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Genetic and Physiological Improvement of Rice for SubmergenceTolerance

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Submergence stress is a major problem in Tamil Nadu during the monsoons. Every year paddy production is greatly affected due to flash floods. A major QTL on chromosome 9, *Sub1* has provided the opportunity to apply Marker Assisted Backcrossing (MAB) to develop submergence tolerant versions of rice cultivars that are widely grown in the region. Present study was at aimed at marker assisted introgression of *Sub1* locus controlling submergence tolerance from FR 13A into the background of CO 43, a popularly grown rice variety in the cauvery delta region. Molecular markers that were tightly linked with *Sub1*, flanking *Sub1*, and unlinked to *Sub1* were used for foreground, recombinant, and background selection, respectively, in backcrosses between a submergence tolerant donor, FR 13A and the widely grown recurrent parent, CO 43. Polymorphic markers for foreground and background selection were identified. Genotyping and phenotyping of BC_1F_1 and F_2 generations revealed the superiority of the plants introgressed with *Sub1* locus. By the BC_2F_2 and BC_3F_3 generations a submergence tolerant plant was identified that possessed 100% CO 43 genome with *Sub1* locus. These backcross progenies were evaluated for their tolerance against flooding. All the plants were able to survive 11 days of complete flooding.

Key words: Rice, Submergence tolerance, Marker assisted breeding, Genotyping, Phenotyping

Rice is grown in a wide range of ecologies ranging from irrigated to uplands, rainfed lowland, deep water and tidal wetlands. Climate projections suggest that temperatures, precipitation and flooding, and sea level rise are likely to increase, with adverse impacts on crop yield and farm income in Southeast Asia (Unnikrishnan *et al.,* 2006; Wassmann *et al*., 2009; INCCA, 2010). India has the largest area (17.2 million hectares) under rainfed lowland amongst the South-East Asian countries. In these areas, flash floods or short-term submergence is a major abiotic stress and flash floods at the seedling stage of rice severely reduced yields of rice grain (Hattori *et al*., 2011). Flash flooding can cover the entire plant for prolonged periods, and most rice cultivars die within 7 days of complete submergence (Xu *et al*., 2006; Bailey-Serres *et al.,* 2010). In Tamil Nadu, flooding is a major problem in the Cauvery delta zone comprising Thiruvarur, Thanjavur and Nagapattinum districts. Every year, 3.0 lakh ha of rice are submerged, leading to heavy production loss.

Studies on molecular genetics of submergence tolerance have shown that a major QTL (*Sub1*) explains about 70 % of phenotypic variation in submergence tolerance has been identified and fine mapped on chromosome 9 in the submergence tolerant cultivar FR 13A (Xu and Mackill 1996; Nandi *et al.,* 1997; Xu *et al.,* 2000). After this discovery, submergence tolerant varieties have been developed through marker-assisted backcrossing (MAB) (Mackill *et al*., 1993). The strategy of marker assisted introgression of target locus through foreground and background selection improves the efficiency of selection. Marker-assisted foreground selection would be effective for the transfer of recessive genes since their classical transfer requires additional recurrent selfing generations, a procedure that is prohibitively slow for most commercial breeders (Welz and Geiger, 2000). Molecular markers such as SSR's have been efficiently utilized in many crop improvement programs *viz*., hybrid identification, testing seed genetic purity and linkage mapping (Pallavi *et al.,* 2011). Developing rice varieties carrying the *Sub1* introgression may substantially improve and stabilize rice production in flood-prone environments. 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 (Tanksley *et al.,* 1989; Hospital, 2003).

To date, the *Sub1* locus of FR 13A has been bred into 10 varieties favored by farmers in different parts of South and Southeast Asia (Bailey-Serres *et al.,* 2012). The rapid adoption of *Sub1* rice by farmers is attributed to its effectiveness, high similarity to the varieties it replaces and involvement of farmers in the varietal selection (Singh *et al.,* 2009; Manzanilla *et al*., 2011). In India, *Sub1* has been successfully introgressed through MAB into a popular high-yielding variety, Swarna (Neeraja *et al*., 2007). The availability of the large effect QTL *Sub1* for submergence tolerance, a theoretical frame-work for MAB and the existence of intolerant varieties that are widely

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accepted by farmers of cauvery delta zone provided an opportunity to develop submergence tolerant cultivars that would be suitable for cultivation in submergence prone rice areas in Tamil Nadu. CO 43 is a medium duration variety popularly grown in the cauvery delta region of Tamil Nadu. It is known for its superior level of salinity tolerance. Improving the submergence tolerance of this genotype will be having an added advantage and hence CO 43 was selected as a target genotype for marker assisted introgression of *Sub1* locus. Therefore, the objectives of study aimed at (1) Marker assisted introgression of *Sub1* locus controlling submergence from FR 13A into the background of CO 43, (2) To develop a panel of tightly linked and flanking markers for converting other popular varieties of rice to flooding tolerance and (3) To develop a submergence tolerant version of CO 43 and screen them for submergence tolerance using physiological traits.

Material and Methods

Plant materials and crossing scheme

FR 13A was used as the donor of *Sub1*. The recipient variety was CO 43, a widely grown cultivar in Tamil Nadu. CO 43 was crossed with FR 13A to obtain F_1 seeds. True F_1 hybrids were selected based on morphological markers and hybridity was confirmed by SSR genotyping, using the genomic DNA isolated from F_1s and parents. Seeds collected from F_1s were used for rising F_2 generation. Parents (CO 43 and FR 13A) were surveyed for polymorphism using SSR markers linked to *Sub1* locus. SSR marker RM219 and CAPS marker namely ART5 has been identified for foreground selection. In the F_2 generations, individual plants that were heterozygous at the *Sub1* locus were identified using foreground marker, RM219 and confirmed by phenotyping. F_1s were backcrossed with CO 43 to obtain a large number of BC_1F_1 seeds. In the BC_1F_1 generation, individual plants that were heterozygous at the *Sub1* locus were identified reducing the population size for further screening (foreground selection). From the individual plants that were heterozygous for *Sub1*, those that were homozygous for the recipient allele at one marker locus (RM219) distally flanking the *Sub1* locus (i.e. recombinant) were identified. Superior BC_1F_1 plants were back crossed with CO 43 to develop BC_2F_1. Superior BC_2F_1 plants possessing >80 % of CO 43 genome were back crossed with CO 43 to develop BC_3F_1. Superior plants of BC_3F_1were used for developing BC_4F_1 generation. Again, selected BC_2F_1 and BC_3F_1 were self pollinated to obtain BC_2F_2 and BC_3F_2 for further phenotypic analysis.

Foreground and background selection

At the initial stages of the experiment, for selection of the *Sub1* locus (foreground), the reported rice microsatellite (RM) markers RM219 which were found to be linked to *Sub1* by 3.4cM was used (Xu *et al.*, 2004). ART5 a closely linked marker when used a 200 bp fragment of *Sub1* found in the promoter region of *Sub1*C in 11 rice genotypes such as Swarna-Sub1, IR64-Sub1, SambaMahsuri-Sub1, INGR04001, INGR08110, AC258830, AC42088, AC20431-W, INGR08109, INGR08111 and FR 13A was also used for foreground selection (Sarkar and Bhattacharjee, 2011). Microsatellite markers unlinked to *Sub1* covering all the chromosomes including the *Sub1* carrier chromosome 9, that were polymorphic between the two parents, were used for background selection to recover the recipient genome. The microsatellite markers that revealed fixed (homozygous) alleles at non-target loci at one generation were not screened at the next BC generation. Only those markers that were not fixed for the recurrent parent allele were analyzed in the following generations.

Evaluation of F_2 generation

Seeds were collected from a single F_1 hybrid plant and F_2 generation (256 plants) was raised along with the parents in the field during Rabi season 2008. Observations on days to 50 % flowering (The number of days from sowing to panicle emergence was counted), plant height (measured from the ground level to the tip of the primary panicle and expressed in centimeters), panicle length (The length of the primary panicle was measured from the base to the tip and recorded in centimeter), number of tillers per plant, number of grains per panicle, 100 grain weight and grain yield expressed in grams/ plant were recorded from the F_2 population and their parents.

Screening for submergence tolerance

Submergence screening was performed in the submergence screening facility constructed in the glass house at the Department of Crop Physiology, TNAU, Coimbatore following standard protocols (Xu *et al.,* 2000). Seeds of the selected plants of BC_2F_3 progenies along with parents were grown in tanks for 21 days and submerged for 11 days. After 11 days of submergence, tanks were de-submerged and recovery was scored after 2 weeks. Survival of plants was scored 14 days after de-submergence (calculated as a percentage) for confirmation of the presence of the *Sub1* locus.

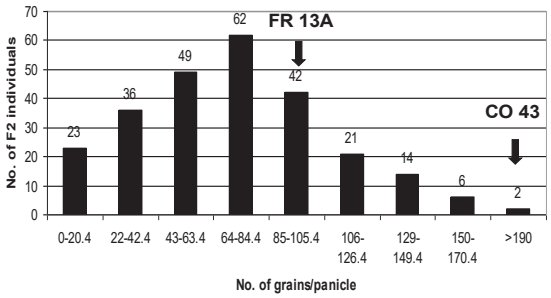
Results and Discussion

Introgression of Sub1 locus controlling submergence tolerance from FR13A into CO 43 and evaluation of parents and F_2 progenies for submergence tolerance

CO 43 is a medium duration variety popularly grown in the Cauvery delta region of Tamil Nadu. It is known for its superior level of salinity tolerance. Improving the submergence tolerance of this genotype will be having an added advantage. Hence CO 43 was selected as a target genotype for marker assisted introgression of *Sub1* locus. Crosses were made between CO 43 x FR 13A and true hybrids were identified by SSR markers F_1s were evaluated in the field and true F_1 hybrids were identified by using both morphological markers and SSR markers. F_1s were maintained by ratooning and back crossed with CO 43. On the other hand, seeds collected from

F_1s were used for rising F_2 generation. A total of 256 F_2 seedlings were raised and evaluated for the morphological traits namely, days to flowering, plant height, number of tillers/hill, number of panicles/plant, panicle length, number of grains per panicle, 100 grain weight and grain yield per plant (Table 1). The morphological traits viz., number of grains/panicle and grain yield in grams/plant showed continuous variation among the F_2 individual (Fig. 1a & 1b).

The introgression of *Sub1* locus was monitored by markers shown to be closely linked with the gene. In the F_2 population, foreground selection using RM219 revealed that 61 F_2 plants were found to possess CO 43 allele, 125 F_2 plants were found to possess both the alleles (heterozygotes) and 64 plants were found to possess FR13A allele (1:2:1 ratio). Phenotyping of selected F_2 plants of all three types (homozygous CO 43 allele, heterozygous and homozygous FR 13A allele harboring plants) revealed the superiority of both homozygous FR 13A and heterozygous plants in terms of submergence tolerance (13 days of submergence) (Plate. A).



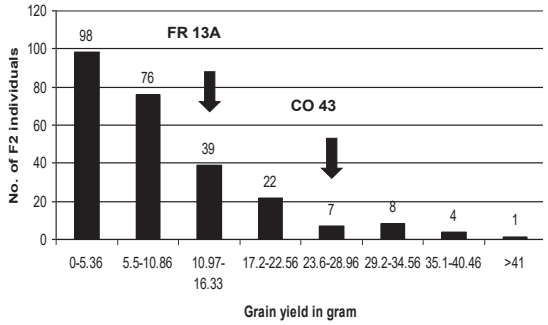


Fig.1 Frequency distribution pattern for morphological traits evaluated in the 256 F populations of CO 43 and FR13A.

(a) Frequency distribution for number of grains/panicle among the 256 F_2 individuals,

(b) Frequency distribution for grain yield among the 256 F_2 individuals showing continuous variation.

Surveying parental polymorphism using SSR markers

Parents (CO 43 and FR13A) were surveyed for polymorphism using SSR markers linked to *Sub1* locus. Five SSR markers namely RM219 (3.4cM), RM464A (0.7cM), RM316 (1.5cM), RM444 (3.2cM) and RM285 (1.8cM) closely linked to *Sub1* locus on chromosome 9 were surveyed for parental polymorphism. Out of these five primers only RM219 showed polymorphism between CO 43 and FR 13A and it was used for foreground selection. Again, CAPS marker namely ART5 have been identified for foreground selection. To identify SSR markers for back ground selection, a total of 232 markers covering all 12 chromosomes in the rice genome were surveyed. Out of these 232 markers, 76 SSRs showed polymorphism between CO 43 and FR13A, this accounts for 32.7% of polymorphism. The maximum numbers of SSR markers (50) were surveyed on chromosome 9 which recorded polymorphism of 36.0 %. These markers are being used for back ground selection.

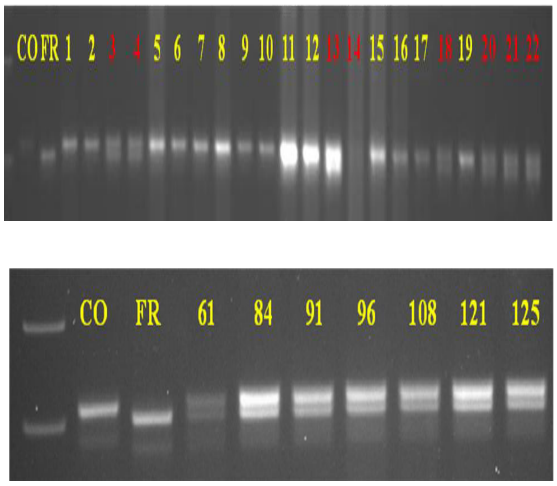


Fig. 2. Genotyping of BC1F1 and BC2F1 progenies with RM 219

Generation of backcross progenies and foreground and background selection

Around 180 crossed seeds were obtained by back crossing the F_1 with CO 43. BC_1F_1 generation was raised and the plants were subjected to foreground selection using RM219. About 26 plants were found to be heterozygous for RM219 (Fig. 2). Superior BC_1F_1 plants were back crossed with CO 43 to develop BC_2F_1. About 130 BC_2F_1 plants were raised under green house conditions and genotyped using RM219 linked to *Sub1* locus. Foreground selection resulted in the identification of 21 BC_2F_1 plants harboring *Sub1* locus from FR13A (Fig. 2). Background selection using 25 genome wide SSR markers resulted in the identification of superior BC_2F_1 plants possessing greater % of CO 43 genome. Superior BC_2F_1 plants (# 15, 23, 26, 36, 55, 57, 61, 84, 91 and 108) possessing >80 % of CO43 genome were back crossed with CO 43 to develop BC_3F_1.

Foreground selection of BC_3F_1 plants using RM219 and ART5 resulted in the identification of 5 BC_3F_1 plants harboring *Sub1* locus from FR 13A. Background genome analysis using 37 SSR markers covering the whole genome revealed that all the BC_3F_1 plants recovered more than 85 % of CO 43 genome. One of the BC_3F_1 plants (# 7) was found to have about 92 % CO 43 genome. These superior plants were used for developing BC_4F_1 generation. Selected BC_2F_1 and BC_3F_1 plants were self pollinated

and these backcross generations were phenotyped for flooding tolerance in the submergence screening facilities.

Screening for submergence tolerance

Backcross progenies were evaluated for their tolerance against flooding/submergence. BC_2F_2 and BC_2F_3 families were grown in tanks for 21 days and then completely submerged for 11 days. After 11 days of submergence, tanks were de-submerged and recovery was scored after 2 weeks. Most of the

Plate A. BC2 F3 Progenies exhibiting complete survive for after 13 days flooding

backcross progenies exhibited complete survival for flooding (Plate A). Survived plants were genotyped again and it was found that most of the plants were homozygyous for FR 13A allele.

Hence, the present study clearly demonstrated that the conversion of the mega variety CO 43, which is grown on around 10,000 ha in Cauvery delta zone of Tamil Nadu, to submergence tolerant within a two year time span for the BC_2 and three year-time span for the BC_3. To the best of our knowledge, this is the first report of the introgression of the *Sub1* QTL to CO 43. Using MABC, a small genomic region containing *Sub1* has been introgressed into modern high-yielding varieties, such as Swarna, Samba Mahsuri, IR64, Thadokkam 1 (TDK1), CR1009, and BR11 (Septiningsih *et al.,* 2009; Iftekharuddaula *et al.*, 2011). MAB strategy has been shown to be an effective means of utilizing QTLs with large effects like *Sub1* in rice breeding programs (Toojinda *et al.*, 2005; Neeraja *et al.*, 2007; Septiningsih *et al.*, 2009). Molecular markers such as SSRs have been efficiently utilized in many crop improvement programs viz., hybrid identification, testing seed genetic purity and linkage mapping. In this study also, the true F_1 hybrids between CO 43 and FR 13A were identified and confirmed by using the method of SSR genotyping. Microsatellite markers that were polymorphic between the two parents were used to ensure that the recurrent parent genome was combined with the *Sub1* region originally from FR 13A on chromosome 9.

Table 1. Morphological traits showing variation in F2 individuals of CO 43 and FR 13A

Traits	Parents		F2 Individuals		
	FR13A	CO 43	Mean	Range	Standard Deviation
Days to flowering	114.0	109	99	75-130	11.59
Plant height	76.9	74	66.26	25-100	17.76
Number of tillers	12.2	14.4	11.60	4-29	5.24
Number of panicles	7.5	11.6	9.43	1-25	4.89
Panicle length	22.7	23.9	18.29	8-30	4.66
Number of grains /panicle	103.5	298.4	72.4	2-204	38.52
100 grain weight	2.34	1.85	2.01	0.8-3.6	0.56
Grain yield	11.7	27.5	10.6	0.12-53.6	13.30

Evaluation of 256 F_2 plants under field conditions revealed the presence of continuous variation for the targeted quantitative traits *viz.,* days to flowering, plant height, panicle size, number of grains per panicle, grain weight etc., This indicated the suitability of population for selection process from the early stage itself. Submergence tolerance, the effect of *Sub1* on growth, maturation, grain production and grain quality was assessed in Swarna-Sub1, IR64-Sub1 and Samba Mahsuri-Sub1 (Sarkar *et al.*, 2006, 2009; Neeraja *et al.*, 2007; Singh *et al.*, 2009). Comparative analysis of the three *Sub1* varieties and their original parents revealed that introgression of *Sub1* does not negatively affect agronomical performance including yield and grain quality under regular growth conditions (Singh *et al.*, 2009).

In this study, the *Sub1* locus was monitored by markers shown to be closely linked with the gene. Initially the *Sub1* locus was monitored by markers shown to be closely linked with the gene (Xu *et al.,* 2000, 2004). In order to select the F_2 segregants harboring the *Sub1* locus, a SSR marker namely RM219 linked to the *Sub1* locus was used for genotyping the F_2 segregants as given by Hospital and Charcosset (1997) and Neeraja *et al.* (2007). Survey on foreground lines indicated that 61 F_2s were found carrying CO 43 allele, 125 F_2 plants carrying both the alleles (heterozygotes) and 64 F_2s carrying FR 13A allele assuring the expected ratio of 1:2:1. The effect of introgression of *Sub1* locus in terms of tolerance against submergence, progenies of 5 F_2 plants possessing CO 43 allele of RM219, progenies of 5 F_2 plants possessing heterozygote alleles of RM219 and progenies of 5 F_2 plants possessing FR 13A allele were screened for their tolerance against submergence. The progenies of F_2 plants possessing FR 13A allele and heterozygote allele showed higher degree of tolerance level than progenies of F_2 plants possessing CO 43 allele. Recovery level after de-submergence were more in lines with FR 13A allele

and heterozygote, where all the plants carrying lines with CO 43 allele were dead, assuring the phenotypic association of submergence tolerance with *Sub1* locus. Multiple evaluations of submergence tolerance in the greenhouse and farmers fields confirmed that all "Sub1" lines exhibit significantly greater tolerance to complete submergence as compared with their original parents (Sarkar *et al*., 2009; Septiningsih *et al*., 2009; Singh *et al*., 2009). These studies indicate that the introgression of the *Sub1* region of FR 13A through MABC is widely applicable to diverse genetic backgrounds.

In order to retain the positive attributes of CO 43, SSR markers were employed for background selection which leads to great acceleration of recipient genome recovery in the present study. In order to identify SSR markers for background selection, about 232 microsatellite (SSR) markers covering all 12 chromosomes in the rice genome were used for genotyping the parents FR 13A and CO 43. Out of 232 SSR primers, 76 primers (minimum of 4-5 markers per chromosome) showed polymorphism between two parents which accounts for 32.7 %. This is in accordance with the conclusion made by Servin and Hospital (2002) and Neeraja *et al.* (2007) to provide adequate coverage of the genome in backcross programs.

Background genome analysis of BC_3F_1 plants using 37 SSR markers lead to identification of one plant (# 7) to have about 92% Co 43 genome (Hospital *et al*., 1992; Hospital, 2003; Servin *et al.,* 2004; Neeraja *et al*., 2007). BC_2F_2 and BC_2F_3 families were evaluated for their tolerance against flooding/submergence. Most of the backcross progenies exhibited complete survival for flooding stress of 11 days. This is in line with the findings of Fukao *et al*. (2011) who demonstrated the survival of *Sub1* containing genotypes under flooding. Sarkar and Bhattacharjee (2011) reported that twenty days submergence caused greater damage even in Submergence 1 (*Sub1*) introgressed cultivars compared to the 14 days of submergence.

In summary, we have developed an enhanced mega variety of rice, CO 43 by using a marker assisted backcrossing approach to incorporate submergence tolerance, which was controlled by a major QTL. The recovery of the recipient parent genome was greatly accelerated emphasizing the increased efficiency of using markers to assist selection of backcross lines.

These varieties can improve farmers productivity by decreasing expected yield losses under unfavourable environments and provide viable income for poor rice farmers. Hence, it is more of a "loss-mitigating" technology for resource poor farmers. This will help in revitalization of marginal rice lands and bring about sustainable rice production, food security and improved standard of living for the farmers of Thiruvarur, Thanjavur and Nagapattinum districts in Tamil Nadu who largely depended on rice for their livelihood.

This approach is currently being used to enhance several other rice mega varieties for submergence tolerance in rice breeding programs. However, additional loci that further improve submergence tolerance are present in rice germplasm and essential for developing robust flooding insurance (Septiningsih *et al.,* 2012).

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