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STS Marker Associated with Iron Toxicity Tolerance in Rice

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ABSTRACT

Iron (Fe) toxicity is one of the limiting factors for rice growth and production in paddy fields. The use of iron tolerant varieties is one of the most efficient ways to solve this problem. Identification of molecular markers associated with the trait is very important to develop marker-assisted selection (MAS) to obtain tolerant lines. The objective of this study was to identify sequence tagged sites (STS) markers associated with iron tolerance trait in double haploid rice population. Forty-five double haploid lines derived from reciprocal double crossing, i.e. IR54/Parekaligolara//Bio110/Markuti, were phenotypically screened in high Fe wetland rice field at Taman Bogo Experimental Station, Lampung. Molecular analysis performed using STS markers. The results of the association between the genetic and phenotypic analysis showed that there were three markers, i.e. *OsIRT1*, *OsIRT2*, and *OsFRO2*, associated with iron tolerance trait in rice. The markers have potential as selection markers for iron tolerant lines.

Keywords: *Fe toxicity, MAS, rice, STS marker*

INTRODUCTION

Iron toxicity is one of the limiting factors in paddy fields which can lead to crop failure [1]. Iron toxicity symptoms in rice leaves subsequently appear from small bronzing starting from the leave edge spread to the base, and followed by leaf color become brown, purple, yellow or orange, and then die. Growth and tillering are stunted with the poor root system, and dark brown root [2]. The use of rice tolerant genotype can be a sustainable solution to solve problems.

Iron tolerant rice varieties can be developed through anther culture technique to produce double haploid lines. The technique is considered as an efficient way to develop high yielding varieties because it can accelerate the formation of pure lines [3]. BMIP is double haploid population developed from anther culture of F1 generation derived from double-crossing between IR54/Parekaligolara and Bio110/Markuti that has been assembled by the Indonesian Center for Agricultural Biotechnology and Genetic Resource Research and Development (ICABIOGRD). The BMIP parents have several agronomic characters, i.e.: Bio110 is resistant to blast disease; IR54 is resistant to blast disease and tolerant to phosphate deficiency; Parekaligolara is resistant to bacterial leaf blight disease; and Markuti is tolerant to iron toxicity.

Tolerance mechanism of plants to iron stress involves the role of several genes. When Fe condition is excessive, these genes play a role in maintaining homeostasis in the plant, which involves in several processes. First, the gene involves in Fe acquisition and mobilization: *OsFRO2*. The gene is up-regulated by the excess of Fe [4]. Second, the gene involves in transportation: *OsNAS*. The gene plays a role in long-distance transportation [5]. Third, the gene acts as the regulatory mechanism in response to various Fe levels: *OsIRT1* [6].

The Iron toxicity tolerance trait is a complex trait controlled by many genes. Quantitative trait loci (QTL) mapping combined with marker-assisted selection (MAS) becomes an efficient selection approach in the breeding program to obtain tolerant genotypes [7]. The identification of molecular markers associated with Fe tolerance character is important to develop marker-

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assisted selection (MAS) tools. It can be achieved by the association between phenotypic data of an Fe tolerant segregation population that is grown in the soil with high Fe levels and molecular marker data. In this research, molecular marker analysis was performed using STS (Sequence Tagged Site) markers derived from relevant target genes associated with Fe tolerance trait. The objective of the study was to identify STS markers associated with iron toxicity tolerance rice using double haploid rice population.

MATERIALS AND METHODS

Plant materials

Forty-five double haploid rice lines derived from reciprocal crosses between Bio110/Markuti and IR54/Parekaligolara, four parental varieties (Bio110, Markuti, IR54, and Parekaligolara), and two control varieties, i.e.: Fe-Tolerant (Mahsuri) and Fe-sensitive variety (IR64).

Screening for iron toxicity tolerant

Rice population was grown at the Taman Bogo Experimental Station, East Lampung on wetland rice field containing 750 ppm Fe [8]. The experiment procedure followed the standard field testing for iron toxicity tolerant conducted by the Indonesian Center for Rice Research (ICRR). Selection method was carried out by stripe check. The field experiment was arranged as a randomized block design with two replications; each line was planted in a plot size of 1 × 3 m^2. Twenty-one to twenty five-day-old seedlings were planted with a planting space of 20 × 20 cm^2 with three seedlings per hole. Urea at a dose of 120 kg/ha and (SP36) at a dose of 60 kg/ha, were applied as the source of N and P, respectively. KCl was not given because it can reduce iron toxicity by strengthening the ability of roots to oxidize the excess of ferrous ion [9]. The phenotypic evaluation was performed by screening the population using a rice bronzing scale followed Standard Evaluation System by IRRI [10].

DNA isolation

The DNA isolation was performed using the CTAB method according to Doyle & Doyle [11]. Plant leaves were ground in liquid nitrogen by TissueLyser. A sample was added with a 750 µL Cetyltrimethyl Ammonium Bromide (CTAB) buffer and incubated with 65oC for 30 min. This suspension was then added with 750 µL CI (chloroform: isoamyl alcohol = 24:1) and centrifuged at 10000 rpm for 15 min. The supernatant was moved to a new tube and then was added with 50 µL Na acetate 2M pH 5.2 and 1 ml absolute ethanol and incubate overnight at -20°C. After that, it was centrifuged at 10000 rpm for 15 min. A pellet was washed with 500 µL 70% alcohol and centrifuged at 10000 rpm for 5 min. After that, A pellet was added with 50 µL 1× TE and 10 µL 10 ng/µL RNase and incubated at 37°C for one hour. Inactivation of RNase was performed by incubation at 65°C for 15 min. DNA quality was tested by electrophoresis on 0.8% agarose gel in 1× TAE buffer at 100 volts for 60 minutes, and then visualized using UV light (BioRad, USA). DNA quantity is determined using the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

Molecular analysis using Sequence Tagged Site (STS) marker

Polymorph markers were identified using PCR. Primers are designed based on the position of the target gene in the genetic map sequence using Primer3. A total of 10 ng/mL of the isolated DNA was amplified using PCR Tetrad 2 MJPTC-240. PCR conditions were as follows: initial denaturation for 5 minutes at 95°C followed by 35 cycles of 95°C for 45 seconds for denaturation, 57°C for 1 minute to annealing, 72°C for 1 minute for elongation; then terminated at 72°C for 15 minutes. PCR products were separated on 2% agarose gel electrophoresis in 1× TAE buffer at 50 volts for 60 minutes, then visualized with UV light (BioRad, USA).

Data analysis

The level of iron toxicity tolerance in rice characterized by levels of leaf bronzing based on standard evaluation system [10]. Association between characters of Iron toxicity tolerance with STS markers was analyzed using Tassel 3.0 program. *P value* < 0.05 indicates that there is an association between STS markers and phenotypic response [12].

Figure 1. Comparison of plant height and root length between (a) Mahsuri (Tolerant control), (b) Markuti, (c) IR54, and (d) IR64 (sensitive control)

RESULTS AND DISCUSSION

Response of rice to iron stress in the field

The observation of phenotypic response in the field indicates that parental lines have variations in the level of iron toxicity tolerance. The tolerance level is determined by leaf bronzing scores, which is considered as the relevant phenotype to screen iron toxicity tolerance [13]. The observation of the phenotypic response of parental lines showed that Markuti and IR54 are tolerant to iron toxicity based on leaf bronzing score (Table 1). However, Markuti develops better root system than that of IR54 (Figure 1).

Phenotypic response variation is also shown in the field testing of the 45 double haploid lines (Table 2). Phenotypic evaluation showed that 12 lines are tolerant to iron toxicity and 33 lines showed medium resistance response. The tolerant lines are BMIP-46-4-1, IPBM-32-1-3-3, BMIP-15-4-2-1, BMIP-20-4-2-1, BMIP-24-4-3-1, BMIP-20-4-3-2, BMIP-40-2-1-1, IPBM-30-1-3-1-1,IPBM-30-1-3-1-2, IPBM -30-1-3-1-3, IPBM-30-1-3-1-4, IPBM-30-1-3-1-5 (data not shown).

The double haploid rice population derived from a cross between Bio110/Markuti//IR54/Parekaligolara contain two tolerant lines with the score of 2, while the double haploid rice lines derived from a cross between IR54/Parekaligolara//Bio110/ Markuti does not have tolerant lines with the score of 2. This result shows that the reciprocal crosses give effect to the Fe tolerance of the double haploid rice lines.

Iron toxicity causes plant leaves become brown and orange with purple spots spread from the edge to the base of the leaf [2]. The rice lines with high tolerance to iron toxicity, leaf bronzing level appears to be low. Tolerant plants develop a tolerance mechanism by producing detoxification enzymes in symplast when excessive Fe^{2+} ions [1]. In contrast, the sensitive plant does not have a detoxification strategy so that the leaves become bronzing when Fe^{2+} becomes abundant in leaf tissue [14].

Genotype evaluation using STS markers

Iron toxicity tolerance trait thought to be controlled by genes that play a role in Fe homeostasis. The genes include *OsIRT*, *OsFRO*, and *OsNAS*. The *OsIRT* is known to play a role in maintaining the Fe homeostasis [6]. The *OsFRO2* involves in the acquisition and mobilization of Fe on the condition of excess Fe [4]. The *OsNAS* has a role in the long-distance transport of Fe in rice plants [5]. STS markers ware developed based on the sequence of those genes (Table 3).

An *OsIRT1* marker detected on chromosome 3 at 27,046,779 bp-27,047,135 bp position, is an OsIRT1 gene based marker (Os03g0667500) (RAP-DB, http://rapdb.dna.affrc.go.jp/index.html). The *OsIRT1* gene (Os03g0667500) including QTL gene clusters that have been detected in BC1F5 population of a cross between Nipponbare/Kasalath and Nipponbare, is on qFETOX-3 QTL region on chromosome 3 (RAP-DB, http://rapdb.dna.affrc.go.jp/index.html) [13]. The *OsIRT2* marker is located on chromosome 7 at 7,313,078 bp-7,310,212 bp position on locus LOC_Os07g12770 (http://rice.plant biology.msu.edu/cgibin/gbrowse/rice/) [15].

The *OsFRO2* markers detected on chromosome 4 at 22,284,474 bp-22,284,883 bp position located in the OsFRO2 gene (Os04g0444800) (RAP-DB, http://rapdb.dna.affrc.go.jp/index.html). The *OsFRO2* marker also detected in chromosome 4 in the BAC clone (OSJNBa0027P08 accession AL7315 93.2) based on nucleutide BLAST (www.ncbi.nlm.nih.gov)with a value of 100% similarity. The *OsFRO2* (AL731593 .2) have also been mapped on chromosome 4 by Gross *et al.* [15].

In this study, seven primer set were used to amplify

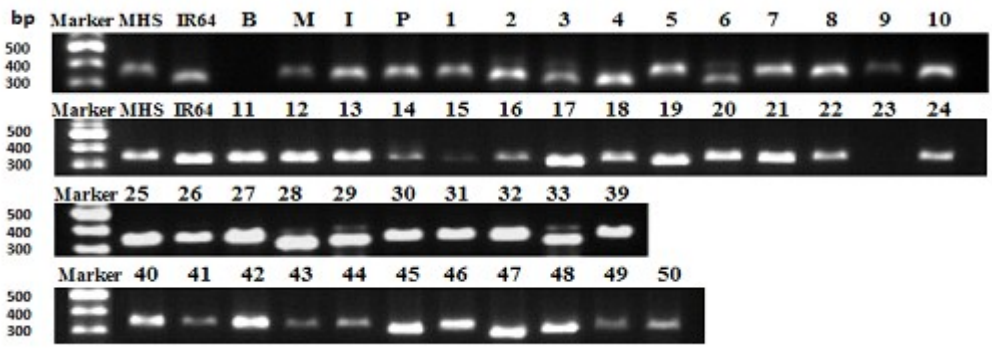

Figure 2. Amplification by PCR using OsIRT1 primers which showed polymorphic DNA bands on varieties. Mahsuri (MHS) which is a tolerant plants, IR64 which is a sensitive crop, 4 parental lines (B=BIO110, M=Markuti, I =IR54, P=Parekaligolara), as well as double haploid lines (numbers 1-33 and 39-50)

Table 1. Leaf bronzing scores on the field-testing for the parental lines

Lines	Average of Leaf Bronzing Percent (%)	Bronzing Score	Notes
Bio110	35	4	Moderate
IR54	15	2	Tolerant
PareKaligora	35	4	Moderate
Markuti	15	2	Tolerant

Table 2. Leaf bronzing score distribution for double haploid rice lines

Score	The Amount of Lines		Notes
	BMIP	IPBM	
2	2	0	Tolerant
3	4	6	Tolerant
4	5	7	Moderate
5	8	13	Moderate
Amount	19	26	

DNA of gene markers in the double haploid rice population (Table 3). The genotypic evaluation showed polymorphic marker between Fe-tolerant genotype (Mahsuri) and Fe-sensitive genotype (IR64). Polymorphic DNA bands were also found in the parental lines. Markuti as a donor to iron toxicity tolerance showed the same banding pattern with Mahsuri. In the double haploid line population, polymorphic DNA bands were also clear (Figure 2).

STS Markers associated with Fe tolerant character

The association between STS markers with phenotypic response shows that there are only three markers among seven markers evaluated that associates with Fe tolerant character (*p value* > 0.05) (Table 4). The markers are *OsIRT1*, *OsIRT2*, and *OsFRO2*, while *AtIRT1*, *OsNAS1*, *OsNAS2*, and *OsNAS3* are not associated with the tolerance character in the double haploid populations.

Based on the results of phenotypic-genotypic association, it is concluded that *OsIRT1*, *OsIRT2*, and *OsFRO2* genes contribute to iron stress tolerance in the double haploid rice population. Although rice is a Gramineae member with a strategy II mechanism to obtain Fe, it seems that rice also has the iron transporters, i.e.: *OsIRT1* and *OsIRT2* [16]. The *OsIRT1* is not only regulated by the Fe-deficient condition but also up-regulated in Fe excess condition [17]. Li *et al.* [18] reported that *IRT1* gene and some homologous genes from *IRT1* that are a member of the ZIP family (Zinc-regulated transporter/Iron-regulated transporter like Protein), are not only related to the Fe absorption but also with detoxification and storage of Fe when Fe excess condition occurred. The same regulation also played by *OsFRO2*, wich active in both the Fe-deficient or excess conditions [19].

An Fe absorption mechanism is initiated with the reduction of Fe^{3+} to Fe^{2+} by *OsFRO2* at the root surface, after which the Fe^{2+} transported across the plasma membrane of epidermal root cells through *OsIRT1* transporters [20]. Subsequently, Fe is translocated into the plant tissues through the radial transport throughout the root tissue (including transport symplastic pass Casparian strip and transport through the xylem and phloem), translocation into deficient tissues, and re-translocation from old tissues [21].

In the rice, the OsIRT1 proteins are located in different locations, i.e. on the plasma membrane of the root epidermis cell, the cortex area around endodermis, and the phloem cell. Promoter analysis of the *OsIRT1* and *OsIRT2* combined with sGFP (synthetic green fluorescent protein) show that both *OsIRT1* and *OsIRT2* play a role in the Fe subcellular localization on the plasma membrane of root cells [16]. At the endodermis, where the Casparian strip acts to prevent the ion entering the stele, the OsIRT1 protein actively takes Fe^{2+} in the apoplast area in the cortex adjacent to the endodermis. The *OsIRT1* localization in phloem cells indicates that the *OsIRT1* also plays a role in the long-distance transport of Fe in rice [16].

Homoeostasis mechanism on the excess Iron condition is also regulated through post-transcriptional regulation. Accumulation of Fe^{2+} will be stopped to prevent toxicity. *FRO2* and *IRT1* genes are regulated by post-transcriptional mechanisms [19]. Another Fe homoeostasis mechanism is through the induction of the *IRT2* expression by the high Fe content in the cytosol. In this condition, Fe can immediately be transported into the vesicles to prevent toxicity [22].

Phenotypic evaluation in the field showed that Markuti was a donor to iron tolerance character to Fe toxicity. This tolerance is contributed by the *OsIRT1* and *OsIRT2* genes [9]. Genotypic evaluation using the *OsIRT1* and *OsIRT2* gene markers showed that Markuti and Mahsuri (Fe-tolerant) have the same DNA band size. The double haploid lines that show a tolerant response in the field-testing, i.e. BMIP-15-4-2-1, BMIP-20-4-2-1, BMIP-24-4-3-1, BMIP-20-4-3-2,

Tabel 3. Primers used in the genotype evaluation using STS Markers

Gene	*DNA Band Size (bp)*	*Chromosome*	*Base Position to (bp)*	*Reference*
OsIRT1	357	3	27046779-27047135	Wu *et al.*, 2014 [13]
OsIRT2	228	7	23905247-23905474	Gross *et al.*, 2003 [15]
AtIRT1	108	1	422565-422672	Connolly *et al.*, 2002 [19]
OsNAS1	264	3	10980195-10980458	Inoue *et al.*, 2003 [5]
OsNAS2	234	3	10978220-10978453	Inoue *et al.*, 2003 [5]
OsNAS3	323	7	29984388-29984710	Inoue *et al.*, 2003 [5]
OsFRO2	410	4	22284474-22284883	Gross *et al.*, 2003 [15]

Tabel 4. Association analysis between STS markers with phenotypic response of double haploid rice population using Tassel 3.0 software

Primer	*P value*	*Primer*	*P value*
OsIRT1	0.0314	*OsNAS1*	0,2425
OsIRT2	0.0226	*OsNAS2*	0,0702
OsFRO2	0.0238	*OsNAS3*	0,0860
AtIRT1	0,5189		

IPBM-30-1-3-1-1, IPBM-30-1-3-1-2, IPBM-30-1-3-1-4, IPBM-30-1-3-1-5. These lines also have the same DNA bands size with Mahsuri and Markuti. Genotypic evaluation using *OsFRO2* gene marker also showed that the DNA band of Markuti has the same size as Mahsuri. However, only two double haploid tolerant lines that have the same size of DNA bands with Mahsuri and Markuti. These lines are BMIP-20-4-3-2 and IPBM-32-1-3-3. Selection of Fe-tolerant lines combined with molecular markers is considered more precise than that of conventional selection because it is directly related to the tolerance genes. However, molecular markers that are designed for a specific cross population should be evaluated before it is applied as selection markers in other cross populations. These markers should be applied in segregation populations derived from a cross between Fe-tolerant and Fe-sensitive parents.

CONCLUSIONS

Based on the phenotypic evaluation in the field, it can be concluded that the double haploid lines vary in iron tolerance levels ranging from moderate tolerant to tolerant. The association between the phenotypic character and STS Markers indicates that the *OsIRT1*, *OsIRT2*, and *OsFRO2* markers are associated with the Fe -tolerant trait. The markers can be potential selection markers to select a rice population to obtain Fe-tolerant lines.

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