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IMPROVEMENT OF THE SUBMERGENCE STRESS TOLERANCE OF LOCAL SOUTH SUMATRAN RICE THROUGH THE INTROGRESSION OF THE *Sub1* GENE BY USING MARKER-ASSISTED SELECTION

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SUMMARY

Submergence stress due to unpredictable soil flooding is one of the main constraints encountered in rainfed growing areas, especially in Southern Sumatran riparian swamps. The development of submergence-stress-tolerant cultivars through the introgression of *Sub1* via marker-assisted backcrossing (MABC) is an ideal solution. This study was carried out during 2020 at Sriwijaya University, Palembang, Indonesia, with the aim to select *Sub1*-introgressed lines in BC_3F_1 generations on the basis of MABC and to screen out the SSR markers that were unlinked to the target gene for application in subsequent background selection studies. Results revealed that almost all the backcrossed progenies segregated from the rice parental cultivars 'FR13A' and 'Pegagan'. The backcrossed lines showed significantly improved submergence stress tolerance and recovery rates compared with their parents. *Sub1* introgression into the BC_3F_1 generation was confirmed by the tightly linked *Sub1* marker *SUB1*C173, and marker RM23915 was used for recombinant selection. These markers followed the expected marker segregation ratio in accordance with the Mendelian single gene model. In the parental polymorphism survey, 84 out of 237 SSR markers that were unlinked to the target loci were found to be available for background study. Twenty-seven backcrossed lines were selected on the basis of foreground selection. Seven plants were selected on the basis of the recombinant marker RM23915. Five backcrossed lines were further selected on the basis of submergence stress tolerance and agronomic performance.

Keywords: Backcrossing, SSR markers, *Sub1* gene, submergence tolerance, *Oryza sativa* L.

Key findings: Twenty-seven out of 50 plants were found to be heterozygous by using the foreground marker *SUB1*C173. Twenty-six out of 27 plants were selected on the basis of phenotypic characteristics. Seven plants were selected on the basis of the recombinant marker RM23915. Furthermore, five lines were further selected for their submergence stress tolerance and agronomic performance.

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INTRODUCTION

Rice is one of the most commonly consumed staple food worldwide, especially in Asia (Muthayya *et al*., 2014). In contrast to rice demand and global population growth, the total production of rice has gradually decreased over the years (Pandey *et al.,* 2010). Climate change and various abiotic stresses are important factors influencing rice crop production (Mishra *et al*., 2015).

South Sumatran riparian wetlands have the highest potential (approximately 298.189 ha) for rice cultivation (BPS, 2021). Despite their potential, these lands have several limitations, such as the lack of nutrients, and biotic and abiotic constraints, including unpredictable submergence and drought stresses (Irmawati *et al*., 2015; Lakitan *et al*., 2018). Among these constraints, submergence stress caused by unpredictable soil flooding is considered to be an important factor that leads to decreased rice production (Septiningsih *et al*., 2013; Irmawati *et al*., 2015). Hence, several strategies are required to sustain and improve rice production (Lakitan *et al*., 2018). An ideal and widely used strategy to tackle this constraint is the development of submergence-stress-tolerant rice cultivars through the introgression of *Sub1* by using marker-assisted backcrossing (MABC) (Oladosu *et al*., 2020).

In rice, submergence stress tolerance is regulated by a major quantitative trait locus (QTL), namely, *Sub1*, which has been mapped on chromosome 9 in the donor cultivar 'FR13A' (Xu and Mackill, 1996; Nandi *et al.,* 1997; Xu *et al.,* 2000). *Sub1* is an ethylene-response-factor-like gene that encodes three transcription factors (*Sub1A*, *Sub1B*, and *Sub1C*). *Sub1A* is a key regulator of submergence tolerance in rice (Xu *et al*., 2006; Fukao and Bailey-Serres, 2008). Previous studies have reported that *Sub1* gene introgression resulted in significant improvement in submergence stress tolerance (Xu *et al*., 2006) without negative effects on rice agronomic traits (Sarkar *et al*., 2009; Singh *et al*., 2009).

MABC is an effective approach to introgressing the *Sub1* gene into susceptible rice cultivars (Neeraja *et al*., 2007; Iftekharuddaula *et al*., 2011; Septiningsih *et al*., 2013). MABC involves the use of molecular markers to select genes that control a desirable trait while maintaining the essential characters of elite rice cultivars (Hasan *et al*., 2015; Oladosu *et al*., 2020). The main objective of MABC is to insert a specific gene from the donor parent into the recipient parent at a certain target locus while minimizing the undesirable donor genome in backcrossed progenies (Hospital and Charcosset, 1997; Frisch and Melchinger, 2005). MABC has been reported to be effective in the introgression of genes for salinity tolerance (Linh *et al*., 2012) and drought tolerance (Batieno *et al*., 2016). IRRI and the Indonesian Centre for Rice Research (ICRR) have developed Indonesian submergence-tolerant rice varieties, such as 'Ciherang-*Sub1*' and 'PSB Rc18-*Sub1*' (Septiningsih *et al.*, 2014, Rumanti *et al*., 2018), as well as 'Inpara 4' and 'Inpara 5' (Hairmansis *et al*., 2012).

Gusmiatun *et al*. (2015) has made considerable progress in developing the BC_1F_1 'Pegagan-*Sub1*' by using the rice cultivar 'FR13A' as the donor parent. Hasmeda *et al*. (2017) developed the rice BC_2F_1 'Pegagan-*Sub1*' by using the markers RM23805 and RM23915. The main objective of this study is to select *Sub1*-gene-introgressed lines in BC_3F_1 generations on the basis of MABC and screen out the SSR markers that are unlinked to the target gene for subsequent studies on background selection.

MATERIALS AND METHODS

Plant material

This study is an expansion of previous studies by Gusmiatun *et al*. (2015) and Hasmeda *et al*. (2017) on developing local South Sumatran submergence-tolerant genotypes. In this study, the recurrent parent cultivar was 'Pegagan', a local rice cultivar from South Sumatra. 'Pegagan' is a high-yielding variety that has good cooking quality and taste but is sensitive to submergence stress (Hanum *et al.,* 2017; Adriansyah *et al.,* 2018). The donor parent was BC_2F_1 'Pegagan' and *Sub1*-derived lines from 'FR13A' (Hasmeda *et al*., 2017). The BC_1F_1 and BC_2F_1 plants were selected by using SSR markers (RM23805 and RM23915). Their submergence stress tolerance has been investigated in a previous work (Hasmeda *et al*., 2017). 'Pegagan' F_1 plants carrying *Sub1* were obtained from a cross between 'Pegagan' × 'FR13A' (*Sub1* donor) by Gusmiatun *et al*. (2015). This study was carried out during 2020 at Sriwijaya University, Palembang, Indonesia. The eight selected BC_2F_1 plants were backcrossed to eight plants of the recurrent parent to obtain the BC_3F_1 rice population. A total of 50 BC_3F_1 plants were genotyped by using the foreground and recombinant markers. Seven selected lines were evaluated on the basis of their phenotypic and agronomic performances and submergence stress tolerance.

DNA extraction

The DNA from 'FR13A', 'Pegagan', BC_2F_1 'Pegagan', and 50 selected plants of the BC_3F_1 'Pegagan' generations were isolated by using a kit from Wizard Genomic (Promega, USA). A total of ±50 mg of 5–10 cm long young leaves of 2-week-old plants was ground in liquid nitrogen and transferred into a 1.5 ml microcentrifuge tube. Then, 600 µl of nuclei lysis solution was added and mixed through vortexing for 1–3 s. The mixture was incubated at 65 °C for 25 min, added with 3 µl of RNase solution, and inverted 2–5 times to lyse cells and remove RNA. Then, the mixture was incubated at 37 °C for 15 min. Before proceeding to the next step, the mixture was cooled at room temperature. Then, 200 µl of protein precipitation solution was added to the mixture, which was then vortexed vigorously for 20 s at high speed. The mixture was centrifuged for 3 min at 13 000 rpm (13 000–16 000 × *g*) to precipitate proteins. The supernatant was transferred into a new clean 1.5 ml microtube containing 600 µl of isopropanol at room temperature. The supernatant was gently mixed through inversion until thread-like strands of DNA formed a visible mass. Then, the contents were centrifuged at 13 000 rpm (13 000–16 000 × *g*) for 1 min at room temperature. The supernatant was carefully decanted. Subsequently, 600 µl of 70% ethanol was added, and the microtube was gently inverted several times to wash the DNA. The contents were centrifuged at 13 000 rpm (13 000–16 000 × *g*) for 1 min at room temperature. The ethanol was carefully aspirated by using a pipette tip. The microtube was inverted onto clean absorbent paper and air-dried for 15–30 min. Subsequently, the DNA was dissolved in 50 µl of ddH_2O, then incubated for 24 h at room temperature. The DNA was stored at 2 °C–8 °C. The DNA was quantified by using a Nanodrop spectrophotometer (ND1000 Spectrophotometer) and then electrophoresed in 1× TAE buffer at 65 V for 30 min on 1% agarose gel stained with 1 µl of 1× Gel Red.

Polymerase chain reaction

All markers were subjected to polymerase chain reaction (PCR) in a single 96-well PCR Biorad (MJ Research Inc., USA) with a total single-locus PCR volume of 25 µl comprising 2 µl of template DNA from 'FR13A', 'Pegagan', BC_2F_1 'Pegagan', and BC_3F_1 'Pegagan' generations; 1 µl each of the forward and reverse primers of the SSR markers; 12.50 µl of MyTaq DNA polymerase (Bioline, BIO); and 8.50 µl of ddH_2O. The amplification was carried out under the following conditions:

predenaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 2 minutes. The PCR products were separated by electrophoresis on 1% agarose gel in 1× TBA stained with 1 µl of DNA dye (GelRed, Biotium Inc., USA). Images were taken by using Kodak Gel Logic 112 (Carestream, USA).

Parental polymorphism and foreground and recombinant study

The objective of the parental polymorphism study is to screen the availability of certain polymorphic SSR markers between two parents that can be used as selection markers in background selection (Hasan *et al*., 2015; Oladosu *et al*., 2020). In the polymorphism study, 237 SSR markers that were unlinked to the target loci and distributed on 12 chromosomes were used to screen for polymorphism between the parental cultivars 'Pegagan' and 'FR13A'. The details of the markers were obtained from the GRAMENE database (http://www.gramene.org/). The markers with clear and reproducible polymorphic banding patterns were used for future background selection. In background selection, the availability of these markers is essential as a tool for screening backcrossed lines to recover the recurrent parent genome of backcrossed recombinant lines (backcrossed recombinant lines with the highest recurrent parent genome) (Hasan *et al*., 2015; Oladosu *et al*., 2020).

Foreground selection is the first step in MABC. The tightly linked QTL markers of interest are used in foreground selection (Hasan *et al*., 2015; Oladosu *et al*., 2020). In this study, the tightly linked marker *SUB1*C173 (exon for *Sub1C*) (Septiningsih *et al*., 2009) and two flanking markers for recombinant selection, i.e., RM464A and RM23958 (Neeraja *et al*., 2007), were genotyped between two parents to identify their availability. The marker SUB1C173 was available and used for foreground selection. *SUB1*C173 was amplified in all backcrossed plants alongside their donor and recurrent parents. The heterozygous plants based on *SUB1*C173 were selected and subjected to phenotypic selection and recombinant selection.

The purpose of recombinant selection is to reduce linkage drag in QTLs by utilizing flanking markers (Hasan *et al*., 2015; Oladosu *et al*., 2020). RM23915, a flanking marker that was found to be polymorphic by Hasmeda *et al*. (2017), was used as the recombinant selection marker. The details of the markers used in the study are provided in Table 1.

Table 1. Tightly linked and flanking markers of the *Sub1* gene.

Primers	Marker type	Primer Sequence (5'–3')		Repeat Motif
		Forward sequence	Reverse sequence	
RM464A	Flanking marker	AAC GGG CAC CTT CTG TCT TC	TGG AAG ACC TGA TGG TTT CC	(CT) 27
RM23958	Flanking marker	CTACCACTGTTTCATTGTGTCTCG	GAATTGAAGGAGAAGCAGGAAGC	(CT)15
RM23915	Flanking marker	GAGGATCCTTACCATCAAACTTCG	CCAAGAACCTGCATTCTTCAAGG	(AC)15
*SUB1*C173	Tightly linked marker	AACGCCAAGACCAACTTCC	AGGAGGCTGTCCATCAGGT	N/A

Phenotypic study, submergence screening, and agronomic evaluation

Phenotypic selection was performed to identify and compare the phenotypic similarity of all selected backcrossed lines to its recipient parent (Iftekharuddaula *et al.* 2012). Phenotypic selection was carried out at the greenhouse of the Faculty of Agriculture, Sriwijaya University, Indonesia. All the plants were evaluated visually at the vegetative stage in accordance with the protocols reported by Iftekharuddaula *et al.* (2012) and IRRI (2013). The phenotypic and agronomic evaluation of the traits days to maturation, plant height, tiller number, productive tillers, flag leaf length, panicle length, grains per panicle, filled grains per panicle, percentage of filed grains, grains weight per panicle, grain length, grain width, 1000-grain weight, and grain yield per plant was performed. These data were analyzed by using the hierarchical clustering method with SPSS23 software. The plants with the highest scores of phenotypic similarity to the recurrent parent were selected for the next selection step. For agronomic evaluation, seven selected BC_3F_1 lines alongside the donor parent and recurrent parent were used as the check varieties. The experiment was laid out with six replications and analyzed through analysis of variance (ANOVA) followed by Honest Significant Difference (HSD) with SAS software.

Submergence-stress-tolerant plants were screened in submergence experimental ponds at the Faculty of Agriculture, Sriwijaya University, Indonesia, in accordance with standard protocols (Neeraja *et al*., 2007; IRRI, 2013). Submergence stress tolerance screening was performed with 10 genotypes that comprised seven lines of the selected BC_3F_1 'Pegagan' and three check varieties, namely, 'FR13A' (tolerant variety), the donor parent (BC_2F_1 'Pegagan'), and the recurrent parent ('Pegagan'). A total of 30 14-day-old rice seedlings of each genotype were submerged for 14 days. The survival and recovery rates of all the plants were recorded on the 6th and 30th day after desubmergence. After 6 days, tolerance was scored as follows: 1, erect dark green leaves and very little elongation; 3, erect green leaves, and little elongation; 5, droopy, pale green leaves, and moderate elongation; 7, long, pale green leaves, elongated, and few surviving plants; 9, long whitish leaves, elongated, and completely dead. Survival percentages were scored as follows: 1, minor visible symptoms of injury, and high tolerance, and 100% survival; 3, some visible symptoms of injury, tolerant, and 95%–99% survival; 5, moderate injury, moderately tolerant, and 75%–94% survival; 7, severe injury, susceptible, and 50%–75% survival; 9, partial to complete death, highly susceptible, and 0%–49% survival.

Statistical analysis

All the data markers were scored as 'A' for the homozygous recipient, 'B' for the homozygous donor allele, and 'H' for the heterozygous allele. The marker data were analyzed by using Graphical Genotypes (GGT 2.0) software (Berloo, 2008). COLONY software was used to analyze the validity of backcrossing (Jones and Wang, 2010). The suitability of the chi-square ($\chi 2$) Mendelian segregation ratio of markers were analyzed with Popgene software (Yeh *et al.,* 1999) by using the formula, $\chi^2 = (O - E)2/E$, where the observed value was O, and the expected value was E. Phenotypic selection was analyzed by hierarchal clustering method with SPSS 23 software. The agronomic performances of the seven BC_3F_1 selected lines alongside with those of their donor parent and recurrent parent were laid out with six replications and analyzed through ANOVA followed by HSD calculated by SAS software.

RESULTS AND DISCUSSION

SSR polymorphism study

A total of 273 SSR markers (non-target loci) were used as the parental polymorphism study markers to screen for SSR markers (unlinked to the QTL) that can be used for background to recover the recurrent parent genome. Among these markers, 84 (35.443%) were found to be polymorphic between two parents (Figures 1 and 2, Table 2). The number of polymorphic SSR markers depends on the total used markers and the rice cultivars (Collard and Mackill, 2008; Hasan *et al*., 2015). In another study, Mojulat *et al*. (2017) found 21.11% polymorphic SSR markers between the rice parental genotypes 'MR263' and 'Swarna-*Sub1'.* Khanh *et al*. (2013) reported that the frequency of SSR polymorphic markers between the rice parental genotypes 'Bacthom 7' and 'IR64' was 15.1%. The availability of these markers is essential for background selection (Hasan *et al*., 2015; Oladosu *et al*., 2020). The background study is essential for determining the size of the rice recurrent parental genome (Transley *et al*., 1989; Frisch and Melchinger, 2005) or selecting against the undesirable genome from the rice donor parent (Hospital, 2001). The observed polymorphic SSR markers will be used in future background studies on rice.

Foreground selection

The main objective of foreground selection is to screen the *Sub1* gene that was introgressed into crossed or backcrossed lines by utilizing tightly linked QTL markers (Hasan *et al*., 2015; Oladosu *et al*., 2020), such as *SUB1*C173 (Septiningsih *et al*., 2009). In this study, 50 plants were obtained from backcrossing and were genotyped by using *SUB1*C173 markers. In foreground selection, the 48 plants produced sufficient DNA yields, and 27 plants were recorded with the heterozygous allele (H score), indicating that the *Sub1* gene had been introgressed into the backcrossed progenies. The results further revealed that 20 plants were identified with the susceptible recipient allele (score 'A'), and only one plant had the fixed resistant donor allele (score 'B'). Figure 3 shows the banding pattern of the BC_3F_1 progeny with the marker *SUB1*C173: the donor parent had a band at 175 bp; the recurrent parent had bands at 150, 200, and 300 bp; and the heterozygote plants (*Sub1* introgressed plants) had bands at 175, 200, and 300. The 27 plants that were recorded with the 'H' score were subjected to phenotypic selection and self-pollinated to obtain the BC_3F_2 rice population. The marker *SUB1*C173 was found to conform to the expected genotypic segregation ratio of 1:1 in accordance with the Mendelian single gene model and had a nonsignificant χ^2value of 0.03 (0.05 of probability level).

In this study, the marker *SUB1*C173 was found to be suitable for foreground selection, whereas markers RM464A and RM23958 did not show any polymorphism. The same findings were also reported by Mojulat *et al*. (2017), who found that that markers RM464A and RM23958 were not polymorphic between the rice parental genotypes 'MR263' and 'Swarna-*Sub1*'. The availability of tightly linked or flanking markers depends on rice genotypes (Amin *et al*., 2019). The marker *SUB1*C173 is widely used to verify the introgression of *Sub1* into various rice cultivars, such as 'Swarna-*Sub1*' (Neeraja *et al*., 2007) and 'Ciherang-*Sub1*' (Septiningsih *et al*., 2009).

The data were analyzed by using COLONY software (Jones and Wang, 2010) to identify the presence of illegitimate individuals. The results revealed that all the backcrossed progenies had segregated from the rice parental cultivars 'FR13A' and 'Pegagan' save for plant number 36, which exhibited an unexpected allele (Figure 4). One plant was exhibited a homozygous donor parent allele. In breeding programs, the BC_3F_1 generation normally produces homozygous susceptible and heterozygous alleles. In this generation, the homozygous donor allele in the rice plants was obtained due

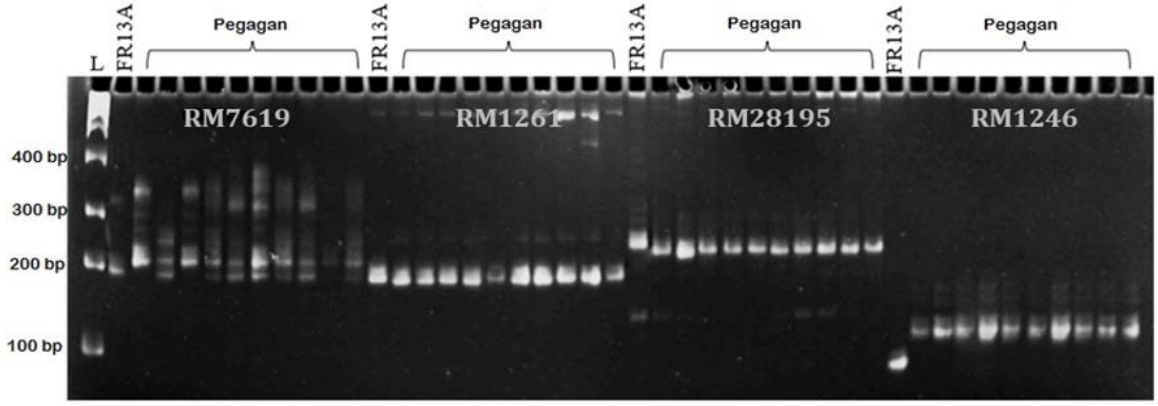

Figure 1. DNA banding pattern of several polymorphic markers surveyed between the rice parental cultivars 'FR13A' and 'Pegagan'.

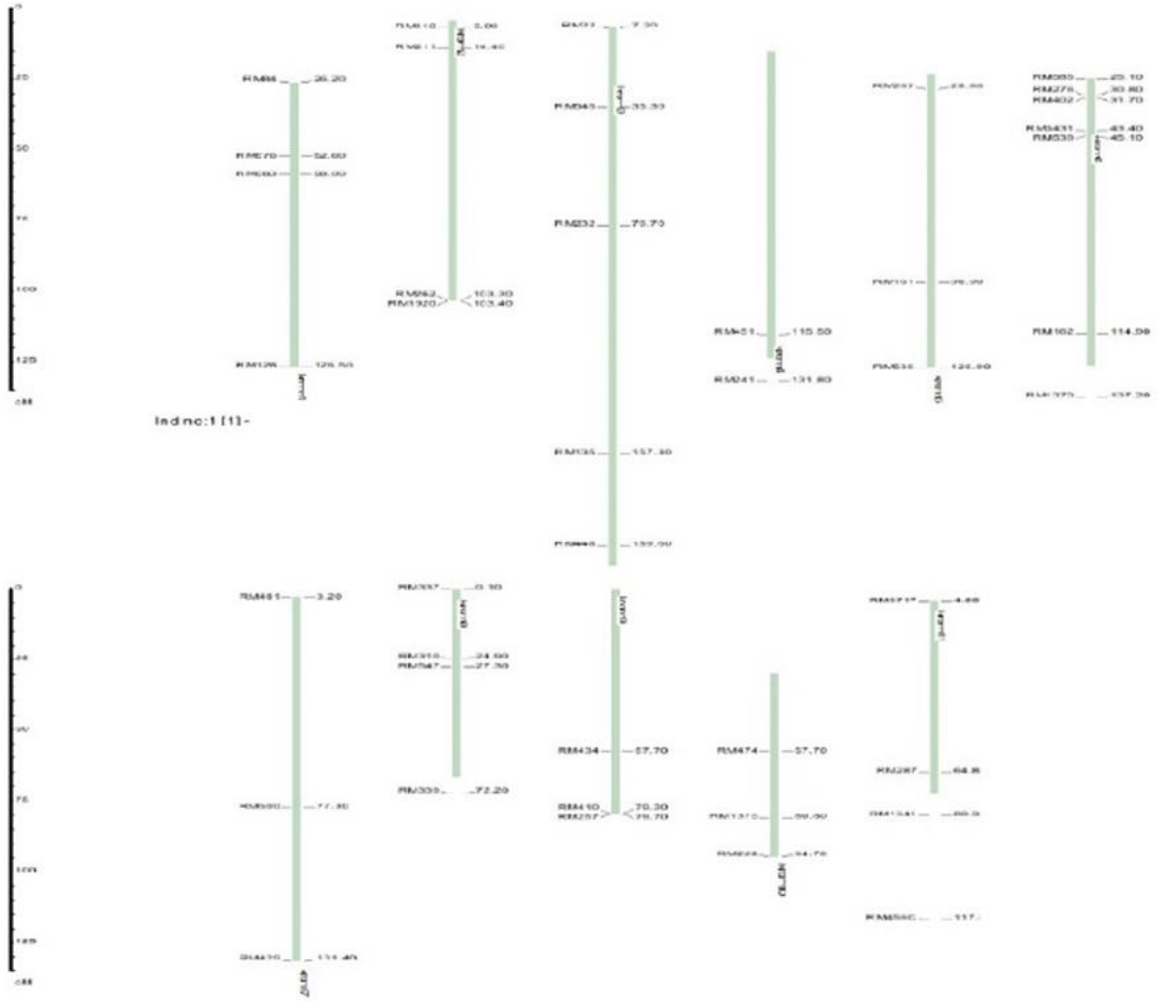

Figure 2. Positions of each polymorphic marker on 12 chromosomes in the rice parental cultivars 'Pegagan' and 'FR13A'.

Table 2. Information on SSR markers that were polymorphic between the parental rice cultivars 'Pegagan' and 'FR13A'.

No.	Chr.	SSR markers	Location (cM)	No	Chr.	SSR markers	Location (cM)
1	1	RM576	52.6	43	7	RM481	3.2
2	1	RM84	26.2	44	7	RM5672	44.1
3	1	RM583	58.9	45	7	RM542	49.7
4	1	RM580	68.2	46	7	RM182	61
5	1	RM24	79.1	47	7	RM560	69.2
6	1	RM128	134.8	48	7	RM429	99.9
7	2	RM154	4.8	49	8	RM337	0.5
8	2	RM211	14.4	50	8	RM1959	1.8
9	2	RM233A	16.3	51	8	RM407	3
10	2	RM262	78.4	52	8	RM1235	13.1
11	2	RM110	100.6	53	8	RM1376	25.9
12	3	RM22	13	54	8	RM547	43.7
13	3	RM585	25.1	55	8	RM72	60.9
14	3	RM545	35.3	56	8	RM339	72.2
15	3	RM282	100.6	57	8	RM531	90.3
16	3	RM135	153.7	58	9	RM23679	0.5
17	3	RM570	158.2	59	9	RM434	56.8
18	3	RM448	189.6	60	9	RM410	64.1
19	4	RM537	8.5	61	9	RM257	65.1
20	4	RM2848	16.7	62	9	RM288	69.5
21	4	RM1869	21	63	9	RM242	73.6
22	4	RM1388	22	64	9	RM108	76.9
23	4	RM273	23	65	10	RM330A	2.4
24	4	RM241	24	66	10	RM474	3
25	4	RM348	25	67	10	RM222	11.3
26	4	RM451	26	68	10	RM1375	44.3
27	5	RM153	27	69	10	RM1873	51.5
28	5	RM267	28	70	10	RM258	70.8
29	5	RM440	29	71	10	RM228	94.7
30	5	RM161	30	72	11	RM4B	3.4
31	5	RM233B	110	73	11	RM20B	3.8
32	5	RM538	132.7	74	11	RM3717	4.8
33	6	RM540	0	75	11	RM287	64.8
34	6	RM585	25.1	76	11	RM229	77.8
35	6	RM276	33.5	77	11	RM1341	80.3
36	6	RM402	40.3	78	11	RM206	88.7
37	6	RM549	42.7	79	11	RM456C	117
38	6	RM539	45.1	80	12	RM7619	3.8
39	6	RM3431	52.3	81	12	RM4A	5.2
40	6	RM402	52.3	82	12	RM20A	9.7
41	6	RM162	104.8	83	12	RM28195	62.2
42	6	RM1370	110.6	84	12	RM1226	109.2

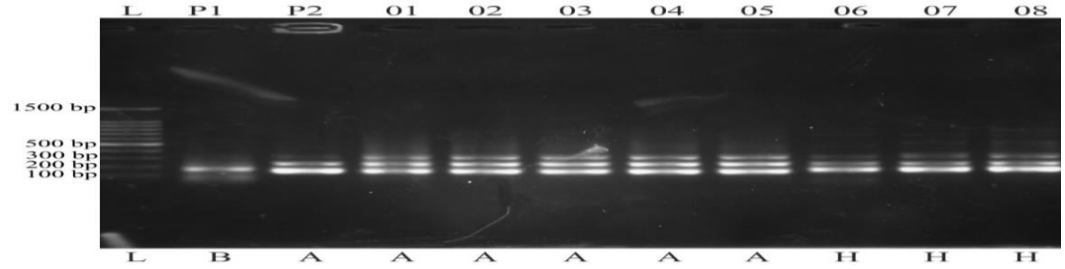

Figure 3A

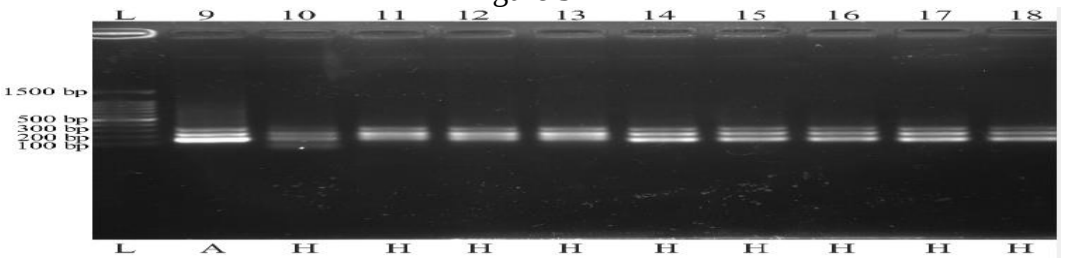

Figure 3B

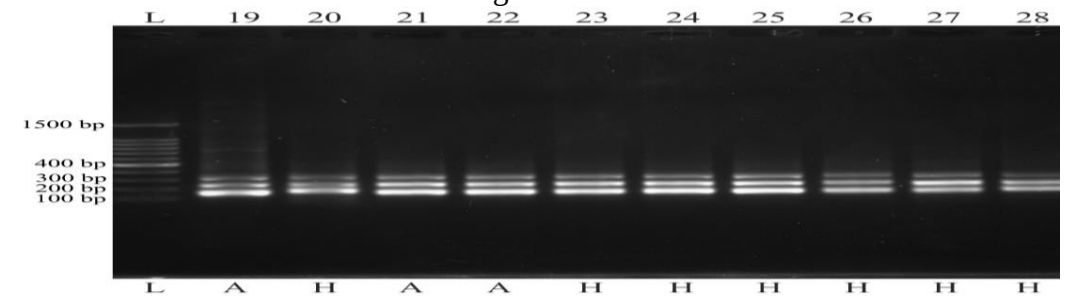

Figure 3C

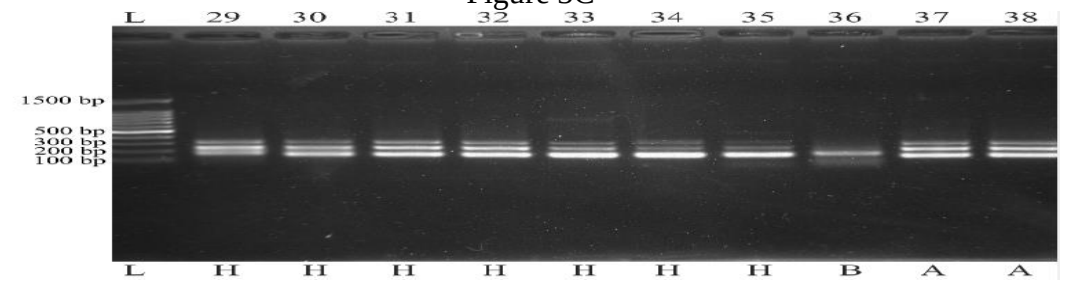

Figure 3D

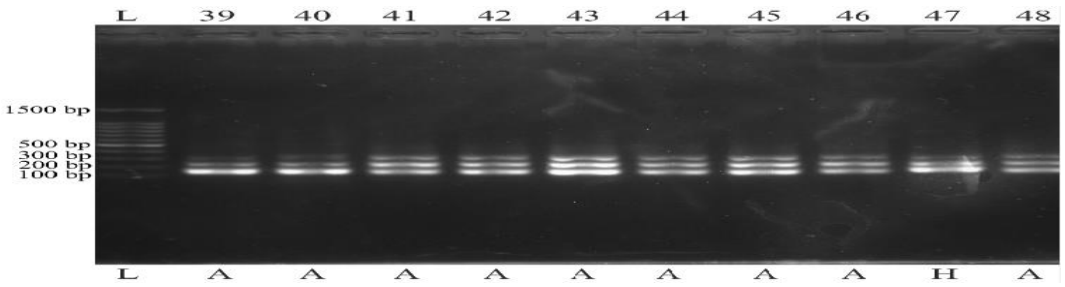

Figure 3E

Figure 3. DNA banding pattern obtained through foreground selection in BC_3F_1 generation with the marker *SUB1*C173. L: ladder; P1: the donor parent; P2: the recurrent parent; 1–48: plant number.

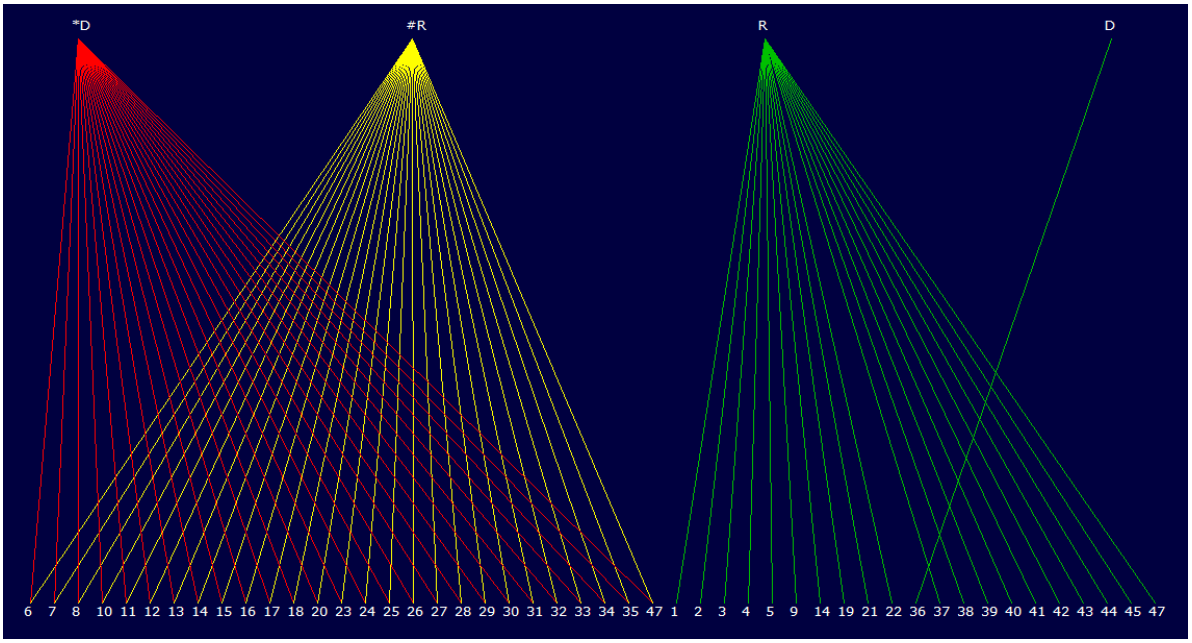

Figure 4. Pedigree analysis between two rice parental cultivars ('Pegagan' and 'FR13A') and their backcrossed progenies based on the *SUB1*C173 marker. *D, red lines are the male or donor parent allele; #R, yellow lines are the female or recurrent parent allele, the combination of red lines and yellow lines indicates the segregation of heterozygous lines; R, green lines indicate self-pollinated for the recurrent parent allele; D, green lines indicate the unexpected allele.

to an error in backcrossing (Acquaah, 2007) as also reported by Iftekharuddaula *et al*. (2015).

Phenotypic selection

Another objective of MABC is to screen backcrossed lines with phenotypes that resemble the phenotype of the recurrent parent, especially for the desirable trait (Hasan *et al*., 2015; Oladosu *et al*., 2020). In other words, MABC aims to reduce undesirable traits from the donor parent (Hospital, 2001). In this selection step, 27 selected BC_3F_1 lines based on *SUB1*C173 along with their parents were phenotypically evaluated in the greenhouse at the Faculty of Agriculture, Sriwijaya University, Indonesia, by following the standard protocol established by Iftekharuddaula *et al*. (2012) and IRRI (2013). Among these plants, 26 were selected on the basis of their similarity scores to the recurrent parent. These 26 plants were self-pollinated to obtain BC_3F_2 seeds. Previous studies on rice indicate that phenotypic selection has a significant correlation with marker selection (Iftekharuddaula *et al*., 2012). However, marker selection is highly effective in different rice populations (Frisch *et al*., 1999; Joshi *et al*., 1999; Frisch and Melchinger, 2005; Collard and Mackill, 2008).

Recombinant study

Recombinant selection with flanking markers is done to minimize the linkage drag from rice donor parents on a specific chromosome having the gene of interest (Hospital, 2003; Collard and Mackill, 2008). In this study, the marker RM23915 was used for recombinant selection. Out

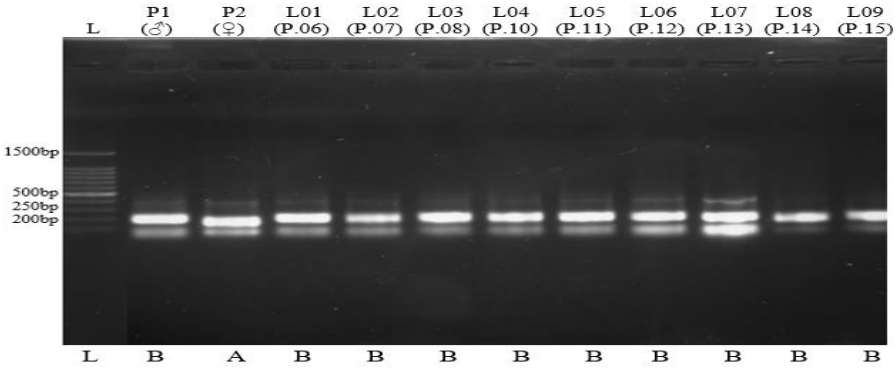

Figure 5A

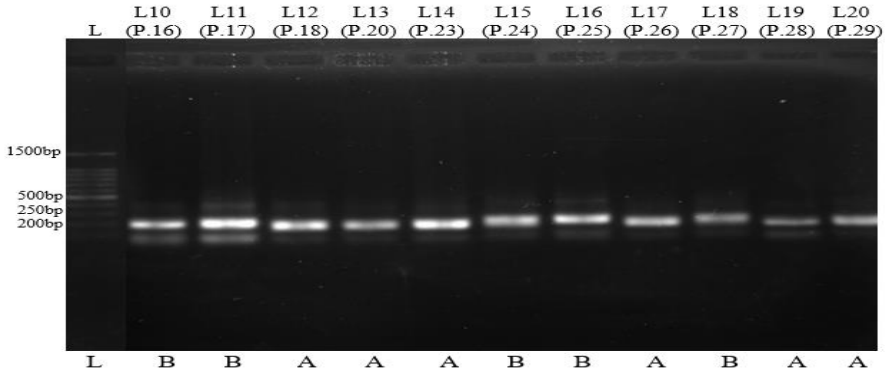

Figure 5B

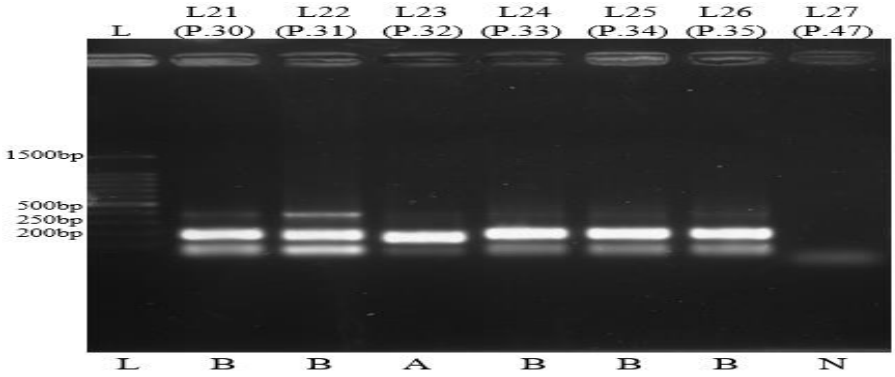

Figure 5C

Figure 5. DNA banding pattern of the recombinant marker RM23915 on electrophoresis gel. L: ladder; P1: the donor parent; P2: the recurrent parent; L01–L27: plant number of BC_3F_1 selected lines.

of 26 selected BC_3F_1 lines, seven produced recurrent alleles; the donor allele had a length of 200 bp, and the recurrent allele had a length of 175 bp (Figure 5). These selected lines were selected and evaluated for submergence stress tolerance and agronomic performance. In this study, the marker RM23915 showed a good fit with the expected genotypic segregation (1:1) in the Mendelian single gene model and a nonsignificant χ^2value of 0.03 (0.05 probability level). The marker RM23915 was located at 7.2 Mb on chromosome 9 (Neeraja *et al*., 2007).

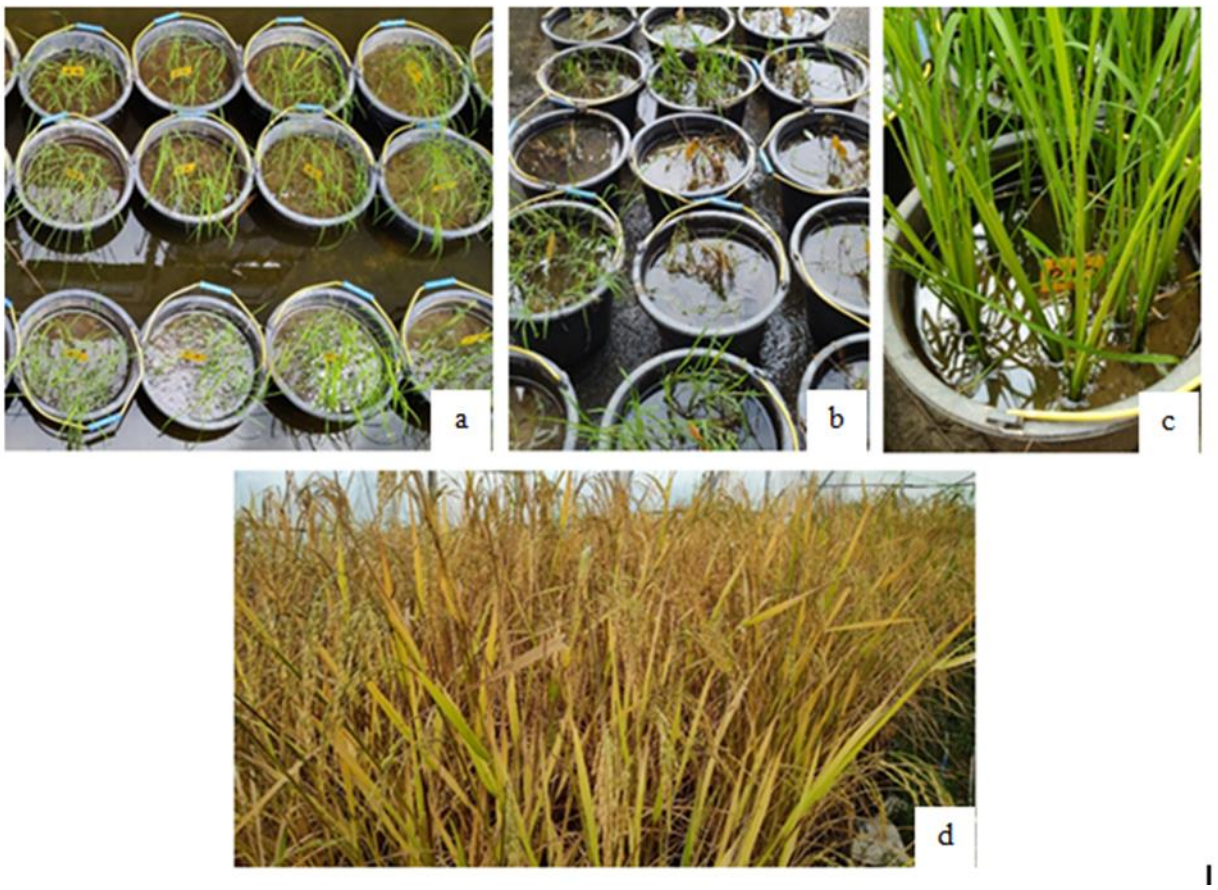

Figure 6. a: BC_3F_1 selected lines, BC_2F_1 lines, 'Pegagan' (recurrent parent), and 'FR13' (submergence tolerant cultivars) plants before submergence, b: BC_3F_1 selected lines, BC_2F_1 lines, 'Pegagan' (recurrent parent), and 'FR13' (submergence-tolerant cultivars) after submergence, c: BC_3F_1 L01 (P.18) plants after recovery, d: agronomic evaluation of BC_3F_1 selected lines.

Submergence screening

Screening for submergence-stress-tolerant cultivars was performed in the submergence experimental pond at the Faculty of Agriculture, Sriwijaya University, Indonesia. The 14-day-old seedlings of seven selected lines were submerged for 2 weeks (Figure 6a). The submergence and recovery performances of all the plants were recorded on the 7th, 14th, and 30th days (Figure 6bc). The submergence stress tolerance scores of various rice populations were also recorded in accordance with the methodology described by Neeraja *et al*. (2007) and IRRI (2013).

The submergence stress tolerance and recovery after desubmergence and survival percentage of the seven selected lines of BC_3F_1 'Pegagan', the donor parent (BC_2F_1 'Pegagan'), the recurrent parent ('Pegagan'), and 'FR13A' were recorded by using 30 14-day-old seedlings per genotype (Table 3). The recurrent parent 'Pegagan' showed no recovery, whereas tolerant plants showed the highest level of survival and recovery capacity. In this study, five selected plants, i.e., L02 (P. 20), L03 (P. 23), L04 (P. 26), L06 (P. 29), and L07 (P. 32), demonstrating submergence stress tolerance and recovery performances of >70% (moderate), indicating the presence of

Table 3. Survival rates and submergence tolerance of selected lines.

Parental cultivars/lines	No. of plants	6 days after desubmergence	30 days after desubmergence	% survival		Submergence tolerance score (after 6 days)
				6 days after desubmergence	30 days after desubmergence	
FR13A	30	30	29	100.00	96.67	1
Pegagan	30	10	8	33.33	26.67	9
BC_2F_1 Pegagan	30	20	18	66.67	60.00	6
L01 (P. 18)	30	24	20	80.00	66.67	5
L02 (P. 20)	30	25	21	83.33	70.00	5
L03 (P. 23)	30	24	22	80.00	73.33	5
L04 (P. 26)	30	26	23	86.67	76.67	5
L05 (P. 28)	30	22	20	73.33	66.67	5
L06 (P. 29)	30	24	23	80.00	76.67	5
L07 (P. 32)	30	24	23	80.00	76.67	5

Note: 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.

Sub1 (Table 3). Backcrossed lines showed significant improvement in submergence stress tolerance and recovery rates compared with their parents, which were applied in recombinant selection to minimize the negative effects due to linkage drag from the donor parents. Submergence-stress-tolerant rice responds to stress via the quiescence strategy and exhibit high recovery rates by undergoing shoot elongation and new leaf development immediately after desubmergence (Fukao and Bailey-Serres, 2008). However, submergence stress tolerance is also influenced by several factors. Submergence stress tolerance, as a polygenic rice trait, is influenced by additive genes and environmental interactions (Mohanty and Khush, 1985; Mishra *et al.,* 1996). Rice genotypes are also affected physiologically and genetically at the seedling stage (Toojinda *et al.,* 2003). Gene interaction also plays an important role in the metabolic mechanism of rice submergence stress tolerance (Mohanty *et al.,* 2000).

Agronomic performance

For agronomic evaluation, the seven selected BC_3F_1 lines alongside the donor parent (BC_2F_1 'Pegagan') and recurrent parent ('Pegagan') as check varieties were laid out with six replications under nonsubmerged conditions at the greenhouse of the Faculty of Agriculture, Sriwijaya University, Indonesia (Figure 6). The agronomic parameters of the selected rice lines, were recorded and analyzed for the evaluation of various yield and yield-contributing factors (Table 4). Significant variation was found among the selected lines for all characters. However, several characters, such as tiller number, productive tillers, and grains per panicle, of several backcrossed progenies showed significant improvement compared with those of their recurrent parent. Mojulat *et al.* (2017) reported the same findings for different rice populations. Hospital (2001) stated that the interaction of size from the donor genome might influence agronomic performance. Past studies have reported a significant improvement in certain agronomic characters due to donor parent introgression in rice (Iftekharuddaula *et al.*, 2015).

Table 4. Means of the agronomic characters of selected lines grown in a greenhouse under normal conditions.

Varia-bles	Genotypes/Lines									HSD (0.05)
	Pegagan (PG)	BC_2F_1 Pegagan	L01 (PG.18)	L02 (PG.20)	L03 (PG.23)	L04 (PG.26)	L05 (PG.28)	L06 (PG.29)	L07 (PG.32)	
DOM	124.50^{f} ± 0.55	129.83^{a} ± 0.75	126.33^{e} ± 0.52	126.66^{de} ± 0.52	127.5^{bc} ± 0.55	126.66^{de} ± 0.52	127.83^{b} ± 0.41	127.16^{bcd} ± 0.41	127.00^{cde} ± 0.63	39.72
PH	146.83^{f} ± 2.64	154.33^{de} ± 5.54	163.50^{bc} ± 6.16	166.50^{b} ± 5.09	173.50^{a} ± 2.95	159.16^{cd} ± 7.41	157.50^{cd} ± 5.43	162.83^{bc} ± 5.04	150.83^{ef} ± 2.64	16.02
TOT	26.00^{abc} ± 1.01	28.00^{a} ± 2.37	25.50^{bc} ± 1.52	27.33^{ab} ± 1.21	26.66^{abc} ± 1.21	27.50^{ab} ± 3.45	26.66^{abc} ± 1.97	25.83^{abc} ± 1.17	24.66^{ab} ± 1.21	1.99
PT	19.66^{c} ± 0.82	22.83^{ab} ± 3.06	22.00^{abc} ± 1.41	23.16^{ab} ± 0.75	23.00^{ab} ± 1.41	24.83^{a} ± 3.87	23.00^{ab} ± 3.46	21.00^{bc} ± 1.10	21.33^{bc} ± 1.86	2.64
FLL	40.50^{b} ± 11.69	39.56^{b} ± 5.33	43.50^{b} ± 6.61	46.38^{ab} ± 4.20	51.55^{a} ± 7.51	40.27^{b} ± 4.96	40.50^{b} ± 3.97	39.27^{b} ± 5.92	38.27^{b} ± 4.23	2.64
PL	27.38^{a} ± 1.47	27.35^{a} ± 1.17	25.83^{b} ± 0.35	25.94^{b} ± 0.71	26.16^{b} ± 0.59	26.44^{ab} ± 0.17	25.39^{b} ± 0.57	25.83^{b} ± 0.18	26.38^{ab} ± 1.10	4.14
TG/P	201.05^{ab} ± 3.34	186.16^{c} ± 4.41	202.39^{ab} ± 4.96	198.72^{b} ± 3.52	205.72^{a} ± 2.76	205.72^{a} ± 4.39	186.88^{c} ± 5.33	202.05^{ab} ± 2.38	200.61^{ab} ± 6.21	17.32
FG/P	180.44^{a} ± 2.63	168.27^{cd} ± 2.27	170.22^{bc} ± 0.75	172.72^{b} ± 1.56	171.66^{b} ± 2.58	170.22^{bc} ± 2.22	162.55^{e} ± 3.29	166.66^{d} ± 2.48	170.94^{bc} ± 2.45	25.77
%G/P	89.75^{ab} ± 1.18	90.42^{a} ± 2.02	84.14^{cd} ± 1.98	86.93^{bc} ± 1.58	83.45^{d} ± 1.69	82.77^{d} ± 2.22	87.00b^{c} ± 1.80	82.49^{d} ± 1.82	85.30^{cd} ± 3.77	10.54
WG/P	3.12^{a} ± 0.06	2.56^{e} ± 0.06	2.99^{c} ± 0.03	3.11^{a} ± 0.02	3.13^{a} ± 0.02	3.11^{a} ± 0.04	2.76^{d} ± 0.04	2.81^{d} ± 0.03	3.04^{b} ± 0.04	163.06
GL	1.05^{c} ± 0.01	1.11^{a} ± 0.02	1.04^{cd} ± 0.01	1.13^{a} ± 0.01	1.08^{b} ± 0.01	1.12^{a} ± 0.02	1.06^{bc} ± 0.01	1.03^{d} ± 0.05	1.05^{c} ± 0.03	17.02
GW	0.24^{ab} ± 0.06	0.22^{b} ± 0.00	0.24^{b} ± 0.00	0.25^{ab} ± 0.01	0.28^{a} ± 0.11	0.24^{ab} ± 0.00	0.24^{ab} ± 0.01	0.25^{ab} ± 0.00	0.24^{ab} ± 0.00	1.23
WG1000	27.10^{a} ± 0.50	26.12^{d} ± 0.30	26.59^{bc} ± 0.15	26.64^{bc} ± 0.15	26.89^{ab} ± 0.18	27.07^{a} ± 0.14	26.42^{cd} ± 0.16	26.67^{bc} ± 0.20	26.83^{ab} ± 0.46	7.35

Note: DOM: Days to maturation (days), PH: Plant height (cm), TOT: Total tiller number (#), PT: Productive tiller number (#), FLL: Flag leaf length (cm), PL: Panicle length (cm), TG/P: Total grain per panicle (#), FG/P: Filled grain per panicle (#), %G/P: Percentage of filled grain per panicle (%), WG/P: Weight of grain per panicle (g), GL: Grain length (cm), GW: Grain width (cm), WG1000: 1000-grain weight (g). Values of mean in the same row followed by the same letter indicate nonsignificant difference in accordance with the HSD test at α: 0.05.

CONCLUSIONS

A total of 84 markers that were unlinked to the target loci were identified for use in future background selection studies on rice. Twenty-seven out of 50 plants were found to be heterozygous by using the foreground marker *SUB1*C173. Twenty-six out of 27 plants were selected on the basis of the phenotypic study. Seven plants were selected on the basis of the recombinant marker RM23915. These lines were selected for their submergence stress tolerance and agronomic performance.

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