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ORIGINAL ARTICLE

Introgression of *Sub1* Locus into Highly Preferred Rice Cultivars (Pooja and Pratikshya) in Eastern Region of India for Submergence Tolerance through Marker Assisted Backcrossing

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ABSTRACT
In this study, two popular semi-lowland rice cultivars Pooja and Pratikshya were developed for submergence tolerance by incorporation of Sub1 locus through marker assisted backcrossing (MABC) within two rounds of backcrossing (BC2F2) with desired genome. Progenies were selected based on its possession of the Sub1 locus and maximum recipient genome. The selected progenies were possessed Sub1 locus with an introgression size of 3.6Mb. And, in the submergence evaluation, more than 75 per cent of survival rate was observed in the BC2F2 generation. Furthermore, in reverse-transcriptase analysis, the expression of Sub1A gene was detected under submergence stress condition in both cultivars having Sub1 locus. These results confirm that the introgressed SUB1 into new genetic backgrounds is well accommodated. In agronomic traits in both recipients, there were non-significant differences were noted in the seed characters compared to original parents, Pooja and Pratikshya.

Keywords: *Conventional backcrossing, marker-assisted selection, sub1, gene expression, submergence stress, drought stress and foreground selection*

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INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population, but it's production is severely being affected by the impact of abiotic stresses due to frequently changing climate. Within completing a life cycle, rice plants are subjected to various abiotic stresses such as submergence, salinity, drought, cold, etc. Among them, flash flooding in rainfed lowlands causes a major loses to rice farmers by submerging rice seedlings for more than 10-days and it leads to death by anoxia. Especially, flooding is a major determining factor in the survival of rice seedlings in rainfed lowland areas. Because prolonged submergence of seedlings causes plant death due to anoxia. According to the nature of flooding there are different types i.e. by flash flood, plants are completely submerged for up to 2 weeks. Stagnant flooding occurs for a longer duration, more than 2 weeks and often several months, at depths up to 50 cm. In deeper stagnant flooding condition, water depth increases throughout the season to depths above 50 cm and often a meter or more.

Fortunately, a major tolerance source called, SUBMERGENCE 1 (Sub1) on chromosome 9 was identified for managing flash flooding in a highly flood tolerant (FR13A) genotype. This Sub1 locus encodes a variable cluster of up to three ethylene-responsive factor (ERF) genes: Sub1A, Sub1B and Sub1C [1, 11]. And, these genes are members of group VII in the ERF gene family [6] and it is reported that they are more closely related to one another than any other rice ERF genes by [3]. Among these three genes, two ERF genes, Sub1B and Sub1C are present in all indica and japonica accessions, whereas Sub1A appears to be limited to a subset of indica accessions. In Sub1A, there are two types of alleles are reported (Sub1A-1and Sub1A-2) which are distinguished by a single amino acid substitution within the coding region. The transcripts of Sub1A-1 and Sub1A-2 are highly and poorly induced under submergence, respectively [11]. In rice breeding, many rice cultivars, so far, improved for submergence tolerance using this Sub1 locus

through marker assisted breeding [4],[8],[10].In this study, we have to improve two rice cultivars (Cv. Pooja and Pratikshya) for submergence tolerance by incorporation of *Sub1* locus through marker assisted backcrossing (MABC). Significantly, these two rice cultivars are farmer adopted varieties in the eastern region of India since they both have very good agronomic characters. But, the states belonging to the eastern region are frequently affected by flooding due to cyclonic storm. Due to this, the rice cultivation is being severely affected by the submergence problem in rice cultivating areas very often. Therefore, we were taken a step to improve submergence tolerance in vastly cultivating rice varieties in this region.

MATERIALS AND METHODS

In this study, high-yielding rice cultivars, Pooja and Pratikshya were used as recurrent parent (RP) for improvement of submergence tolerance through conventional breeding with help of marker assisted selection and Swarna-Sub1 (SS1) was used as male parent (Donor) having SUB1 locus. This work was carried out in Central Rice Research Institute (CRRI), Cuttack, Odisha state, INDIA.

In the first step of submergence development, F1 population were derived by making a cross between Pooja or Pratikshya (recipient) and Swarna-Sub1 (Donor). The positive plants in the F_1 population were selected in the foreground selection using gene-specific markers (IYT1, IYT3, Sub1BC2, ART5 and Sub1C173) in the PCR screening (foreground selection) [10].Then, the positive plants were backcrossed with recurrent parent, (Pooja or Pratikshya) to derive BC_1F_1 population. For PCR amplification, a crude DNA preparation suitable for PCR screening was prepared using a simplified miniscale procedure [5]. PCR amplification was performed using genomic DNA at 56-59°C (annealing temperature). In the BC_1F_1 generation, positive plants were selected through foreground (only with IYT1 and Sub1BC2 markers) and recombinant selection. For recombinant selection, two flanking markers, RM23679 (0.8Mb) and RM219 (7.8Mb) located at both sides of *Sub1* locus were used. Thus selected desired plants in the BC_1F_1 generation were once again backcrossed with respective recurrent parent (Pooja or Pratikshya) to derive BC_2F_1 population. In the BC_2F_1 generation, desired progenies were selected in the foreground and recombinant selection [(RM23679 (0.8Mb), RM23662 (0.6Mb), RM23778 (3.9Mb) and RM23788 (4.2Mb) located at proximal end of *Sub1* locus and RM219 (7.8Mb), RM23957 (7.9Mb) and RM24071 (10.4Mb)] **[8]** and 50 per cent of the progenies were used for backcrossing purpose to get BC_3F_1 population and the remaining progenies were allowed for self-pollination to get BC_2F_2 population. In the BC_2F_2 population, the background selection was done using 3-5 microsatelite (SSR) markers per chromosome and the percentage of gene alleles of recurrent parent against donor was calculated [7]. Details of the polymorphic primers used for in the background selection is given in Table -1.

Table-1 Details of SSR markers used in the background selection in BC_2F_2 population.

S.No.	SSR markers	Chromosome	S.No.	SSR markers	Chromosome
1	RM12061	1	46	RM20834	7
2	RM11764	1	47	RM8035	7
3	RM10047	1	48	RM2966	7
4	RM8003	1	49	RM22175	7
5	RM11312	1	50	RM22905	8
6	RM11070	1	51	RM22720	8
7	RM8085	1	52	RM22459	8
8	RM10009	1	53	RM23345	8
9	RM6321	1	54	RM23645	8
10	RM6842	2	55	RM22720	8
11	RM13910	2	56	RM22905	8
12	RM12548	2	57	RM5891	8
13	RM1497	2	58	RM22688	8
14	RM12941	2	59	RM5899	9
15	RM13366	2	60	RM24904	9
16	RM6374	2	61	RM8058	9
17	RM14723	3	62	RM24663	9
18	RM14377	3	63	RM24037	9
19	RM15558	3	64	RM23911	9
20	RM15317	3	65	RM23778	9
21	RM15838	3	66	RM25239	10
22	RM16153	3	67	RM25086	10

23	RM14272	3	68	RM25427	10
24	RM551	4	69	RM25735	10
25	RM16699	4	70	RM5708	10
26	RM6679	4	71	RM6364	10
27	RM3473	4	72	RM5841	10
28	RM17611	4	73	RM26893	11
29	RM16577	4	74	RM3863	11
30	RM17417	4	75	RM27180	11
31	RM3853	5	76	RM27353	11
32	RM18004	5	77	RM27123	11
33	RM17780	5	78	RM6094	11
34	RM19183	5	79	RM27392	11
35	RM8215	5	80	RM26885	11
36	RM18384	5	81	RM3721	11
37	RM7329	6	82	RM26660	11
38	RM20003	6	83	RM28305	12
39	RM20158	6	84	RM27879	12
40	RM20667	6	85	RM28047	12
41	RM439	6	86	RM28610	12
42	RM21349	7	87	RM28616	12
43	RM1362	7	88	RM28070	12
44	RM5583	7	89	RM27900	12
45	RM22168	7	90	RM27446	12

In agronomic traits analysis, the seed character of BC_2F_2 population was analyzed in terms of seed width, length and 1000 grain weight with original recurrent parents using ANOVA method.

Furthermore, in the BC_2F_2 generation, the gene expression analyze was done for SUB1A and SUB1C genes through reverse transcriptase (RT)-PCR. For analysis, samples were collected from rice seedlings of BC_2F_2 population with *Sub1* locus with homozygous condition and recurrent parent (Pooja or Pratikshya) under submergence condition at 0 h and 48 h and 100 mg leaf tissue was ground in liquid nitrogen using mortar and pestle. Total RNA was extracted using TRIzol reagent according to manufacturer's instructions. The RNA pellet was dissolved in 50µl RNase free water and stored at –20ºC. The quality and quantity of total RNA were analysed by gel visualization in a 1.5% Tris–boric–EDTA–agarose gel stained with ethidium bromide and by spectrophotometric analysis. cDNA sysnthesis was done using SuperScript™ III Reverse Transcriptase according to manufacturer's protocol (Invitrogen, California, USA) in a reaction mixture containing 50–75 ng RNA with the final volume completed to 20µL using RNase free water. PCR amplification was carried out at 56ºC using cDNA with primer sequences of SUB1A and SUB1C primers [2]**.** The primer sequence of Rubisco was used as a loading control.

Additionally, the drought tolerance screening was done in Pooja and Pratikshya with Sub1 locus or without it. For this, seedlings grown in pots were stress imposed at 21-day old seedling stage by withholding the irrigation for 10-days. Then, the seedlings were re-irrigated and allowed for the regeneration of seedlings and the survival percentage was calculated. Simultaneously, leaves were collected for RNA extraction to analysis the expression of drought-inducible genes under control and drought stress condition through RT-PCR [2]

RESULTS AND DISCUSSION

In the first cross between recipient and donor parent, more than one hundred F1 seeds were derived for both cultivars. In the selection of positive plants through PCR screening in F1generation, we got gene amplification with CAPS markers, IYT1 and IYT3 in plants those having only Sub1 QTL and no gene amplification was found for none of the recipient genome. With these markers, we got only single band in positive plants, but not double band (Heterozygous) in F1 population. But, with other markers, Sub1BC2, ART5 and Sub1C173, there were positive plants with heterozygous condition (double band). However, the positive result obtained only with Sub1BC2 marker were coincided with that of IYT1 and IYT3 markers located in the promoter region of SUB1A gene which is the major determinant for submergence tolerance, but not with ART5 and Sub1C173 markers. Generally, Sub1A is more closely related to Sub1B than Sub1C due to the high amino acid sequence similarity at the N-terminus shared by Sub1A and Sub1B,

but not Sub1C**[1]**. Furthermore, we found that the results of IYT1, IYT3 and Sub1BC2 markers coincided with the phenotypic results also compared to ART5 and Sub1C173 **(Fig.1).**

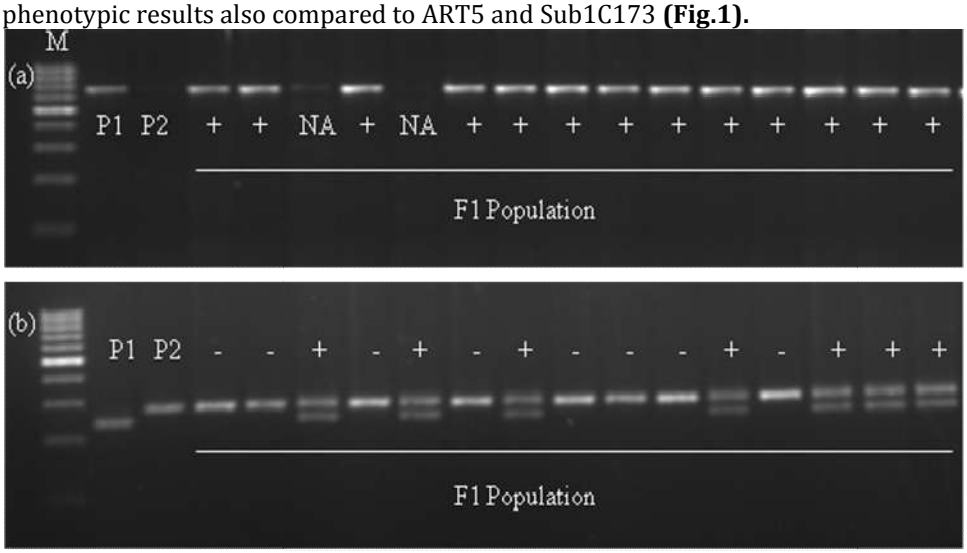


Figure 1. Selection of positive plants from F1 population using intragenic DNA markers (a) IYT1 and (b) Sub1BC2. P1: Donor (SS1), P2: recipient (Pooja), **'+'** – positive plants and **'-'** – negative plants; **NA** – No amplification (negative plants).

In the F1 generation, 17 plants for Pooja and 19 plants for Pratikshya were selected as positive in the foreground selection. And, in the backcrossing with respective recurrent parent, 131 BC_1F_1seeds for Pooja and 136 BC_1F_1 seeds for Pratikshya were derived. In the BC_1F_1 generation, 61 plants for Pooja and 74 plants for Pratikshya were selected as positive with IYT1 and Sub1BC2 markers. Among these, 10 progenies having fixed alleles of recipient genome for Pooja and 3 progenies for Pratikshya were selected in the recombinant selection with flanking marker, RM219 located on distal side of the *Sub1* locus. These plants were found for possessing alleles in heterozygous condition on other side (Proximal side) of the locus with RM23679 marker. And, the rest of the plants were found to be possessed alleles in heterozygous condition on both sides of the *Sub1* locus. Thus selected plants (10 plants for Pooja and 3 plants for Pratikshya) were again backcrossed with respective recurrent parent and 129 BC_2F_1 seeds for Pooja and 318 BC_2F_1 seeds for Pratikshya were derived.

In the BC2F1 generation, 61 plants from 129 plants for Pooja and 153 plants from 318 plants for Pratikshya were selected as positive in the submergence screening and foreground selection (Fig.2). Subsequently, 11 progenies for Pooja and 5 progenies for Pratikshya were selected based on its possession of the *Sub1* locus and maximum recipient genome in the recombinant selection. Finally, in the selected plants the introgression size of *Sub1* locus was identified in between RM23788 located at 4.2Mb and RM219 located at 7.8Mb.

Alongwith these markers, thus selected plants were found for having recipient's alleles in fixed condition with RM23662 (0.6Mb), RM23679 (0.8Mb), RM23778 (3.9Mb), RM23957 (7.9Mb) and RM24071 (10.4Mb) markers (Fig.3). Then, these plants were allowed to self-pollination to obtained BC_2F_2 population. In this way, many mega rice varieties have been so far developed for submergence-tolerance having an introgression size of 6.5-7.3Mb for Swarna-Sub1, 6.5-3.4Mb for Samba Mahsuri-Sub1, 6.5-7.8Mb for IR64-Sub1 and 2.7-6.4Mb for CR1009-Sub1 in BC2 generation. The developed marker assisted backcrossing(MAB) system is used efficiently in converting additional tolerant varieties derived from one of the mega varieties such as Swarna, IR64, etc., as early as the BC_1F_2 generation. In the present study also, one the recipients Cv. Pratikshya is closely related to Swarna. Similarly, IR64-Sub1 is being widely used in breeding in south and South-east Asia and in Africa. Moreover, it has been reported that in cases where the recipient genome is closely related to the Sub1 donor, the MAB approach facilitates the identification of ideal plants without using recombinant selection [10].

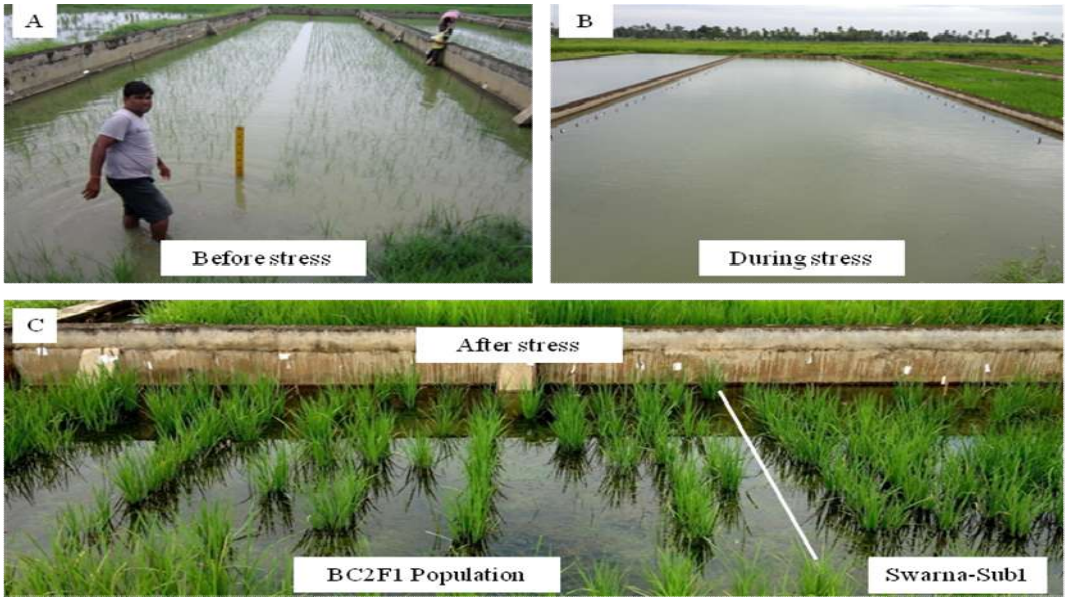


Figure 2. Submergence screening for selection of tolerant plants in BC_2F_1 population of Pooja and Pratikshya. **A:** 21-days old seedlings before submergence stress; **B:** the water level maintained at 95cm during stress and **C: s**urvived rice seedlings of BC_2F_1 population and the Donor (SS1) followed by de-submergence.

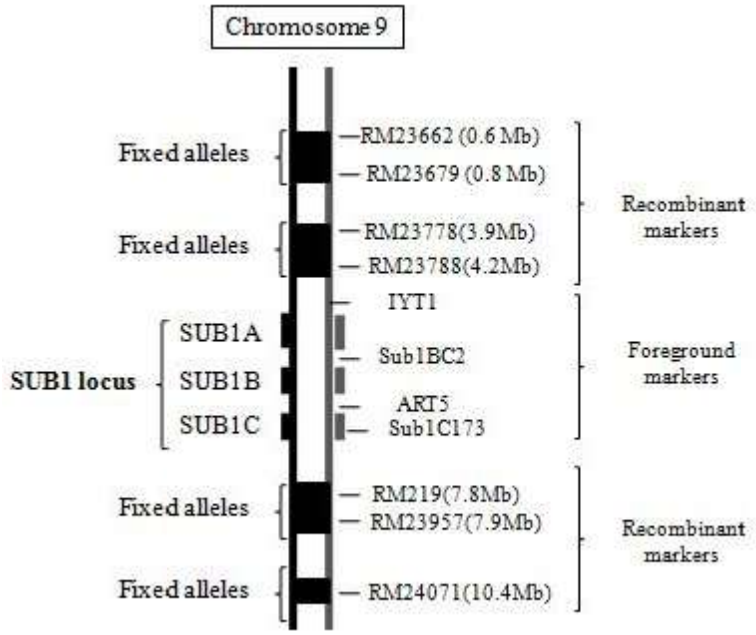


Figure 3. Details of gene-specific and SSR markers used in the foreground and recombinant selection and their position of chromosome 9. Black boxes indicate the fixed allele of recipient with flanking markers located on both sides of Sub1 locus.

In the RT-PCR analysis, the expression of Sub1A and Sub1C genes is undetectable under submerged condition at 0h in both Sub1 and non-Sub1 plants. But, at 48H, it was observed that in Sub1 plants expression of Sub1A in Pooja-Sub1 and Pratikshya-Sub1 was found to be induced compared to Sub1C as well as with that of Pooja and Pratikshya (Fig.4). The limited expression of Sub1C is associated with tolerance by the process of repression of shoot elongation and carbohydrate consumption by Sub1A under submergence stress due to reduced ethylene-mediated gibberelin (GA) biosynthesis and Sub1C supression [2],[9]**.** From this gene expression analysis, it is confirmed that the introgressed Sub1 locus has accommodated into two different genetic backgrounds (Cv. Pooja and Pratikshya) and it performs well.

In the background selection, an effort of checking the allele's percentage of recurrent parent and donor alleles in BC_2F_2 population using polymorphic SSR markers throughout 1-12 chromosomes, we found that the percentage of donor's alleles was found less than 20 per cent and the recurrent parent's allele was found more than 90 per cent in many plants (Fig.5) and also the rate of segregating alleles (heterozygous condition) were found in less than 10 per cent.

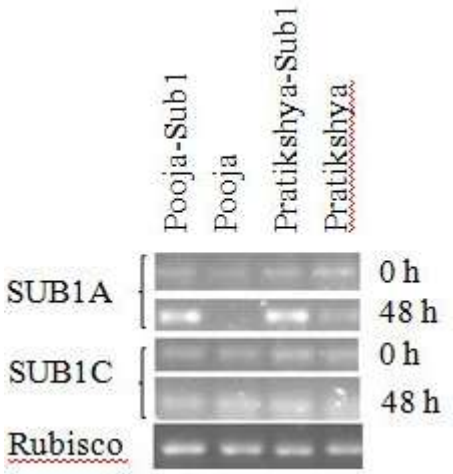
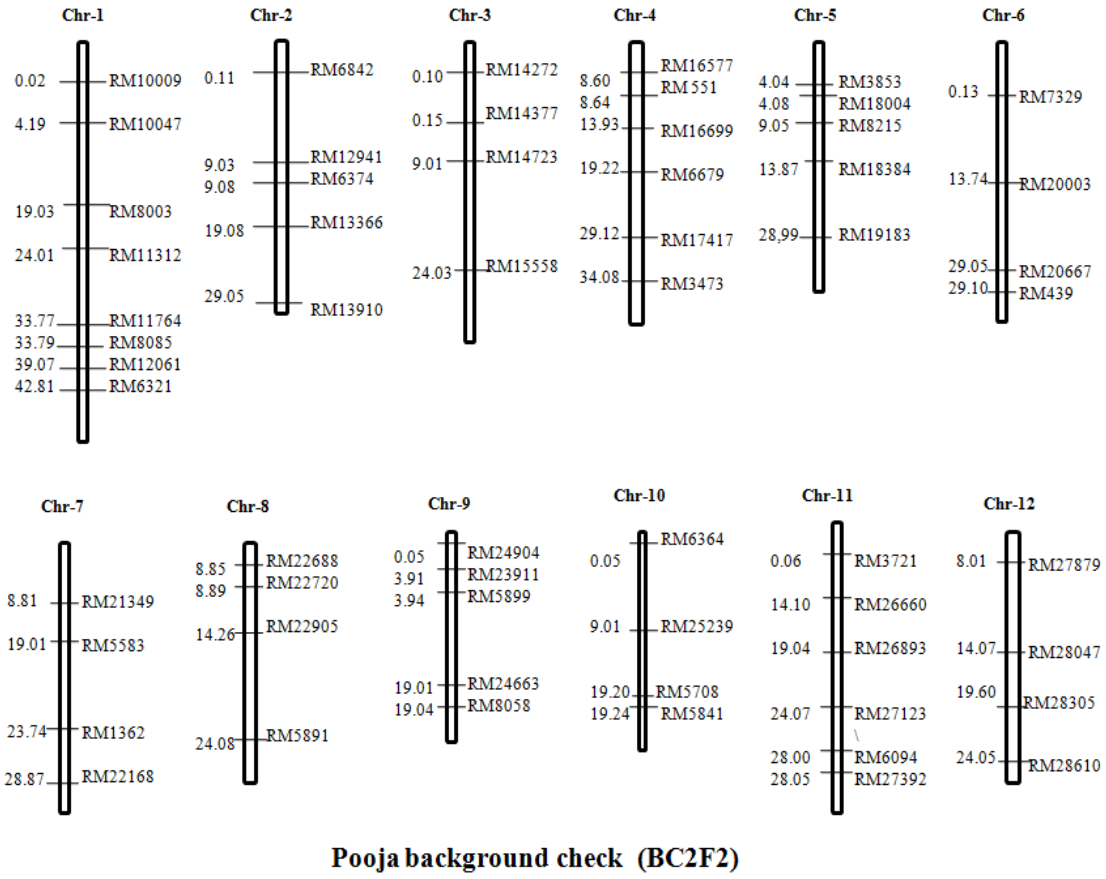


Figure 4. Expression of Sub1A and Sub1C genes in Pooja and Pratikshya with Sub1 locus or without it was analysed through RT-PCR technique at 0 h and 48 h under submergence condition. Rubisco primer was used as loading control



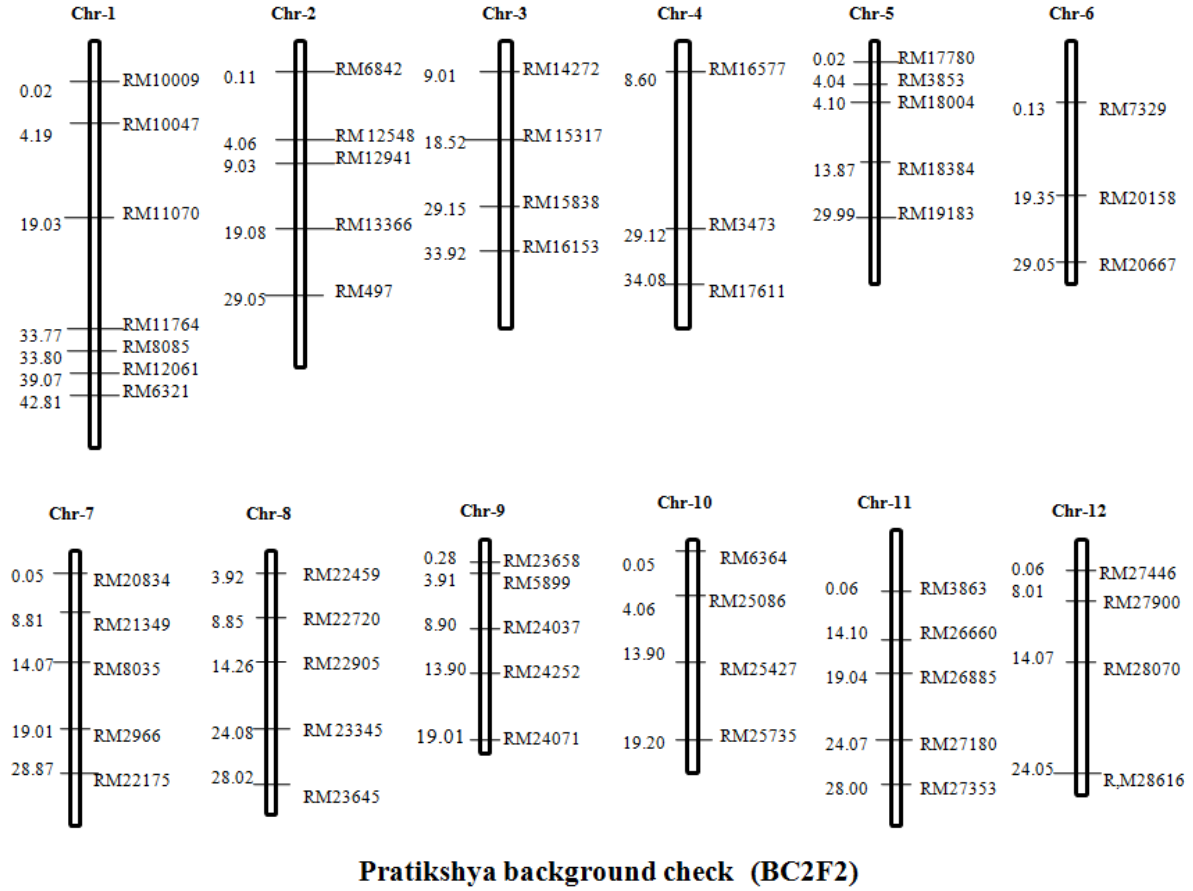


Figure 5. Graphical genotype of Pooja-Sub1 and Pratikshya-Sub1 plants (BC2F2) that is homozygous for the recipient genome. The distances were represented based on published map of Narsimulu G, et al. (2010)

In agronomic traits, particularly seed length, width and grain weight of developed BC2F2 plants were found to be more or less similar to recurrent parental characters in both cases of *Pooja* and *Pratikshya* (Table -2a-c; Fig. 6). Besides, it was noted that the day of flowering of BC population did not affect by crossing and the day of their flowering coincided to respective recurrent parent.

Table-2a shows the mean value of agronomic trait in terms of seed length of developed BC2F2 population and its recurrent parent.

Source	Degree of Freedom	TYPE III SS	Mean Square	F Value	Pr>F	Significant
Variety	1	0.0241	0.0241	3.0021	0.2253	NS
Replication	2	0.0009	0.0004	0.0561	0.9469	NS
Error	2	0.0160	0.0080	-	-	-

** - significant at 1%; * - significant at 5%; NS – Non Significant

Table-2b shows the mean value of agronomic trait in terms of seed width of developed BC2F2 population and its recurrent parent.

Source	Degree of Freedom	TYPE III SS	Mean Square	F Value	Pr>F	Significant
Variety	1	0.2400	0.2400	43.2432	0.0224	*
Replication	2	0.0090	0.0045	0.8138	0.5513	NS
Error	2	0.0111	0.0056	-	-	-

** - significant at 1%; * - significant at 5%; NS – Non Significant

Table-2c shows the mean value of agronomic trait in terms of 1000 seed weight of developed BC2F2 population and its recurrent parent.

Source	Degree of Freedom	TYPE III SS	Mean Square	F Value	Pr>F	Significant
Variety	1	0.0105	0.0105	0.8344	0.4575	NS
Replication	2	0.0331	0.0165	1.3136	0.4322	NS
Error	2	0.0252	0.0126	-	-	-

** - significant at 1%; * - significant at 5%; NS – Non Significant

Furthermore, in an additional effort to check drought tolerance in SUB1 lines, the regeneration rate was higher in Cv.Pooja-Sub1 (80%) compared to Cv.Pratikshya-Sub1 (75%). But, in both cultivars with no SUB1 did not regenerate (0%) followed by re-irrigation **(Fig.7a).** Following this screening, in the gene expression analysis with drought-induced genes (DREB1A, DREB1E, SalT, LIP9, LEA3 and Rab16A), DREB1A gene expression was strongly induced in all rice lines with SUB1 (Pooja, Pratikshya and Swarna) rather than other genes **(Fig.7b).** In a previous study also, the association of SUB1 with tolerance to drought stress has been demonstrated through expression of drought-inducing genes in rice line with SUB1 and not in non-SUB1 line under drought condition [2].

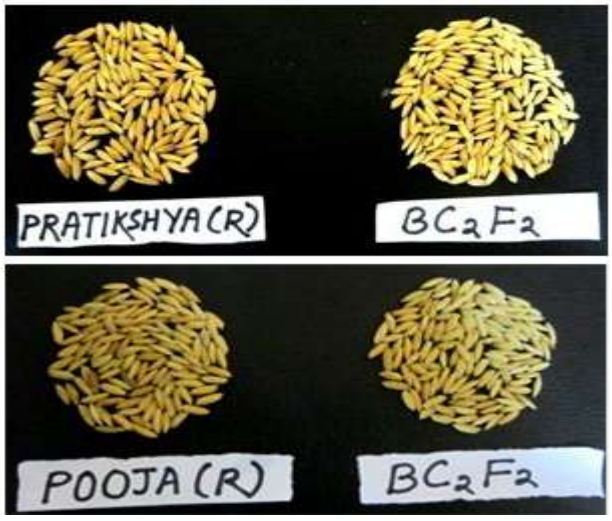


Figure 6. Shows the grain appearance of BC2F2 population of Pooja and Pratikshya and their original parent

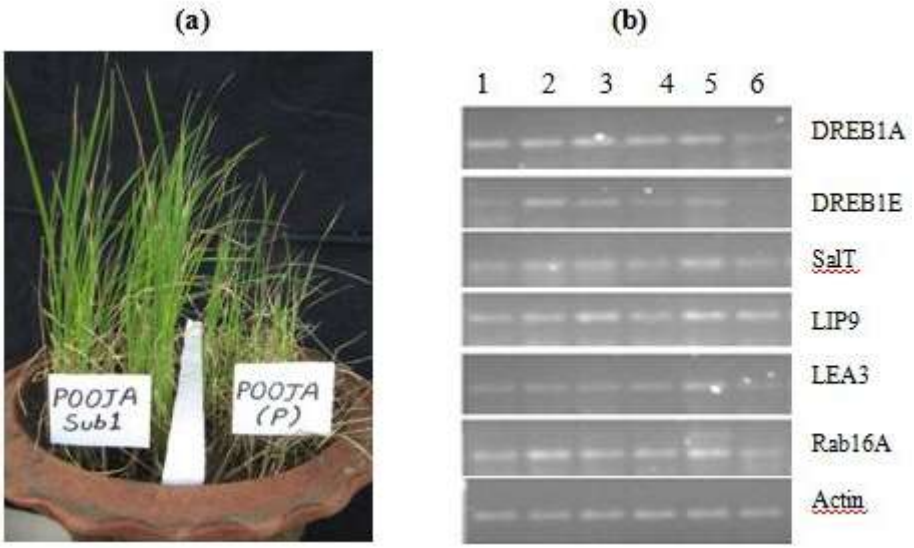


Figure 7. (a) Drought experiment. Regeneration of Pooja-Sub1 and Pooja (RP) without Sub1 followed by drought stress. Stress was imposed on seedlings at twenty-one days-old for 10 days. **(b). Reverse Transcriptase (RT) PCR analysis.** Expression of drought related genes in response to drought stress and

normal condition in BC2F2 plants, their recurrent parents and donor parent. Lane: **1-**Swarna-Sub1; **2-**Pooja (RP); **3**-Pooja-Sub1 (BC2F2); 4-Pratikshya (RP); 5-Pratikshya-Sub1; 6-control condition.

Thus, in the present study, the popular high-yielding rice cultivars, Pooja and Pratikshya are developed within two rounds of backcrossing and one generation of self-pollination following the marker assisted backcrossing (MABC) procedure using Swarna-Sub1 as donor. Similarly, many popular rice cultivars such as *Swarna-Sub1* [8], *IR64-Sub1*, [10] *BR11-Sub1* [4]**,** etc have been developed through marker assisted backcrossing for submergence tolerance. Therefore, our effort in the development of submergence tolerant varieties (Pooja-Sub1 and Pratikshya-Sub1) will be a big challenge to the increasing vulnerability of rice farming to frequent flash floods in eastern region of India.

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