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RESEARCH ARTICLE

Morpho-molecular investigation of recurrent parent genome recovery of *Sub1* introgressed advance lines of rice (*Oryza sativa* L.)

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Abstract

Abiotic stresses are known to affect rice productivity and production world over. Developing abiotic stress tolerant rice varieties with sustainable production is a major challenge for the rice breeders. Flooding damages the rice crop in coastal areas the world over. The present investigation was carried out to estimate genome recovery in 32 BC_2F_3 lines comprising of 24 deriving from the cross Ranjit × Swarna-Sub1 and 8 from of the cross Bahadur × Swarna-Sub1. The BC_2F_3 introgressed Sub1 lines exhibited tolerance in submerged conditions. Background recovery of the recurrent parent was accomplished in terms of sixty-two morpho-physiological characters recorded. The best lines with >74 % background recovery in morpho-physiological characters were selected and analyzed with 69 polymorphic molecular markers which exhibited >86.68 % background recovery of the recurrent parent genome. The advance BC_2F_3 line E-6 and 1189-1 in the background of Ranjit and Bahadur exhibited the highest similarity towards the recurrent parents. Significant variation was recorded in the *Sub1* introgressed advanced BC_2F_3 lines on that account. These advanced BC_2F_3 lines are expected to benefit the flood-affected farmers of Assam and other flood-affected parts of India.

Keywords: Advanced BC_2F_3 lines, Bahadur, background recovery, GGT, Ranjit, *Sub1* QTL

Introduction

For over half of the global population, rice is the most vitally important source of food. As the global population is rising at a rapid pace every year will necessitate increasing food production (Muthu et al. 2020) to which rice contributes very significantly. In order to fulfil the demand, the total production, of rice must reach 160 million tonnes by 2050 (Ashikari et al. 2015). Global rice production is declining under the pressures of a constantly changing climate (Kumar et al. 2020), and these are significant constraints on meeting the ever-increasing food demand for an exponentially growing population and maintaining global food security (Lesk et al. 2016). Coastal rice ecosystems covering over 16% of the world's rice fields ($20×10^6$ ha) are significantly impacted by annual floods (Ismail et al. 2013). Rice fields are either vulnerable to sudden floods or long-term floods in these flood-prone lowlands (Rumanti et al. 2018). Flash floods affect rice plants to submerge few days to two weeks, while long-term partial submergence at close depth 50 cm (medium to semi-deep), typically regarded as stagnant flooding, normally takes several weeks to certain months (Septiningsih et al. 2018). Submergence damage (Yield loss) varies from 10 to 100% based on the rice variety, period of the flood, intensity, and conditions of the flood. Assam is a state which has a rich center of genetic diversity (Sahoo et al. 2019), and a major state of rice growing in north-eastern India (Sharma et al. 2017), occupies the area of 2.6 million ha, about one million ha of which is affected by flash flooding (Chetia et al. 2018). Farmers continue to use their traditional varieties in this region, which meet their taste and ensure crop health.

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Traditional rice varieties suited to these conditions are of low yielding (0.5 to 2.0 t/ha), which is below the yield of more appropriate suitable lowland cultivars (Mackill et al. 2012). Therefore, the incorporation of submergence tolerance into existing common elite rice cultivars have been a promising solution to cope with the consequences of submergence and decreases yield losses during flood conditions. A single major quantitative trait locus (QTL) on chromosome 9 controls submergence tolerance, together with some minor effect QTLs (Toojinda et al. 2003). An important major effect QTL *Sub1* from a landrace FR13A has been reported (Xu et al. 1996; Septiningsih et al. 2014), and describing a 69 % of phenotypic variance that gives tolerance up to two weeks under submergence situation. Using marker-assisted backcross breeding, mega varieties like Swarna (Neeraja et al. 2007), Ciherang (Septiningsih et al. 2014; Toledo et al. 2015), and PSB Rc18 (Septiningsih et al. 2014), now can withstand submergence effectively. Marker-assisted backcrossing (MABC) is a specific and efficient approach to introgress a single loci that governs the trait of interest while maintaining the important traits or background genome of the recurrent parent (RP) (Collard et al. 2008). This strategy has been used to improve rice varieties for various traits of interest such as submergence tolerance through the transfer of *Sub1* genomic region (Septiningsih et al. 2009). North-East India, including Assam, is regarded to be one of the main rice origin centers representing a precious rice gene pool reservoir. *Sali* rice is cultivated as a rainfed crop, covering about 1.8 million hectares. Flood impacts one million hectares of the cropped area in particular years in Assam (Chetia et al. 2018) and therefore it is the most important issue, affecting all three courses of rice (Gautam et al. 2022).

One of the simplest and cost-effective alternatives to solve the problem of saving the crop from floods is to breed high yielding submergence tolerant varieties to be introduced in flood-prone areas. The most popular elite rice varieties in Assam are Ranjit and Bahadur, which comprise approximately 65 % of the *Sali* rice production in the state (Chetia et al. 2018). Unfortunately, these varieties are susceptible to flooding conditions and suffer major yield losses. Therefore, the development of *Sub1* genomic region introgressed version of Ranjit and Bahadur will be addressed in the present investigation. The identification of flood tolerant lines from the populations derived from backcross breeding may hasten the process of selection. Marker-assisted backcrossing methodology was followed utilizing SSR markers administering two backcrosses and three selfing generations BC_2-F_3 population was developed. Therefore, The present study was conducted to select suitable advanced lines with *Sub 1* gene in the background of recipient varieties Ranjit and Bahadur to mitigate the yield losses in the flood-prone areas of Assam and elsewhere.

Materials and methods

Plant materials

A traditional high-yielding, semi-tall statured and susceptible to flooding rice varieties, Ranjit and Bahadur were chosen as recurrent parent. These varieties are recommended for rainfed shallow lowland conditions for submergence improvement (Singh et al. 2016; Chetia et al. 2018). Swarna-*Sub1* variety harboring the *Sub1* genomic region (Neeraja et al. 2007; Singh et al. 2016) was used as the donor parent (DP). The plant material for the present investigation consisted of 32 *Sub1* introgressed advanced BC_2F_3 lines (24 *Sub1* introgressed advance lines in Ranjit and 8 *Sub1* introgressed advanced lines in Bahadur) with three parents i.e. Ranjit, Bahadur, (RP) and Swarna-*Sub1* (DP). Resistant checks (Swarna-*Sub1*, FR13A) and susceptible checks (Swarna, Ranjit, Bahadur and IR42) were used in phenotypic evaluation of lines (Table 1). The field trials using standard protocols (Xu et al. 2000), were carried out at Regional Agricultural Research Station Titabar, Assam, India.

Background recovery of recurrent parent

Morpho-physiological characters were used to assess recurrent parent background recovery. Data were recorded for 62 morpho-physiological characters (Table 2) for Ranjit and Bahadur lines as per the protection of plant varieties and farmers right authority (PPV and FRA). Evaluation was done to identify the best line that resembles morphologically similar to the recurrent parents, Ranjit, and Bahadur. The calculation for background recovery with morpho-physiological traits was done by using the following formula:

Dissimilarity with recurrent parent (%) = (No of traits which shows variation among the DUS characters with the recurrent parent)/Total number of DUS observations recorded) × 100 and similarity with recurrent parent (%) = (100 - Dissimilarity with recurrent parent).

Based on the per cent recovery in morpho-physiological characters, advance lines similar to RP were selected for background recovery analysis with the aid of SSR markers.

Molecular marker analysis

Genomic DNA was extracted from 3 weeks old seedlings of BC_2F_3 advance lines including parents with minor modifications of miniprep protocol (Dellaporta et al. 1983). Quality of DNA was checked and resolved in 0.8% agarose gel. PCR amplification was carried out in 10 µL reactions containing 10 ng/µL of DNA template, 1 µL TBE buffer (containing 200 mM Tris-HCl with pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 0.25 µL of 1 mM dNTP, 0.5 µL of 5 µM forward and reverse primers, and 0.25 µL of Taq DNA polymerase (4 U/µL) (Initial denaturation for 5 minutes at 94°C, followed by 35 cycles for 1-minute denaturation at 94°C, 1-minute annealing at 55°C, and 2 minutes extension at 72°C with a final extension for 5 minute at 72°C). PCR products have

Table 1. List of Genotypes used in the investigation

Plant material	Genotypes
Resistant check varieties	Swarna-Sub1, FR13A
Susceptible check varieties	IR 42, Swarna, Ranjit, Bahadur
Test entries	Y-374, C-210, 1160-2, Y-219, N-6, S-4, 1160-1, N-9, 1316-2, W-167, J-329, C-376, Y-350, L-2, C-196, U-86, N-3, J-164, L-3, D-160, C-367, Z-1, E-6, D-65, Z-8, D-136, A-193, 1241-1, 1189-1, 1324-1, 1398-1 and 1384-1.

Table 2. List of different morpho-physiological characters used in the study

S. No.	Characteristics	S. No.	Characteristics
1	Coleoptile: Colour	32	Stem: Anthocyanin colouration of internodes
2	Basal leaf: Sheath colour	33	Panicle: Length of main axis
3	Leaf: Intensity of green colour	34	Flag leaf: Attitude of blade (late observation)
4	Leaf: Anthocyanin colouration	35	Panicle: Curvature of main axis
5	Leaf: Distribution of anthocyanin colouration	36	Panicle: Number per plant
6	Leaf Sheath: anthocyanin colouration	37	Spikelet: Colour of tip of lemma
7	Leaf sheath: Intensity of anthocyanin colouration	38	Lemma and Palea: Colour
8	Leaf: Pubescence of blade surface	39	Panicle: Awns
9	Leaf: Auricles	40	Panicle: Colour of awns (late observation)
10	Leaf: Anthocyanin colouration of auricles	41	Panicle: Length of longest awn
11	Leaf: Collar	42	Panicle: Distribution of awns
12	Leaf: Anthocyanin colouration of collar	43	Panicle : Presence of secondary branching
13	Leaf: Ligule	44	Panicle: Secondary branching
14	Leaf: Shape of ligule	45	Panicle: Attitude of branches
15	Leaf: Colour of ligule	46	Panicle: Exertion
16	Leaf: Length of blade in (cm)	47	Time maturity (days)
17	Leaf: Width of blade (cm)	48	Leaf: Senescence
18	Culm: Attitude (for floating rice only)	49	Sterile lemma: Colour
19	Culm: attitude	50	Grain: Weight of 1000 fully developed grains
20	Time of heading (50% of plants with panicles)	51	Grain: Length
21	Flag leaf: Attitude of blade (early observation)	52	Grain: Width
22	Spikelet: Density of pubescence of lemma	53	Grain: Phenol reaction of lemma
23	Male sterility	54	Decorticated grain: Length
24	Lemma: Anthocyanin colouration of keel	55	Decorticated grain: Width
25	Lemma: Anthocyanin colouration of area below apex	56	Decorticated grain: Shape (in lateral view)
26	Lemma: Anthocyanin colouration of apex	57	Decorticated grain: colour
27	Spikelet: Colour of stigma	58	Endosperm: Presence of amylose
28	Stem: Thickness(cm)	59	Endosperm: Content of amylose
29	Stem: Length (excluding panicle; excluding floating rice)(cm)	60	Varieties with endosperm of amylose absent only Polished grain: Expression of white core
30	Stem: Anthocyanin colouration of nodes	61	Gelatinization temperature through alkali spreading value
31	Stem: Intensity of anthocyanin coloration of nodes	62	Decorticated grain: Aroma

been mixed with bromophenol blue gel loading dye and analyzed on 3.5% agarose gel. The bands were visualized after 0.5 mg/mL EtBr gel staining and were documented using gel imager (UVP, UK).

Background recurrent parent genome (RPG) selection with SSR markers

For background selection, 69 polymorphic SSR markers were selected from a parental polymorphism survey done

Table 3. Per cent background recovery of recurrent parent based on morpho-physiological data

Similarity in percentage	*Sub1* introgressed lines
>74	E-6, C-210, C-196, C210, 1160-1, 1189-1, 1324-1, 1241-1, N-6, D-160, L-2, L-3
66-73	1160-2, N-3, Z-8, 1316-2, 1398-1, S-4, 1384-1, Y-374, C-367, N-9, J-164, C-376, D-136, Z-1
<65	Y-350, U-86, Y-219, D-65, J-329, A-193, W-167

Table 4. Background recovery of recurrent parent genome based on the morpho-physiological data and SSR markers genotyping analysis

S. No	Entries	Background recovery with morpho-physiological traits (%)	Background recovery with SSR markers (%)
1	E-6	79.04	93.47
2	C-210	75.81	89.85
3	C-196	74.20	86.68
4	1189-1	77.42	97.82
5	1160-1	75.81	89.85

in elite rice varieties of Assam (Verma et al. 2017). These polymorphic SSR/microsatellite markers, which were well distributed on the 12 linkage group of rice, were used for analyzing background recovery of RPG in five genotypes.

Statistical analysis

Based on a known molecular weight ladder, the molecular weight of PCR products obtained from SSR analysis for each primer was determined. During band scoring, intense bands are scored according to presence or absence of an amplified particular DNA fragment. Scoring for the genotyping data was done by use of the GGT user manual. Ranjit type band was designated as "A" and Swarna-*Sub1* type band was designated as "B". Similarly scoring for Bahadur type band was designated as "A" and scoring for the heterozygous band was designated as "H". To mitigate the risk of mis-scoring markers PCR products, showing stability in subsequent amplifications were chosen. The SSR data were analyzed by using the software package, Graphical Genotype (GGT) pc Version 2.0 (Van et al. 2008), to calculate the background recovery of RPG in the advance *Sub1* introgressed BC_2F_3 lines with the background of Ranjit and Bahadur.

Results

Background recovery of recurrent parent with morpho-physiological characters

The various observations recorded for background recovery of a RP based on the morpho-physiological traits and significant variations were found in the *Sub1* genomic region introgressed BC_2F_3 advance lines. The per cent background recovery of RP ranged from 57 to 79.04% (Table 3).

Background selection with SSR markers genotyping analysis

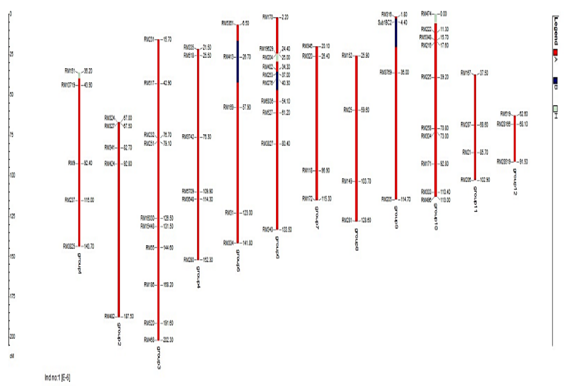
The three *Sub1* introgressed advanced lines in Ranjit background and two *Sub1* introgressed advanced in Bahadur background were selected based on the morpho-physiological background recovery from test entries and were analyzed by using 69 polymorphic SSR markers. The recovery percentage of background markers for the parents ranges from 86.69 to 97.82% in the selected plants The maximum RPG recovery percentage was 93.47 in E-6 and 97.82 in 1189-1 (Table 4). A graphical map with all the polymorphic markers shown in Fig.1 was constructed.

Background recovery analysis

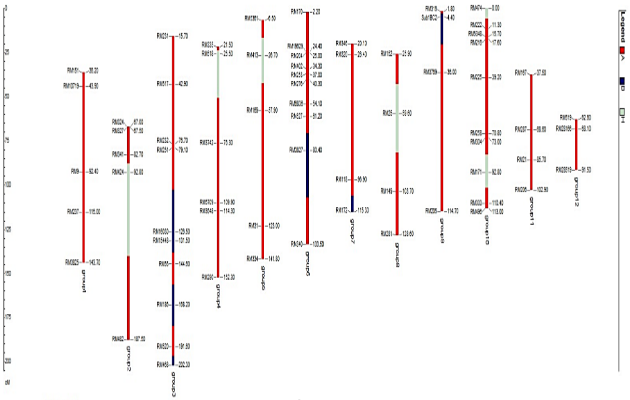
In the present investigation, the genotype E-6 showed 79.04% background recovery of RPG by phenotypic evaluation, whereas in genotyping analysis showed 93.47% recovery (Fig. 1a). The genotype C-210 showed 75.81% recovery of RPG by phenotypic evaluation and genotyping analysis showed 89.85% recovery (Fig. 1b). In genotype C-196, the recovery % of RPG was found to be 74.20% by phenotypic evaluation. In a genotyping analysis, it was found as 86.68% (Fig. 1c). The background recovery of RPG of the genotype 1189-1 was found to be 77.42% by phenotypic evaluation and 97.82% was found in genotyping analysis (Fig. 1d). The % background recovery of RPG of the genotype 1160-1 has been found 75.81 by phenotypic evaluation and 89.85% in genotyping analysis (Fig. 1e). The genotypic screening of *Sub1* genomic region introgressed BC_2F_3 advance lines including parents has been summarized. Some representative gel pictures of genotypic screening are given in Fig. 2.

Discussion

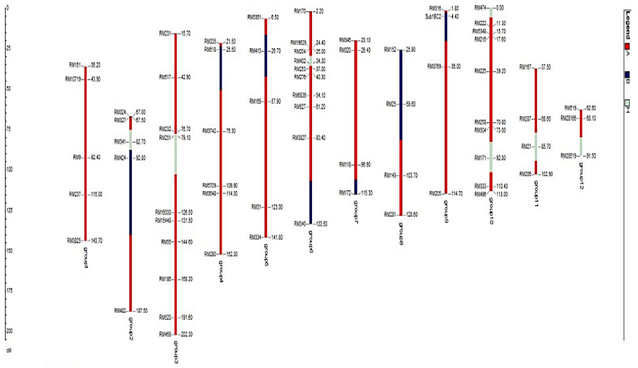
Development of suitable varieties with higher levels of tolerance against different biotic and abiotic stresses provides a better option, if the sources of tolerance are effective at different stages of plant growth. Genetic introgressions are playing a significant role in the improvement in different crops including rice (Zhang and Xie 2014; Basavraj et al. 2022). The rice varieties Ranjit and Bahadur are highly popular in Assam the eastern region of the country. The rice is being cultivated in lowland rainfed and marginal areas of Assam where flooding is a major problem, A pioneering work on *Sub1* QTLs linked to sub-mergence tolerance is



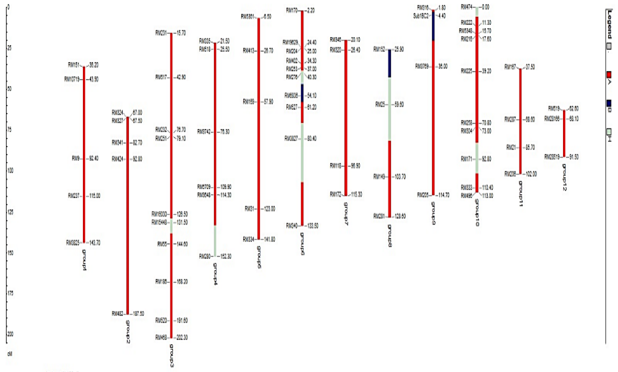
1a. E-6



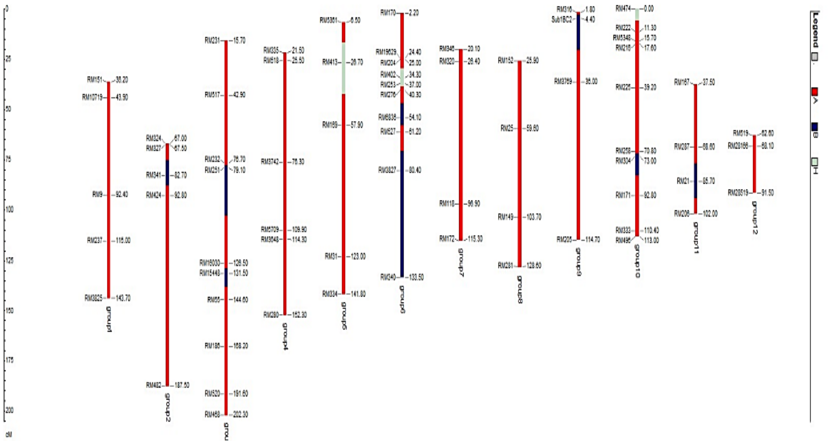
1b. C-210



1c. C-196



1d. 1189-1



1e. 1160-1

Fig. 1. Graphical representation of background recovery map of *Sub1* genomic region introgressed BC_2F_3 advance lines genotypes (2a.E-6, 2b.C-210 and 2c. C-196) derived from Ranjit × Swarna-*Sub1* cross and genotypes (2d. 1189-1 and 2e. 1160-1) derived from Bahadur × Swarna-*Sub1* cross

being pursued for the last several decades (Siangliw et al. 2003; Xu et al. 2004; Toojinda et al. 2008). Several varieties are now successfully incorporated with sub-mergence tolerance gene *Sub-1* to increase rice production. The present study showed an effective improvement in selected BC_2F_3 advance lines in the background of Ranjit and Bahadur for submergence tolerance. The advanced BC_2F_3 lines identified were further evaluated by using for morpho-physiological traits. A set of selected 35 genotypes, including parents and checks were evaluated for background recovery of RPG based on 62 morpho-physiological traits. These characteristics have been used to identify the advanced lines having maximum RPG recovery, and such characters are also used for varietal protection. Phenotypic evaluation of morpho-physiological traits and selection carried out in the advanced back cross-generation facilitated identification of lines showing maximum similarity with Ranjit and Bahadur. Among these introgressed advanced BC_2F_3 lines

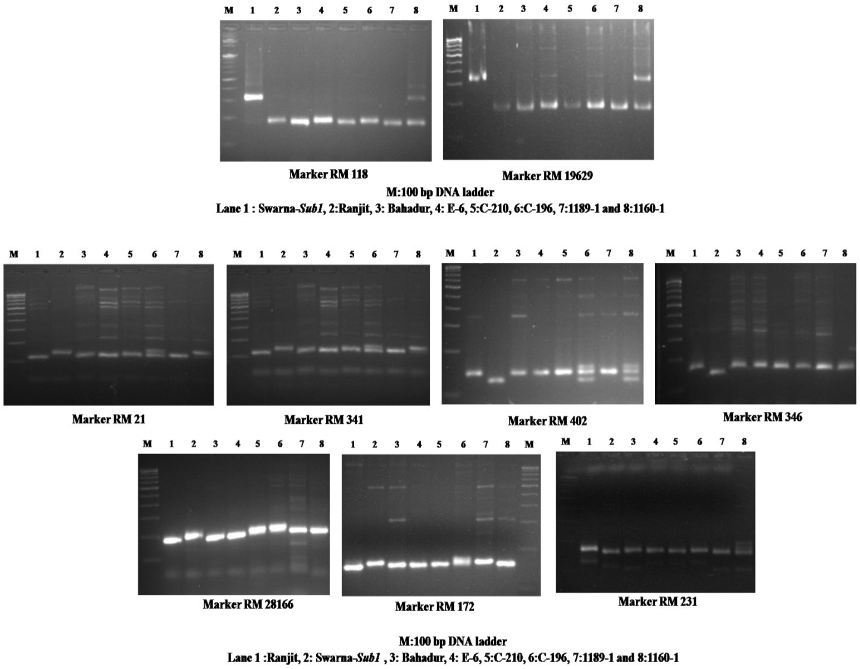


Fig. 2. Genotypic screening of BC_2F_3 advance lines including parents with SSR marker.

ten lines had 10 to 18 traits in respect of dissimilarity out of 62 morpho-physiological traits; 15 lines had 19 to 21 traits of dissimilarity and 7 were dissilar in 23 to 25 traits. The characters namely, leaf sheath anthocyanin colouration, leaf auricles, leaf length of blade, width of leaf blade, leaf length of blade, anthocyanin colouration of apex (lemma), stem length, stem anthocyanin colouration, intensity of anthocyanin colouration of nodes (stem), lemma and palea colour, panicle number/plant, grain width and decorticated grain width are the phenotypic traits were not found to be recovered. Earlier studies reported that phenotypic selection in the early back cross-generation using morphological traits showed maximum similarity with a RP helped in reducing the population size (Chetia et al. 2018). The per cent background recovery based on the morpho-physiological characters of RP ranged from 57 to 79.04 per cent. The lines such as E-6 (79.04 %), 1160-1 (75.81%), C-196 (74.2%), C-210 (75.81%) and 1189-1(77.42 %) recorded higher background recovery (RPG) amongst the *Sub1* introgressed advanced lines indicating that phenotypic selection in segregating generations was effective as also reported earlier (Chetia et al. 2018). Therefore, the morpho-physiological characters can successfully be utilized to get maximum recovery of the RP. The *Sub1* genomic region introgressed advanced lines which exhibited >74% background recovery of RPG based on the morpho-physiological characters were selected for genotypic screening and it was performed with SSR marker for background recovery analysis in selected BC_2F_3 advance lines. A set of 69 polymorphic SSR markers used recorded the background recovery ranging from 86.68 to 97.82%. A recent study showed that for certain BC_2F_2 and BC_3F_2 versions of Swarna-*Sub1*, 32 selected background markers were used to ensure RPG recovery (Neeraja et al. 2007). Because recombination occurs over time, the number of donor chromosomal segments increases as their length decreases. Thus, more markers are needed to trace them at later backcross generations (Collard et al. 2008; Hospital et al. 1997).

The basic purpose of using the modified MABC is to reduce the selection activities by skipping recombinant selection with the aid of molecular markers. Therefore, in the present study recombinant selection was chosen to skip and selection of the background that was assigned to generation BC_2F_2 (Chetia et al. 2018). Similar results have been reported that the mega-variety, Swarna with 3 backcrosses and two selfing generations had 96.2% RPG recovery, with introgressed fragments of 2.3-3.4 MB, which has been developed using MABC (Neeraja et al. 2007). Similarly, the 20 BC_2F_2 lines showed 89.52 to 96.54% RPG recovery and 91.74 to 94.89% similarity for chromosome 9, which also showed a very high RPG recovery (Chetia et al. 2018). The submergence tolerance form of BR11-*Sub1* was developed with the aid of MABC with 2 backcrossing and 2 selfing generations, in which the RPG recovery of 99.8% with an introgressed fragment of 800 Kb (Iftekharuddaula et al. 2011). The introgression of genomic region of size 0.3 Mb flanked by markers ART5 and SC3 into elite rice variety-AS996 of Vietnam in which the RPG recovery was achieved after

3 generations of backcrossing followed by two selfing generation (Cuc et al. 2012). The advanced backcross line, E-6 in the Ranjit background with a RPG recovery of 93.47 and 1189-1 in the background of Bahadur with recovery of 97.82 were developed which wereidentified with highest RPG recovery among the best selected advanced lines. However, Chetia et al (2018) reported a still higher (96.54%) background recovery of RP. The estimated background recovery for the BC_2F_2 generation was recorded by Ahmed et al. (2016) was 95.37 per cent suggesting a maximum similarity between the RP (MR219) and the subsequent lines. The background recovery depends on the number of backcrosses and subsequent selfing. The best plant among the test entries had 67.9 per cent of the RPG by BC_1F_1, 87.2-94.2 per cent by BC_2F_1, and 96.2 per cent by BC_3F_1 (Neeraja et al. 2007).

The development of the *Sub1* tolerant form of Ranjit and Bahadur with *Sub1* introgressed and the advance lines selected in BC_2F_3 has been achieved conventional and MABC . The selected advanced lines carrying submergence tolerance genomic region (*Sub1*) were superior in agronomic performance as compared to the elite Ranjit and Bahadur varieties (RP), with an improved trait of flooding tolerance and therefore, if released, they are likely to benefit farmers of the flood-affected regions of Assam. Compared to parents, the performance levels of all the *Sub1* introgressed advanced lines were considerably higher. It was also observed that *Sub1* genomic region introgressed advanced lines in the BC_2F_3 generation had lower elongation per cent during submergence as compared with parents and susceptible check IR42, as well. The identified advanced lines with *Sub 1* gene may serve as a potential source of submergence tolerance in rice breeding.

Authors' contribution

Conceptualization of research (SKC, AG); Designing of the experiments (SKC, AG); Contribution of experimental materials (SKC); Execution of field/lab experiments and data collection (AG); Analysis of data and interpretation (AG, SKC, VS); Preparation of the manuscript (AG, SKC, VS, MKM, TA and MP).

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