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Full Length Research Paper

Rapid and high-precision marker assisted backcrossing to introgress the *SUB1* QTL into the Vietnamese elite rice variety

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Submergence stress regularly influences about 15 million hectares of rice growing areas in South and Southeast Asia. Vietnam is one of the most vulnerable countries affected by submergence stress in Asia. Submergence caused by typhoons and floods is one of the major reasons for rice production losses in this country. A major quantitative trait locus (QTL) on chromosome 9, *SUB1*, has provided the opportunities to apply marker-assisted backcrossing (MABC) to develop submergence tolerant for an elite variety that is popularly grown in Vietnam. Improving rice tolerance with submergence is vital to minimize the risks from submergence stress. In this study, we reported a successful application of MABC method to select a line number 19 in BC_2F_1 populations that has genetic background up to 89.8%.

Key words: Bac Thom 7 (BC7), marker-assisted backcrossing (MABC), submergence tolerance.

INTRODUCTION

Increases in submergence stress caused by climate change are the major impediments to enhancing production in rice growing areas worldwide. The most common and damaging type of flooding is short-term inundation (up to 2 weeks), also referred to as flash floods. This type of flooding is estimated to affect about 20 million ha of rice growing areas in Asia (excepted China) as well as significant areas of lowland rice production in Africa (Bailey-Serres et al., 2010; Mackill et al., 2012). Moreover, due to adverse effects from climate change, these seasonal flash floods are extremely unpredictable and may occur at any growth stage of the rice crop (Ronald, 2012). Submergence of rice (*Oryza sativa*) during the monsoon flooding season severely limits rice production in South and Southeast Asia, causing annual losses of over one billion U.S. dollars (Xu et al., 2006; Manzanilla et al., 2011). Vietnam is one of the countries hardest hit by climate change in Asia. Adverse effects cause by annual floods is a big problem in this country; by the year 2011, more than 10.000 hectares of rice areas were inundated by floods, which caused significant economic losses (MARD, 2011). Temperature would rise by about 2.3°C and sea level rise by 75 cm relative to the average of 1980 to 1999 by the end of the 21st century. Vast portions of the food producing regions in the country will be inundated by flood including rise of sea water, expected at about 19 to 37.8% of the Mekong River Delta (MRD) and about 1.5 to 11.2% of the Red River Delta (RRD). With water rise by 1 m, approximately 40,000 km^2 will be inundated (MARD, 2005).

Rice is the most important food crop for over half of the world's population and supplies 20% of daily calories (WRS, 2010). Rice is a major crop in Vietnam, as the world's second-largest rice exporter after Thailand, and together accounting for 50% of the world rice trade. However, rice yield and its cultivating areas are adversely

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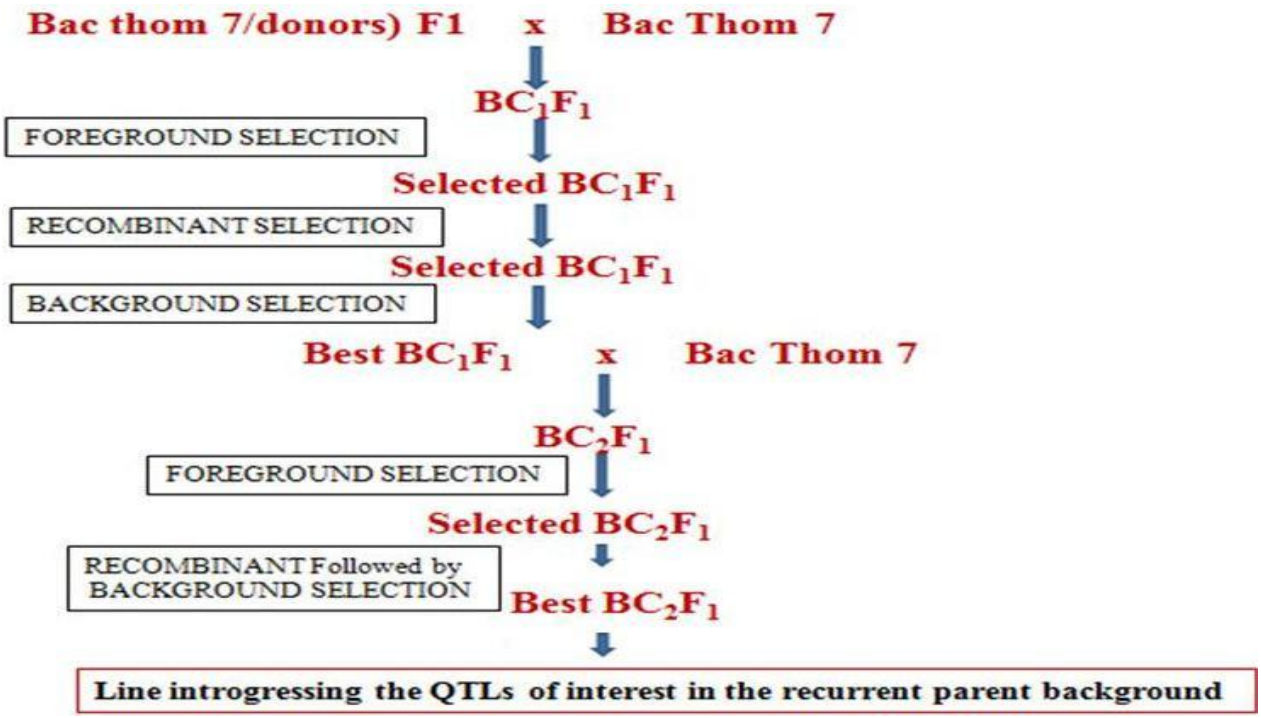

Figure 1. The scheme of breeding strategy by MABC method to improve submergence in BC7.

affected by the threats of devastation caused by typhoon and flash flooding which are inducing significantly economic loss in this country. Complete submergence for 1 to 2 weeks leads to death of most rice cultivar (Xu et al., 2006; Perata and Voesenek, 2007). However, tolerant varieties like the Indian landrace FR13A can survive up to 2 weeks of complete submergence. Previous quantitative trait locus researches indicated that the Submergence 1 (*SUB1)* locus on chromosome 9 is the major source affecting submergence tolerance in lowland rice, which accounts for 35 to 69% of phenotypic variance in tolerance in diverse background.

The *SUB1* locus is comprised of three ethylene-responsive factor (ERF) transcriptional regulators (*SUB1* A, *SUB1B*, and *SUB1*C) (Xu et al., 2006; Jung et al., 2010; Xu and Mackill, 1996; Bailey-Serres and Voesenek, 2008). A study by Fukao et al. (2006) revealed that introgression of the FR13A *SUB1* locus into a submergence-intolerant background (*Japonica* M202) using maker-assisted selection (MAS) led to the improvement of the M202 (*SUB1*) submergence-tolerant near-isogenic line. Marker assisted backcrossing (MABC) is an accurate and effective method to introgress a single locus controlling a trait of interest while retaining the essential characteristics of the recurrent parents (Hospital, 2003). Mackill (2006) suggested that adoption of a completely new variety could take considerable time, while the chances of rapid adoption of popular varieties converted via MABC were relative higher. Specifically, a major *SUB1* QTL explaining about 70% of phenotypic variation in submergence tolerance has been identified and mapped on chromosome 9 in submergence tolerant cultivar FRI3A (Xu et al., 2000). Recent studies conducted at the International Rice Research Institute (IRRI) have been successfully transferred *SUB1/*QTL into some modern rice varieties (Neeraja et al., 2007; Cuc et al., 2012). The *SUB1* rice varieties had reached more than 1,000,000 farmers, and rapid seed multiplication now targets production areas of 5 million ha by 2014 in South Asia alone (IRRI, 2012; Mackill et al., 2012)

The main objective of this study was to improve the submergence tolerance of Bac Thom 7 (BT7), an elite Vietnamese rice variety by using the MABC method. The improved cultivar may be useful for growing submergence prone areas of Vietnamese Deltas.

MATERIALS AND METHODS

Plant materials and crossing scheme

The high flood tolerance rice variety IR64-*SUB1* derived from submergence tolerance breeding line was used as the donor of *SUB1*, whereas the widely grown cultivar with aromatic and good quality in Vietnam was used as the recipient for the breeding program. The MABC scheme for breeding program is shown in Figure 1. The donor variety was screened and reconfirmed the submergence ability and yield potential under the laboratory and natural conditions of Vietnam. IR64-*SUB1* showed high submergence tolerance compared with the other rice variety without carrying *SUB1/*QTL. Pursuing the MABC breeding strategy, BC7 was crossed with IR64-*SUB1* to produce F_1 seeds (Figure 1). F_1

Table 1. Details of markers for foreground and recombinant selection.

No.	Name of markers	Mb	Forward and reverse primers	PCR band size (bp)	Annealing temperature (°C)
1	RM23662	0.4	GAGAGGACGATGGCACTATTGG CGAGGAACTTGATTCGCATGG	149	60
2	RM5688	1.7	GCAGTGTCCAACCATCTGTG ATCTGGTCACCCTTTGCTTG	150	55
3	ART5	2.6	CAGGGAAAGAGATGGTGGA TTGGCCCTAGGTTGTTTCAG	124	55
4	SC3	6.8	GCTAGTGCAGGGTTGACACA CTCTGGCCGTTTCATGGTAT	217	55
5	RM23877	6.3	TGCCACATGTTGAGAGTGATGC TACGCAAGCCATGACAATTCG	327	60

seeds were backcrossed with BC7 to obtain a number of BC_1F_1 seeds. In this generation, individual plants that were heterozygous at the *SUB1* locus were identified by 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 two markers locus namely SC3 and ART5, which are distally franking the *SUB1* locus were identified. Some markers used in detail are shown in Table 1. From these recombinant plants, individuals with the fewest number of markers from the donor genome were selected (background selection). In the following BC generations, the same strategy was followed for selection of individual plants with the desired allele combination at the target loci including selection for recombinants between *SUB1.* The selected BC_1F_1 plants were self-pollinated as BC_2F_1 for further analyses.

Molecular marker analysis

DNA was extracted from juvenile leaves of 2-week-old plants using a modified protocol as described by Zheng et al. (1995). Polymerase chain reaction (PCR) was performed in 10 µL reactions containing 5 - 25 ng of DNA template, 1 µL 10 X TB buffer (containing 200 mM Tris–HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 µL of 1 mM dNTP, 0.50 µL each of 5 µM forward and reverse primers and 0.25 µL of Taq DNA polymerase (4 U/µL) using an MJ Research single or dual 96-well thermal cycler. After initial denaturation for 5 min at 94°C, each cycle comprised 1 min denaturation at 94°C, 1 min annealing at 55°C, and 2 min extension at 72°C with a final extension for 5 min at 72°C at the end of 35 cycles. The PCR products were mixed with bromophenol blue gel loading dye and were analyzed by electrophoresis on 8% polyacrylamide gel using mini vertical polyacrylamide gels for high throughput manual genotyping (CBS Scientific Co. Inc., CA, USA). The gels were stained in 0.5 mg/ml ethidium bromide and were documented using Alpha Imager 1220 (Alpha Innotech, CA, USA). Microsatellite or simple sequence repeat (SSR) markers were used for selection (McCouch et al., 2002).

Screening for submergence tolerance

Submergence screening was conducted in the greenhouse at the experimental farm of Thanh Tri, Hanoi, Vietnam, following the standard protocols of Xu et al., (2000) and HilleRisLambers and Vergara (1982). Seeds of the selected plants of BC_2F_1 (BT7/IR64-*SUB1*) along with parents and susceptible check IR42 were generated in rows in 20 cm × 15 cm × 10 cm trays. Fifteen-day-old seedlings were submerged for 14 days. The percentage of survival of plant were recorded 14 days after de-submergence for confirmation of the presence of *SUB1* locus.

Statistical analyses

The molecular weights of the different alleles were calculated by Alpha Ease Fc 5.0 software. The marker data was analyzed using the software Graphical Genotyper (Van Berloo, 2008). The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as ‘A’, ‘B’ and ‘H’, respectively. The percent markers homozygous for recipient parent (%A) and the percent recipient alleles including heterozygous plants (%R) were calculated. All experimental analyses of the agronomic traits were performed in a completely randomized design with at least thrice. Data were analyzed with the use of the Duncan’s multiple-range test ($P<0.05$).

RESULTS AND DISCUSSION

Foreground and recombinant selections

The present study indicated that MABC strategy is an effective means of utilizing QTLs with large effects in rice breeding programs. SSR markers were used in this study because of its predominant use in mapping and introgressing agronomically important QTLs into elite rice variety by MABC method. BC7 is an elite Vienamese variety that was first introduced in Vietnam in 1992, and widely grown in many areas in this country due to it stable yield, aromatic and good quality. Hence, BC7 was selected as a recipient parent. In this step, 24 SSR markers at the target region on the chromosome 9 were

used to identify polymophism between the parents (BC7 and IR64-*SUB1*). It showed that 11 markers were polymophism, of which 5 markers (RM23662, RM5688, ART5, SC3 and RM23877) were tighly linked to QTL/*SUB1*. Detailed information on the polymophic markers is shown in Table 1. The *SUB1* was previously monitored by markers demonstrated to be closely linked with the gene (Xu et al., 2000). Six individual plants of F_1 were identified by 2 SSR markers (ART5 and RM25181) which linked with QTL/*SUB1*.

In the next backross generation (BC_1F_1 and BC_2F_1), the target locus *SUB1* was recorded by the markers which linked to the QTL/*SUB1*. The individual backcross generation plants were selected on the heterozygous of all the target loci at *SUB1* region. Hence, few selected plants that obtained the least donor alletes of the background markers were chosen to backcross with BC7. In the advanced backrossing, polymorphic markers SC3 and ART5 and ART5 and RM23877 that are tighly linked with *SUB1* were used to assess the individual BC_1F_1 and BC_2F_1 plants, respectively. The result showed that in 235 plants of BC_2F_1 generation, 57 individual plants were confirmed in the introgression of *SUB1* using by ART5 marker. Similarly, 87 out of 259 individual plants of BC_2F_1 were validated for introgression of *SUB1* by RM23877. The recombinant selection was performed by applying heterozygous flanking markers to select the best individual plants for background selection.

The use of tighly linked and flanking markers for *SUB1* were suggested by Hospital and Charcosset (1997) to ensure efficient foreground and recombinant selection to reduce the linkage drag. On the other hand, the F_1 was backcross to the the mega-variety and large BC_1F_1 populations were generated. These polulations were first screened with a marker closely linked to the *SUB1* QTL. The plants with *SUB1* were screened with the markers flanking the *SUB1* locus and only those with recombination near the gene were selected. This stage was termed recombinant selection (Collard and Mackill, 2008). The combinant plants were screened with background markers on all the chromosomes, the fewest donor markers of the plants were selected and backcrossed to produced the BC_2F_1. But, it is difficult to identify a recombinant on both sides of *SUB1* by the BC_2F_2 generation, so the first set of plants had reombination on only one side. This may be explained that the *SUB1* gene is near the end of chromosome 9; these plants still had a relatively small segment of the donor surrounding the *SUB1* gene (Mackill et al., 2012).

Background selection

A total of 384 SSR markers distributed on the 12 chromosomes were used to screen the parental polymorphism. Among these, 58 markers (15.1%) showed polymorphisms on 4% polyacrylamide between the parents (Figure 2). The 58 polymorphic markers were further used for background selection. The result for polymorphism by SSR markers analysis is illustrated in Figure 2. In BC_2F_1 generation, 53 polymorphic markers were used for background selection in BC_2F_1 plants. Specifically, 8 individual plants were of the genetic background (70 to 75%) of BC7, 19 plants were arranged 80 to 85%, 12 plants were 80 to 85%, and 3 plants had the highest genetic background by 85 to 90%, respectively (Figure 3).

Rice breeding programs have been developed to improve submergence tolerant cultivars that have been going on for more than three decades (HilleRisLambers and Vergara, 1982; Mackill, 1986; Neeraja et al., 2007). Since the discovery of the tolerance gene *SUB1* which enabled marker assisted backcrossing to transfer it into rice mega varieties, eight "*SUB1* varieties" have recently developed in the background of popular Asian cultivars. The improved cultivars are being tried to release in submergence prone areas in Asia. To date, five varieties have been officially approved for distribution to farmers and the remaining three are under advanced evaluation (Mackill et al., 2012). In this study, we have successfully applied MABC method to select a line number 19 in BC_2F_1 population, with 85 to 90% genetic background. An ongoing work is being intensively carried out to produce next BC generations, and hopefully select the best plants (*SUB1*) with background genetic (100%) from the recipient parents will be achieved. The development of submergence tolerant rice verities with acceptable grain quality and wide adaptability offers a model for an alternative to the time-consuming, labor intensive task of developing new varieties in a conventional crossing program. Neeraja et al. (2007) indicated that the mega variety Swarna could be efficiently converted to a submergence tolerant variety (Swarna-*SUB1*) in three backcross generations, involving a time of two to three years. Interestingly, Swarna-*SUB1* plants appear identical to the recurrent parent Swarna in the important characteristics, such as grain yield and yield (Neeraja et al., 2007; Mackill et al., 2012).

Seeds from the BC_2F_1 selection were available earlier and most of the characterization of submergence tolerant plants, were performed with this section, which we designated "BT7-*SUB1*". Fifteen-day-old seedlings of the selected plant of BC_2F_1 were scored as tolerant and survived the 14 days submergence confirming the introgression of the *SUB1* locus. BT7-*SUB1* showed a survival percentage of plants similar with IR64-*SUB1* and was significant higher than intolerant parent and susceptible check variety (Table 2; Figure 4). This implies that *SUB1* locus was successfully transferred into BT7, which is similar with the report of Neeraja et al. (2007). The plants expressing *SUB1* often show suppression of elongation and, upon submergence, they recover rapidly and continue to grow by producing more new leaves and early tillers after de-submergence (Fukao and

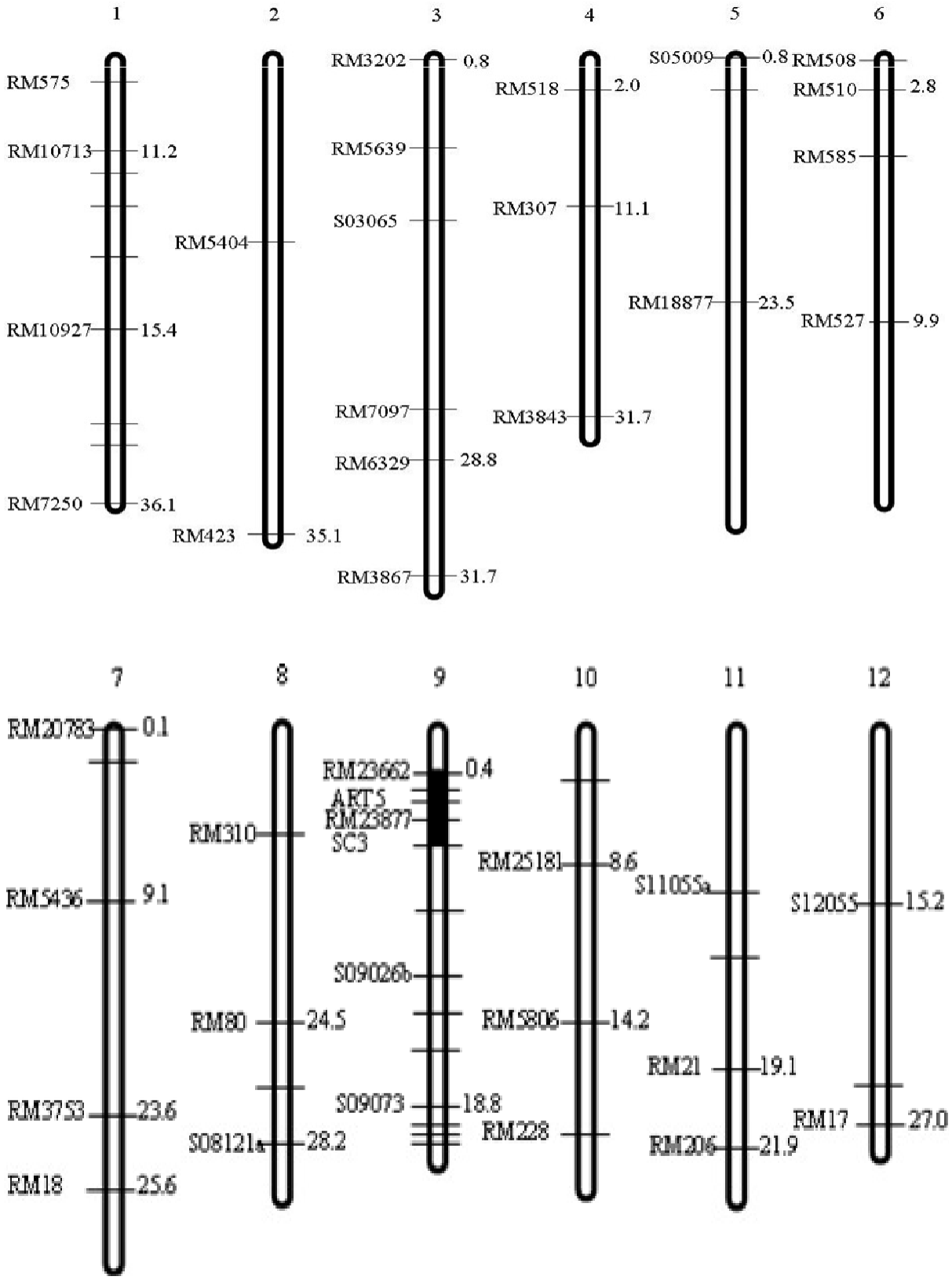

Figure 2. Graphical representation of mapping; chromosome numbers are at the top of the bars. White portions of the bars are derived from BC7 and dark regions with the SSR markers linkage the *SUB1*. Markers polymorphism between BC7 and IR64-*SUB1* are labeled on the left of the chromosomes. The estimated distances in kb between the SSR markers and their order were followed (SSR Marker, 2011).

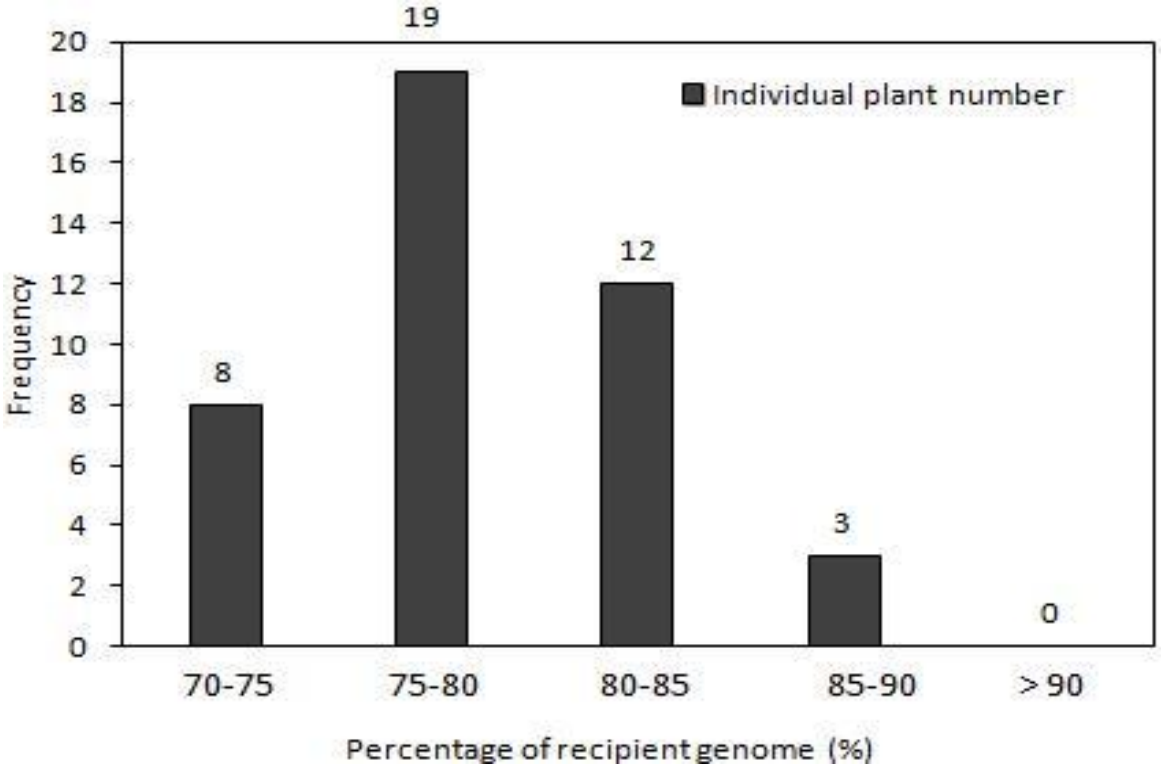

Figure 3. Frequency distribution of the percentage of recurrent parent genome (BC7) in the BC_2F_1 population derived from the cross between BC7 and IR64-*SUB1*. The vertical axis of each figure represents the relative numbers of BC_2F_1 plants.

Table 2. Screening submergence of BT7-SUB1 compared to the parents and susceptible check IR42 variety.

No.	Variety or line	*SUB1* present	Percentage of survival (plant)	Tolerant rank
1	BT7-Sub1	+	18^a	Good
2	IR42 (SC)	-	3.3^b	Intolerance
3	BT7 (C)	-	0^c	Intolerance
4	IR64-*Sub1* (C)	+	18.7^a	Good
CV (%)			8.9	
LSD (0.05)			0.7	

+: SUB1 present; -: non-SUB1 present; SC: susceptible check. Means with the same letters in column are not significantly different at P<0.05.

Bailey-Serres, 2008). Some trials were conducted in IRRI under the controlled flooding conditions to determine whether *SUB1* introgression had any effect on grain yield and/or grain quality when submergence stress was not encountered (Mackill et al., 2012). The findings suggest that *SUB1* does not have other effects on growth and yield in the absence of stress, and this can be explained to be due to ethylene-responsive factor (ERF) (Fukao et al., 2006; Xu et al., 2006; Mackill et al., 2012).

In conclusion, we have successfully improved a submergence tolerance of BT7 cultivars by using MABC method, which were controlled by the major *SUB1* QTL. The recovery of the recurrent parent genome by molecular genotyping and selection could increase the efficiency of the MABC, and this was achievable in a short span of time in rice breeding strategy. The obtained results suggest that MABC should be applied not only for the target segment but also for the background selection. This study could have a good impact in rice breeding and it is applicable for the introduction of important agronomic traits into the genomes of popular rice cultivars.

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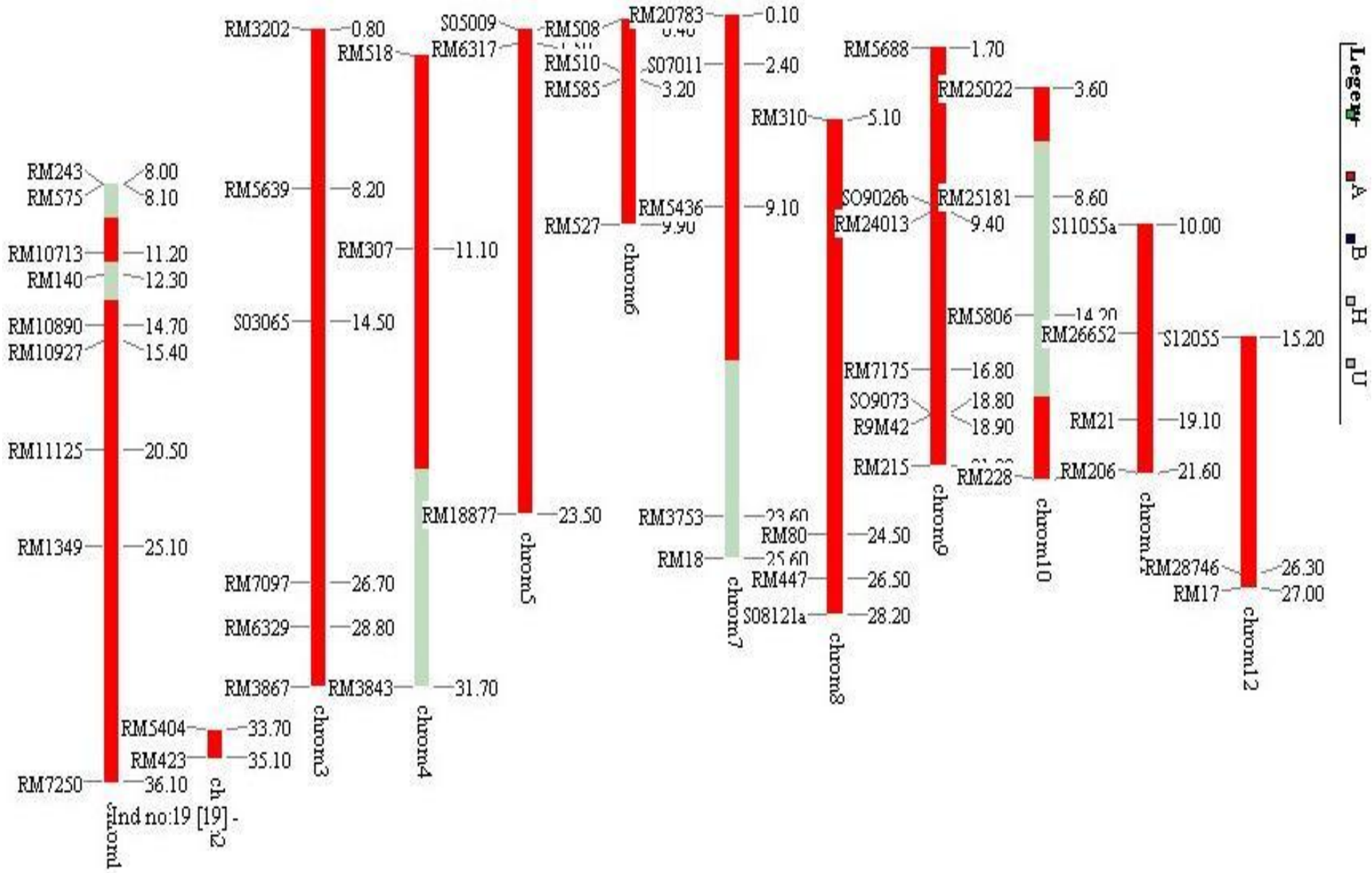

Figure 4. Graphical representation of the plant IL- 19. Chromosome numbers are located at the top of the bars. Black portions of the bars are derived from BC7 and slash regions indicated the *SUB1* and IR64-*SUB1* introgressions. Markers are labeled on the right side of the chromosomes.

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