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Introgression of the *Saltol* into AS996, the elite variety of Vietnam, using Marker Assisted Backcrossing

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Abstract. This study focus on developing new salinity tolerance and high yielding rice lines, using markers assisted backrossing (MABC) as a technological tool for breeding. Total of 500 SSR markers on 12 rice chromosomes were screened for parental polymorphic markers. Of which, 52 primers in the *Saltol* region were examined with the two parents varieties to identify polymorphic primers for screening the *Saltol* region of the breeding populations. An analysis of 63 SSR markers on approx. 500 plants for each backcross generation of ASS996/FL478 for three steps selection. The two BC_1F_1 plants P284 and P307 which had the highest recipient alleles up to 89.06% and 86.36%, were chosen for the next backcrossing. Three BC_2F_1 plants with the recipient alleles up to 94,03 and 93,18% were used to develop BC_3F_1 generation. The best BC_3F_1 plant was P284-112-209 with all the recipient alleles and *Saltol* region. The four plants P307-305- 21, P284-112-195, P284-112-198, P284-112-213 were the second ranking with only one locus heterozygous (applied 63 markers covere on 12 chromosomes). These five plants were chosen as the breeding lines as the result of *Saltol*-AS996 introgression. The breeding line BC_4F_1 having 100% genetic background of donor variety is ready for develop new salinity tolerant variety ASS996-Saltol to cope with climate change.

Keywords: AS996, marker assisted backcrossing, rice, *Saltol*, QTL.

1. Introduction

Rice is the most important food source for half of the world's population and also the main staple food for most of the country's 86 millions people. Vietnam is the world's second-largest rice exporter, along with the top exporter Thailand, both counted for 50 percent of the world rice trade. Developing adaptation rice varieties to cope with climate change and sea level rise for the Red River Delta and Mekong River Delta is crucial to Vietnam economy and food security, it also contributes to the global food security.

Research at IRRI resulted in the development of high yielding rice varieties tolerant of abiotic stresses such as submergence and salt stress, and these varieties can help the unfavorable coastal areas less vulnerable to climate change impacts [1]. These improved varieties were developed using both conventional and modern breeding methods.

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Breakthroughs in salinity tolerance breeding became feasible after the identification of major chromosomal regions (Quantitative trait loci, QTLs) underlining salinity (*Saltol*) stresses, and the development and use of a marker system for their speedy incorporation into modern high yielding and popular varieties through marker assisted backcrossing (MABC) [1]. The foundation of MABC strategy is to transfer a gene/QTL from a donor line to a recipient line while selecting against donor introgressions across the retained genome [2, 3, 1]. MABC breeding strategy was applied for developing new salt tolerance Vietnam's varieties.

2. Materials and Methods

2.1. Plant materials and crossing scheme

- FL478 was used as the donor of *Saltol*. The recipient variety was AS996, which is widely grown cultivars in the South of Vietnam. For the MABC breeding strategy, AS996 was crossed with FL478 to obtain F1 seeds. F1 was backcrossed to AS996 to obtained a large number of BC_1F_1. Total 573 BC_1F_1 plants were screened for foreground, recombinant and background selections. The plants carrying target QTLs and the biggest recepient genom were selected for the next BC generation. Over five hundreds BC_2F_1 and then 371 BC_3F_1 plants were screened for foreground, recombinant and background selections. The BC_2F_2 or BC_3F_1 individuals carrying target genes and almost recepient genom were obtained.

2.2. Parents SSR polymorphism screening

Approx. 500 SSR markers distributed in the 12 chromosomes including foreground, recombinant and background markers were screened.

2.3. Genotype data analyses

Genotype data anlyses were obtained by analyzing DNA with SSR markers using 15 µL PCR reactions on 96-well plates. After initial denaturation for 4 min at 94^0C each cycle comprised 1 min denaturation at 94^0C, 1 min annealing at 55^0C, and 1 min extension at 72^0C with a final extension for 5 min at 72^0C at the end of 30 cycles (Eppendoft thermal cyclers). The PCR products were mixed with bromophenol blue gel loading dye and were analyzed by electrophoresis on 4.5% acrylamide gel at 1500V (Biorad system) followed by silver stainning steps and scoring; or electrophoresis on 6% -8% acrylamide gels at 100v (Dual Triple-Wide Mini-Vertical System, C.B.S.Scientific, CA, USA) followed by SYBR-Safe staining (Invitrogen), gel documentation (Alpha Innotech), and manual scoring of the gel pictures.

2.4. Data analyses

The molecular weights of the different alleles were scored using Alpha Ease Fc 5.0 software. The marker data was analyzed using the software Graphical Genotyper (GGT 2.0) [4]. The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as 'A', 'B' and 'H'. The percent markers homozygous for recipient parent (%A) and the percent recipient alleles including heterozygous plants (%R) were calculated.

2.5. Evaluation of salinity tolerance

Pre-germinated BC_2F_2 and BC_3F_1 seeds were sown in holes on styrofoam floats with a

net bottom suspended on trays filled with Yoshida nutrient solution [5]. Three replications were used for each experiment, with nine individual plants per line evaluated for each replication. Salt stress was imposed 14 days after germination by adding NaCl to an EC of 12 dS m−1 in Yoshida nutrient solution until final scoring. IR29 (sensitive) and FL478 (highly tolerant) were used as checks. The pH of the nutrient solution was adjusted daily to 5.0, and the culture solutions were replaced every 5 days. Entries were scored based on visual symptoms using IRRI's Standard Evaluating Score (SES) for rice, with ratings from 1 (highly tolerant) to 9 (highly sensitive) [6].

3. Results and discussion

3.1. Parental SSR polymorphism screening

A number of about 500 SSR markers on 12 rice chromosomes were screened for parental polymorphic markers for all foreground, recombinant and background analyses. Of the 500 SSR markers, 52 primers in the *Saltol* region were checked with the two parental varieties to find out more polymorphic primers to use for screening the *Saltol* region of the populations.

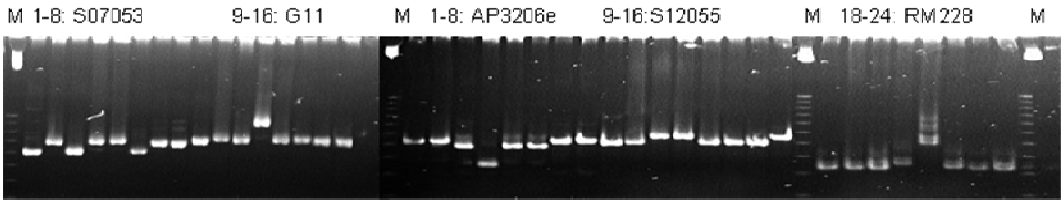

Figure 1. Parental screening on 6% polyacrylamide gel
DNA for each primer:1.Q5; 2.Q5DB; 3.OM5472; 4.FL478; 5.IR64SUB1; 6.AS996; 7.KDDB; 8,BT,
M: 25 bp ladder Primers: SO7053; G11; AP3206e; S12055; RM228

Total 63 polymorphic primers for the cross AS996/FL478 were identified. The result showed that frequence SSR markers for DNA polymorphisms between parental AS996/FL478 was very low. All those markers were used for screening the BC_1F_1, BC_2F_1 and BC_3F_1 generations.

3.2. Genotyping

3.2.1 . Genotyping BC_1F_1

We have already applying MABC on foreground selection, recombinant selection folowed by background selection. MABC is a precise and effective method to introgress a single locus controlling a trait of interest while retaining the essential characteristics of the recurrent parent [2]. MABC has three main advantages over conventional backcrossing. Firstly, DNA markers can be used for simple and efficient selection of the target locus ('foreground selection'). Secondly, the size of the donor chromosome segment containing the target locus can be minimized ('recombinant selection'). Thirdly, the recovery of the recurrent parent can be accelerated by selecting backcross lines with a higher proportion of recurrent parent genome ('background selection').

Saltol is a major QTL associated with the Na–K ratio and seedling-stage salinity

tolerance, was identified on chromosome 1. This QTL was tested in a hydroponic screen at the seedling stage revealed that this QTL explained 43% of the variation for seedling shoot Na–K ratio in the population [7]. One highly salt tolerant RIL from this population, FL478 (IR 66946-3R-178-1-1), has been promoted as an improved donor for breeding programs, as it has a high level of seedling stage salinity tolerance and is photoperiod insensitive, shorter and flowers earlier than the original Pokkali landrace.

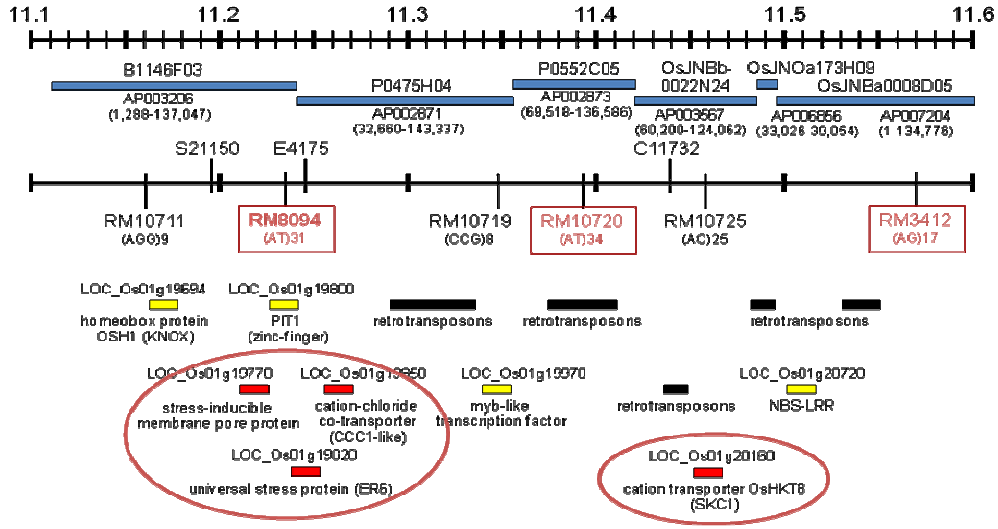

Figure 2. Physical map of Salton region 11.1-11.6Mb on chr.1.

Several global gene expression profiling studies have investigated transcriptional differences between the susceptible IR29 compared with FL478, revealing the up-regulation of genes in FL478 under salt stress for ion transport and cell wallrelated genes [8, 9], while differential expression was observed in roots for cation transport proteins [10] and kinases and phosphatases [11]. Furthermore, an analysis of single feature polymorphism in the *Saltol* region suggested that FL478 contained a DNA fragment smaller than 1 Mb from Pokkali at 10.6–11.5 Mb on chromosome 1, flanked by IR29 alleles [12]. In 2010, based on result from IRRI scientist, more STS markers were developed for used in MABC. The physical map of *Saltol* region was shown in figure 2.

Based on the map of *Saltol* QTL region, the best markers within the *Saltol* QTL region were AP3206 and RM3412, the most useful markers flanking the *Saltol* region were RM10694 (telomeric to *Saltol*) and RM493 and RM10793 (centromeric to *Saltol*), while nearby markers that can be used for negative selection are RM490 above *Saltol* and RM7075 below. Microsatellite markers unlinked to *Saltol* covering all the chromosomes, that were polymorphic between the two parents, were used for recombinant and background selection to recover the recipient genome. Among 500 SSR primers surveyed, 42 markers were analysed for selection initially on BC1F1 individuals. For foreground selection, AP3206, RM3412 and RM10793 were used for screening heterozygous plants. After that step, another flanking markers were used to identified the recombinant plants.

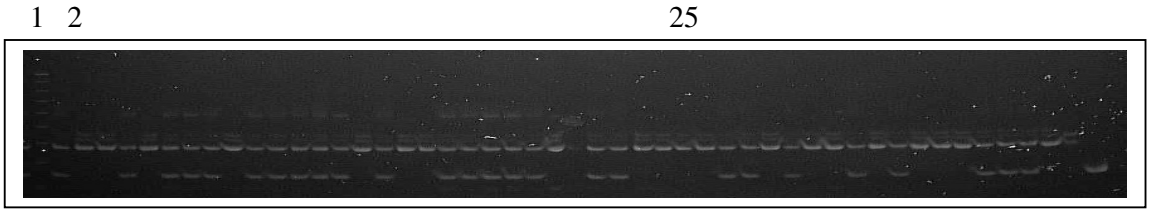

Figure 3. Screening individuals on crossed BC_1F_1(AS996/FL478) using primer AP3206.
Lane 1: 25bp marker, 2-25 and 26-47: BC_1F_1 in dividuals, Lane 48:AS996, Lane 49: FL478

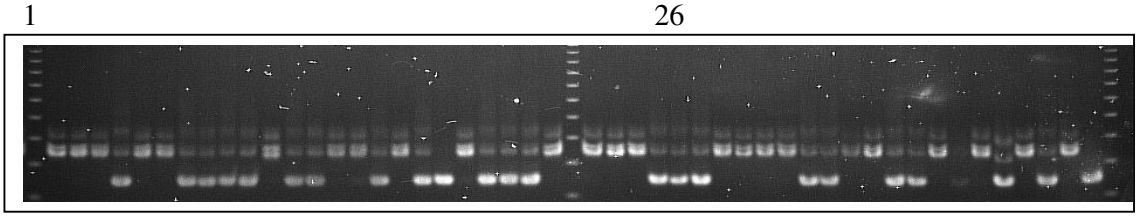

Figure 4. Screening individuals on crossed BC_1F_1(AS996/FL478) using primer RM310.
Lanes 26, 51: 25bp marker, 2-25 and 27-48: BC_1F_1 in dividuals, lane 49:AS996, lane 50: FL478

In table 1 was the recipient allele of the twelves BC_1F_1 recombinant individuals, the percent markers homozygous for recipient parent was "A%", the percent markers heterozygous was "H%' and the percent recipient alleles including heterozygous plants was "R%'.

Table 1. The recippient allele of the twelves BC_1F_1 recombinant plants

Plant number	65	149	228	238	281	**284**	305	**307**	311	401	**411**	426
A %	55.26	51.43	60.53	44.74	56.25	**78.13**	66.67	**75.76**	63.64	73.68	**66.67**	63.64
H %	34.38	37.93	36.36	34.38	15.63	**21.88**	33.33	**21.21**	36.36	0.00	**33.33**	36.36
R %	72.45	70.39	78.71	61.92	64.06	**89.06**	83.33	**86.36**	81.82	73.68	**83.33**	81.82

Total of 12 recombinant plants were used for background selection. Two plants P284 and P307 having the highest recipient alleles up to 89.06 and 86.36% were used to develop BC_2F_1 populations. In case the ordinary breeding was applied, frequency of recipient genome was only 75% in the BC_1F_1, lower than in this study 11-14%.

3.2.2 .Genotyping BC_2F_1

Approx. five hundred BC_2F_1 individuals of the cross (AS996xFL478) were grown and analysed. The same procedures were applied to screen the foreground selection again with AP3206, RM3412, RM10793, RM10711. The recombinant selection was done with RM10694,RM562, RM7075 along the *Saltol* region on chromosome 1. From 250 heterozygous plants, 26 recombinant plants were identified.

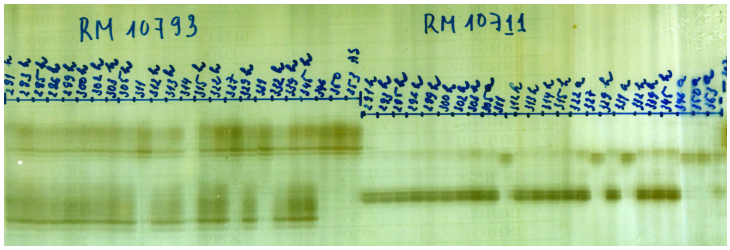

Figure 5. BC_2F_1 (AS996xFL478) individuals screening using primer RM10793 - left, and RM10711- right.

For background selection, the primers shown heterozygous DNA bands from previous generation with 10 more additional primers were used. Plant P307-322, P284-112 and P307-305 were the best plants with the recipient alleles up to 93,18% and 94,03% respectively. These three plants were used to cross with recipient variety for BC_3F_1 generation. In each individuals, half of the tillers were used for BC_3F_1 crossing, the others were used for BC_2F_2 selfing. In the case where the ordinary breeding was applied, frequency of receipient genome was only 87,5% in the BC_1F_1, but in here, the best plants were selected with the recipient alleles about 5,7- 6,5% higher than those of conventional method.

3.2.3. Genotyping BC_3F_1

From the above results, three populations from three plants were analysed. Total of 371 plants were screened for the four markers located in the *Saltol* region. Only 94 plants were used for recombinant selection. In background selection, 25 primers were used. In figure 6 was the graphical of 14 BC_3F_1 individuals, which was given by GGT2.0 software.

Figure 6. Graphical of the 14 recombinant BC_3F_1 plants using GGT2.0 software.

Plant P284-112-209 was the best BC_3F_1 individual with all the recipient alleles screened based on total of 63 markers (figure 7). The four plants P307-305- 21, P284-112-195, P284-112-198, P284-112-213 were the second ranking with only one loci heterozygous. All those 5 plants were chosen as the breeding lines for result of *Saltol*-AS996 introgression.

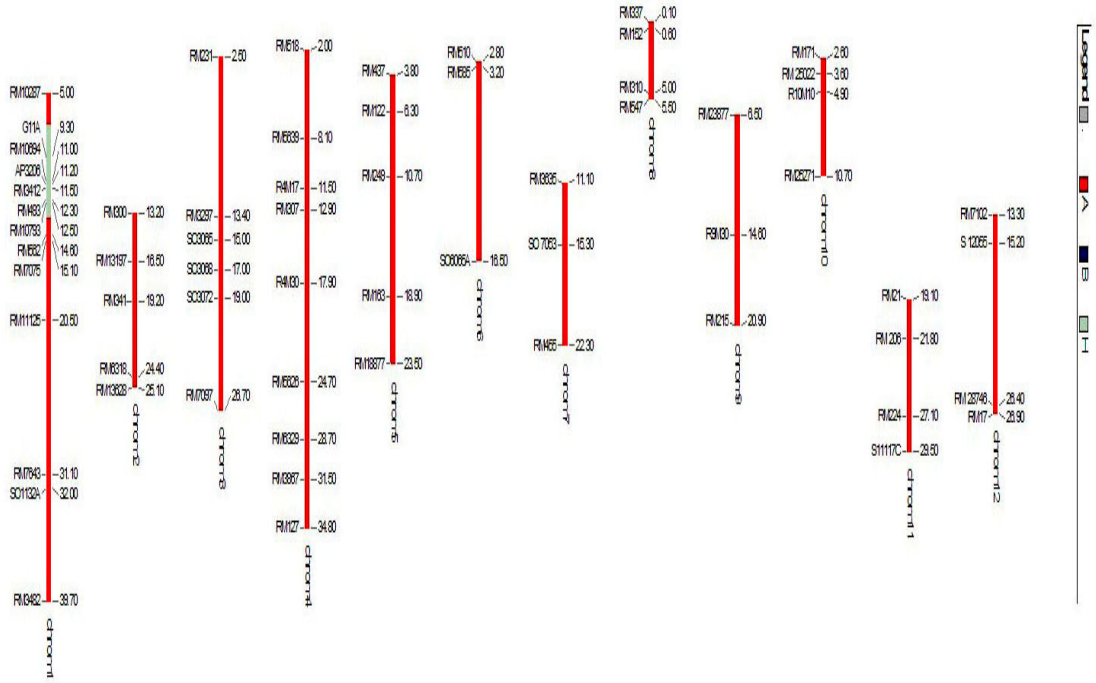

Figure 7. Graphical of the best BC_3F_1 plant P284-112-209 using GGT2.0 software.

3.2.4. Evaluation of salinity tolerance

As described in the part of method, BC_2F_2 and BC_3F_1 seeds were screening to evaluate the introgression of *Saltol* fragment into AS996. Salt stress was imposed 14 days after germination by adding NaCl to an EC of 12 dS m−1 in Yoshida nutrient solution until final scoring. Based on visual symptoms using IRRI's SES for rice, when the suceptible variety IR29 (sensitive) scored 9 and variety FL478 were used as highly tolerant checks scored 3, all the BC_2F_2 of the selected plants P284-112, P307-305 and P307-322 having the same score as the tolerant checks. It means that the homozygous *Saltol* fragment working well in BC2F2 generation. The BC3F1 plants scored 3-5 because of the heterozygous of *Saltol* fragment.

The original IR29/Pokkali QTL study using 80 extreme RILs identified *Saltol* as the QTL with the highest significance for shoot Na–K ratio with an LOD of 14.5 and R2 of 64%, based on selective genotyping [13]. A follow-up study categorized the RILs into sensitive and tolerant groups and mapped the position of *Saltol* between RM23 and RM140 (10.7–12.2 Mb on chromosome 1), and confirmed the effect of the shoot Na–K ratio with an LOD of 6.6 and R2 of 43% using 54 RILs [7]. While neither of these studies presented the percent variation explained for visual SES. tolerance scores or survival, it was assumed that by controlling the key mechanism of Na+/K+ homeostasis under stress, *Saltol* is a major contributor to seedling stage tolerance. The data

from the current study confirmed that *Saltol* contributes to Na+/K+ homeostasis with an LOD of 7.6 and R2 of 27% across the 140 RILs and a 30% decrease in the shoot Na–K ratio, from 1.7 to 1.2 in the IR29/Pokkali backcross lines, while the *Saltol* effect on SES scores in the QTL population and backcross lines was much smaller. The fact that *Saltol* affected the Na–K ratio more than other traits supports the possibility that the sodium transporter SKC1 gene underlying the *Saltol* QTL [8]. SKC1 was found to encode a sodium transporter that helps control Na+/K+ homeostasis through unloading of Na+ from the xylem [15], which has been suggested to function primarily in roots to reduce the amount of Na+ ions that are transported to the leaves [16]. Although the SKC1 QTL was originally detected using Nona Bokra, more research is needed to characterize the Pokkali allele at SKC1 to determine if it serves a similar function to maintain Na+/K+ homeostasis in the shoots. Interestingly, a recent study identified a QTL for Na–K ratio between 11.1 and 14.6 Mb on chromosome 1 from the upland japonica variety Moroberekan [4] suggesting that the *Saltol* region may have functional significance for salt tolerance across both indica and japonica varieties. In this study, all the BC_2F_2 of the selected plants P284-112, P307-305 and P307-322 having the same score as the tolerant check. It means that the homozygous *Saltol* fragment working well in BC_2F_2 generation. The next generation will be used to check the function of *Saltol* in the following BC_3F_2, BC_2F_3.

In conclusions, approximately 500 SSR markers distributed in the 12 chromosomes were screened for parental polymorphism. Of which, 63 polymorphic markers were identified. The result showed that frequence SSR markers for DNA polymorphisms between parental AS996/FL478 was very low. Two BC_1F_1 plants P284 and P307, having the highest recipient alleles up to 89.06 and 86.36%, were identified for the next backcrossing. Frequency of recepient genome in selected plants was 16-19% higher than the ordinary breeding. In BC_2F_1, three plants with the recipient alleles up to 94,03 and 93,18%. were used to cross with recipient variety for BC_3F_1 generation. Plant P284-112-209 was the best BC3F1 individual with all the recipient alleles screened based on total of 63 markers. The four plants P307-305-21, P284-112-195, P284-112-198, P284-112-213 were the second ranking with only one loci heterozygous. All those 5 plants were chosen as the breeding lines for result of *SALTOL*-AS996 introgression. Conventional breeding will be applied on the BC_4F_1, BC_3F_2 for selection of the new salt tolerance rice lines with all receipient genome.

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Quy tụ QTL *Saltol* vào giống lúa ưu tú của Việt Nam - AS996 bằng phương pháp chọn giống nhờ chỉ thị phân tử và lai hồi giao

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Mục tiêu của nghiên cứu là góp phần chọn tạo giống lúa chịu mặn, năng suất cao, sử dụng phương pháp chọn giống nhờ chỉ thị phân tử kết hợp lai hồi giao (MABC-Marker assisted backcrossing) như là một kỹ thuật cao trong chọn tạo giống. Tổng số 500 chỉ thị SSR nằm rải rác trên 12 NST được sử dụng để sàng lọc đa hình các giống bố mẹ, trong đó có 52 chỉ thị trong vùng gen Saltol. Chỉ tìm được 63/500 chỉ thị đa hình, được sử dụng để sàng lọc cá thể của các quần thể hồi giao BC_1F_1, BC_2F_1 và BC_3F_1. Qua ba thế hệ chọn lọc, đã thu được một dòng BC_3F_1 - P284-112-209 có chứa vùng gen *Saltol* và 100% nền di truyền của giống nhận gen và bốn dòng BC_3F_1 khác P307-305- 21, P284-112-195, P284-112-198, P284-112-213 chỉ có một locut dị hợp tử trong số 63 chỉ thị sàng lọc. Dòng BC_4F_1 đã được lai tạo mang 100% hình thái và nền gen của cây nhận gen AS996 sẵn sàng cho phát triển giống lúa mới ASS996-*Saltol* chịu mặn ứng phó với biến đổi khí hậu.