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Analysis of Simple Sequence Repeat Markers Linked to Submergence Tolerance on Newly Developed Rice Lines Derived from MR263 × Swarna-*Sub1*

(Analisis Penanda Ulangan Jujukan Ringkas terhadap Titisan Padi Baharu Dibangunkan yang Toleransi Tenggelam daripada Kacukan MR263 × Swarna -*Sub1*)

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ABSTRACT

*Nowadays, in extreme changing environments, development of submergence tolerance variety is necessary for ensuring crop production stability where, it is known that Malaysian commercial rice varieties such as MR219, MR220 and MR263 were severely susceptible to submergence. First step towards the development of submergence tolerance variety starts with the breeding program by crossing MR263 and Swarna-*Sub1*. Marker-assisted selection (MAS) was carried out through the utilization of simple sequence repeats (SSR) markers, considering its reliability as pre-selection tools to conduct this research. F_1 generations plants were confirmed by tightly linked markers. In case of background study, out of 180 SSR markers, 38 were found polymorphic between two parents. Association of molecular markers and submergence tolerance were determined using Chi-square test. MR263 × Swarna-*Sub1 F_2 lines were tested for *Sub1 *gene conformation using the markers RM8300 and RM219. These markers showed a good fit to the expected marker segregation ratio (1:2:1) in a Mendelian single gene model (DF=1.0,* p≤*0.05). Eleven homozygous lines with *Sub1 *gene out of 256 were selected for future development of submergence tolerant varieties. Eleven lines were selected based on phenotypic study and agronomic performance.*

Keywords: Marker-assisted selection; rice; Sub1*; submergence tolerance; SSR*

ABSTRAK

*Pembangunan varieti padi yang toleransi tenggelam adalah penting bagi memastikan kestabilan pengeluaran tanaman akibat perubahan melampau alam sekitar yang tidak menentu. Varieti padi komersial Malaysia seperti MR219, MR220 dan MR263 telah dikenal pasti terjejas teruk akibat tenggelam. Langkah pertama ke arah pembangunan varieti yang toleransi tenggelam bermula dengan program pembiakbakaan MR263 dan Swarna-*Sub1*. Dalam kajian ini, pemilihan berbantukan penanda (MAS) telah dijalankan dengan menggunakan penanda ulangan jujukan ringkas (SSR) yang disahkan kebolehpercayannya sebagai alat pra-pemilih. Generasi pokok F_1 telah disahkan oleh penanda yang terkait erat. Dalam kajian latar belakang, daripada 180 penanda SSR, 38 penanda adalah polimorfik antara kedua-dua induk. Hubungan antara penanda ulangan jujukan ringkas dan toleransi tenggelam ditentukan dengan menggunakan ujian Chi-square. Titisan F_2 MR263 × Swarna-*Sub1 *telah diuji untuk korformasi gen Sub1 dengan menggunakan penanda RM8300 dan RM219. Penanda ini menunjukkan keserasian yang baik dengan kadar segregasi yang dijangka (1:2:1) dalam model gen tunggal Mendel (DF=1.0,* p≤*0.05). Sebelas titisan homozigot yang mempunyai gen *Sub1 *telah dipilih daripada 256 titisan untuk pembangunan masa depan varieti padi yang toleransi tenggelam. Sebelas titisan ini dipilih berdasarkan kajian fenotip dan prestasi agronomi.*

Kata kunci: Padi; pemilihan berbantukan penanda; Sub1*; toleransi tenggelam; SSR*

INTRODUCTION

Rain-fed lowlands account for about 25 million hectares of rice area in South and Southeast Asia. The total production of rice has also become static over the years with the gradual decrease of crop-growing area for non-rice crops, other enterprises and infra-structures. The static production of rice is still attributable to the lack of suitable improved cultivars for different agro-climatic conditions, particularly for unfavorable ecosystems. In order to increase total rice production, we must explore the development possibility of high yielding varieties with tolerance against abiotic stresses for unfavorable ecosystems. Among the abiotic stresses, submergence is one of the important factors in the flash flood prone rice growing environment. Catling (1992) defined submergence tolerance of a rice plant as its ability to survive 10-14 days of complete submergence and renew its growth when the water subsides.

Submergence tolerant varieties have been long developed but it is not widely adopted by the farmers because its lack of many desirable traits of widely grown varieties (MacKill 2006) such as high yield and short stature. Submergence tolerance in rice was reported to

be a quantitative trait controlled by a major locus. The effect of environment on this trait was high in spite of its high heritability. Additive gene action was found more preponderant in the inheritance of the trait (Mishra et al. 1996; Mohanty & Khush 1985).

The introgression of submergence tolerant *Sub1* gene into susceptible rice variety improves submergence tolerance significantly, thus it has no negative side effect in terms of yield, harvest index and grain quality when grown under non-submerged condition (Xu et al. 2006). To date, no submergence tolerant local variety has been developed in Malaysia and also no report of submergence tolerance study on local variety have been found. MAS has previously been used in rice breeding to incorporate the bacteria blight resistance genes (Chan 2001; Chen 2000), blast resistance genes (Hasan et al. 2016; Miah et al. 2015) waxy gene (Zhou et al. 2003), fragrance gene (Lau et al. 2017) and submergence tolerance (Ahmed et al. 2016; Neeraja et al. 2007) into elite varieties. The markers used in this study to identify submergence tolerance lines are also used by Neeraja et al. (2007) and Toojinda et al. (2005). Molecular markers such as SSRs have been efficiently utilized in many crop improvement programs *viz.*, hybrid identification, testing seed genetic purity and linkage mapping. Even though a single gene namely *Sub1A* controlling tolerance against submergence has been identified, the transfer of this gene through conventional breeding combined with MAS is still the most effective way to develop submergence tolerant rice cultivars (Xu et al. 2006). The effectiveness of MAS depends on the availability of closely linked markers and/or flanking markers for the target gene, the size of the population, the number of crosses and the position and number of markers for background selection (Frisch & Melchinger 2005). The objectives of this study were to analyze SSR markers associated with submergence tolerant gene up to F_2 generation derived from a cross between MR263 and Swarna-*Sub1* varieties and, to select improved lines from the cross carrying the *Sub1* gene.

Materials and Methods

Planting materials and crossing scheme

The recipient variety was MR263, high yielding variety, identified as susceptible to submergence. Swarna-*Sub1*, submergence tolerant variety was used as the donor for *Sub1* gene. It is an improved submergence variety released in India with high yield. A single cross between the varieties MR263 and Swarna-*Sub1* was performed to obtain F_1 seeds. F_1 seeds were tested with tightly linked SSR marker linked to *Sub1* gene . Positive plants were planted again to obtain F_2 seeds.

DNA extraction

DNA was extracted from young leaves of 4-week-old F_1 and the parental plants using a modified protocol as described by McCouch et al. (1988). The procedure involved grounding of 1 g leaf tissue and then transferred it into an Eppendorf tube containing 15 mL CTAB extraction buffer (100 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 1.25 M NaCl and 30 µL β-mercapoethanol). It was then incubated in a hot water bath for 1 h at 65°C (gently shaken at 5 min interval). Then, 15 mL chloroform-isoamyl alcohol was added. The contents were mixed gently for 2-3 min and centrifuged at 5000 rpm for 10 min at 25°C using centrifuge 5810 (Eppendorf, Germany). The supernatant was transferred to a fresh sterile 50 mL Falcon tube and 0.6 volume of ice-cold isopropanol was added. The DNA was pelleted by centrifugation at 12,000 rpm for 1 min at room temperature. After centrifugation, the supernatant was discarded and the DNA pellet was washed twice with 70% ethanol. The pellets were air dried for 1 h and dissolved in 100 µL of sterile TE buffer (10 mM Tris HCl, pH 8.0 and 1mM EDTA, pH 8.0). 2 µL RNAse was added into each DNA samples to remove RNA.

Quantification of DNA was done using nanodrop spectropfotometry (ND1000 Spectrophotometer); followed by gel electophoresis using 1× TAE buffer at 5-8 V/cm for 30 min on 1% agarose gel .Then it was visualized using an UV transluminator where, ethidium bromide was used as staining agent. The DNA sample was then kept in a freezer at -20°C for polymerase chain reaction (PCR) analysis.

Polymerase chain reaction

PCR was carried out in a thermocycler (PTC-200 Peltier Thermal Cycler DNA Engine) using primers related to *Sub1* gene. PCR reaction mix included the following: 40 ng template DNA, 100 µM dNTPs (0.2 mM each of dATP, dTTP, dGTP and dCTP), 1.5 mM $MgCl_2$, 1× PCR buffer (10 mM Tris-HCl and 50 mM KCl, pH 8.3), 1.0 µM of each primer and 0.2 U *Taq* DNA polymerase. PCR profile, started with initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 min, followed by rapid cooling to 4°C prior to analysis. The PCR product was electrophoresed in a 3.0% Metaphor agarose gel in 1× TBE buffer (0.05 M Tris, 0.05 M boric acid and 1 mM EDTA, pH 8.0) at 90 V for 1 h after it was mixed with with 2.5 µL 6× loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol in a Tris-EDTA buffer) and visualized under UV light. PCR product will be observed on a UV transilluminator. The PCR products were scanned using the Bio-Imaging System (Chemi-Genius, USA) for further analysis.

Foreground, recombinant and background study

One hundred and eighty SSR markers covering all the 12 chromosomes in rice genome were used for genotyping the parents MR263 and Swarna-*Sub1*. Eight markers were used for foreground and recombinant selection based on previous MAS program on rice (Iftekharuddaula et al. 2011; Neeraja et al. 2007; Septiningsih et al. 2009; Xu & Mackill 1996) (Table 1).

TABLE 1. Foreground and recombinant SSR Marker

Primer	Primer Sequence (5'-3')		Repeat motif
	F: forward primer	R: reverse primer	
RM219	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	$(CT)_{17}$
RM8300	GCTAGTGCAGGGTTGACACA	CTCTGGCCGTTTCATGGTAT	$(ACCATTAT)_3$
RM23679	TCACAGCTTAGTGCATGTTGAGC	GATTCACCTGGCAATGAGAACG	$(AGAA)_{10}$
RM23805	GAGACAGATGTGTACGGTTTGGTG	TTGACAAGGAAGGAGAAG	$(TG)_{13}$
RM23915	GAGGATCCTTACCATCAAACTTCG	CCAAGAACCTGCATTCTTCAAGG	$(AC)_{15}$
RM23958	CTACCACTGTTTCATTGTGTCTCG	GAATTGAAGGAGAAGCAGGAAGC	$(CT)_{15}$
ART5	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG	N/A
Sub1C173	AACGCCAAGACCAACTTCC	AGGAGGCTGTCCATCAGGT	N/A

DATA ANALYSIS

The markers were scored for presence or absence of the corresponding bands between two parents. The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as 'A', 'B' and 'H', respectively. The marker data was analyzed using the software Graphical Genotyper (van Berloo 2008). Mendelian segregation ratio was analyzed by chi-square ($\chi 2$) test. The chi-square analysis for the genotypic and phenotypic ratio was calculated using the formula, $\chi^2 = (O-E)^2/E$ where, O is the observed value and E is the expected value.

SCREENING FOR SUBMERGENCE TOLERANCE

Phenotypical screening for submergence tolerance was done at the glasshouse at Ladang 10, UPM following standard protocols (Xu et al. 2000). Selected F_2 seeds, along with their parents were grown in trays where FR13A were used as check variety. Fourteen days old seedlings were submerged for two weeks. After de-submergence, survival and recovery data were taken at 6 days and 30 days following standard protocol (IRRI 2002) for the confirmation of the *Sub1* locus.

RESULTS AND DISCUSSION

PARENTAL POLYMORPHISM SURVEY USING SSR MARKERS

Out of 180 SSR primers, 38 primers showed polymorphism between two parents (Figure 1, Table 2). In this study, the frequency of polymorphic SSR markers was 21.11%. Cuc et al. (2012) reported 12.6% frequency of polymorphic SSR markers. In another study between PRR78 and Pusa 1460, Basavaraj et al. (2010) reported a frequency of 17.47%. A study of polymorphism in two parents, Bac Thom 7 and IR64, showed that 15.1% of the SSR markers were found to be polymorphic (Khanh et al. 2013).

FOREGROUND SELECTION

Rice microsatellite markers RM219, RM8300, RM23679, RM23805, RM23915, RM23958, ART5 and *Sub1* C173 which

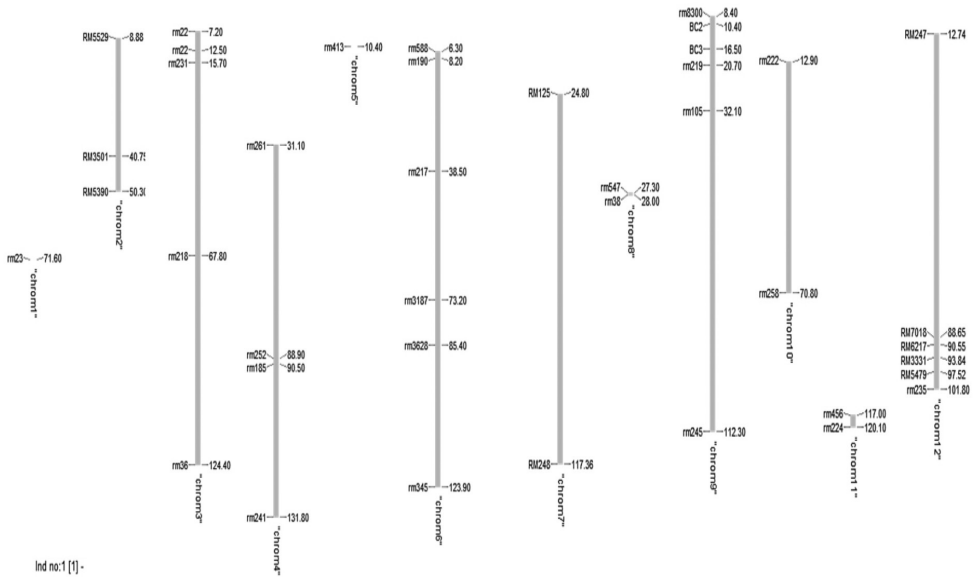

FIGURE 1. Chromosome wise distribution of each polymorphic marker between MR263 and Swarna-*Sub1*

TABLE 2. List of polymorphic SSR markers with their chromosome location

No.	Chromosome	SSR Primer	Location (cM)
1	1	RM23	71.6
2	2	RM5529	8.8
3	2	RM3501	40.75
4	2	RM5390	50.3
5	3	RM22	7.2
6	3	RM231	15.7
7	3	RM218	67.8
8	3	RM36	124.4
9	4	RM261	31.1
10	4	RM252	88.9
11	4	RM185	90.5
12	4	RM241	131.8
13	5	RM413	10.4
14	6	RM588	6.3
15	6	RM190	8.2
16	6	RM217	38.5
17	6	RM3187	73.2
18	6	RM3628	85.4
19	6	RM345	123.9
20	7	RM125	24.80
21	7	RM248	117.36
22	8	RM547	27.3
23	8	RM38	28
24	9	RM105	32.1
25	9	RM245	112.3
26	9	RM8300	8.40
27	9	BC2	10.40
28	9	BC3	16.50
29	9	RM219	20.70
30	10	RM222	12.9
31	10	RM258	70.8
32	11	RM456	117
33	11	RM224	120.1
34	12	RM235	101.8
35	12	RM5479	97.52
36	12	RM3331	93.84
37	12	RM6217	90.55
38	12	RM7018	88.65

were previously found to be closely linked to *Sub1* gene on chromosome 9 were surveyed for parental polymorphism. Out of these two markers RM8300 and RM219 showed clear polymorphism and were used for foreground selection in F_1 and F_2 populations.

In order to select F_2 plants carrying the *Sub1* gene, genomic DNA was isolated from all the 256 F_2 individuals and genotyped using the SSR markers RM219 and RM8300. Eleven best F_2 lines based on their genotypic data were selected from 125 F_2 heterozygotes plants on the basis of presence of *Sub1* gene (Figures 2-4). In earlier study of MAS, few diagnostic markers were used to select submergence-tolerant varieties, such as markers RM23805, RM8300, RM219 (Xu et al. 2006). Introgression of QTLs associated with submergence tolerance into different varieties have been carried out by several researchers (Neeraja et al. 2007; Siangliw et al. 2003; Toojinda et al. 2005; Xu et al. 2004).

In F_2 generation, recombinant selection was done to get recombinant at each side of the *Sub1* QTL to reduce the size of the inserted segment. For the determination of the size of the *Sub1* region of chromosome 9 one flanking markers were used in F_2 generation. Neeraja et al. (2007) used RM316 (1.8cM) and RM219 (11.7cM) at the proximal and distal end, respectively, in case of development of Swarna-*Sub1*. Selection of flanking marker should be chosen to keep the donor segment as closely as possible to reduce the linkage drag (Hospital 2001). Young and Tanksley (1989) proposed the optimal number of population and the generation to obtain the double recombinant for closely linked markers. The distance between the two closest markers (RM8300 and RM219) at both sides of the *Sub1* QTL was 12.3 cM. SSR markers were linked to *Sub1* were analyzed for the alleles existing in F_2 plants. All of these markers exhibited visible polymorphic bands and showed consistent band sizes between resistant

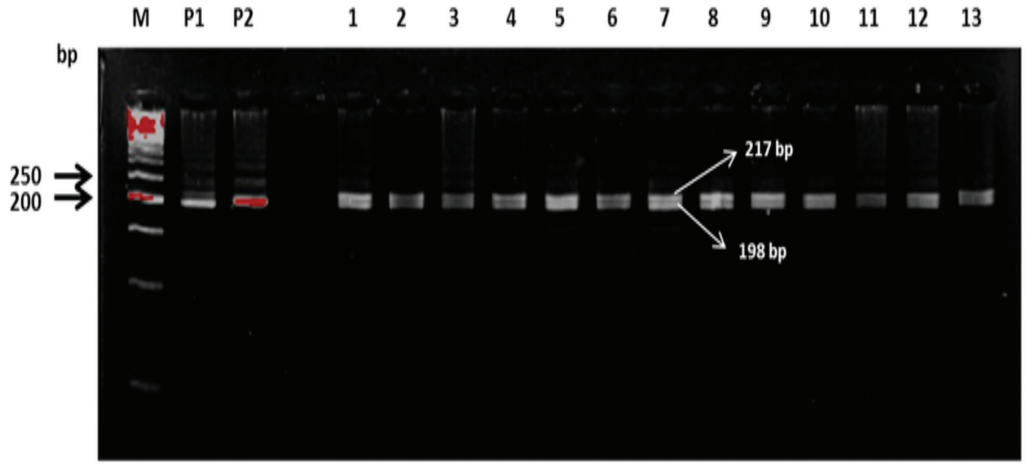

FIGURE 2. Banding pattern of F_1 plants of MR263 × Swarna-*Sub1* for RM8300 marker linked to *Sub1* gene

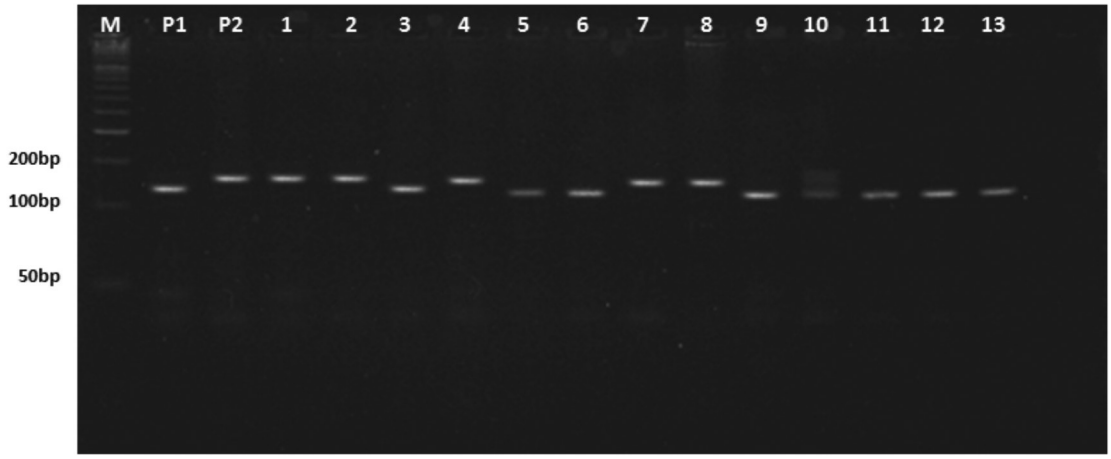

FIGURE 3. Banding pattern of F_2 plants of MR263 × Swarna-*Sub1* for RM8300 marker linked to *Sub1* gene

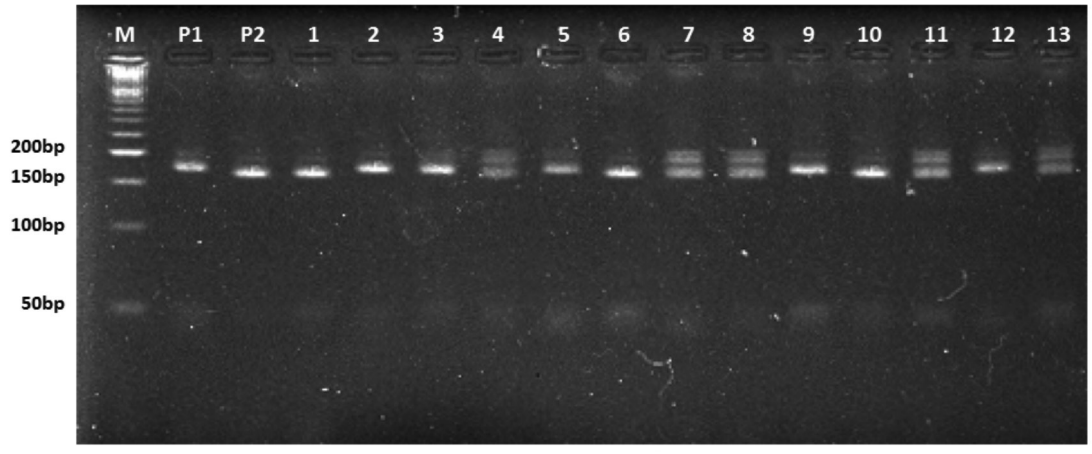

FIGURE 4. Banding pattern of F_2 plants of MR263 × Swarna-*Sub1* for RM219 marker linked to *Sub1* gene

or susceptible accessions. Segregation ratio for used markers is shown in Table 3. The segregation analysis showed that the F_2 plants containg *Sub1* gene associated with the markers RM219 and RM8300. These markers showed a good fit to the expected genotypic segregation ratio (1:2:1) in the Mendelian single gene model (Df=1.0, $p \leq 0.05$). From this study, it was shown that the markers RM8300 and RM219 could be used for the introgression of the *Sub1* gene into MR263 rice variety which is prone to submergence stress.

SUBMERGENCE SCREENING

Selected 11 lines showed submergence tolerance and also recovery after desubmergence. MR263 did not recover from submergence stress, while resistant checks and all the selected lines showed a very good survival capacity and recovery. Selected lines along with their donor parent showed very good survival percentage (above 80%), during testing on glass house. The above result confirms the presence of *Sub1* on the selected lines (Table 4). In submergence screening at glasshouse all the selected lines showed tolerance, which is similar to its donor parent Swarna-*Sub1*. In agronomical performance study, all the seleced newly developed lines showed the performance close to that of high yielding variety MR263.

AGRONOMICAL PERFORMANCE OF F_2 LINES

Out of selected lines from F_2, 11 were selected based on their agronomical characteristics in 2013-2014 seasons at Ladang 10 UPM, Malaysia. Agronomical performance of the 11 selected lines were evaluated regarding different yield and yield contributing factors. Significant variation

TABLE 3. Marker analysed in the F_2 population derived from cross between MR263 × Swarna-*Sub1* indicates that the observed F_2 segregation ratios of this population were not significantly different from the expected 1:2:1 single dominant gene segregation

Markers	Marker segregation analysis (no. of lines observed)			χ^2 (1:2:1)	*p* value
	A	AB = SG	B		
RM219	70	133	53	2.65	0.265
RM23679	54	166	36	25.09	<0.001
RM8300	69	125	62	0.52	0.771
RM23805	81	150	25	32.06	<0.001
RM23915	73	142	41	11.06	0.004

According to a model on asingle dominant gene, (A): Resistant; (B): Susceptible; and (AB or SG): Segregant.
df =1.0; $\chi 2$ (0.05, 1)=3.84; $\chi 2$ (0.01, 1)=6.633

TABLE 4. Submergence screening of MR263 × Swarna-*Sub1* lines in field condition

Variety/Lines	% Survival		Submergence tolerance score
	6 days after de-submergence	30 days after de-submergence	
FR13A	100.0	99.9	1
Swarna-*Sub1*	93.0	84.5	3
BR11-*Sub1*	91.8	80.8	5
MR263	15.0	10.0	9
MR220	8.3	3.3	9
MR219	5.3	2.3	9
L01	87.0	79.0	6
L02	81.0	81.0	7
L03	81.0	75.0	6
L04	84.0	74.0	6
L05	79.0	75.0	6
L06	78.3	76.3	6
L07	75.0	75.0	6
L08	85.0	85.0	7
L09	78.0	75.0	6
L10	82.0	72.0	6
L11	86.8	76.8	6

Score for tolerance after 6 days: 1, erect dark green leaves, very little elongation; 3, erect green leaves, little elongation; 5, droopy, pale green leaves, moderate elongation; 7, long, pale green leaves, elongated, few survived; 9, long whitish leaves, elongated, completely dead

TABLE 5. Different Yield and yield contributing characters of the newly developed submergence tolerance lines

	Days of maturation	Plant height	Tiller number/ hill	Panicle number/ hill	Panicle length	Filled grain	Grain length	Grain width	1000-grain weight	Yield/ hill
MR263	100.07	66.58	29.49	31.40	24.27	72.77	9.57	2.24	27.39	55.39
L05 (MR263-*Sub1*)	99.63	66.10	25.56	27.89	26.18	58.46	9.29	2.20	26.22	53.01
L09 (MR263-*Sub1*)	110.34	70.19	25.80	27.89	26.18	60.30	8.84	2.31	24.65	49.84
HSD (0.05)	7.6	6.45	21.404	3.78	5.56	7.86	0.008	0.010	6.06	0.766

was found among all the characters except grain width and grain length with the parent MR263 (Table 5).

CONCLUSION

In this study, the rapid and precision introgression of *Sub1* gene into MR263 was done by F_2 generation. The specific aim of the present study was to keep the donor segment size as small as possible with the help of flanking markers (recombinant selection) within F_2 generation. Thirty eight out of 180 evenly distributed SSR markers were screened for parental polymorphism between MR263 and Swarna-*Sub1*. RM8300 and RM219 showed clear polymorphism between the parental lines and it was used for foreground selection in F_1 and F_2 generations. Out of 256 F_2 plants, 125 plants were heterozygous as identified by RM8300 and RM219. Only 11 F_2 plants were selected considering the primers and agronomical performances. The selected plants were subjected for further submergence and morphological evaluation. The MAS approach described in this study was successfully used to introgress *Sub1* gene into MR263 and could be utilized to introgress *Sub1* gene into other important Malaysian rice varieties with minimum introgression segment and within shorter time frame.

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