Introgression of *Sub1* QTL (Submergence tolerant QTL) into the elite rice variety Jaya by Marker Assisted Backcross Breeding

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Abstract

Rice (*Oryza sativa* L.) is the most important staple food crop of Asia. Submergence is the third important abiotic stress affecting rice crop in coastal ecosystems of Kerala due to heavy showers of South-West monsoon. The most viable solution to overcome this problem is to introgress submergence tolerant gene into high yielding elite rice varieties. Hence, the present study was undertaken to introgress submergence tolerant gene (*Sub1* gene) into the elite rice variety Jaya through Marker Assisted Backcross Breeding (MABB). Polymorphism assay using 625 SSR markers could select two polymorphic foreground markers, seven polymorphic recombinant markers and 76 polymorphic genome wide markers. The genotypic selection of heterozygous plants was made in F_1 and in each backcross generation and in selfed progeny homozygous plants were selected using the selected foreground and recombinant markers. The *Sub1* region of the selected BC_2F_2 progeny ranged from 0.5-5.6 mb and had ≥ 85% homozygosity to that of recurrent parent. These selected progeny were screened for submergence tolerance *in vitro* and scored according to SES by IRRI which showed similar score to that of the donor parent. Our work could introgress the abiotic stress submergence tolerance controlled by *Sub1* gene into the popular rice variety Jaya. The developed abiotic stress tolerant rice lines are under field evaluation and can be cultivated profitably in the coastal ecosystem where flood is the major constraint limiting rice production.

Keywords: Marker Assisted Backcross Breeding, Polymorphic SSR markers, Rice, Submergence tolerance.

Introduction

Rice is the major staple food crop in India which feeds more than half of its populations. Among the various abiotic stresses, submergence is the third important stress affecting rice yield adversely. Frequent flash flood of about 10-14 days is a common occurrence in coastal rice fields and even in irrigated paddy fields of Kerala due to heavy and continuous showering of south west monsoon during *Kharif* season. Most of the high yielding rice varieties are not tolerant to submergence and hence flash flood causes substantial reduction in rice yield. It is reported that shallow flash floods can result in less than 10% production loss while deeper and stagnant water for about 2 weeks duration and > 100 cm in depth can cause damage ranging from 40% to 77% (Manzanilla et al., 2011). Now the flood prone paddy fields are increasing due to unpredicted cyclone rains and due to the adverse effect of climate change. Hence, submergence is a serious problem as far as the rice farmers in coastal agro - ecosystems of Kerala and South India is concerned. During complete submergence, the entire rice plant including the tips of the longest leaves remain under water for a time period which leads to low light, limited gas diffusion, effusion of soil nutrients, mechanical damage, increased susceptibility to pests

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and diseases and finally results in considerable reduction of rice yield. Studies show that 5642.68 sq.km of area of Kerala, which is 14.52% of the total area, is prone to floods (CESS, 2010). In Allepey district more than 50% percentage of area is identified as flood prone.

Submergence tolerance is the ability of rice plant to survive 10 to 14 days of complete submergence and again renew growth when the water subsides (Toojinda et al., 2003). It is controlled by a single major quantitative trait locus (QTL) called as *Sub1* on chromosome 9 (Xu and Mackill, 1996), along with a number of minor QTLs (Nandi et al., 1997). Plant breeders have used the landrace FR13A, the most submergence tolerant rice variety, to introgress *Sub1* QTL into the elite variety, Swarna (Neeraja et al., 2007). Though FR-13A is the most submergence tolerant variety, it is tall, lodging and a poor yielder. Hence, development of submergence tolerant high yielding rice varieties is an urgent need to address the field problem of submergence. As conventional breeding takes a long period for the development of a tolerant variety, a molecular approach had been attempted in the present study to introgress the major *Sub1* gene into the high yielding mega rice variety Jaya, so as to convert it into a submergence tolerant rice variety for cultivation in flood prone areas.

Marker Assisted Backcross Breeding strategy facilitates the transfer of a target gene/ QTL from a donor line to a recipient elite variety which lacks the attribute. During this breeding process, the presence of donor genome across the rest of the recipient genome is eliminated by negative selection through background molecular screening. Among the molecular markers, Simple Sequence Repeat (SSR) markers were used because it is easy to access and has high reproducibility. Screening using microsatellite/SSR markers permits the genetic dissection of progeny in each generation, thereby increasing the speed of the selection process and genetic gain per unit time (Tanksley et al., 1989; Hospital, 2003).

The objective of the present study was to introgress submergence tolerant gene (*Sub1* QTL) into the popular rice variety Jaya and thereby help the farmers to get sustained yield in the flood affected ecosystems of South India.

Materials and Methods

Plant materials

The rice variety Jaya developed by Indian Institute of Rice Research (IIRR), Hyderabad (TN1 x T-141) was used as the recurrent parent (RP) for improvement and the seed was collected from IIRR, Hyderabad. Jaya is the national yield check rice variety and widely accepted among the farmers of South India because of its good palatability and high yield potential of 5-6 tons/ha (KAU, 2016). Swarna *Sub1* which is the first *Sub1* introgressed rice variety developed by the International Rice Research Institute, Philippines, was used as the donor parent (DP), and submergence tolerance is controlled by single major quantitative trait locus (*Sub1*) on chromosome 9 (Neeraja et al., 2007). The seed of Swarna *Sub1* was collected from Tamil Nadu Agricultural University, Coimbatore.

Molecular breeding strategy

The recurrent parent Jaya was used as the female parent and crossed with donor parent Swarna *Sub1* and the F_1 generation was raised. Genotypic analysis was carried out using selected polymorphic foreground markers and the heterozygous plants in the target locus were selected. The selected heterozygous F_1 plants were backcrossed with RP Jaya to raise BC_1F_1 generation so as to regain recurrent parent genome recovery. Genotypic analysis of the BC_1F_1 progeny was carried out using foreground, recombinant and background polymorphic markers. The selected heterozygous BC_1F_1 plants were backcrossed with RP to raise BC_2F_1. Genetic analysis was done similarly to BC_1F_1 generation. The selected BC_2F_1 progenies were selfed to raise BC_2F_2 generation. Genetic analysis was done as described above and introgressed homozygous plants in the target locus with maximum background genome recovery were

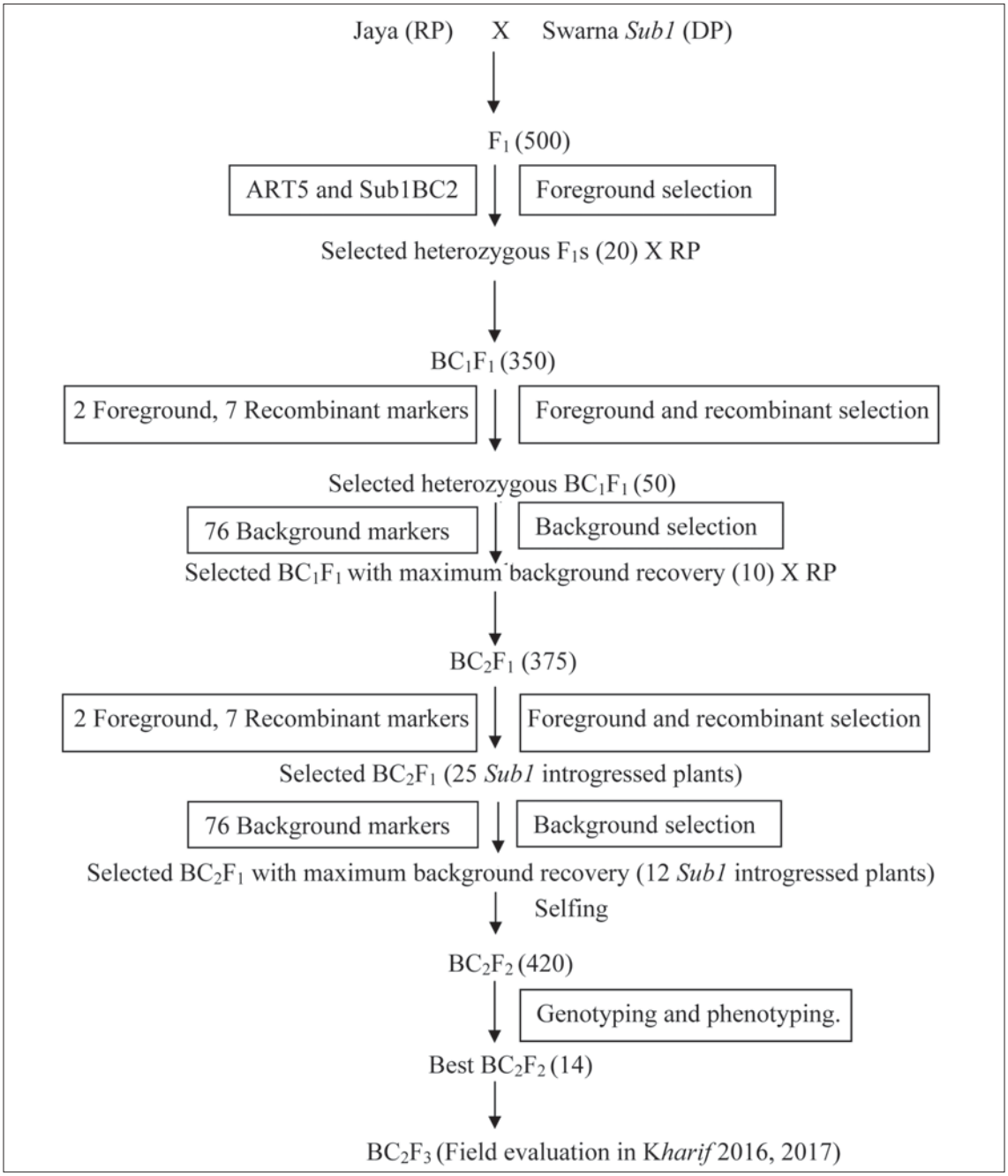

Figure 1. Breeding schedule for developing Jaya *Sub1* lines. RP (Recurrent Parent) - Jaya, DP (Donor Parent) - Swarna *Sub1*.

selected. These selected plants were phenotyped under *in vitro* condition and the plants surviving under submergence stress similar to donor parent with respect to introgressed gene were alone selected for further advancement and field evaluation (Fig.1). Positive molecular selection similar to recurrent parent genome was done in all other chromosomes and the remaining part of the chromosome 9, apart from the target locus.

DNA Isolation and PCR assay

Genomic DNA was extracted from young leaves of 3-4 week old plants using CTAB method (Doyle and Doyle, 1987). The quality and quantity of the extracted DNA was assessed using Nano Drop 2000c (Thermo Scientific).

PCR was performed in total volume of 20 µl reactions containing 2 µl (25 - 50 ng) of DNA template, 10 µl of 2X PCR Master Mix (comprising of 1X *Taq* buffer with 4 mM $MgCl_2$, 0.4 mM of each dNTPs and 0.05 U/µl *Taq* DNA polymerase enzyme, Thermo Scientific),1 µl each of 1.0 µM forward and reverse primers. Amplification of microsatellite markers was performed using standard 96 well thermo cycler (Bio- Rad T100™) with initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturing at 94°C for 40 seconds, annealing at 55-65 °C for 40 seconds and extension at 72 °C for 40 seconds with a final extension for 5 minutes at 72 °C.

Electrophoresis and DNA visualization

The PCR products were mixed with 6x gel loading dye (containing 0.25% bromo-phenol blue, 0.25% xylene cyanol and 30% glycerol in water) and were analyzed by electrophoresis on 8% polyacrylamide gels using mini vertical polyacrylamide gel electrophoresis for high throughput manual genotyping (Biorad system). The gels were stained using silver stain method (Benbouza et al., 2006) and were documented using Gel DOC™ XR^+ (Bio-rad). Microsatellite or Simple Sequence Repeat (SSR) markers were used for selection (Temnykh et al., 2001; McCouch et al., 2002; IRGSP 2005).

Polymorphism assay using SSR markers

A total of 625 numbers of reported rice SSR markers were used for parental polymorphic assay which includes foreground, recombinant and background SSR markers (http://www.gramene.org).

Foreground and Recombinant Screening

At the beginning of the polymorphism assay, for the foreground selection of the *Sub1* locus, reported nine microsatellite markers viz., IYT1, IYT3, AEX, ART3, ART5, Sub1AB1, Sub1BC1, Sub1BC2 and Sub1BC3 were used (Septiningsih et al., 2009) to select polymorphic foreground markers.

For recombinant selection, flanking markers of about 5 mb region on each side of the *Sub1* locus was targeted. Sixteen flanking markers viz., RM23668, RM23679, RM316, RM8303, RM23770, RM23778, RM23788, and RM23805 on telomeric end and RM23887, RM464, RM23917, RM23922, RM23928, RM219, RM23958, and RM24005 on centromeric end were used to select polymorphic recombinant markers (Neeraja et al., 2007).

Background Screening

A total of 600 SSR markers covering the 12 chromosomes and unlinked to the target loci were used for polymorphic assay between the recurrent and donor parents in background selection to recover the recipient genome. At least four polymorphic microsatellite markers per chromosome need to be used for efficient background selection. The microsatellite markers that revealed fixed (homozygous) alleles at non-target loci at one generation were not screened at the next backcross generation. Only those markers that were not fixed for the recurrent parent allele were genotyped in the following generations (Cuc et al., 2012).

Statistical analysis

The excel sheet with the marker data of *Sub1* introgressed Jaya lines was put into the Graphical Genotyper (GGT 2.0) software (Ralph, 2008) and analysed. The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as 'A', 'B' and 'H' respectively. GGT software analysis was used to calculate recurrent parent genome recovery in the selected *Sub1* introgressed BC_2F_2 lines of Jaya. Similarly, the size of the *Sub1* locus was also calculated using this software.

Table 1. Standard Evaluation System (IRRI, 1988) for scoring submergence tolerance of rice

Survival percentage (%)	Score	Observation	Tolerance
100%	1	Minor visible symptoms of injury	Highly tolerant
95-99%	3	Some visible symptoms of injury	Tolerant
75-94%	5	Moderate injury	Moderately tolerant
50-75%	7	Severe injury	Susceptible
0-49%	9	Partial to complete death	Highly susceptible

Table 2. Polymorphic SSR markers used for Foreground selection (Septiningsih et al., 2009)

Primer name	Sequence	Position	Band size (bp)	
			Donor parent allele	Recurrent parent allele
ART5 F	CAGGGAAAGAGATGGTGGA	Sub1C	217	201
ART5 R	TTGGCCCTAGGTTGTTTCAG	promoter		
Sub1BC2 F	AAAACAATGGTTCCATACGAGAC	Between Sub1	238	280
Sub1BC2 R	GCCTATCAATGCGTGCTCTT	B and C		

In vitro screening for submergence tolerance

Submergence screening was conducted in a well maintained greenhouse following standard protocols (Xu et al., 2000). For submergence screening, 14 days old *Sub1* introgressed BC_2F_2 lines along with parents, tolerant (FR13A) and susceptible check (IR64) varieties were planted into small pots in three replicates. Equal numbers of plants (40 nos.) were maintained for *in vitro* screening. These pots were submerged in tank containing water of 1m depth. After 14 days of submergence, the pots were de-submerged. The survival of the plants was scored after 14 days of de-submergence using Standard Evaluation System (IRRI, 1988) for the confirmation of introgression of *Sub1* locus in the survived plants as shown in table 1.

Results and Discussion

Marker selection

Parental polymorphism assay is a measure of genetic diversity among parents used in the study. From the parental polymorphic assay, two foreground SSR markers viz., ART5 and Sub1BC2 showing clear polymorphism between parents were selected. The tightly linked marker to *Sub1* locus viz., RM464A and flanking markers RM219 and RM316 as suggested by Hospital and Charcosset (1997) for efficient foreground and recombinant

Table 3. Polymorphic SSR markers used for Recombinant selection (Neeraja et al., 2007)

Primer name	Sequence	Position (mb)	Band size (bp)	
			Donor parent allele	Recurrent parent allele
RM8303 F	AGGGGAGAGGACACACACAC	2.3	133	140
RM8303 R	GGATCCTCCTGCAAAATCAA			
RM23770 F	GACCTTGTCCAGAGTGATTTTG	3.7	280	292
RM23770 R	ATTTGAGAATAACTTTTCCTACTTCG			
RM23788 F	ACCTTCACATAGCAGGGTTGAATC	4.2	330	332
RM23788 R	ACTCTAAGCCCCTGGATAATCTGC			
RM23805 F	CACATAGTTTCCATGCTCGTTCAC	4.5	241	250
RM23805 R	GGTAGAATCCATGACCGTCTCATC			
RM23917 F	CTCAGCTGTCTGTTCAGCTCTCAC	7.3	187	185
RM23917 R	CTTTGGTGCTGAGGTAGGTATTGG			
RM23922 F	TGGAGGGAGTATCATTATTAGCCG	7.4	273	236
RM23922 R	CTTGGATAGATTTGGTGGGATGAC			
RM23958 F	GAGACAGATGTGTACGGTTTGGTG	7.9	233	205
RM23958 R	TTGACAAGGGAATTGAAGGAGAAG			

selection of *Sub1* locus were monomorphic between parents in the present study and hence could not be selected for genotyping. The polymorphic indel marker ART-5 in the present study had been reported as diagnostic foreground marker for *Sub1* locus (Septiningsih et al., 2009; Iftekharuddaula et al., 2011; Sarkar and Bhattacharjee, 2012). Also, the marker Sub1BC2 has been used as polymorphic foreground marker as reported by Pradhan et al.,(2015), and Das and Rao (2015). These two polymorphic SSR markers used for foreground selection of target loci in all the generations are given in table 2.

Sixteen out of eighteen recombinant markers used for *Sub1* introgression into Swarna by Neeraja et al.(2007) were analysed for polymorphism in the present study. Among the sixteen recombinant markers used, seven markers were found to be polymorphic between recurrent and donor parents and were used for recombinant screening in each generation as shown in Table 3. The position of the selected seven polymorphic recombinant markers ranged from 2.3 - 7.9 mb in the 9th chromosome.

Out of 600 genome wide SSR markers, 76 were found to be polymorphic between donor parent

Table 4. Polymorphic SSR markers used for Background selection

Chromosome No.	Background Markers	Position (cM)	Chromosome No.	Background Markers	Position (cM)
1	RM 3252	0.6	6	RM 402	40.3
1	RM 490	51.0	6	RM 3	74.3
1	RM 583	58.9	6	RM 541	75.5
1	RM 600	61.3	6	RM 30	125.4
1	RM 9	92.4	6	RM 340	133.5
1	RM 246	115.2	7	RM295	0.0
1	RM 543	145.6	7	RM 125	24.8
1	RM 212	148.7	7	RM 336	61.0
1	RM 486	153.5	7	RM 18	90.4
2	RM 3340	0.0	7	RM 1362	116.1
2	RM 279	17.3	8	RM 337	1.1
2	RM 1347	26.6	8	RM 310	57.0
2	RM 1352	48.1	8	RM 42	78.4
2	RM 71	49.8	8	RM 458	124.6
2	RM 324	66.0	9	RM 296	0.0
2	RM 6	154.7	9	RM 524	13.2
2	RM 240	158	9	RM 105	32.1
2	RM 250	170.1	9	RM 410	64.4
2	RM 7485	175.7	9	RM 245	112.3
3	RM 231	15.7	10	RM 216	17.6
3	RM 36	40.5	10	RM 311	25.2
3	RM 7	64.0	10	RM 2504	35.9
3	RM 218	67.8	10	RM 304	73.0
3	RM 232	76.7	10	RM228	96.3
3	RM 251	79.1	10	RM 333	110.4
3	RM 282	100.6	10	RM 591	118.3
3	RM 468	202.3	11	RM 286	0.0
4	RM 551	8.5	11	RM 7120	56.2
4	RM 335	21.5	11	RM 287	68.6
4	RM 142	68.5	11	RM 1341	80.2
4	RM252	99.0	11	RM 6965	101.9
4	RM 280	152.3	11	RM 224	120.1
5	RM 413	26.7	11	RM 144	123.2
5	RM 1089	37.2	11	RM 2136	136.8
5	RM 169	57.9	12	RM 19	20.9
5	RM 39	78.7	12	RM 247	32.3
5	RM 26	118.8	12	RM 235	91.3
6	RM 469	2.2	12	RM1296	108.2

Swarna *Sub1* and recurrent parent Jaya and these markers were used for background screening in each backcross generation (Table 4).

Foreground Screening

It involves the selection of plants with target loci using polymorphic foreground markers. True hybrids expressed heterozygous alleles, one allele of recurrent parent and the other allele of donor parent in F_1 generation (Figs. 2 & 3). A total of 20 superior performing heterozygous progeny were selected from 500 progeny in F_1 generation using ART5 and Sub1BC2 foreground markers.

In BC_1F_1 generation, 155/350 plants and in BC_2F_1 generation 140/375 plants showed clear heterozygosity using the foreground SSR markers ART5 and Sub1 BC2 which are tightly linked to the *Sub1* gene (Figs. 4,5,6 & 7). The homozygosity for the target locus could be achieved only by selfing. Hence after two generations of backcrossing, the target gene introgressed BC_2F_1 plants were selfed to raise BC_2F_2 generation. In BC_2F_2 generation, 145 plants out of 420 plants were found to be homozygous for the target locus. In the foreground screening of BC_2F_2 generation, progeny which showed homozygous bands in the *Sub1* locus like that of donor parent alone were selected (Figs. 8 & 9).

Recombinant screening

The heterozygous plants confirmed with *Sub1* gene introgression were screened with flanking markers. In BC_1F_1 generation, 50/155 plants and in BC_2F_1

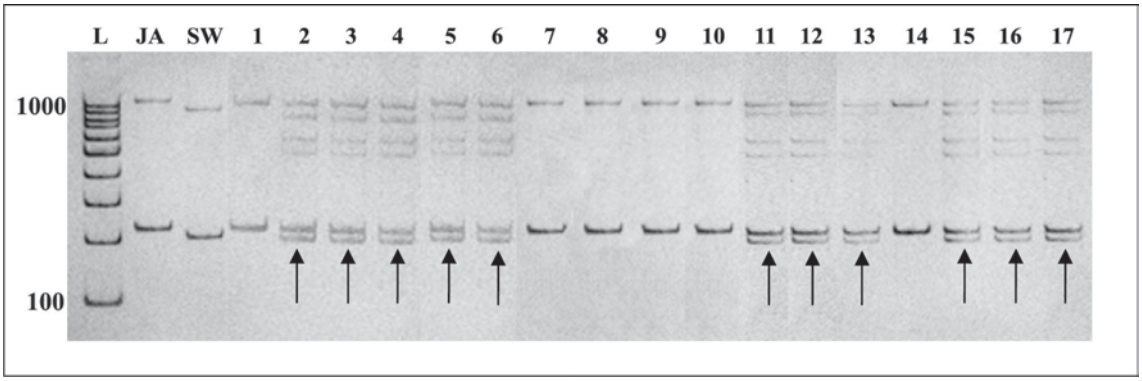

Figure 2. Genotypic screening of F_1 progenies with foreground marker ART5. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-17: F_1 progeny. Arrows indicate heterozygous F_1 progeny.

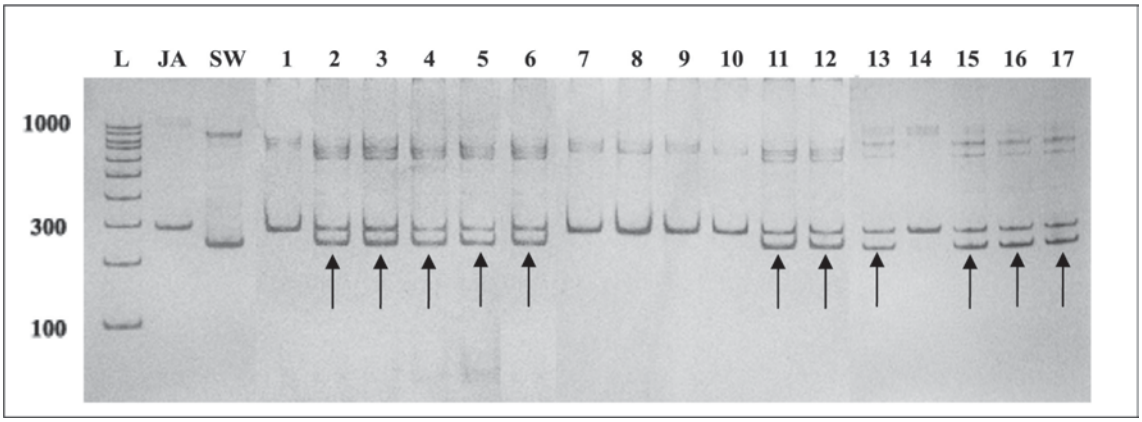

Figure 3. Genotypic screening of F_1 progenies with foreground marker Sub1BC2. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-17: F_1 progeny. Arrows indicate heterozygous F_1 progeny.

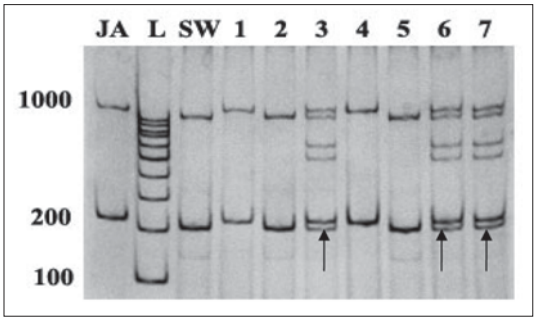

Figure 4. Genotypic screening of BC_1F_1 progenies with foreground marker ART5. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-7: BC_1F_1 progeny. Arrows indicate heterozygous BC_1F_1 progeny.

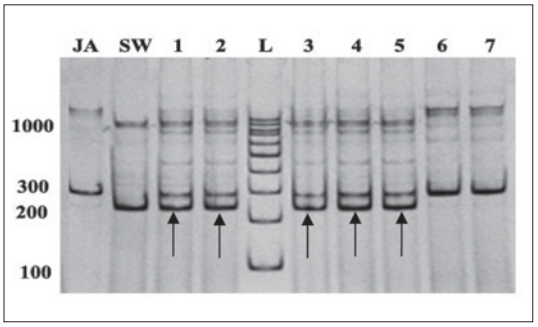

Figure 5. Genotypic screening of BC_1F_1 progenies with foreground marker Sub1BC2. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-7: BC_1F_1 progeny. Arrows indicate heterozygous BC_1F_1 progeny.

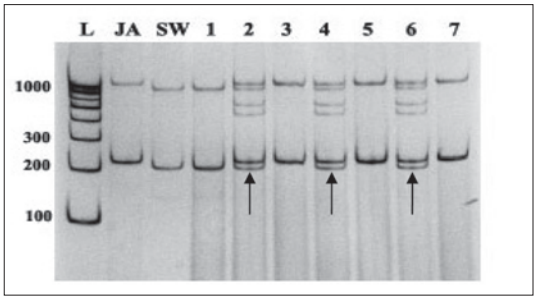

Figure 6. Genotypic screening of BC_2F_1 progenies with foreground marker ART5. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-7: BC_2F_1 progeny. Arrows indicate heterozygous BC_2F_1 progeny.

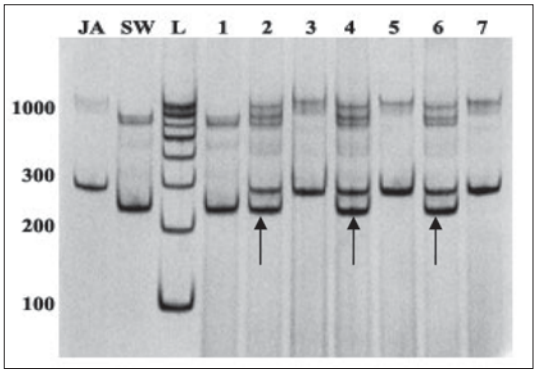

Figure 7. Genotypic screening of BC_2F_1 progenies with foreground marker Sub1BC2. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-7: BC_2F_1 progeny. Arrows indicate heterozygous BC_2F_1 progeny.

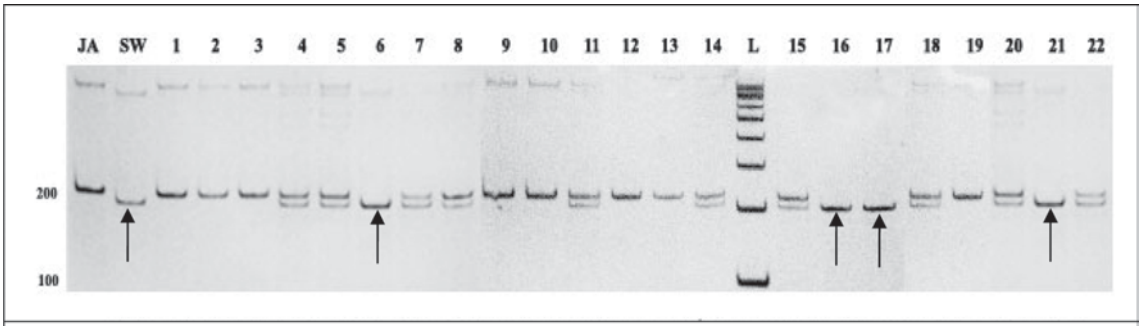

Figure 8. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with foreground marker ART5. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-22: BC_2F_2 progeny. Arrows indicate homozygous *Sub1* locus similar to donor parent

generation, 25/140 plants showed clear heterozygosity using the recombinant markers. Four markers, viz., RM8303 (2.3mb), RM23770 (3.7mb), RM23788 (4.2mb) and RM23805 (4.5mb), were used as telomeric end markers as where three makers, viz., RM23917 (7.3mb), RM23922 (7.4mb) and RM23958 (7.9mb), were used as centromeric end recombinant markers. In BC_1F_1, BC_2F_1, BC_2F_2 generations, plants introgressed with *Sub1* gene were screened with the above mentioned flanking

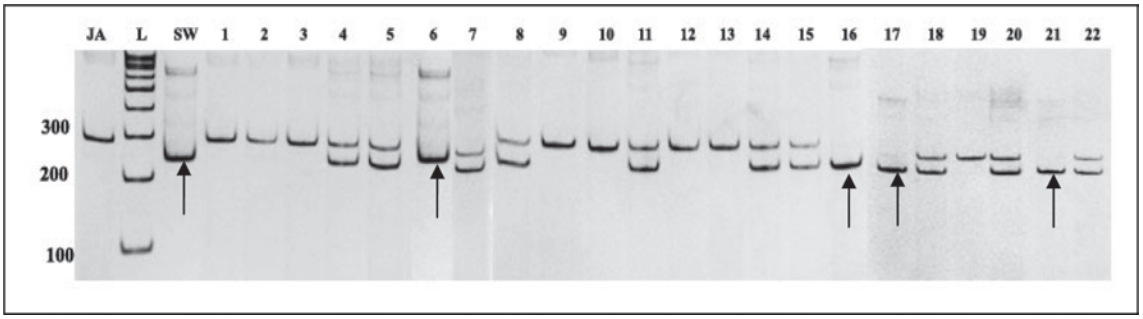

Figure 9. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with foreground marker Sub1BC2. Lane L: 100bp Ladder, Lane JA : Jaya, Lane SW: Swarna *Sub1*, Lane 1-22: BC_2F_2 progeny. Arrows indicate homozygous *Sub1* locus similar to donor parent.

markers in the upstream and downstream of the *Sub1* locus. Progeny with heterozygous bands expressed both the recurrent and donor parent alleles. In the selected BC_1F_1, BC_2F_1 generations, screening with the polymorphic recombinant markers showed the presence of both the recurrent and donor parent alleles. But in the selfed BC_2F_2 plants (80/145) the introgressed region of the selected BC_2F_2 plants was of donor type. In BC_2F_2-3-2-13 and BC_2F_2-3-2-21, telomeric and centromeric end markers showed homozygous allele like that of donor parent. In BC_2F_2-5-21-12 telomeric end markers showed homozygous allele like that of donor whereas centromeric end markers showed homozygous allele like that of recipient parent with markers, RM23922 (7.4mb) and RM23958 (7.9mb) respectively. Figures 10, 11, 12 & 13 show the banding pattern of *Sub1* introgressed Jaya BC_2F_2 progeny with recombinant markers. The segment size of introgressed donor *Sub1* region into the selected BC_2F_2 progeny was estimated to be 0.5-5.6 mb. The plants BC_2F_2-3-2-13 and BC_2F_2-3-2-21 showed 5.6 mb while plant BC_2F_2-5-21-12 showed slightly lesser size of about 5.1 mb, donor fragment (Table 3).

In the *Sub1* region, three similar genes encode the ethylene response factor (ERF) domain: *Sub1 A*, *Sub1 B*, *Sub1 C*. In submergence intolerant cultivars, this locus encodes only two ERF genes *Sub1 B* and *Sub1 C*. The function of *Sub1 B* is not clear. *Sub1 A* diminishes ethylene production and GA

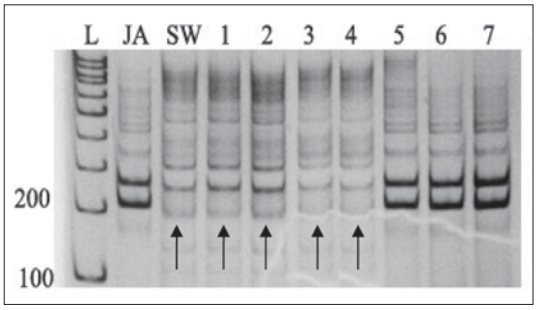

Figure 10. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with recombinant marker RM23922. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1*, Lane 1-7: BC_2F_2 progeny. Arrows indicate homozygous bands similar to donor parent.

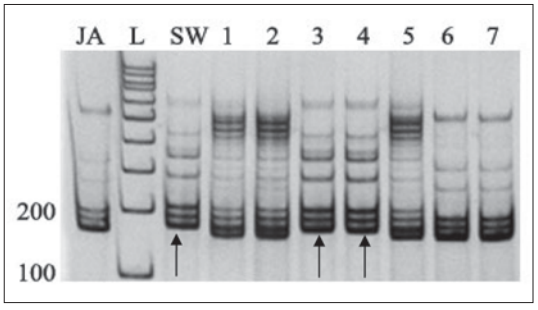

Figure 11. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with recombinant marker RM23958. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna *Sub1,* Lane 1-7: BC_2F_2 progeny. Arrows indicate homozygous bands similar to donor parent.

responsiveness causing quiescent growth under submergence. *Sub1 C* on the other hand increases ethylene production and GA responsiveness causing greater elongation of shoot, greater exhaustion of

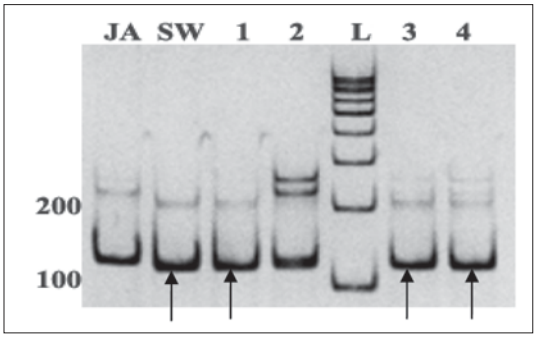

Figure 12. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with recombinant marker RM8303. Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna-*Sub1*, Lane 1-4: BC_2F_2 progeny. Arrows indicate homozygous bands similar to donor parent.

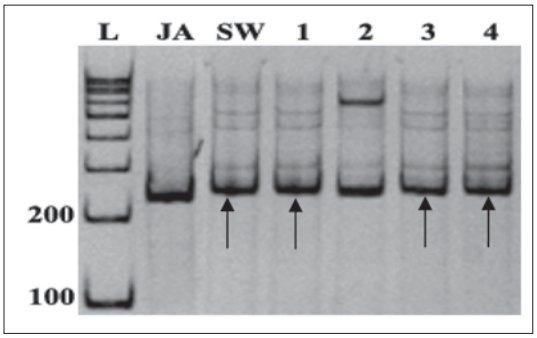

Figure 13. Genotypic screening of *Sub1* introgressed Jaya BC_2F_2 progenies with recombinant marker RM23788 Lane L: 100bp Ladder, Lane JA: Jaya, Lane SW: Swarna-*Sub1*, Lane 1-4: BC_2F_2 progeny. Arrows indicate homozygous bands similar to donor parent.

carbohydrate and poor survival. Several reports suggested that *Sub1 A* dominates over *Sub1 C* (Xu et al., 2006; Fukao et al., 2006). It was likely that presence of *Sub1 A* restricted shoot elongation.

In the study reported by Neeraja et al. (2007), they concentrated on reducing the linkage drag (2.3-3.4mb) to the minimum level because they used the wild variety FR13A derived line as the donor parent. Also, they tried to introgress *Sub1 A* alone to the progeny, as *Sub1 A* is the main determinant of submergence tolerance and *Sub1 B* and *Sub1 C* are already present in Swarna. But in our study we have concentrated on introgressing all the three ethylene response factor genes into the recurrent parent Jaya and the reduction of linkage drag was not much considered as both the recurrent parent and donor parent used were improved varieties. The presence of small portion of Swarna *Sub1* did not affect the phenotype and yield of the introgressed lines.

Background screening

Background selection involves successive backcrossing to remove the genetic background of the donor while recovering genetic properties of recurrent parent as much as possible. Except target locus, all genomic regions of the recurrent parent can be recovered in background selection using recurrent parent marker alleles (Hasan et al., 2015). After background selection, 10/50 plants in BC_1F_1 generation, 12/25 plants in BC_2F_1 generation and 14/80 plants in BC_2F_2 generation were selected. In BC_1F_1 generations, the percentage of recurrent parent genome recovery ranged from 36.5 to 70.3%, of which BC_1F_1-3 had the highest recurrent parent genome recovery of 70.3%, followed by BC_1F_1-5 (68.6%) and BC_1F_1-25 (66.6%) respectively. All these three plants were forwarded to next generation by backcrossing with recipient parent Jaya. In BC_2F_1 generation, recurrent parent genome recovery percentage ranged from 68.6 to 78.5%. BC_2F_1-3-2 had the highest recurrent parent genome recovery percentage of 78.5%, followed by BC_2F_1-3-5 (76.2%) and BC_2F_1-5-21(75.3%) respectively. In the selfed BC_2F_2 generation, the percentage of recurrent parent genome recovery ranged from 72.0 to 86.9% of which BC_2F_2 -3-2-13 had the highest recurrent parent genome recovery of 86.9% followed by BC_2F_2-5-21-12 (86.0%) and BC_2F_2-3-2-21 (85.9%) respectively. In BC_2F_2-3-2-13, the chromosome nos. 4, 5, 7, 8 and 10 were found to be similar to the recurrent parent Jaya. The percentages of homozygous markers for recipient alleles (A %), donor alleles (B %) and heterozygous (H %) of the selected *Sub1* introgressed BC_2F_2 progeny are shown in Table 5. Graphical genotype of the best plant BC_2F_2-3-2-13 had maximum recurrent parent genome recovery of 86.9% (Fig. 14). As backcross generation increased, the percentage recovery of the recurrent parent genome would also be increased upto 94-95%. The next generation is being

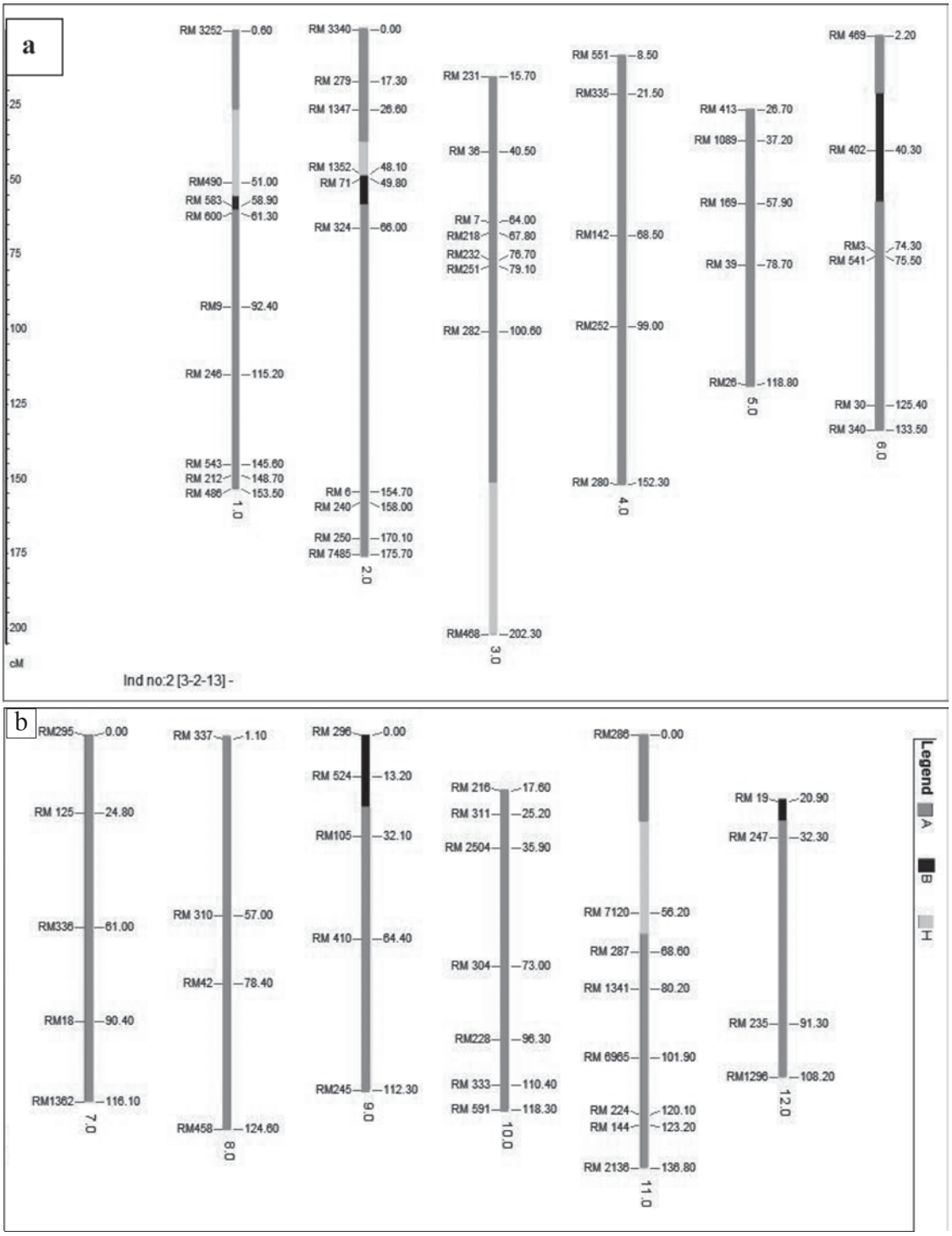

Figure 14. Graphical genotype of plant number 3-2-13 of BC_2F_2 population. 'a' indicate GGT of 1-6 chromosomes and 'b' indicate 7-12 chromosomes. The grey colored regions (A) on the chromosomes indicate homozygous regions for the recipient genome while the light grey colored regions (H) indicate the heterozygous regions and the black coloured regions (B) indicate the homozygous regions for the donor genome The distances were represented in cM based on published map of Temnykh et al. (2001).

Table 5. Percentage of recurrent parent genome recovery after background screening of BC_2F_2 progenies using GGT software

Sl. No.	Plant No.	Recurrent parent (A %)	Donor parent (B %)	Heterozygous (H %)
1.	BC_2F_2-3-2-13	86.9	5.0	8.1
2.	BC_2F_2-5-21-12	86.0	9.4	4.6
3.	BC_2F_2-3-2-21	85.9	4.3	9.8
4.	BC_2F_2-5-21-6	81.3	5.3	13.4
5.	BC_2F_2-5-21-1	80.7	9.2	10.1
6.	BC_2F_2-3-5-16	80.6	15.7	3.7
7.	BC_2F_2-5-21-14	79.5	2.9	17.6
8.	BC_2F_2-3-5-22	78.1	15.6	6.2
9.	BC_2F_2-3-2-6	78.0	10.9	11.1
10.	BC_2F_2-3-5-19	77.4	10.9	11.7
11.	BC_2F_2-3-5-4	75.0	12.1	12.8
12.	BC_2F_2-3-5-13	74.3	16.5	9.2
13.	BC_2F_2-3-5-3	74.2	15.5	10.3
14.	BC_2F_2-3-5-23	72.0	13.0	15.0

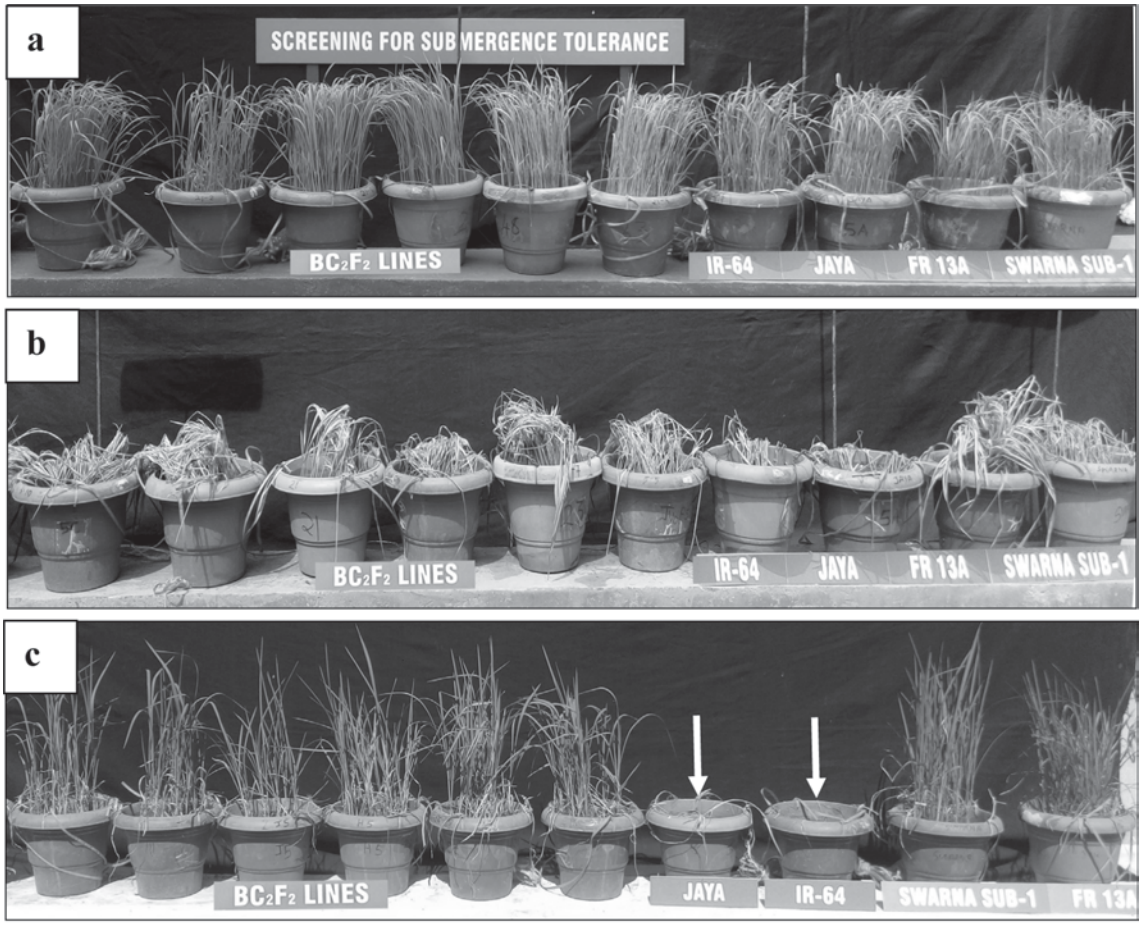

Figure 15. *In vitro* screening for submergence tolerance. **a** 14 day old plants, **b** de - submerged plants after 14 days of submergence, **c** survived plants after de - submergence. Arrows indicate susceptible check and recurrent parent which could not survive after de - submergence.

Table 6. SES Score after 14 days of de-submergence followed by 14 days of complete submergence

Plant no.	Number of plants before submergence screening	Number of plants survived after submergence screening	Survival percentage (%)	Mean value after arc sine transformation	Score
BC_2F_2-3-2-6	40	38.0[c]	95.0	77.079[c]	3
BC_2F_2-3-2-13	40	39.0[a]	97.5	80.903[a]	3
BC_2F_2-3-2-21	40	38.6[ab]	96.5	79.628[ab]	3
BC_2F_2-3-5-3	40	38.3[bc]	95.7	77.079[c]	3
BC_2F_2-3-5-4	40	38.3[bc]	95.7	78.354[bc]	3
BC_2F_2-3-5-13	40	38.0[c]	95.0	77.079[c]	3
BC_2F_2-3-5-16	40	38.0[c]	95.0	77.079[c]	3
BC_2F_2-3-5-19	40	38.3[bc]	95.7	78.354[bc]	3
BC_2F_2-3-5-22	40	38.0[c]	95.0	77.079[c]	3
BC_2F_2-3-5-23	40	38.0[c]	95.0	77.079[c]	3
BC_2F_2-5-21-1	40	38.6[ab]	96.5	79.628[ab]	3
BC_2F_2-5-21-6	40	38.6[ab]	96.5	79.628[ab]	3
BC_2F_2-5-21-12	40	39.0[a]	97.5	80.903[a]	3
BC_2F_2-5-21-14	40	38.6[ab]	96.5	79.628[ab]	3
Jaya	40	0.0[d]	0.0	0.716[d]	9
Swarna *Sub1*	40	39.0[a]	97.5	80.903[a]	3
CD(0.01)	-	0.818	-	2.915	-
CD(0.05)	-	0.608	-	2.164	-

developed and evaluated for better recovery percentage.

In vitro screening for submergence tolerance

The selected BC_2F_2 plants were screened to evaluate the introgression of *Sub1* QTL. After de-submergence, tolerant plants were selected based on the visual symptoms using IRRI's SES for submergence tolerance screening in rice (Fig.15). Plants which scored '3' showed similar pattern to that of Swarna *Sub1* and were found to be tolerant thus confirming the successful introgression of *Sub1* QTL (Table 6) whereas the recipient parent, Jaya showed zero survival. The data was subjected to analysis of variance using WASP 2.0 software, the first Web Based Agricultural Statistics Software Package developed by ICAR (www.icargoa.res.in). The plant numbered BC_2F_2-3-2-13 and BC_2F_2 -5-21-12 showed significantly higher survival rate after de - submergence and was statistically on par with the donor parent Swarna *Sub1*.

In the present study, 14 lines introgressed with *Sub1* locus could be generated in the BC_2F_2 generation. Out of these eight lines with above 78% background genome recovery of the recurrent parent were selected and backcrossed with donor parent Swarna *Sub1* to raise BC_3F_1 generation. The *Sub1* region in the range of 0.5-5.6 mb was introgressed in the developed lines. Simultaneously, these lines are being field evaluated. In recent years, incidence of flooding have increased due to the extreme weather events such as unexpected cyclonic heavy rains and consequent outflow of rivers which cause inundation and flooding of paddy fields, particularly in coastal and low lying areas. As rice is the only crop adapted to waterlogged areas, development of flood tolerant rice varieties is the only solution for cropping in the flooded / submerged areas. The *Sub1* gene introgression into the existing high yielding mega rice varieties suited to each location can meet such demand. After successive field evaluations, the *Sub1* introgressed lines developed in the present study can be released as high yielding submergence tolerant rice varieties.

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