Identification of SNPs Associated with Iron Toxicity Tolerance in Rice

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Abstract. Iron (Fe) toxicity is one of the limiting factors that can lead to the decrease of rice yield in paddy fields. Association studies to identify potential alleles or markers linked to iron toxicity tolerant trait can be carried out using high throughput single nucleotide polymorphisms (SNPs). We conducted an association study for Fe toxicity tolerance characters, using Forty-five double haploid lines derived from reciprocal double-crossing, i.e. IR54 / Parekaligolara // Bio110 / Markuti in high Fe wetland rice field. Genome-wide association study was carried out using 384 SNP-plex markers distributed on 12 rice chromosomes. A total of 77 SNPs were significantly associated with the Fe toxicity tolerance-related traits. Functional annotation allowed us to shortlist four SNP markers associated with Fe toxicity tolerance trait, i.e.: **TBGI204006**, **TBGI310247**, **id9006377**, and **id10000498**. The research suggests that association studies followed by functional annotation can effectively detect potential alleles and candidate genes for the trait. The identified QTL and genes provided valuable sources for future genetic improvement of Fe tolerant rice lines.

Keyword: Rice, SNPs, Fe Toxicity Tolerance, QTL.

1. Introduction

Iron (Fe) is an important micronutrient, which can be limiting factor for rice production and rice growth in paddy fields. Fe toxicity can be caused by several conditions, such as the differences in soil landscape, soil types, and high Fe concentrations in the soil [1]. Symptoms of Fe toxicity can be observed at an early stage of development. Yield loss due to Fe toxicity can range up to 100% [2].

Iron toxicity in rice has become general symptoms, begin with purplish-brown spots on the leaves then followed by drying. Sympton of Fe toxicity in rice plants can be seen from red-brownish leaf discoloration (bronzing) on leaves [3] that develop from the edge of the leaf and then spread to the

base and change color to brown, purple, yellow to orange, and then die. A sustainable solution is needed to overcome the problem. One approach is the use of rice tolerant genotypes [4].

One effort to improve superior varieties in rice plant breeding is the application of anther culture techniques. The technique is considered efficient in assembling superior varieties because it can accelerate obtaining pure lines [5]. The development of Fe tolerant rice lines through anther culture techniques has been carried out by the Indonesian Center for Research and Development on Biotechnology and Genetic Resources for Agriculture (BB BIOGEN). BMIP is a double haploid rice population developed from anther culture of F1 generation derived from double-crossing between rice lines derived from IR54/Parekaligolara and Bio110/Markuti. The BMIP parents have several agronomic characters, i.e.: Bio110 is resistant rice to blast disease; IR54 is resistant rice to blast disease and tolerant to phosphate deficiency; Parekaligolara is resistant rice to bacterial leaf blight disease; and Markuti is tolerant rice to iron toxicity.

The identification of molecular marker associated with Fe toxicity tolerance trait is important as the preliminary step of the selection and breeding of rice crop. SNP markers have been widely used in identifying alleles associated with a disease or other biotic and abiotic stress tolerance related traits in plants [6]. High throughput genotyping and phenotyping technology is widely used in the Genome-Wide Association Selection (GWAS) project [7]. The complete rice genome database is an advantage for researchers to predict whether SNP is in the expected gene or it causes functional changes in protein products as a result of gene changes [8] .In this research, we conducted an association study for Fe toxicity tolerance characters using 384 SNP-plex markers followed by functional annotation to detect candidate genes for Fe toxicity tolerance in rice.

2. Materials and Methods

2.1 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 sensitive (IR64) and Fe Tolerant (Mahsuri) varieties were used in this research.

2.2 Screening for iron toxicity tolerant

The experiment procedure followed the standard of the field testing for iron toxicity tolerant conducted by the Indonesian Center for Rice Research (ICRR). Rice population was grown on wetland rice field containing 750 ppm Fe [9]. Selection was carried out using stripe check method. The field experiment was arranged as a randomized block design with two replications; each line was planted in a plot size of 1 × 3 m2. Twenty one day old seedlings were planted with a planting space of 20 × 20 cm2 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. In this research, KCl was not given because it can reduce iron toxicity by strengthening the ability of roots to oxidize the excess of ferrous ion [10]. The Phenotypic evaluation was performed by screening the population using a rice bronzing scale followed Standard Evaluation System by IRRI [11].

2.3 DNA isolation

The DNA isolation was performed using the CTAB method. Plant leaves were ground in liquid nitrogen by a Tissue Lyser. A sample was added with a 750 µL Cetyltrimethyl Ammonium Bromide (CTAB) buffer and incubated at 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 50µL 2M Na acetate pH 5.2 and 1 ml absolute ethanol, and incubated at -20ºC overnight. The DNA was then 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, an air-dry pellet was added with 50 µL 1×TE and 10 µL 10 ng/µL RNase and incubated at 37ºC for one hour. Inactivation of RNase was performed by incubation at 65ºC for 15 min. DNA quality was checkedon 0.8% agarose gel electrophoresis 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).

2.4 SNPs detection

SNP was detected using Illumina BeadChip GoldenGate technology Assay [12]. We used 384 SNP-plex. SNP primers is placed on the BeadChip, contains 2 micron bead so that it can be hybridized with plant DNA samples during annealing. The principle of the methode is oligonucleotide hybridization, ligation, and specific allele extensions followed by universal PCR amplification. Beadchip is then read by neon readers using an iScan reader. The GenomeStudio software from Illumina was used to group alleles based on the ratio of the cy3/cy5 signal ratio generated to collect the three classes of SNP genotypes.

2.5 Data analysis

The level of Fe toxicity tolerance in rice was characterized by levels of leaf bronzing based on standard evaluation system [11]. We used Flapjack to visualize high-throughput SNP genotype data [13]. Association between SNP markers and Fe toxicity tolerance characters was analyzed using Tassel 3.0 program. P-value <0.05 indicates that there is an association between SNP markers and phenotypic response [14].

3. Results and Discussion

3.1 The screening of rice lines for Fe toxicity tolerance

A Field experiment was carried out to observe the phenotypic responses of the rice population to Fe toxicity. The results showed that there were a wariations among rice lines in Fe toxicity tolerance level. The fe toxicity tolerance level is determined based on leaf bronzing scores. Leaf bronzing scores is considered as the relevant phenotype to screen iron toxicity tolerance [1]. We measured the percentage of leaves bronzing and grouped them according to the IRRI Standard Assessment System [11]. To evaluate whether there are variation of the data between the two replications, we tested the mean of the bronzing score using ANOVA test. The test results showed that the two replications did not show a significant difference between the two average leaf bronzing score (t-table (1.67303) <tcount (1.14) <table (1.67303)) (Table 1). Thus, leaf bronzing scores obtained from both replications were then used for further analysis in this research (Table 2).

Table 1. The mean score of bronzed leaves of rice lines tested with a confidence level of 95%

Replications	Number of Lines	Mean	St Dev	SE Mean
1	51	36.4	12.8	1.8
2	51	33.6	11.5	1.6

Table 2. Leaf bronzing scores in the field testing for double haploid lines

Lines	ID	Bronzing Score		Mean	Category
		Group A	Group B		
BMIP1	BMIP-46-4-1	3	3	3	Toleran
BMIP2	IPBM-2-3-2	5	3	4	Moderat
BMIP3	IPBM-32-1-2-1-1	5	3	4	Moderat
BMIP4	IPBM-32-1-2-1-2	5	5	5	Moderat
BMIP5	IPBM-32-1-2-1-3	5	5	5	Moderat
BMIP6	IPBM-32-1-2-1-4	5	5	5	Moderat
BMIP7	IPBM-32-1-2-2-1	5	5	5	Moderat
BMIP8	IPBM-32-1-2-2-2	3	5	5	Moderat
BMIP9	IPBM-32-1-2-2-3	5	5	5	Moderat
BMIP10	IPBM-32-1-2-2-4	5	3	5	Moderat
BMIP11	IPBM-32-1-2-3-1	5	5	5	Moderat
BMIP12	IPBM-32-1-2-3-2	5	5	5	Moderat
BMIP13	IPBM-32-1-2-3-3	5	5	5	Moderat
BMIP14	IPBM-32-1-2-3-4	5	3	4	Moderat
BMIP15	IPBM -32-1-2-3-5	5	3	4	Moderat
BMIP16	IPBM -32-1-2-3-6	5	5	5	Moderat
BMIP17	IPBM -32-1-3-1	5	5	5	Moderat
BMIP18	IPBM -32-1-3-2	5	5	5	Moderat
BMIP19	IPBM -32-1-3-3	3	3	3	Toleran
BMIP20	BMIP -15-4-2-1	3	3	3	Toleran
BMIP21	BMIP -17-1-4-1	3	5	4	Moderat
BMIP22	BMIP -18-4-4-1	5	5	5	Moderat
BMIP23	BMIP -18-4-4-2	5	5	5	Moderat
BMIP24	BMIP -20-4-2-1	1	3	2	Toleran
BMIP25	BMIP -24-4-3-1	1	3	2	Toleran
BMIP26	BMIP -20-4-3-2	3	3	3	Toleran
BMIP27	BMIP -24-1-2-1	3	5	4	Moderat
BMIP28	BMIP -24-1-4-1	5	5	5	Moderat
BMIP29	BMIP -24-1-4-2	5	5	5	Moderat
BMIP30	BMIP -40-2-1-1	3	3	3	Toleran
BMIP31	BMIP -40-2-1-2	5	3	4	Moderat
BMIP32	BMIP -44-4-3-1	5	3	4	Moderat
BMIP33	BMIP -44-4-3-2	5	3	4	Moderat
BMIP39	IPBM-32-2-1-1-2	5	3	4	Moderat
BMIP40	IPBM-32-2-1-1-3	5	3	4	Moderat
BMIP41	IPBM-32-2-1-1-4	5	3	4	Moderat
BMIP42	BMIP -24-1-1-1-1	5	5	5	Moderat
BMIP43	BMIP -24-1-1-1-2	5	5	5	Moderat
BMIP44	BMIP -24-1-1-1-3	5	5	5	Moderat
BMIP45	BMIP -24-1-1-1-4	5	5	5	Moderat
BMIP46	IPBM-30-1-3-1-1	3	3	3	Toleran
BMIP47	IPBM-30-1-3-1-2	3	3	3	Toleran
BMIP48	IPBM-30-1-3-1-3	3	3	3	Toleran
BMIP49	IPBM-30-1-3-1-4	3	3	3	Toleran
BMIP50	IPBM-30-1-3-1-5	3	3	3	Toleran

Tolerance levels based on leaf bronzing scores are commonly used in phenotypic evaluations of Fe toxicitystress. The leaf bronzing score was assessed as a relevant phenotypic in the screening activity of tolerance to Fe– [1]. Phenotypic responses based on leaf bronzing score of the parental lines showed that rice var. Markuti and IR54 are tolerant to Fe toxicity (Figure 1).

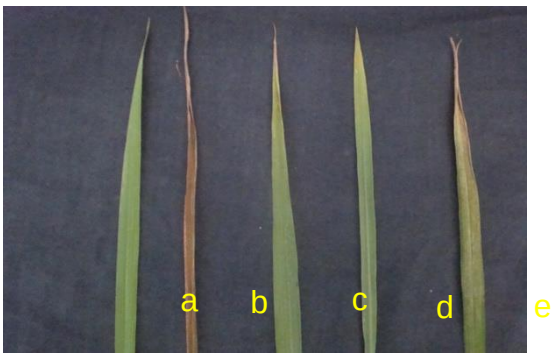

Figure 1. Phenotipic responses of parental lenes; a. Mahsuri as tolerant control (bronzing score1); b. IR64 as sensitive control (bronzing score 9), c. Markuti (bronzing score 2), d. IR54 (bronzing score 2), and e. BIO110 (bronzing score 4).

Based on the bronzing score variations among the rice in the field, the rice lines are groupe into 6 groups of Fe tolerance levels (Table 3), i.e.: 1) a group with bronzing score of 1 (very tolerant) consisted only of var Mahsuri; 2) a group with bronzing score of 2 (tolerant) consisted of 4 rice lines; 3) a group with bronzing score of 3 (moderate tolerant) consisted of 10 rice lines; 4) a group wirh bronzing score of 4 (moderate sensitive) consisted of 14 rice lines; 5) a group with bronzing score of 5 (sensitive) consisted of 21 rice lines); and 6) a group with bronzing score of 9 (very sensitive) that only consist of IR64 which are very sensitive. Double haploid lines with high tolerance to fe toxicity are IPMB-32-1-3-3, BMIP-46-4-1, BMIP-20-4-2-1, BMIP-15-4-2-1, BMIP-24-4-3-1, BMIP-20-4-3-2, BMIP-40-2-1-1, IPBM-30-1-3-1-2, IPBM-30-1-3-1-1, IPBM-30-1-3-1-4, IPBM-30-1-3-1-3, and IPBM-30-1-3-1-5

Table 3. Groups of rice lines based on leaf bronzing scores in field testing

Bronzing Score	Lines	Total
1	Mahsuri	1
2	BMIP 25, BMIP 24, IR54, MARKUTI	4
3	BMIP 50, BMIP 49, BMIP 48, BMIP 47, BMIP 46, BMIP 30, BMIP 26, BMIP 20, BMIP 19, BMIP 1	10
4	BMIP 41, BMIP 40, BMIP 39, BMIP 33, BMIP 32, BMIP 31, BMIP 27, BMIP 21, BMIP 15, BMIP 14, BMIP 3, Bio110, Parekaligolara	14
5	BMIP 45, BMIP 44, BMIP 43, BMIP 42, BMIP 29, BMIP 28, BMIP 23, BMIP 22, BMIP 18, BMIP 17, BMIP 16, BMIP 13, BMIP 12, BMIP 11, BMIP 10, BMIP 9, BMIP 8, BMIP 7, BMIP 6, BMIP5, BMIP 4	21
9	IR64	1

3.2 SNPs Genotyping

The Fe toxicity tolerance in the BMIP double haploid rice population is suggested to be due to the presence of potential genes related to the tolerant trait that spread throughout the rice chromosome. The potential genes must be homozygous for its alleles. Heterozygous alleles need to be avoided in the linkage studies because they allow allele segregation that will affect the Fe toxicity tolerance. We used Flapjack to visualize high-throughput genotype data. The genotyping of the rice lines with 384 SNPs showed that there are numerous heterozygous alleles distributed along the loci on the rice chromosomes (Figure 2).

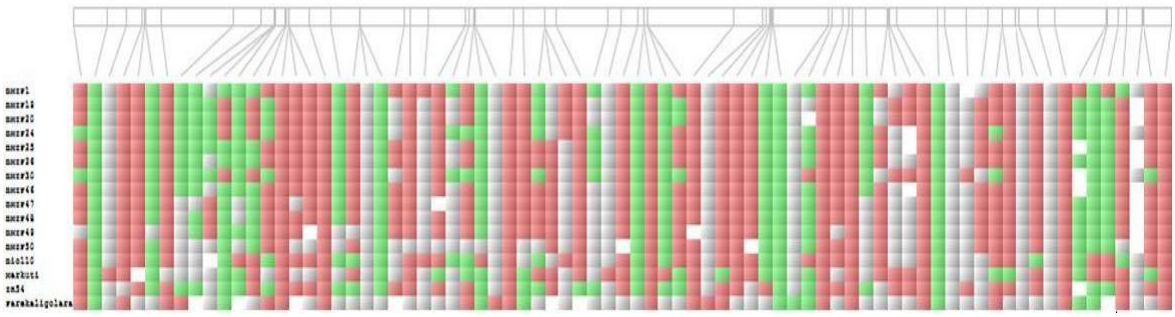

Figure 2. Genotype visualization 384 SNPs. Gray color shows heterozygous alleles

3.3 SNP marker association with leaf bronzing

The genotypic and phenotypic data were then tested for their association using TASSEL 3.0 program. The results showed that there were 77 SNPs associated with phenotypic response (bronzing score) with *P*-value <0.05 (Figure 3). Of the 77 selected SNPs, 18% were heterozygous alleles.

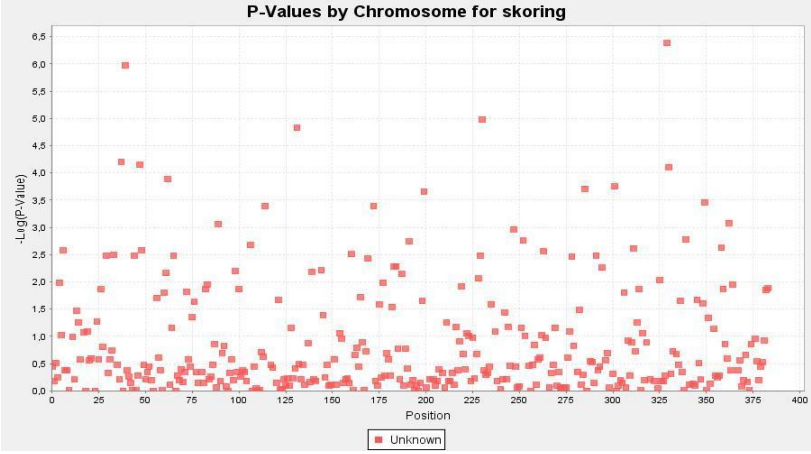

Figure 3. Manhattan Plot from p_Values of Association between Leaf Bronzing with SNPs marker

Selected SNPs associated with Fe toxicity tolerance were then annotated for related genes through the Oryza SNP Browser. This browser provides a graphical display of the SNP that could identify both the gene model version of the TIGR 5 pseudomolecules and RAP. Based on this identification, we know SNPs position on genes related with Fe toxicity tolerance. In this study, we obtained four sellected SNPs, i.e **TBGI204006**, **TBGI310247**, **id9006377**, and **id10000498** (Table 4).

TBGI204006 is located on chromosome 4. Markuti (Parental line, donor for Fe toxicity Tolerance) was homozygous for this SNP (TT). The tolerant lines, such as BMIP -15-4-2-1, IPBM -32-1-3-3, BMIP -20-4-2-1, BMIP -24-4-3-1, BMIP - 20-4-3-2, IPBM-30-1-3-1-1, IPBM-30-1-3-1-2, IPBM-30-1-3-1-3, BMIP -40-2-1-1, IPBM-30-1-3-1-4, and IPBM-30-1-3-1-5 were also homozygous for the alleles. **TBGI204006** was found in the transmembrane ferric reductase domain (*FRD*) gene, which has two forms, i.e.: ferric reductase (*FRE*) and NADPH oxidase (*NOX*). The *FRE* and *NOX* genes are homologous genes. *NOX2* involves in the defense response of ROS dependent plants [15]. **TBGI204006** was found in the *OsFRO2* gene. Previous study reported that OsFRO2 STS markers were associated with the Fe tolerance trait. Genotypic evaluation using *OsFRO2* gene marker in this population also showed that the DNA band of Markuti has the same size as a tolerant control, Mahsuri [16]. The *FRO* gene is differentially expressed at the tissue level and *FRO2* works

Table 4. Selected SNP markers located at the gene locus that play a role in the Fe toxicity tolerance.

SNP	Alel	Position		chrom.	P value	ID SNP	RAP-DB[3)]	
		IRGSP.v4[1)]	MSU.v6[2)]				Locus	Description
TBGI204006	T	22285012	21966410	4	0,0221	**TBGI204006**	**Os04g0444800**	Cytochrom b-245, heavy chain family
TBGI310247	A	29335182	28457182	6	0,0112	**TBGI310247**	**Os06g0682800**	Zn-finger
id9006377	A	19020266	18365554	9	0,0132	**id9006377**	**Os09g0479100**	Cyclin-like F-box domain containing protein.
id10000498	G	2038800	-	10	0,0033	**id10000498**	**Os10g0133100**	Cyclin-like F-box domain containing

1)*International Rice Genome Sequencing Project* (IRGSP)

2)*The MSU Rice Genome Annotation Project Database and Resource*

3)*Rice Annotation Project-Data Base*, gene annotation based on IRGSP (http://rapdb.dna.affrc.go.jp/ index.html)

4)*The* TIGR *genetic map browser-Data Base*, gene annotation based on MSU (http://www.tigr.org/tdb/e2k1/osa1/BACmapping/description.shtml)

specifically for roots [17]. We observed the root length in tolerant and sensitive plants (Figure 4). The Figure 4 indicates that rice var. Markuti has better root length and growth under Fe toxicity than that of the IR64 roots.

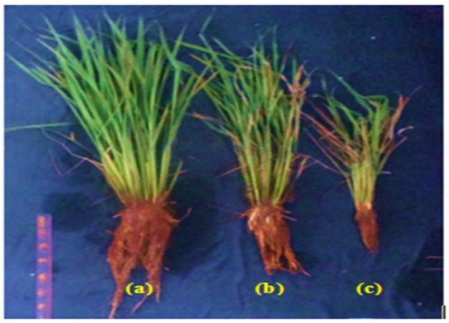

Figure 4. Comparison of root damage (a) Mahsuri as a tolerant control), (b) Markuti, and (c) IR64 as a sensitive control.

The **TBGI310247** was located at the **Os06g0682800** locus on chromosome 6. In Markuti, the **TBGI310247** was AA homozygous alleles. Homozygous alleles were also found in all tolerant lines, except for the IPBM-30-1-3-1-5 line. **TBGI310247** was also found in a Zn-Finger encoding gene. The *Zn-Finger gene* is known as a gene that is up-regulated in Fe excess conditions in the root [18]. The Zn-finger protein also known to regulate resistance mechanism for abiotic stresses [19][20]. In addition, Zn-Finger protein is also known to play a role as a transcription factor in the regulation of plant defense genes related ROS [21].

SNP **id9006377** was foun on chromosome 9 and SNP **id10000498** was located on chromosome 10, which is inthe *Cyclin-like F-box domain containing protein*. Both SNPs **id9006377** and **id10000498** are already homozygous. *Cyclin-like F-boxes* have been shown to be involved in homeostasis of Fe, especially in regulation of Fe uptake in rice [18]. Fe^{2+} accumulation will be removed to stop toxicity when the Cyclin like F-boxes protein work. Cyclin F-box is also known to play a role in the regulation of protein degradation as a post-transcription factor.

Conclusions.

The double haploid lines varies in Fe toxicity tolerance levels ranging from sensitive to tolerant based on the phenotypic evaluation in the field. The association between the phenotypic character and SNP followed by functional annotation allowed us to shortlist four SNP markers associated with Fe tolerance, i.e.:**TBGI204006**, **TBGI310247**, **id9006377**, and **id10000498**, which are located on chromosomes 4, 6, 9, and 10, respectively.

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