 2015). A major QTL for salt tolerance at the seedling stage, *Saltol*, was identified using F8 recombinant inbred lines (RILs) of Pokkali/IR29 (Gregorio *et al*, 1997). The basis of a marker-assisted backcrossing (MAB) strategy is to transfer a specific 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-478 through marker-assisted backcross breeding. The donor and recipient parents, both were selected from salinity screening at seedling stage (Figure 1). The results of the study are described and discussed below.



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

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 *SaltolQtl* 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 (Figure 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.

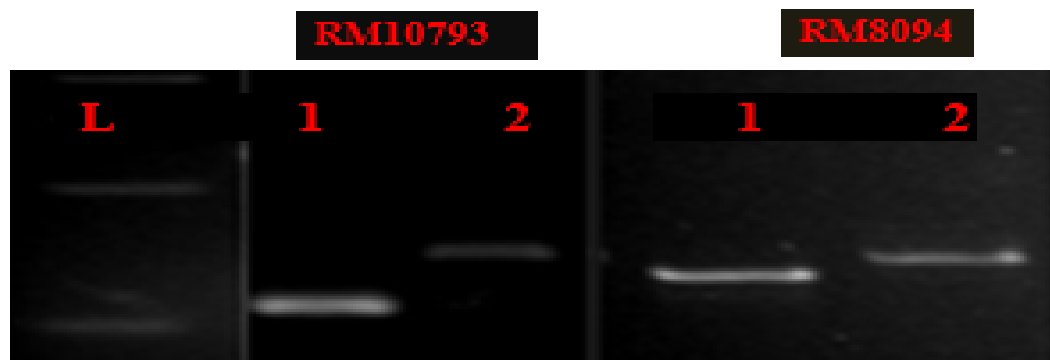


Fig 2 Agarose gel showing polymorphism between the parents by SSR markers 1-FL-478 and 2-APMS-6B

The polymorphic SSR markers selected (RM8094 and RM10793 for *SaltolQtl*) 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.

Introgression of *saltol* gene in rice variety APMS-6B

The genotypes, FL-478 (donor for salinity tolerance *SaltolQtl*) were used to generate salt tolerant lines. The recipient parent APMS-6B was crossed with FL-478 and the F₁s 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 (Figure 3 & 4). It identified four true hybrids with both markers and identified true hybrids were similar with APMS-6B type.

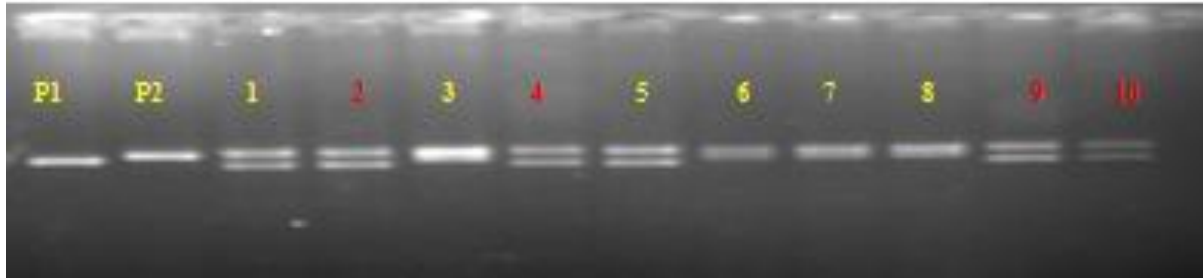


Fig 3 Hybridity confirmation study for the APMS-6B x FL-478 by using RM8094 (P₁:FL-478; P₂:APMS-6B; 1-10: F₁s)

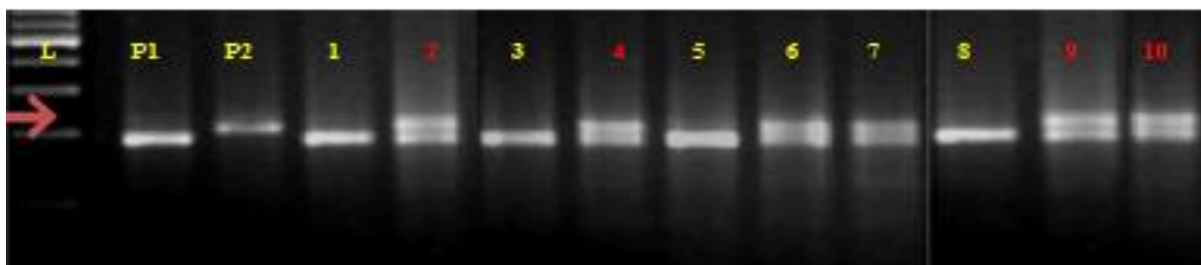


Fig 4 Hybridity confirmation study for the APMS-6B x FL-478 by using RM10793 (P₁:FL-478; P₂:APMS-6B; 1-10: F₁s)

Generation of BC₁F₁ plants and their foreground selection using the gene linked markers

The identified positive F₁s possessing *SaltolQtl* were back crossed with recurrent parent, APMS-6B and BC₁F₁ lines derived from these crosses were checked for the presence of *SaltolQtl* using the linked molecular markers RM8094 and RM10793 respectively (Figure 5&6). Among the 50 BC₁F₁ plants derived from true F₁s and recurrent parent APMS-6B cross, screened with gene linked markers, 24 plants were identified positive for *SaltolQtl* using RM10793 and 27 plants were identified for *SaltolQtl* using RM10793 and 15 plants were identified positive for both markers RM10793 and RM8094.

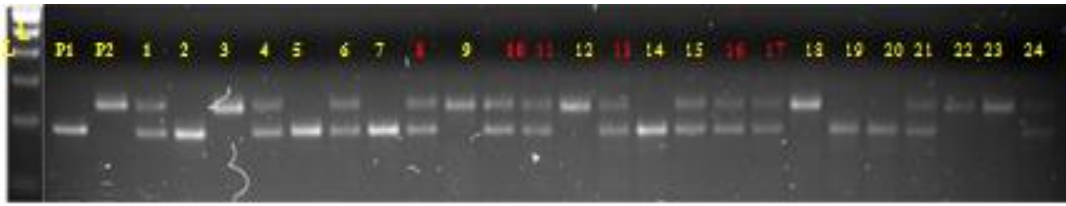


Fig 5 Screening of the BC₁F₁ plants for *SaltolQtl* by using RM10793 marker (P₁: FL-478; P₂: APMS-6B 1-50: BC₁F₁s)



Fig 6 Screening of the BC₁F₁ plants for *SaltolQtl* by using RM8094 marker (P₁: FL-478; P₂: APMS-6B 1-50: BC₁F₁s)

Development of BC₂F₁ lines from the selected BC₁F₁ plants and their marker-assisted selection

Strict phenotypic selection was carried out among the 15 positive BC₁F₁ plants, one plants possessing similar plant type characters to recurrent parent were selected and once again backcrossed with recurrent parental line. A total 60 BC₂F₁ seeds were produced from the cross between BC₁F₁ and recurrent parent APMS-6B. Out of 60 BC₂F₁ seeds, only 42 seeds were germinated when sown in pots. Foreground selection was practiced in BC₂F₁ to identify the plants possessing the resistance genes *SaltolQtl* using the linked molecular markers RM10793

and RM8094 respectively (Figure 7 and 8). A total eleven BC₂F₁ 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 BC₁F₁ and recurrent parent APMS-6B, were identified to be positive for *SaltolQtl*. The selected BC₂F₁ 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.

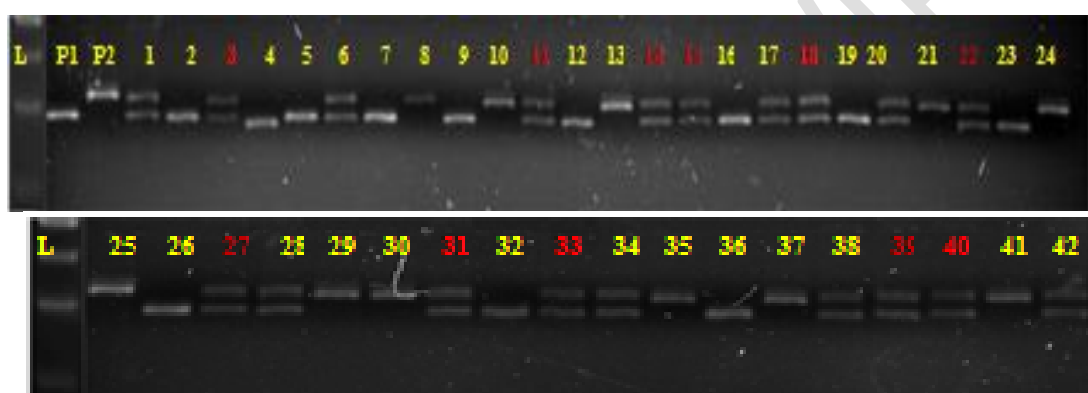


Fig 7 Screening of the BC₂F₁ plants for *SaltolQtl* by using RM10793 marker (P₁: FL-478; P₂: APMS-6B 1-50: BC₂F₁s)

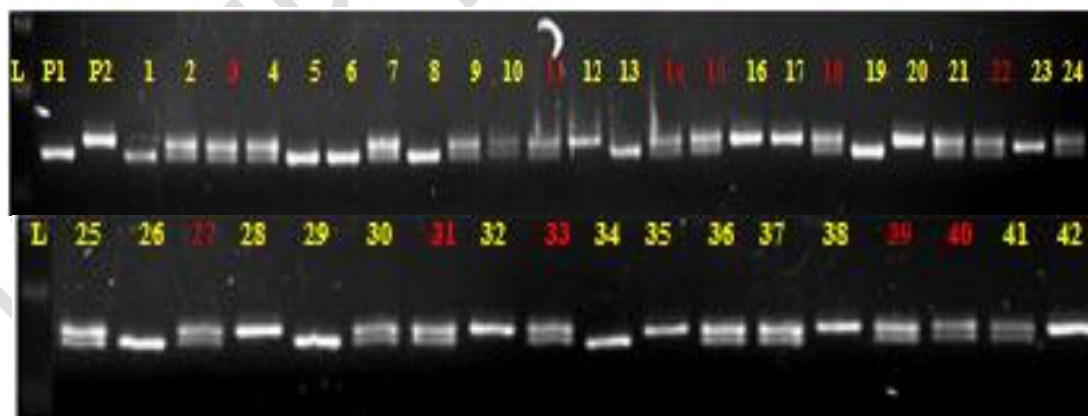


Fig 8. Screening of the BC₂F₁ plants for *SaltolQtl* by using RM8094 marker (P₁: FL-478; P₂: APMS-6B 1-50: BC₂F₁s)

Identification of BC₂F₂ plants homozygous for *SaltolQtl* using the gene linked markers

Among the 11 BC₂F₁s, 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 BC₂F₂ population. A total of 200 seeds were produced from P-15 selfing. Genotyping of 200 BC₂F₂s with gene linked markers was done to identify those BC₂F₂ plants possessing *SaltolQtl* 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 *SaltolQtl* (Figure 9 and Figure 10). Identification of homozygous BC₂F₂ lines is very important because if the selected BC₂F₂ lines 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 *SaltolQtl* genes associated with salinity tolerance.

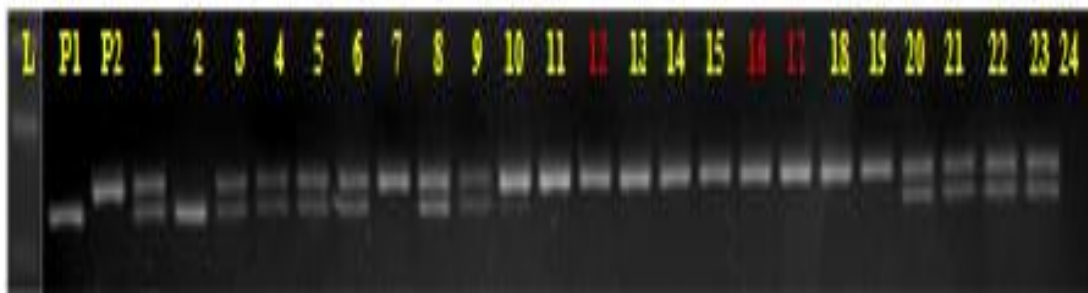


Fig. 9: Screening of the BC₂F₂ plants for *SaltolQtl* by using RM8094 marker (P₁: FL-478; P₂: APMS-6B 1-24: BC₂F₂s)

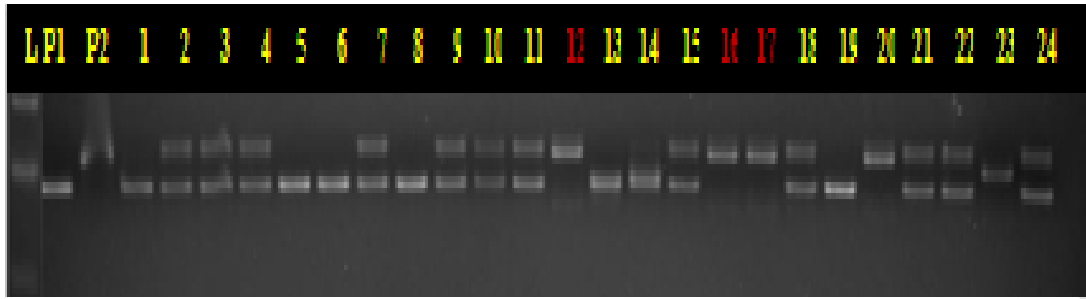


Fig. 10: Screening of the BC₂F₂ plants for *SaltolQilby* using RM10793 marker (P₁: FL-478; P₂: APMS-6B 1-24: BC₂F₂s)

Screening of BC₂F₂ lines for salinity tolerance at seedling stage

BC₂F₂ lines obtained from the cross between BC₂F₁ and recurrent parent APMS-6B were screened for salinity tolerance as described in materials and methods. 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 BC₂F₂ 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 but leaf tips or few leaves whitish and rolled score of ‘3’ (Figure 1).

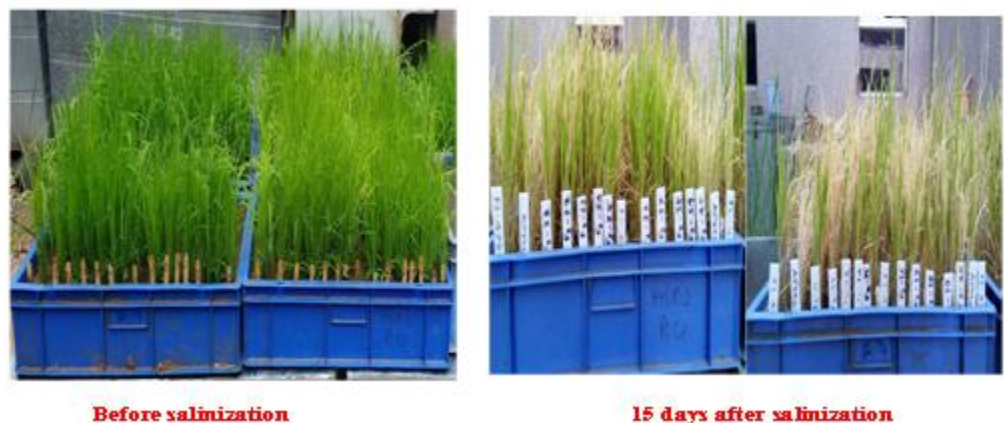


Fig 11 Screening of BC₂F₂ lines for salinity tolerance at seedling stage

The results obtained from the introgression of *SaltolQtl* into the genetic background of APMS-6B is in conformity with the outcomes of Hoqueet *al.*, (2015) where ‘*Saltol*’ QTL was introgressed into the genetic background of BRRI dhan49 through marker-assisted backcrossing from FL478.

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 BC₂F₂ 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 BC₂F₂ under salt-affected soil will reveal, other than agronomic performance, physiological improvements such as photosynthetic efficiency gained by incorporation of salt tolerance.

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