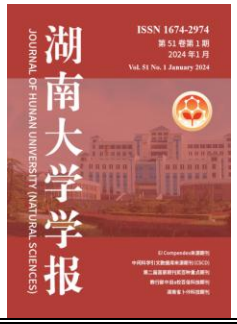

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Marker-Assisted Selection for Leaf Bronzing Associated with Fe Tolerance in Rice (*Oryza Sativa* L.)

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Abstract: Iron (Fe) toxicity characterized by bronzing leaves is a significant factor that reduces rice productivity. The severity of this disease varies in rice fields, making molecular selection with markers associated with Fe tolerance an important process. Therefore, this study aimed to analyze the diversity of BMIP rice population lines based on their response to Fe toxicity in the field and combine molecular evaluation to obtain Fe-tolerant lines. The results showed that the BMIP lines inherited Fe-tolerant traits from its parent, Markuti. Phenotypic response analysis showed that there were 5 clusters of tolerance variation in the BMIP population. Furthermore, the phylogenetic tree constructed based on phenotypic response as well as OsIRT1, OsIRT2, and OsFRO2 markers indicated the existence of 3 large clusters. This analysis identified BMIP 24, BMIP 25, BMIP 20, BMIP 26, BMIP 46, BMIP 47, BMIP 49, and BMIP 50 lines as tolerant lines.

Keywords: Fe toxicity, marker-assisted selection, bronzing leave, rice, molecular marker.

与水稻(水稻)耐铁性相关的叶片古铜色的标记辅助选择

摘要：以叶子呈古铜色为特征的铁（铁）毒性是降低水稻产量的一个重要因素。这种疾病的严重程度因稻田而异，因此利用与铁耐受性相关的标记物进行分子选择成为一个重要过程。因此，本研究旨在根据田间铁毒性反应分析 BMIP 水稻群体系的多样性，并结合分子评价获得耐铁系。结果表明，BMIP 品系从其亲本马库蒂遗传了耐铁性状。表型反应分析表明，BMIP 群体中存在 5 个耐受性变异簇。此外，基于表型反应以及奥斯 IRT1、奥斯 IRT2 和 OsFRO2 标记构建的系统发育树表明存在 3 个大簇。该分析确定 BMIP 24、BMIP 25、BMIP 20、BMIP 26、BMIP 46、BMIP 47、BMIP 49 和 BMIP 50 品系为耐受品系。

关键词：铁毒性，标记辅助选择，烫金叶，水稻，分子标记。

1. Introduction

Global rice production is experiencing a consistent decline every year. In 2022/2023, it is expected to decrease by more than 2% compared to the previous

year. The decline is due to smaller production forecasts for several countries, including Mexico, the United States, China, Ukraine, Russia, and Mexico [1]. Therefore, global rice production is currently estimated at around 512 million tons (milled basis), which is 1.2 million tons lower than the December forecast and 2.6% from the all-time high in 2021 [2]. These significant changes in production figures demand greater availability of food.

The challenge of low rice productivity can be overcome by opening new lands, such as tidal swamps and peatlands. Tidal swamp lands have the potential to be developed as food barns, specifically as rice suppliers [3]. However, these lands face limitations due to their inherent characteristics, encompassing chemical, physical, and biological properties of the soil [4]. Tidal marshlands are reported to have low fertility and often suffer from certain nutrient toxicity. The opening of new land for rice cultivation is also often faced with iron (Fe) toxicity, thereby hindering optimal plant growth and productivity [5].

Iron (Fe) toxicity is a widespread issue in various soil types, particularly in areas with prolonged inundation during crop growth. Land with high Fe levels typically exhibits poor drainage, low macronutrient content, and a soil pH range of 4-7. Therefore, Fe toxicity is a major concern in newly opened rice fields, tidal lands, and basin areas. This toxicity can affect both the vegetative and generative phases of plant growth. During the vegetative phase, poisoned plants are characterized by dryness and abnormal height. The dry weight of the plant decreases and there are fewer tillers and less chlorophyll, resulting in poor plant appearance. In rice plants, Fe toxicity manifests visually as distinctive symptoms, namely bronzing or yellowing of leaves caused by the accumulation of oxidized polyphenols [6]. This condition causes a reduction in rice productivity.

The severity of diseases caused by Fe toxicity in the field varies due to several environmental conditions, This makes phenotypic evaluation less effective, emphasizing the crucial role of molecular selection with markers associated with Fe tolerance in rice breeding [7]. The continuous development of Fe-tolerant markers for breeding derived from QTL mapping provides valuable tools for effective selection [8-10]. Therefore, this study analyzed the diversity of rice population genotypes based on the response to Fe toxicity in the field and used molecular markers for genotype evaluation to obtain tolerant lines.

2. Material and Methods

2.1. Genetic Materials

The genetic materials used were 45 double haploid rice lines, derived from a reciprocal cross between IR54/Parekaligolara and Bio 110/Markuti. A total of 4 rice lines that served as the parents of the cross were Bio 110, Markuti, IR54, and Parekaligolara. Furthermore, Mahsuri was used as a control plant tolerant to Fe toxicity and IR64 as a plant sensitive to Fe toxicity.

The identification of Fe toxicity tolerance genes in the tested lines was carried out using molecular markers. Furthermore, OsIRT1, OsIRT2, OsNAS1, OsNAS2, OsNAS3, and OsFRO2 genes were identified using STS (Sequence-Tagged Sites) molecular markers.

2.2. Planting of Rice Population

Rice cultivation was carried out using the stripe check method, where sensitive and tolerant comparison varieties were planted around the perimeter of the plot. The tested rice populations were placed in rows on the plots, measuring 1 m x 3 m each, with a spacing of 20 cm x 20 cm, and three seedlings per planting hole, as illustrated in Fig. 1. The rice population was planted in paddy fields with Fe levels of 750 ppm [11]. The experimental design used a Randomized Block Design (RAK), with two replications.

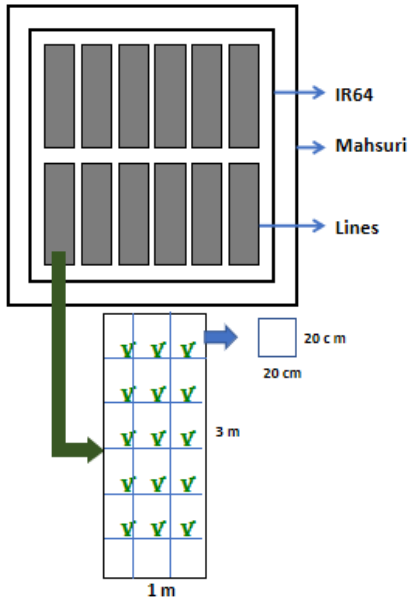


Fig. 1 The scheme of planting lines in the field using the stripe check method (The authors)

2.3. Phenotypic Analysis

The phenotypic evaluation was conducted by screening rice populations using bronzing scale by IRRI to characterize tolerance to Fe toxicity. Bronzing leaves were scored in two replications on each test line based on the IRRI Standard Evaluation System [12]. Subsequently, bronzing leaves data from both replicates were tested for homogeneity by independent t-test at a 95% confidence level. Hierarchical Cluster Analysis was carried out using a dendrogram to categorize Fe tolerance levels in the field.

2.4. Total DNA Isolation

Total DNA was isolated from rice leaves using the modified method of [13]. Approximately 0.5 grams of

rice leaf pieces were put into a 2 ml Eppendorf tube along with 2 beads, which were immersed in liquid nitrogen for 5 minutes. The Eppendorf was put into a tissuelyser machine for 2 minutes with a frequency of 25/second. The crushed rice leaves were added with 750 µl CTAB and sodium bisulfite buffer (0.38 g sodium bisulfite in 100 ml CTAB). The mixture was incubated at 60°C for 20 minutes, with the Eppendorf tube flipped every 5 minutes. The 750 µl CI (Clorofrom: Isoamil-alcohol) was added into the sample to be centrifuged at 12,000 rpm for 10 min in a Beckman Microfuge 12.0 machine. Subsequently, 500 µl of supernatant was taken and mixed with 100 µl Na Acetate (pH 5.2, 3M) as well as 500µl isopropanol. The resulting mixture was precipitated in the freezer overnight and the samples were centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the precipitate formed was added to 200 µl of 70% ethanol and centrifuged at 12,000 rpm for 10 minutes. After drying the pellet, 100 µl TE and 1.5 µl RNAse were added, followed by overnight incubation at 37°C. To inactivate the RNAse, the samples were further incubated at 65°C for 15 minutes.

2.5. DNA Quality and Quantity Test

DNA quality was determined using electrophoresis with TAE 1X buffer solution, 0.8% agarose gel for 60 minutes at 100 Volts, and visualized under UV light (Biorad, USA). Subsequently, the DNA quality obtained was known through Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.6. Dilution of DNA Concentration

Dilution of DNA concentration was carried out until the best concentration for the amplification process was obtained, namely 10 ng/µL.

2.7. PCR Amplification

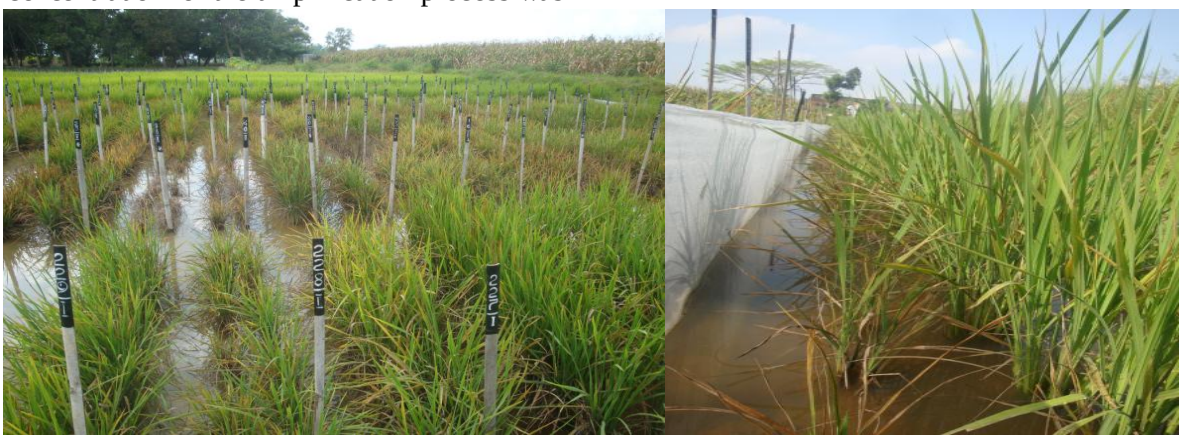
Polymorphism genotypes were obtained by amplifying DNA with STS primers for OsIRT1, OsIRT2, AtIRT1, OsFRO2, OsNAS1, OsNAS2, and OsNAS3 genes. The amplification process was carried out using a Tetrad-2 MJ PTC 240 PCR machine. The PCR products obtained were visualized with UV light, followed by electrophoresis in a 2% agarose gel in TAE 1x buffer at 50 Volts for 60 minutes.

2.8. Genotype Analysis

The amplicon bands were sized and compared with phenotype data using the Tassel 3.0 program to assess the association indicated by a p_value < 0.05 [14]. Furthermore, a phylogenetic tree was constructed for the selection of tolerant lines using the UPGMA method.

3. Results

The results of observations on the response of rice plants to Fe stress in the field showed variations in the level of tolerance to Fe toxicity, as presented in Fig. 2. The level of tolerance was determined by evaluating the bronzing character through the presence of brown spots and damage on the leaves. The assessment of the leaf bronzing score was used in the phenotypic evaluation of abiotic stress. This score was considered a relevant phenotypic activity in screening for tolerance to Fe toxicity [15]. Based on the observation of the phenotypic response, it was discovered that the BMIP and the parental lines had varied leaf bronzing responses to Fe stress.



A B C

Fig. 2 Phenotyping tests in the field with more than 750 ppm Fe showed varying tolerance: A) BMIP lines, B) negative control (IR64), C) positive control (Mahsuri) (The authors)

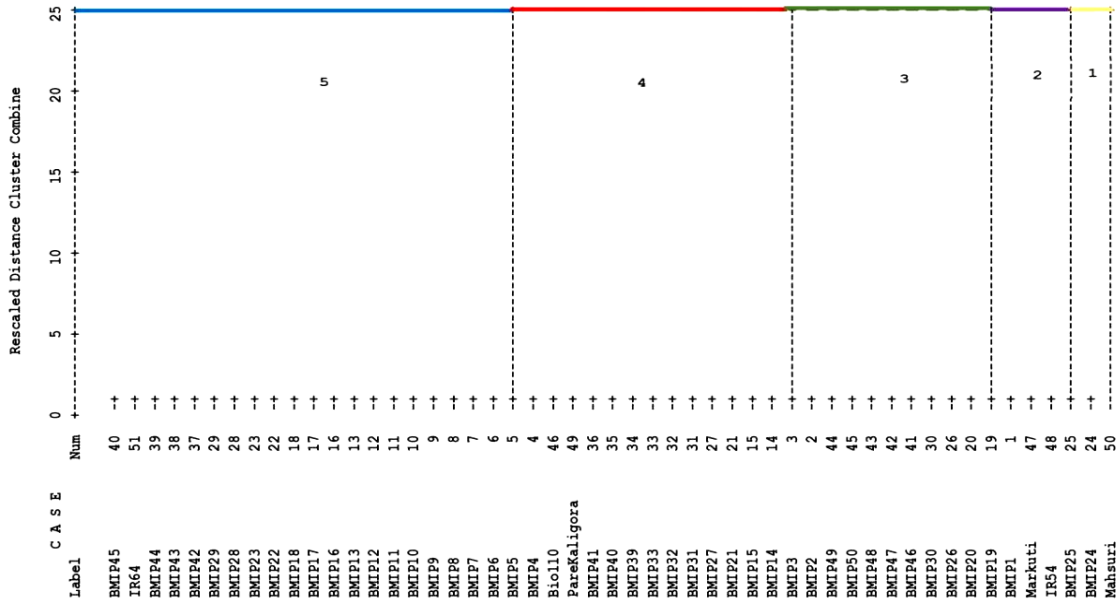


Fig. 3 Dendrogram using hierarchical cluster analysis for clustering the level of Fe tolerance in the parental lines, BMIP, IR64 (sensitized control), and Mahsuri (positive control) (The authors)

Hierarchical Cluster Analysis of the phenotypic test showed that there were five groups of lines based on Fe resistance scores, as determined by the level of leaf bronzing. Cluster 1 described the tolerant group, while Cluster 5 represented the less tolerant group. Furthermore, the BMIP 25 and BMIP 24 lines were in the same cluster as Mahsuri (Fe-tolerant control line). In the second cluster, BMIP 1 and BMIP 19 lines were in the same cluster as Markuti (Fe-tolerant donor parent), showing good Fe tolerance in the phenotype test. The third cluster contained a total of 10 BMIP lines, while the fourth cluster, the tolerant-intermediate, consisted of 12 BMIP lines. The sensitive control line, IR64, was in the fifth cluster with 19 total BMIP lines.

Genotype testing using the OsIRT1, OsIRT2, AtIRT1, OsNAS1, OsNAS2, OsNAS3, and OsFRO2 gene primers showed a single DNA band. As shown in Table 1, analysis of amplicons on amplification using primers OsIRT1, OsIRT2, and AtIRT1 showed its appearance in each line. However, the primers of OsNAS1, OsNAS2, OsNAS3, and OsFRO2 genes, for some lines, did not appear.

Table 1 Analysis of amplicons using the OsIRT1, OsIRT2, AtIRT1, OsNAS1, OsNAS2, OsNAS3, and OsFRO2 gene primers (The authors)

No.	Galur	ID	Amplicon appearance on primer amplification						
			Os*IRT1*	Os*IRT2*	At*IRT1*	Os*NAS1*	Os*NAS2*	Os*NAS3*	Os*FRO2*
1	BMIP1	BMIP-46-4-1	+	+	+	+	+	+	+
2	BMIP2	IPBM-2-3-2	+	+	+	+	+	+	+
3	BMIP3	IPBM-32-1-2-1-1	+	+	+	+	+	+	-
4	BMIP4	IPBM-32-1-2-1-2	+	+	+	+	+	+	+
5	BMIP5	IPBM-32-1-2-1-3	+	+	+	+	+	+	+
6	BMIP6	IPBM-32-1-2-1-4	+	+	+	+	+	+	+
7	BMIP7	IPBM-32-1-2-2-1	+	+	+	+	+	+	+
8	BMIP8	IPBM-32-1-2-2-2	+	+	+	+	+	+	+
9	BMIP9	IPBM-32-1-2-2-3	+	+	+	+	+	-	+
10	BMIP10	IPBM-32-1-2-2-4	+	+	+	+	+	-	-
11	BMIP11	IPBM-32-1-2-3-1	+	+	+	-	+	+	+
12	BMIP12	IPBM-32-1-2-3-2	+	+	+	+	+	+	-
13	BMIP13	IPBM-32-1-2-3-3	+	+	+	+	+	-	+
14	BMIP14	IPBM-32-1-2-3-4	+	+	+	+	+	+	-
15	BMIP15	IPBM -32-1-2-3-5	+	+	+	+	+	+	+
16	BMIP16	IPBM -32-1-2-3-6	+	+	+	+	+	+	+
17	BMIP17	IPBM -32-1-3-1	+	+	+	+	+	+	-
18	BMIP18	IPBM -32-1-3-2	+	+	+	+	+	-	+
19	BMIP19	IPBM -32-1-3-3	+	+	+	+	+	+	+
20	BMIP20	BMIP -15-4-2-1	+	+	+	+	+	+	+
21	BMIP21	BMIP -17-1-4-1	+	+	+	+	+	+	+
22	BMIP22	BMIP -18-4-4-1	+	+	+	+	+	-	-
23	BMIP23	BMIP -18-4-4-2	-	+	+	+	+	+	+
24	BMIP24	BMIP -20-4-2-1	+	+	+	-	+	+	-
25	BMIP25	BMIP -24-4-3-1	+	+	+	+	+	+	+
26	BMIP26	BMIP -20-4-3-2	+	+	+	-	+	+	+

Continuation of Table 1

27	BMIP27	BMIP -24-1-2-1	+	+	+	+	+	-	+
28	BMIP28	BMIP -24-1-4-1	+	+	+	+	+	-	+
29	BMIP29	BMIP -24-1-4-2	+	+	+	-	+	+	+
30	BMIP30	BMIP -40-2-1-1	+	+	+	-	+	-	-
31	BMIP31	BMIP -40-2-1-2	+	+	+	+	+	+	+
32	BMIP32	BMIP -44-4-3-1	+	+	+	-	+	+	+
33	BMIP33	BMIP -44-4-3-2	+	+	+	+	+	+	+
34	BMIP39	IPBM-32-2-1-1-2	+	+	+	+	+	+	+
35	BMIP40	IPBM-32-2-1-1-3	+	+	+	+	+	+	-
36	BMIP41	IPBM-32-2-1-1-4	+	+	+	-	+	+	+
37	BMIP42	BMIP -24-1-1-1-1	+	+	+	-	+	+	-
38	BMIP43	BMIP -24-1-1-1-2	+	+	+	+	+	+	-
39	BMIP44	BMIP -24-1-1-1-3	+	+	+	-	+	+	-
40	BMIP45	BMIP -24-1-1-1-4	+	+	+	+	+	+	-
41	BMIP46	IPBM-30-1-3-1-1	+	+	+	+	+	+	+
42	BMIP47	IPBM-30-1-3-1-2	+	+	+	-	-	+	-
43	BMIP48	IPBM-30-1-3-1-3	+	+	+	+	+	+	-
44	BMIP49	IPBM-30-1-3-1-4	+	+	+	-	+	-	-
45	BMIP50	IPBM-30-1-3-1-5	+	+	+	+	+	+	-
46	BIO110		-	+	+	+	+	-	-
47	Markuti		+	+	+	+	+	+	+
48	IR54		+	+	+	+	+	+	+
49	Parekaligolara		+	+	+	+	+	+	+
50	Mahsuri		+	+	+	+	+	+	+
51	IR64		+	+	+	+	+	+	+

Notes: + DNA band appears, - DNA band disappears.

The genotypes were evaluated using molecular markers to assess their association with Fe toxicity tolerance traits using the General Linear Model (GLM). Markers associated with tolerance response to Fe toxicity were considered as potential candidates to assist selection activities in the formation program of tolerant rice lines. The results of the association between STS markers with phenotypic responses showed that there were three primers associated with phenotypic responses (p_value < 0.05), as shown in Table 2. These three primers included gene markers OsIRT1, OsIRT2, and OsFRO2. Meanwhile, gene markers AtIRT1, OsNAS1, OsNAS2, and OsNAS3 were not associated with tolerance responses in the double haploid population tested.

Table 2 Marker test results using general linear model (GLM) for selected markers (p_value < 0.05) (The authors)

marker_F	marker_p	markerR2	markerDF	markerMS	errorDF	errorMS	modelDF	modelMS
5.54E + 15	0.02264	0.10157	1.0	5.79E + 15	49.0	1.04E + 16	1.0	5.79E + 15
5.44E + 15	0.02386	0.09988	1.0	5.69E + 15	49.0	1.05E + 16	1.0	5.69E + 15
4.91E + 14	0.03143	0.09101	1.0	5.19E + 14	49.0	1.06E + 16	1.0	5.19E + 14

Phylogenetic trees were constructed based on genotyping with selected markers and phenotyping, resulting in the separation of three groups. The IR64 as a sensitive control was in group 1, while Mahsuri as a tolerant control was in group 3. The Markuti elders, known as Fe donors, clustered in group 3 with 8 tolerant lines in the phenotyping test. These lines included BMIP 24, BMIP 25 (cluster 1 on the phenotypic test) as well as BMIP 20, BMIP 26, BMIP 46, BMIP 47, BMIP 49, and BMIP 50 (Cluster 3 on the phenotypic test).

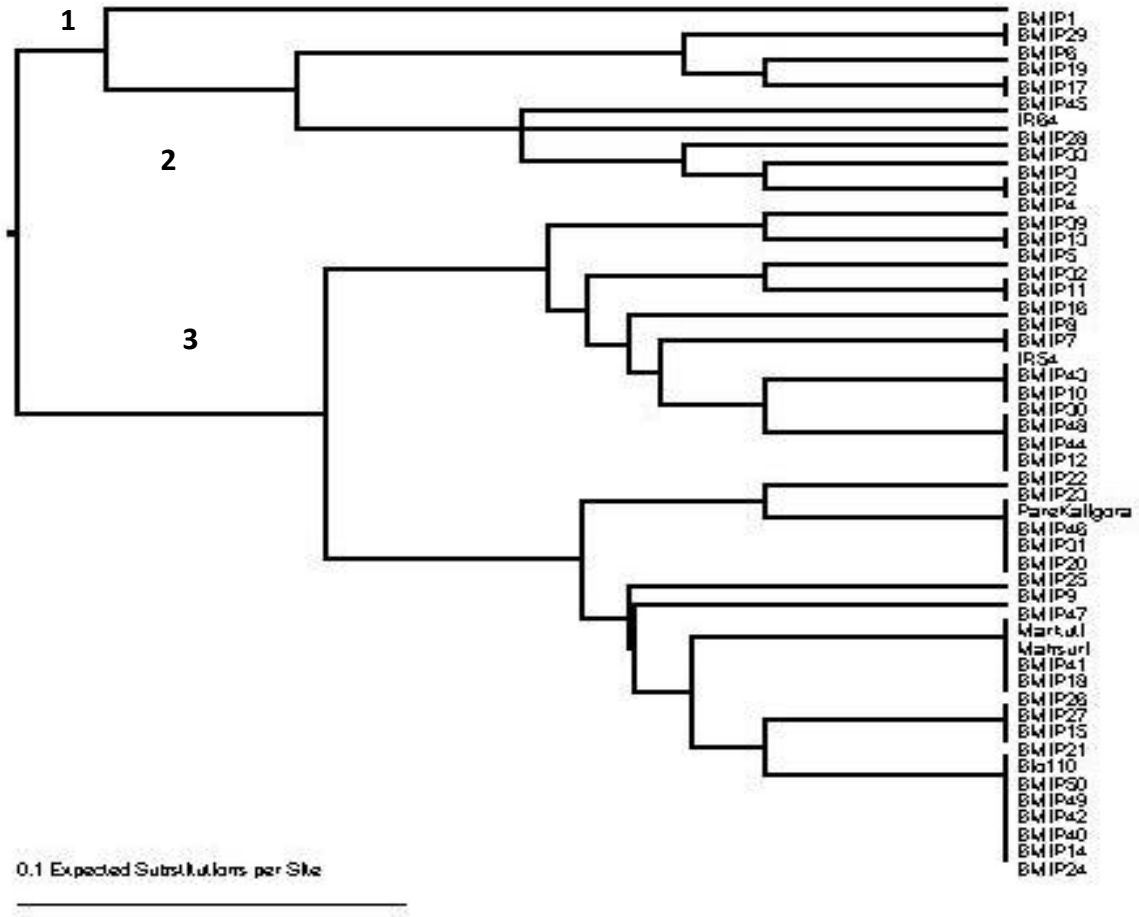


Fig. 4 Phylogenetic trees constructed based on the STS marker and phenotype (The authors)

4. Discussion

The BMIP germplasm used in this study had Markuti, an elder that was tolerant to Fe toxicity. Phenotyping tests placed Markuti in the second cluster, which showed a high level of tolerance to Fe in the field. Mahsuri which was used as the positive control occupied the first cluster, offering the lowest bronzing score. Nugraha et al. [16] reported that Mahsuri showed shoot-based tolerance through high shoot Fe concentration, reduced leaf varnish, and high grain production. Moreover, IR64 which was used as the negative control was in the fifth cluster with the lowest Fe tolerance.

The BMIP 1 and BMIP 19 lines, which exhibited the same position as Markuti (second cluster) in the field test showed a different placement in the phylogenetic grouping, even clustered with IR64 (negative control). BMIP 24 and BMIP 25 lines that were in the same cluster as Mahsuri in phenotyping tests also clustered together with Mahsuri and Markuti on the phylogenetic tree (Group 3), indicating that the lines had a Fe-tolerant response. In the third cluster on phenotypic testing that showed a tolerant response, only BMIP 20, BMIP 26, BMIP 46, BMIP 47, BMIP 49, and BMIP 50 were on the same branch as Mahsuri and Markuti. Meanwhile, the BMIP 6, BMIP 17, BMIP 28, and BMIP 29 lines were in the same position as IR64 in phenotyping testing (Cluster 5) and the phylogenetic tree, indicating sensitivity to Fe toxicity.

The tolerant rice lines in the field test showed a low percentage of bronzing compared to the sensitive lines. Leaf bronzing, caused by Fe toxicity, induced oxidative damage through the generation of ROS in chloroplasts [17]. This oxidative damage caused permanent damage to DNA, membranes, proteins, and cell structures [18]. Moreover, an excess Fe can increase cytochrome b6/f in thylakoids, threaten photosynthesis II, inhibit photosynthesis, and accelerate oxygen production [19]. In rice lines with lower Fe tolerance levels, symptoms of the reticulum on the leaves cannot be avoided, resulting in low yield production [15].

The formation of Fe tolerance traits in rice involved the role of multiple genes. In this study, the molecular marker approach was employed to characterize three genes that contributed to the formation of Fe tolerance. The genotype association analysis with the results of field testing showed three genes that contributed to Fe tolerance in the BMIP rice population, namely OsIRT1, OsIRT2, and OsFRO2. Markuti as Fe donor elders in BMIP lines were known to have potential alleles of Fe toxicity tolerant genes, namely OsIRT1 and OsIRT2 [20]. These genes contributed to the homeostasis mechanism in Fe-stressed rice plants. The QTL qFETOX-3, which marked the OsIRT1 gene was identified in the Nipponbare/Kasalath population as a region associated with alleles, thereby contributing to

Fe tolerance [15]. According to the Microarray analysis, the expression of OsIRT1 and OsIRT2 genes, involved in Fe uptake and transport, was highly suppressed in roots with low to high Fe concentrations that caused Fe toxicity [21]. To prevent Fe toxicity, the expression of genes involved in Fe utilization, absorption, translocation, and storage was tightly regulated [22]. Plants induced the expression of various genes under Fe-deficient conditions, while suppression occurred under Fe-sufficient conditions [23]. Moreover, polymorphisms in this gene indicated responsibility for the Fe exclusion mechanism.

According to Martín-Barranco [24], IRT 1 interacted with FRO 2 in the acidification-reduction-transport strategy for iron acquisition. The process involved the acidification by plasma membrane H+-ATPase (P-ATPase), which enhanced the solubility of Fe3+. The presence of insoluble Fe3+ around the root rhizosphere membrane was reduced by OsFRO2 to Fe2+ which is more soluble. Subsequently, the Fe2+ ions were carried into the root tissue by the OsIRT1 transproter [25]. The accumulation of Fe2+ in the root cell cytosol induced OsIRT2 to immediately distribute into the vesicles [26]. Fe2+ rich in root tissues must be immediately distributed to others to prevent accumulation. This transportation occurred through the vascular network for long-distance transportation [27].

5. Conclusions

In conclusion, the Hierarchical Cluster Analysis of Fe-stressed rice populations in the field grouped BMIP rice populations into five clusters, indicating variations in response to Fe toxicity. Genotype testing associated with phenotypic responses showed OsIRT1, OsIRT2, and OsFRO2, which contributed to the Fe-tolerant trait. Furthermore, the phylogenetic tree combining phenotypic response and selected genotypes showed the presence of 3 large clusters. This consisted of Markuti and Mahsuri in the same cluster, while IR64 was in a different cluster. The evaluation of phenotypic response combined with genotyping identified BMIP 24, BMIP 25, BMIP 20, BMIP 26, BMIP 46, BMIP 47, BMIP 49, and BMIP 50 lines as tolerant lines.

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