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Marker assisted selection approach for identification of salinity-submergence tolerant rice genotypes through microsatellite markers

A. Sultana, M.M. Islam[1], L. Hassan[2], R. Rahman and M.M. Haque[1]
Department of Biotechnology, [2]Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, [1]Plant Breeding Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh-2202, Bangladesh, E-mail: afroza_ag@yahoo.com

Abstract: Development and continuation of the dual tolerance for salinity and submergence is a fruitful entry point for improving the rice productivity in coastal areas. A major quantitative trait locus (QTL) was identified bestowing salt tolerance on chromosome 1 and designated *Saltol*. Submergence tolerance is controlled by a single major QTL (*Sub1*) on chromosome 9. This study aims to unite *Saltol* and *Sub1* genes within one genetic background. Crosses have been made between one advanced line (RC-251) of International Rice Research Institute as donor (salinity-submergence tolerant) with popular salt tolerant rice variety, Binadhan-10 as recurrent parent. A total of 25 microsatellite markers for *Saltol* and 15 markers for *Sub1* have been screened for hybridization test and foreground selection. Total 80 heterozygous genotypes have been selected by hybridization test with highly polymorphic markers RM7075 (for *Saltol*) and RM5688 (for *Sub1*). Selected F_1 progenies have been backcrossed and indentified a total number of 54 BC_1F_1 genotypes, genotyped with tightly linked salt tolerant markers (RM7075, RM490 and RM493) and submergence tolerant markers (RM5688, RM23662 and SC3). These selected genotypes be used for further back crossing and MAS for the development of salinity and submergence tolerant rice varieties to cope with common abiotic stresses such as salinity-submergence in coastal areas.
Key words: Marker-assisted selection, QTL, SSRs, Salinity Submergence and Rice.

Introduction

Rice or paddy (*Oryza sativa* L.) has not only been the staple food for more than half of the humankind but also shaped the culture, diet and economy of the majority of the world's population, especially the east and south-east Asian continents (Singha *et al.*, 2015).

Abiotic stress is a major factor limiting productivity of rice crops in large areas of the world. The major abiotic stresses worldwide causing risks to food security are high salinity, drought, submergence and cold (Sanghera *et al.*, 2011). Salt tolerance is generally a sustained growth of the plant in the soil environment impregnated with NaCl or other salt combinations. Submergence occurs when a large proportion of the pore spaces in the soil are occupied by water which limits the diffusion of oxygen and gas exchange between the soil, plants and atmosphere and resulted in decreased growth of roots and their functioning. Presently around 31 upazillas of Jessore, Satkhira, Khulna, Narail, Bagerhat and Gopalganj districts are facing severe tidal movement and salinity problem.

The success of MAS (Marker Assisted Selection) is influenced by the relationship between the markers and the genes of interest. Microsatellites are the most widely used markers in major cereals (Gupta and Varshney, 2000). They are highly reliable (i.e. reproducible), co-dominant in inheritance, relatively simple and cheap to use and generally highly polymorphic.

A major QTL associated with salinity tolerance, named *Saltol*, was identified on chromosome 1, while submergence tolerance is controlled by a single major quantitative trait locus (QTL), named *Sub1* on chromosome 9 (Toojinda *et al.*, 2003). MAS hastened the selection to identify recombinants with *Saltol* and *Sub1* introgressed into one genetic background.

Agricultural activities in coastal areas are dominated by rice farming, so the ideal rice genotype for coastal areas needs the dual tolerance for salinity-submergence for better conformation. This study aims to combine salinity tolerance conferred by *Saltol* with submergence tolerance conferred by *Sub1* within one genetic background. This research will provide some promising rice lines used for further back crossing and MAS for the development of salinity-submergence tolerant rice varieties to cope with common abiotic stresses such as salinity-submergence in coastal areas.

Materials and Methods

Plant materials and crossing scheme: inadhan-10, a widespread salt tolerant variety of Bangladesh Institute of Nuclear Agriculture (BINA) was selected as recipient parent while IRRI developed dual (salt-submergence) tolerant advanced line RC-251 was used as donor of *Saltol-Sub1* gene. The experiment was conducted during the period from Aman, 2013 to Aman, 2014 in the experimental field and Laboratory of Biotechnology Division and Plant Breeding Division of BINA, Mymensingh. For the MAS strategy, Binadhan-10 was crossed with RC-251 to obtain F_1 seeds. F_1 was backcrossed to Binadhan-10 to obtain a huge number of BC_1F_1 population. BC_1F_1 plants were screened for foreground selection. The plants carrying target gene were selected for the next back cross generation (Fig. 1).

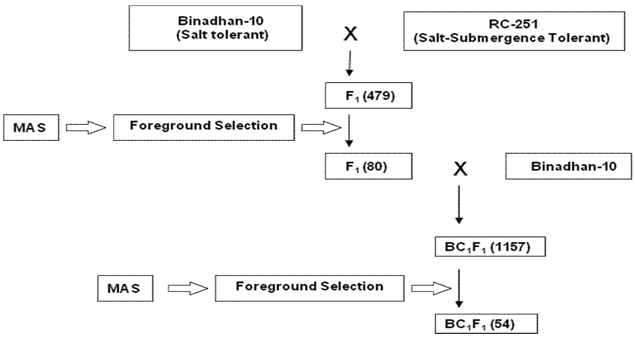

Figure 1. A flow diagram depicting the development of salt-submergence tolerant BC_1F_1 population through MAS.

Molecular Marker Analysis:

Collection of leaf sample and genomic DNA extraction: Genomic DNA was extracted from young leaves of 21 days old plants. Leaf tissues were frozen with liquid nitrogen and grinded using vortex. DNA extraction was

followed a modified protocol as described by Pervaiz *et al*. (2011).

Polymerase Chain Reaction (PCR): 2.88 µl of sterilized ddH2O, 1.0µl of dNTPs (Deoxynucleotide Triphosphates), 1.0µl of Taq buffer B (without MgCl2), 1.0µl of MgCl2, 2.0µl of primer (forward + reverse) 0.12µl of *Taq* DNA polymerase were mixed for one reaction. Thus, PCR cocktail (8.0µl) was added to each tube of PCR plate containing 2.0µl DNA template. The PCR plate was set on the thermocycler (Biometra T_3 thermal cycler) and the machine was run according to the program. After initial denaturation for 5min at 94°C, each cycle comprised 1min denaturation at 94°C, 0.45 min annealing at 55°C to 60°C and 2min extension at 72°C with a final extension for 7 min at 72°C at the end of 35 cycles.

Polyacrylamide Gel Electrophoresis (PAGE): The PCR products were mixed with 10X bromophenol blue gel loading dye (Invitrogen) and were analyzed by electrophoresis on 8% polyacrylamide gels at 90-100 v for high throughput manual genotyping (CBS Scientific Co. Inc., CA, USA). The gels were stained in 0.5mg/mL ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA) and manual scoring of the gel pictures. SSR markers were used for selection.

Identification of Polymorphism: A widespread primer survey is prerequisite for successful Marker Assisted Selection. Polymorphic markers are essential for a marker assisted back crossing scheme. Parents that provide adequate polymorphism are selected on the basis of the level of genetic diversity between parents. A total of 25 microsatellite markers for *Saltol* and 15 markers for *Sub1* were screened for hybridization test and foreground selection (Table 1 and 2).

Table 1. Details of SSR markers for MAS of salinity tolerance in rice.

Markers	Chr. 1 (Mb)	Forward primer	Reverse primer
RM140	12.3	CTTGCACAAGAGATGATGATGAGC	CATGCTGAGAAATAGTACGCTTGG
RM10825	13.3	GGACACAAGTCCATGATCCTATCC	CTTTCCTTTCCATCCTTGTTGC
RM490	6.7	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG
RM1287	10.9	GGAAGCATCATGCAATAGCC	GGCCGTAGTTTTGCTACTGC
RM10694	11	TTTCCCTGGTTTCAAGCTTACG	AGTACGGTACCTTGATGGTAGAAAGG
AP3206	11.2	TTCTCATCGCACCATCTCTG	GGAGGAGGAGAGGAAGAAG
AP3206f	11.2	GCAAGAATTAATCCATGTGAAAGA	ATGCTCTGGCTCCCTCAAG
RM8094	11.2	AAGTTTGTACACATCGTATACA	CGCGACCAGTACTACTACTA
RM3412b	11.5	TCATGATGGATCTCTGAGGTG	GGGAGGATGCACTAATCTTTC
RM10748	11.7	CATCGGTGACCACCTTCTCC	CCTGTCATCTATCTCCCTCAAGC
RM493	12.2	TAGCTCCAACAGGATCGACC	GTACGTAAACGCGGAAGGTG
RM10793	12.5	GACTTGCCAACTCCTTCAATTCG	TCGTCGAGTAGCTTCCCTCTCTACC
SalT1	13.8	TGCCCATGGATAGTGATACC	TTGCACCGATCCGTAGTACA
RM562	14.6	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC
RM7075	15.1	TATGGACTGGAGCAAACCTC	GGCACAGCACCAATGTCTC
RM10711	11.2	GCTTCGATCGATGAGAAAGTAGAGG	GAATCTCCCATCCTTCCCTTCC
G11A	9.3	AGCTGGTAGGAAGGCTGAAAG	TGCCAGCAGCTCAGTAGAAG
RM10745	11.7	TGACGAATTGACACACCGAGTACG	ACTTCACCGTCGGCAACATGG
RM10764	12.1	AGATGTCGCCTGATCTTGCATCG	GATCGACCAGGTTGCATTAACAG
RM10772	12.2	GCACACCATGCAAATCAATGC	CAGAAACCTCATCTCCACCTTCC

Table 2. Details of SSR markers for MAS of submergence tolerance in rice.

Markers	PCR band size (bp)	Forward primer	Reverse primer
IYT1	150	TAGGGGCCCATGAGTACTTG	TCAGACAGCTAGCTCGCAAC
IYT3	231	GTTGATAACCGGAGGAGACG	GTAACCCGACTGGTCTCAGG
AEX	203	AGGCGGAGCTACGAGTACCA	TCTGAACCGGATCATCATTG
ART3	330	AGTTTGTCTCCATTCGAAGTCA	CAGGGAAAGAGATGGTGGA
ART5	217	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG
Sub1C173	126	AACGCCAAGACCAACTTCC	AGGAGGCTGTCCATCAGGT
Sub1AB1	148	CATGTTCCATAGCCATCGACT	GAGCGAAGAGAGCTACCTGAA
Sub1BC1	170	CAATCGATGCGTGCTTCTT	CGCAACAAGGCAGAAAAATA
Sub1BC2	235	AAAACAATGGTTCCATACGAGAC	GCCTATCAATGCGTGCTCTT
Sub1BC3	217	CATGGGTAAAATTGCCATCC	GCTTGAGGGTGAGTGGAGAG
RM23662	150	GAGAGGACGATGGCACTATTGG	CGAGGAACTTGATTCGCATGG
RM5688	180	GCAGTGTCCAACCATCTGTG	ATCTGGTCACCCTTTGCTTG
ART5	124	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG
SC3	200	GCTAGTGCAGGGTTGACACA	CTCTGGCCGTTTCATGGTAT
RM23877	337	TGCCACATGTTGAGAGTGATGC	TACGCAAGCCATGACAATTCG

Hybridization test: DNA samples were extracted from 479 F_1 progenies and genotype with highly polymorphic markers RM7075 (for *Saltol*) and RM5688 (for *Sub1*). PCR bands of heterozygous alleles for donor and recipient were scored as "H".

Foreground selection: Markers which were found to be tightly linked to *Saltol* and *Sub1* genes, were used for foreground selection. Tightly linked salt tolerant marker (RM7075, RM490 and RM493) and submergence tolerant markers (RM5688, RM23662 and SC3) were used for the selection.

Data analysis: The molecular weights of the different alleles were measured using Alpha Ease Fc 5.0 software. The marker data was analyzed using the software Power Marker. The heterozygous alleles were scored as "H".

Results

Introgression of the saltol-sub1 gene into Binadhan-10 using MAS: The main aim of this study was to introgress the *saltol-sub1* gene into Binadhan-10 using a marker assisted backcrossing approach in order to maximize the recovery of all of the desirable characteristics of the recurrent parent. The results of these selection strategies were shown on table 3.

Table 3. Crossing combination and number of F_1 and BC_1F_1 seed.

Recurrent parent	Donor parent	Total no. of F_1 seeds	Total no. of BC_1F_1 seeds
Binadhan-10	RC-251	479	1157

Parental DNA polymorphism survey: In this study, a total of 40 SSR markers were used to identify DNA polymorphic markers between two parents, from which 25 markers for salinity tolerance and 15 for submergence tolerance. The results showed that the 11 polymorphic SSR markers in *Saltol* locus and 7 markers in *Sub1* loci were detected and the ratio of polymorphic markers on parental survey for *Saltol* is approximately 44% and for sub1 is 46%.

Confirmation of F_1 plants: DNA samples were collected from 479 F_1 progenies and PCR was carried out with highly polymorphic markers RM7075 for *Saltol* and RM5688 for *Sub1* and total 80 heterozygous genotypes were selected by hybridization test. PCR bands from all the F_1 plants were scored as "H". Score "H" represented heterozygous alleles for donor and recipient parent (Fig.1).

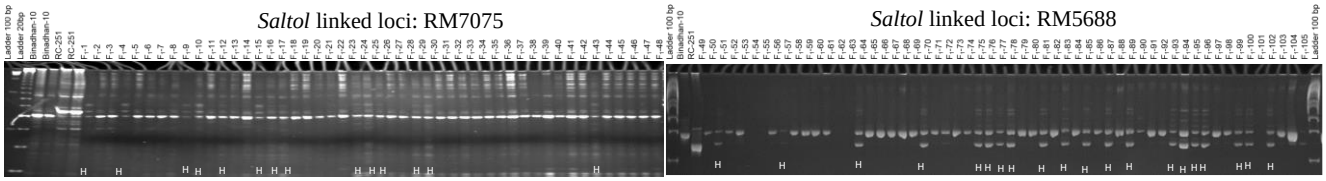

Figure 1. Hybridization test of the genotypes (F_1) of cross combination Binadhan-10×RC-251

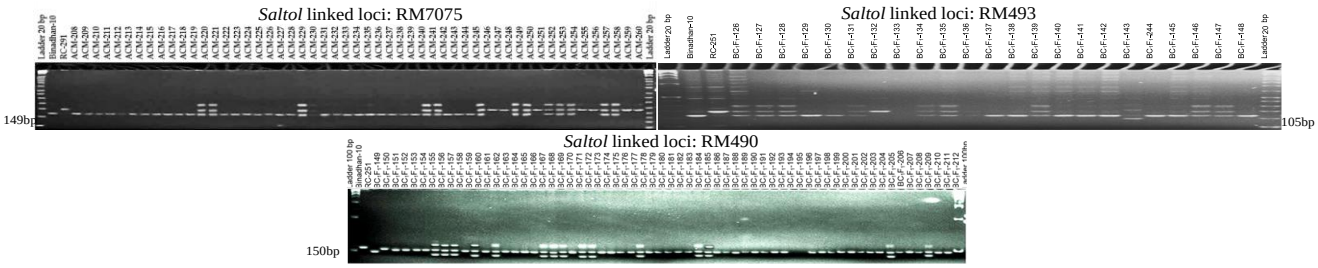

Figure 2. DNA banding profiles of the genotypes (BC_1F_1) of cross combination of Binadhan-10 and RC-251 for salinity tolerant SSR markers

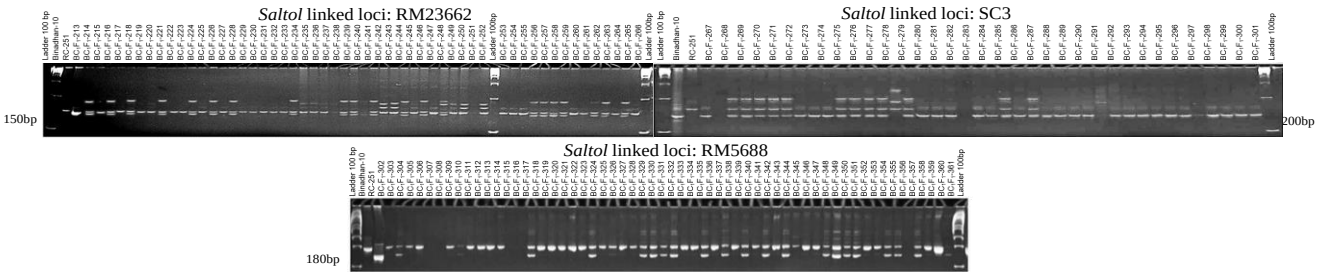

Figure 3. DNA banding profiles of the genotypes (BC_1F_1) of cross combination of Binadhan-10 and RC-251 for submergence tolerant SSR markers.

Foreground selection at BC_1F_1 generation: Selected F_1 progenies have been backcrossed with Binadhan-10 and produced a total of 1157 BC_1F_1 seeds. A total number of 54 BC_1F_1 genotypes were identified by genotyping with tightly linked salt tolerant markers (RM7075, RM490 and RM493) (Fig. 2) and submergence tolerant markers (RM5688, RM23662 and SC3) (Fig. 3).

Discussion

Climate change is severely stimulant the protesting impacts of abiotic stresses on rice production. Maximum rice cultivated lands in coastal areas are already being affected by the rising sea level, increasing the occurrences of salinity and submergence. It is crucial to develop salinity and submergence tolerance rice cultivars.

The current study indicates that MAS strategy is an effective method of utilizing QTLs with large effects in rice breeding programs. The codominant nature of SSR markers could be very useful for the introgression of the *Saltol as* well as *Sub1* locus into recipient elite cultivars. In backcross generation (BC_1F_1), the target locus *Saltol* was monitored by markers linked to the *Saltol* genes and *Sub1* locus monitored by markers linked to the *Sub1* genes. In each backcross generation, 2-3 polymorphic markers within the QTL region in the range of 11.16-12.6 Mb on chromosome 1, which were used for foreground selection to determine the heterozygous plants (Xu *et al.,* 2000; Thomson *et al.,* 2010). Screening for *Sub1* with molecular markers on chromsome 9, were done based on information of genetic map of Mackill *et al.,* (2006). In this study, we used a MAS system to transfer the *Saltol* and *Sub1* alleles into Binadhan-10. Our results verified that the polymorphic markers between two parents in target region of *Saltol* on chromosome 1 (RM490, RM493 and RM7075) and submergence tolerant markers (RM5688, RM23662 and SC3) on chromosome 9. PCR was carried out with highly polymorphic markers RM7075 for *Saltol* and RM5688 for *Sub1* of 479 F_1 progenies and total 80 heterozygous genotypes were selected. Selected F_1 progenies were backcrossed and developed a total of 1157 BC_1F_1 seeds and identified a total of 54 BC_1F_1 genotypes with tightly linked salt tolerant marker (RM7075, RM490 and RM493) and submergence tolerant markers (RM5688, RM23662 and SC3). Vu *et al.,* (2012) used two SSR markers (RM3412 and RM493) for foreground selection to get heterozygous plants from BC_1F_1 population. Cuc *at el., (*2012) confirmed foreground selection by the use of markers from the *Sub1* genes ART5 (6.3 Mb) and SC3 (6.6 Mb) in all the plants. They used of two precise primers located in the *Sub1* region absolutely resulted in minimized the introgression size of the *Sub1* in AS996 variety. It was expected that 54 lines possessed the *Saltol-Sub1* alleles. So, these near isogenic lines (NILs) were promoted for further backcross with Binadhan-10 in order to develop salinity-submergence tolerant rice variety.

Conclusion

Salinity and submergence tolerant rice variety can acclimate better to the coastal areas. We have developed salt-submergence tolerance BC_1F_1 generation by using MAS. This study could have a good impact on rice breeding and it is applicable for further backcrosses and MAS for the development of salinity-submergence tolerant rice variety to cope with common abiotic stresses such as salinity and submergence.

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