MOLECULAR EVALUATION FOR DROUGHT TOLERANT USING MARKER ASSISTED BREEDING METHOD

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Abstract

The sustainability and increasing the national rice production require the readiness of food and agriculture sector cope with the impacts of climate change, land degradation, drought area, sloping production and the raising of population growth. Adaptation plays an important role in ensuring the sustainability of food security. This research aimed to develop drought-tolerant variety of Inpari 30 (submergence tolerance variety) and Situ Bagendit through marker-assisted backcrossing-through pyramiding gene of identified QTLs for foreground selection and to explore SSRs and 6K SNPs for background selection distributed in 12 rice chromosome of drought tolerant donor (Cabacu) and recipient rice (Inpari 30 and Situ Bagendit). The foreground selection revealed that flanking SSRs of each QTLs (*qRPF2.1, qGPP2.1, qSPP4.1* and *Sub1*) was less than 2 cM. The background selection through polymorphic survey of Rice 6K SNP primers revealed 2457 (53,3%) polymorphic SNPs on Inpari 30 vs Cabacu and 2563 (55,6%) polymorphic SNPs on Situ Bagendit vs Cabacu with the average distance about 0.74 cM/chromosome. The genotypic selection of F_1 Inpari 30/Cabacu and F_1 Situ Bagendit/Cabacu have already in heterozygote condition for these 4 QTLs target. These lines was continued for backcross breeding to develop BC_1F_1 Inpari 30/Cabacu and BC_1F_1 Situ Bagendit/Cabacu generation.

Keywords: Cabacu, Inpari 30, marker assisted backcrossing, pyramid, Situ Bagendit

Introduction

Prolonged droughts, the shift of rainy season and heavy rainfall causing floods are the impacts of climate change in Indonesia that hinder growth and development of rice plants. These two abiotic stresses (drought and flood) decline rice production and the crop failure leading to significant yield losses. Thus, it will affect the national food security program. Ruminta *et al.* (2016) reported that the impact of climate change has reduced harvested area and production in central rice production of West Java such as Karawang, Subang, and Indramayu. The decrease in harvested area of rice in average of 77-100 ha per year and the decline in rice production average of 926.1-1000 tons per year. Wahyunto (2005) reported the level of vulnerability of agricultural land to drought was variated among the regions. The evaluated rice paddy plantation around 5,14 million ha of which 74 thousand ha was very vulnerable and one million ha was vulnerable to drought. Ministry of Agriculture (2012) reported the rice paddy plantation with flood, drought and pest and disease were 333.000 ha, 319.500 ha and 428.600 ha respectively and the yield loss around 997.300, 984.200, and 352.300 ton respectively.

In anticipating the climate change and its impacts on crop production, the development of new rice varieties to improve crops by selecting for the desired traits through gene pyramiding scheme is suggested. Joshi & Nayak (2010) suggested to cumulate several traits from multiple parents into a single genotype using marker assisted breeding approach that permits complete gene identification of the progeny at each generation (Hasan *et al*., 2015).

The sustainability and increasing the national rice production requires the readiness of food and agriculture sector to cope with the impacts of climate change. Adaptation plays

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an important role in ensuring the sustainability of food security through the development of adaptive varieties that respond to these abiotic stresses. These adaptive varieties will respond to minimize the damage, survive in the stress while keeps growing and maintain yield. This research aimed to develop drought-tolerant varieties of Inpari 30 (submergence tolerance variety) and Situ Bagendit (rain fed variety) crossed with Cabacu (drought tolerance variety) through marker-assisted backcrossing (MABc) and pyramiding gene approach.

Materials and Methods

Plant Materials and Breeding Scheme.

Two recipient parents, Inpari 30 (Ciherang-*Sub1*) and Situ Bagendit, were crossed with Cabacu as a donor parent for drought tolerant from Brazil. The MABc scheme for Inpari 30 and Situ Bagendit was crossed with Cabacu to obtain F_1 seeds (Figure 1 and Figure 2). The selected F_1 was backcrossed with the recipient parent to develop the next generation (BC_1).

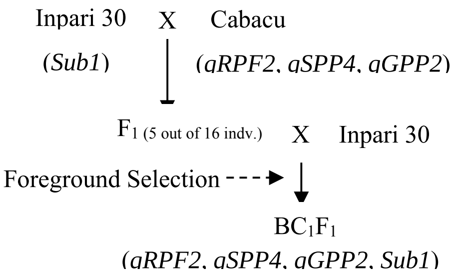

Figure 1. Scheme for the development of pyramided 4 QTLs lines of Inpari 30/Cabacu using marker assisted backcrossing.

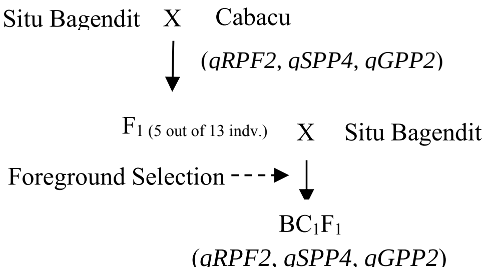

Figure 2. Scheme for the development of pyramided 3 QTLs lines of Situ Bagendit/Cabacu using marker assisted backcrossing.

Molecular Marker Analysis.

Total genomic DNA was extracted using Dellaporta (1983) method. PCR mix contained a total volume of 20 µl containing 50 ng of DNA template, 10 pmole of each primer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP and 1U of Taq polymerase. The PCR amplification was performed using Biorad Thermal Cycler PCR machine using the following conditions: (i) an initial denaturation step of 2 min at 94 ^{0}C, (ii) 30 cycles of 45 second at 94 ^{0}C, 45 second at 55 ^{0}C, 1 min at 72 ^{0}C and (iii) a final extension step for 5 min at 72^{0}C. Amplified products was separated by electrophoresis in 8% polyacrylamide gel at 100 v (C.B.S. Scientific) for 90 minutes. It was observed with ethidium bromide or silver staining and photographed under ultraviolet light using the gel documentation system (BioRad).

Foreground Evaluation.

Cabacu is the donor parent for three QTLs (Quantitative Trait Locus) of drought tolerant (*qRPF2.1, qGPP2.1,* and *qSPP4.1*) (Trijatmiko *et al.*, 2014). It was surveyed among 24 primers near the flanking region of those QTLs (4 SSR primers up and below the targeted SNP ID) for foreground selection. Additional four SSR markers that linked with submergence tolerant (*Sub1*) was conducted using FR13A variety as control (Gonzaga *et al.*, 2015) (Table 1).

Table 1. List of QTLs used in ths study

No.	Trait	QTL	Chr.	Donor	SNP ID	Reference
1	*Root Pulling force*	*qRPF2.1*	2	Cabacu	id2009319	Trijatmiko *et al.*, 2014
2	*Grains per panicle*	*qGPP2.1*	2	Cabacu	wd200409	Trijatmiko *et al.*, 2015
3	*Spikelets per panicle*	*qSPP4.1*	4	Cabacu	id4010621	Trijatmiko *et al.*, 2016
4	*Submergence*	*Sub1*	9	Inpari 30	ART5	Gonzaga *et al.*, 2015

Background Evaluation.

Two hundred and seventy two SSR primers were surveyed for polymorphic markers between the two parents that well distributed on all twelve chromosomes (Fatimah *et al.*, 2017) meanwhile rice 6K SNP assayed in three parents (Inpari 30, Situ Bagendit and Cabacu) was conducted using the BeadXpress

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(Illumina®) platform and RiceOPA2.1 primer in IRRI, Philipines.

Drought Evaluation.

The drought evaluation was conducted using root penetration test (RPT) according to Suardi (2000). The 50 F_1 seeds of Inpari 30/Cabacu and 50 F_1 seeds of Situ Bagendit/Cabacu, parents (Inpari 30, Situ Bagendit and Cabacu), tolerant control (Gajah Mungkur) and susceptible control (IR20) were sowed in *petridish* for 3 days. The plants were transplanted into medium consisted of soil, sand and organic fertilizer (6:3:1). The medium was placed in ± 230 cc root penetration plastic pot whose bottom was layered with paraffin and vaselin (60%:40%) with 3 mm thick or similar with 12 bar. After 1 week the plastic pots were arranged on the bigger plastic pot filled with water (Figure 3). After 4 weeks, the plants were evaluated for plant height (PH), tiller number (TN), root penetration length (RPL), root penetration number (RPN), and root weight (RW). The selected individual plants were transplanted and maintained until harvest.

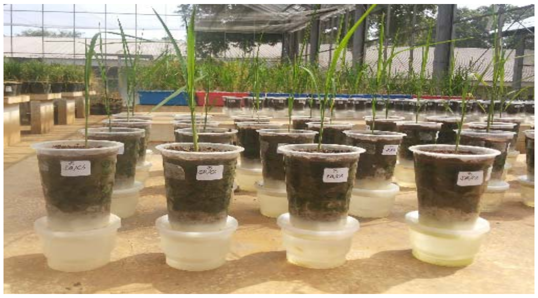

Figure 3. Performance of F_1 Inpari 30/Cabacu and F_1 Situ Bagendit/Cabacu on drought selection using root penetration test.

Data Analysis

Polymorphisms appeared in the DNA profiles were scored visually by comparing with two parents and a standard DNA ladder. The homozygous recipient allele, homozygous dominant allele and heterozygous allele were scored as “A”, “B” and “H”. The agronomic data revealed of each line were written into Ms Excel (Microsoft, 2007) and calculated.

Results

Foreground Evaluation.

Among surveyed 24 primers surround the flanking region (4 SSR primers up and below the targeted SNP ID) of *qRPF2.1*, *qGPP2.1* and *qSPP4.1* and four SSR markers that linked with submergence tolerant (*Sub1*) revealed polymorphic primers between three parents (Figure 4). The polymorphic primers flanked the targeted SNP ID of the QTLs with the distance less than 2 cM was selected (Table 2).

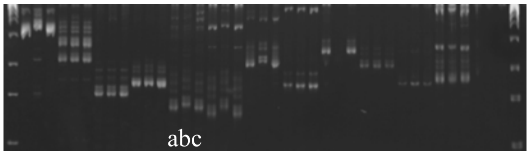

Figure 4. Electrophoregram of surveyed SSR primer flanked on QTL target for foreground selection in polyacrilamid gel electrophoresis 8%. a = Inpari 30, b = Cabacu, dan c = Situ Bagendit.

Table 2. List of selected SSR primers for foreground selection.

No	Trait	QTL	Chr	Donor	SNP ID & SSR	Range (cM)
1	*Root Pulling force*	*qRPF 2.1*	2	Cabacu	RM13596	0,22
					id2009319	0
					RM13599	0,785
2	*Grains per panicle*	*qGPP 2.1*	2	Cabacu	RM12865	0,17
					wd2000409	0
					RM3680	0,12
3	*Spikelets per panicle*	*qSPP 4.1*	4	Cabacu	RM5473	0,24
					id4010621	0
					RM6629	0,15
4	*Submergence*	*Sub1*	9	Inpari 30	RM23805	1,8
					ART5	0
					RM8300	0,3

Genotyping.

The selected primers were continuedly used for foreground selection in F_1 Inpari 30/Cabacu and F_1 Situ Bagendit/Cabacu lines (Figure 5). The result of genotyping analysis revealed heterozygote condition in 23 lines with 4 QTLs target of F_1 Inpari 30/Cabacu lines (46%) and 34 lines with 3 QTLs target of F_1 Situ Bagendit/Cabacu lines (68%). These lines were the candidates for backcrossed with the recipient parent to develop the next generation (BC_1).

Parental Polymorphism Survey. SSR Evaluation.

The surveyed SSR primers showed clear polymorphisms between the two parents and well distributed on all twelve chromosomes. The polymorphic markers in Inpari 30 vs Cabacu was 192 primers (70,5%) while in Situ Bagendit vs Cabacu was 187 primers (68,8%). The average polymorphic primer per chromosome was 15 primers with the average distance was 14 cM (Fatimah *et al.,* 2017).

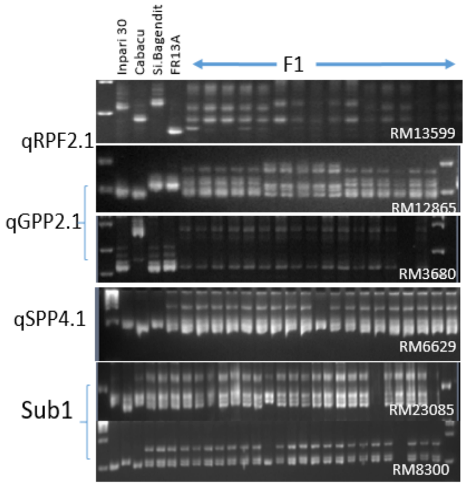

Figure 5. Electrophoregram of flanking SSR primer on 4 targeted QTLs on Inpari 30, Cabacu, Situ Bagendit, FR13A, F_1 Inpari 30-Cabacu and F_1 Situ Bagendit-Cabacu.

SNP Evaluation.

Examination on SNPs distributed in all twelve rice chromosomes between three parents showed that 2457 out of 4606 SNPs screened (53,3%) were polymorphic between Inpari 30 and Cabacu. Chromosome 11 had >60% polymorphism and chromosome 6 had the lowest polymorphism of 40.2%. The average polymorphic primer per chromosome was 205 SNPs with the average distance was 0.76 cM (Table 3). Whie in Situ Bagendit and Cabacu, 2563 SNPs (55,6%) were polymorphic and the chromosome 9, 11, and 1 had >60% polymorphism and chromosome 6 had the lowest polymorphism of 41.5%. The average polymorphic primer per chromosome was 214 SNPs with the average distance was 0.72 cM (Table 4).

Drought Evaluation.

RPT evaluation F_1 Inpari 30/Cabacu was differed with Inpari 30 on plant height and tiller number and not differed with Inpari 30 on root length, root number and root weight (Table 5). F_1 Situ Bagendit/Cabacu was differed with Situ Bagendit on plant height and tiller number and not differed with Situ Bagendit on root length, root number and root weight (Table 6). However 16 F_1 Inpari 30/Cabacu lines (32%) and 13 F_1 Situ Bagendit/Cabacu (26%) have similar root penetration ability to Cabacu variety (Figure 6).

Table 3. Percentage of polymorphic SNP markers surveyed in Inpari 30 and Cabacu.

Chr.	∑ Total Primer	∑ Polymorphic	% Polymorphic	Distance Length (cM)	Distance Ave (cM)
1	514	300	58,4	210,37	0,70
2	457	256	56,0	177,79	0,69
3	486	281	57,8	175,83	0,63
4	421	233	55,3	173,44	0,74
5	378	175	46,3	144,76	0,83
6	376	151	40,2	151,16	1,00
7	367	208	56,7	143,39	0,69
8	345	170	49,3	136,48	0,80
9	300	169	56,3	111,66	0,66
10	276	126	45,7	112,04	0,89
11	346	215	62,1	141,39	0,66
12	340	173	50,9	137.00	0,79
Total	4606	2457	53,3	1815,31	8,67
Ave	383,8	204,8	53,3	151,276	0,76

Table 4. Percentage of polymorphic SNP markers surveyed in Situ Bagendit and Cabacu.

Chr.	Total Primer	∑ Polymorphic	% Polymorphic	Distance Length (cM)	Distance Ave (cM)
1	514	309	60,1	211,49	0,68
2	457	258	56,5	177,79	0,69
3	486	283	58,2	175,83	0,62
4	421	227	53,9	173,44	0,76
5	378	161	42,6	144,76	0,90
6	376	156	41,5	151,16	0,97
7	367	214	58,3	143,39	0,67
8	345	194	56,2	139,04	0,72
9	300	198	66,0	111,66	0,56
10	276	150	54,3	112,04	0,75
11	346	213	61,6	141,39	0,66
12	340	200	58,8	137.00	0,69
Total	4606	2563	55,6	1818,99	8,67
Ave	383,8	213,6	55,6	151,583	0,72

Five individuals of the improved lines of F_1 Inpari 30/Cabacu and F_1 Situ Bagendit/Cabacu were selected based on genotyping (foreground selection) and phenotyping (root penetration test). These lines was continuedly used for backcross breeding to develop BC_1F_1 Inpari 30/Cabacu and BC_1F_1 Situ Bagendit/Cabacu generation.

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Table 5. The average measurement of agronomic characters of Cabacu, Inpari 30, dan F_1 Inpari 30/Cabacu on root penetration test.

No	Variety/ Lines	PH (cm)	TN	RPL (cm)	RPN	RW (g)
1	Cabacu	64,1 ±3,4	3,7± 0,6	33,8 ±9,9	23,3 ±3,1	11,3 ±1,1
2	Inpari30	48,3 ±1,5	5,7± 0,6	12,7 ±6,3	15,3 ±7,8	10,6 ±2,1
3	F_1 Inpari30 /Cabacu	58,8 ±5,1	4,0± 0,5	17,0 ±5,3	14,6 ±6,6	12,6 ±2,1

(PH: Plant Height, TN: Tiller Number, RPL: root penetration length, RPN: root penetration number and RW: root weight.

Table 6. The average measurement of agronomic characters of Cabacu, Situ Bagendit, dan F_1 Situ Bagendit/Cabacu on root penetration test.

No	Variety/ Lines	Plant Height (cm)	Tiller number	Root length (cm)	Root number	Root weight (g)
1	Cabacu	64,1±3,4	3,7±0,6	33,8±9,9	23,3±3,1	11,3±1,1
2	Situ Bagendit	49,4±0,4	6,3±0,6	14,5±2,8	11,7±5,0	10,0±0,2
3	F_1 Situ Bagendit /Cabacu	62,0±3,2	4,0±0,6	16,9±4,4	14,3±6,9	12,1±2,2

(PH: Plant Height, TN: Tiller Number, RPL: root penetration length, RPN: root penetration number and RW: root weight.

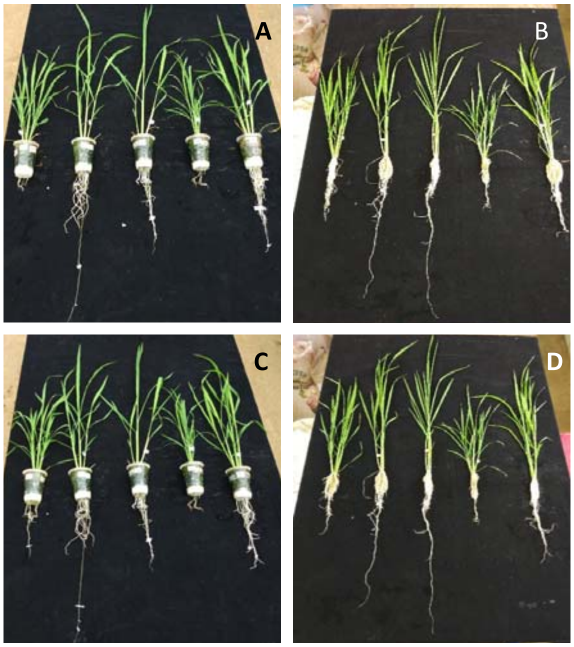

Figure 6. Root penetration length as response to root penetration test after 4 weeks treatment. A & C: plants in root penetration pot, B & D: unloaded plants. From left to right: (A & B) Inpari 30, Cabacu, Gajah Mungkur, IR20, F_1 Inpari 30/Cabacu #56. (C & D) Situ Bagendit, Cabacu, Gajah Mungkur, IR20, F_1 Situ Bagendit/Cabacu #93.

Discussion

Developing new crop varieties with resistance to biotic and abiotic and yield potential is a challenging task in nowadays in plant breeding. Dixit *et al.* (2014) developed an advanced backcross (AB) QTL approach on a large backcross population developed from a cross between tropical japonica, drought- and rice blast-tolerant donor Moroberakan with highly popular recipient indica rice variety Swarna.

In this study, three QTLs of drought tolerant (*qRPF2.1, qGPP2.1,* and *qSPP4.1*) were incorporated into Inpari 30 and Situ Bagendit rice variety. Zhang dan Xiao (2018) identified 4 out of 14 genes in DNA repair co-localized in drought stress related QTLs. Three genes were located in the region of QTL *qGPP2.1* and encoded RAD23 DNA repair protein, RNA recognition motif protein, and peptidyl-prolylcis-trans isomerase and another gene encoded a RNA recognition motif protein and collocated with *qRPF2.1*. While Zhang *et al.* (2009) reported that SPP is highly associated with GPP, which is a very important component of yield. SPP stays stable after panicle differentiation. It is not affected by environment during flowering.

The foreground selection of the individual plants with desired allele at the target QTL reducing the population size for further screening. Thus, from these selected plants backcrossed with the recipient parent to develop the next generation (BC_n). Servin *et al.*, (2004) reported that the frequency of favorable alleles were higher when markers were located exactly on the QTL rather than when marker-QTL distance was 5 cM. Result of this study showed that the distance of polymorphic primers of the targeted QTLs were less than 2 cM.

In molecular backcrossing, the parental polymorphism survey for background selection, is important to have evenly markers with approximately 5 Mb or around 5-10 markers per chromosome. It was generally sufficient to have an efficient recovery of the recipient parent during background selection (Gonzaga *et al*., 2015). Prasetiyono (2010) reported that the distance between the primers in background selection around 10 cM to minimize the effect of linkage drag. Moeljopawiro *et al*., (2009) reported that the ideal number of polymorphic primers for background selection was 10-15 primers. The average polymorphic primer per chromosome obtained from this study was 15 primers with the average distance was 14 cM. Hence, the polymorphic SSR primers could be used for background selection.

Cabacu is an upland tropical japonica with wide compatibility (Hairmansis *et al.,* 2005) while Inpari 30 and Situ Bagendit are indica type based on Rice 6K SNP (Prasetiyono *et al*., 2018). The parental SNP polymorphism between indica (Inpari 30 and Situ Bagendit) and japonica (Cabacu) resulted from this study was around 50%. Yu *et al.* (2014) reported that the SNP from RICE6K revealed 35.5% polymorphism between japonica and indica (with an allele frequency >0.9 in one subspecies and <0.1 in the other), 42.1% between two random indica accessions and 16.9% between two japonica accessions. Singh *et al.* (2015) had developed the single copy gene based 50K SNP chip for genetic studies and molecular breeding.

Drought resistance include three mechanism: drought avoidance, drought tolerance, and drought recovery (Luo, 2010). Comas *et al.* (2013) suggested that root traits associated drought condition include small fine root diameters, long specific root length, and considerable root length density.

The root penetration test was used for screening of root traits at early stages in plant development to estimate the possibility of its resistance at mature stages but it is still need validation under drought condition.

The root length, root number and root weight of F_1 Inpari 30/Cabacu lines and F_1 Situ Bagendit/Cabacu revealed from this study were not differed with their recipient parent however 32% of F_1 Inpari 30/Cabacu lines and 26% of F_1 Situ Bagendit/Cabacu lines have similar root penetration ability to Cabacu variety. This indicated that the QTL target (*qRPF2.1*) for drought tolerant from Cabacu rice variety has been incorporated into their progenies. Five improved lines of F_1 Inpari 30/Cabacu and F_1 Situ Bagendit/Cabacu that have similar root performance with Cabacu variety on root length, root number and root weight were selected and continued for backcross breeding. Wardoyo *et al.* (2017) evaluated the effect of *Pup1* locus using drought stress condition by RPT on BC_2F_6 Kasalath/NIL-C433 lines revelead that the longer penetrated root, the higher number of penetrated root, the more root weight, more tolerant to drought stress.

This study has conducted the molecular evaluation and phenotyping assay of Inpari 30/Cabacu and Situ Bagendit/Cabacu. The foreground selection revealed that flanking SSRs of each QTLs (*qRPF2.1, qGPP2.1, qSPP4.1* and *Sub1*) was less than 2 cM. The background selection through parental polymorphic survey of Rice 6K SNP primers revealed that the polymorphic SNPs on Situ Bagendit vs Cabacu was 2,3% higher than Inpari 30 vs Cabacu. The root penetration assay revealed that the progenies of Inpari 30/Cabacu and Situ Bagendit/Cabacu were differed in plant height and tiller number. The five selected improved lines were continued for backcross breeding.

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