Indonesian Journal of Agricultural Science
Vol. 21 No. 1 June 2020: 17–29

DOI: http//dx.doi.org/10.21082/ijas.v.21.n1.2020.p.17–29

IDENTIFICATION OF IRON TOLERANT CANDIDATE LOCI IN RICE DETERMINED THROUGH GENOME-WIDE ASSOCIATION STUDY

Identifikasi Lokus Kandidat Toleran Besi pada Padi Menggunakan Genome-Wide Association Study

Dwinita W. Utami[a*], Ida Rosdianti[a], Lili Chrisnawati[b], Subardi[c], Siti Nurani[a], and Suwarno[d]

[a]*Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development*
Jalan Tentara Pelajar No. 3A, Bogor.16111, West Java
Phone; +62-251-8337975; Fax.: +62-251-8338820
[b]*Master Student of Plant Biology, Department of Biology, Bogor Agricultural University, Bogor, West Java*
[c]*Indonesian Soil Research Institute, Taman Bogo Experimental Station, East Lampung, Lampung*
[d]*Indonesian Center for Rice Research, Jalan Raya 9, Sukamandi, Subang, West Java*

**Corresponding author: dnitawu@windowslive.com*

Submitted: 27 March 2019, Revised: 25 April 2020, Accepted: 13 May 2020

ABSTRACT

Iron (Fe) toxicity is a significant abiotic stress in swamp land. The study aimed to identify the candidate loci related to Fe toxicity tolerance through Genome-Wide Association Study (GWAS) approach. The study used 242 rice accessions consisting of 192 breeding lines and 50 local landraces, and custom-designed 384 rice SNPs-chips. A field evaluation was conducted in inland swamp for two season periods (2014 and 2015). Phenotypic data and association mapping were analyzed using XLSTAT and TASSEL 3.0. The candidate loci were analyzed by functional gene detection of the significant SNPs aligned to the Rice Annotation Project and the Institute for Genomic Research databases. Three linkage disequilibrium (LD) blocks were detected in the Fe tolerant population around the significant SNPs. The first LD block was mapped in chromosome 1 (the *AtIRT* gene and *qFETOX1*; *qFETOX1-3* QTLs loci) resembled partitioning of Fe-toxicity tolerant mechanism. The second LD blocks located in chromosome 2 (*qFETOX-2-1* and *qFETOX-2* QTLs loci) and chromosome 3 (*qFETOX-3* QTL, *OsNAS1* and *OsNAS2* loci), probably contributed to Fe exclusion mechanism. The third LD blocks located in chromosome 4 (*OsFRO2* and *qFETOX-4* QTL loci) and chromosome 7 (*OsIRT2* and *NAS3* loci). The third LD block was found on tolerant genotypes both on vegetative and generative stages. This condition indicated that these loci were presumed playing a role for Fe toxicity tolerance in rice. Results of the study are beneficial for determining the strategy on developing Fe-toxicity tolerant rice for specific swamp land type through breeding programs.

[**Keywords**: GWAS, SNP markers, iron toxicity tolerance, rice landrace]

ABSTRAK

Keracunan besi merupakan kendala abiotik utama di lahan rawa. Penelitian ini bertujuan untuk mengidentifikasi kandidat lokus gen/QTL karakter toleran besi melalui pendekatan Genome-Wide Association Study *(GWAS). Penelitian dilakukan pada 242 aksesi padi terdiri atas 192 galur pemuliaan dan 50 varietas padi lokal, menggunakan* custom designed 384 SNPs-chip *padi. Pengamatan dilakukan di lahan rawa lebak dalam selama dua musim (2014 dan 2015). Analisis asosiasi data fenotipe dan analisis pemetaan dilakukan masing-masing menggunakan program XLSTAT dan TASSEL 3.0. Kandidat lokus gen/QTL dianalisis berdasarkan marka SNPs yang signifikan dengan didukung hasil penjajaran ke pangkalan data Rice Annotation Project dan The Institute for Genomic Research. Tiga blok LD terdeteksi terkait karakter toleransi keracunan besi di sepanjang marka SNPs yang signifikan. Blok LD-1 terpetakan di kromosom 1, pada gen* AtIRT *dan pada lokus QTL* qFETOX1*;* qFETOX1-3*, yang terindikasi berkontribusi pada proses partisi kadar toksik* Fe^{2+} *(strategi toleransi I). Blok LD-II terpetakan di kromosom 2, pada lokus QTL* qFETOX-2-1 *dan* qFETOX-2*, serta di kromosom 3 pada QTL* qFETOX-3*, lokus gen* OsNAS1 *dan* OsNAS2*, yang terindikasi berkontribusi pada proses eksklusi* Fe^{2+} *(strategi toleransi II). Blok LD-III terpetakan di kromosom 4 pada lokus gen* OsFRO2 *dan QTL* qFETOX *dan di kromosom 7 pada lokus gen* OsIRT2 *dan* NAS3*. LD blok ini terdeteksi pada genotipe toleran, baik pada fase vegetatif maupun generatif, dan diduga berkontribusi dalam mekanisme toleransi keracunan Fe pada tanaman padi. Hasil penelitian ini bermanfaat untuk menentukan strategi pengembangan varietas padi toleran keracunan besi untuk tipe lahan rawa spesifik melalui program pemuliaan.*

[**Kata kunci**: GWAS, marka SNP, ketahanan keracunan besi, varietas padi lokal]

INTRODUCTION

Ferrum/iron (Fe) is a micronutrient essential for plants because it plays a role in the metabolic processes such as DNA synthesis, respiration, and electron transport support photosynthesis process. Iron also acts as an electron acceptor in the redox reaction and activator for important enzymes in plant metabolism. Nevertheless, in acid soils, the soluble Fe could be available excessively (more than 300 mg kg^{-1}) resulted in toxic effects to plants (Dobermann and Fairhurst 2000). Iron toxicity is one

of the important abiotic stresses that can decrease rice production. Millions of hectares of rice fields in Asia, Africa, and Latin America were reported suffering iron toxicity (Matthus et al. 2015). In Indonesia, rice fields suffering from iron toxicity is spread on suboptimal soils, such as swamp area, tidal land, red-yellow podsolic land, lowland with poor drainage, and new crop areas scattered in many islands of Indonesia. The estimated hectarage of rice fields with a high content of Fe in Indonesia reached one million hectares (Suhartini 2004).

Development of iron-toxicity tolerant rice variety through breeding program seems to be a practical approach in dealing with iron toxicity stress in rice. In principle, physiological strategies can be targeted to address the iron toxicity problem. The strategies include: (1) Fe^{2+} exclusion mechanism on the root surface through the Fe^{2+}oxidation process into insoluble Fe^{3+}. This strategy leads to the plaque formations on the root surfaces. Lateral roots contain large amounts of aerenchyma, allowing oxygen diffusion into the rhizosphere (Becker and Asch 2005; Wu et al. 2014); (2) Partitioning of Fe^{2+} into organs and subcellular tissues (Moore et al. 2014) of different plants so the plants are more tolerant to the iron excess conditions.

Genomic mapping technologies, such as cytogenetics, molecular genetics, and physical mapping to complete rice genome sequence are essential breakthroughs for uncovering the functional part of the rice genome (rice functional genomic) for many critical complex characters such as tolerance to abiotic stress (Tyagi et al. 2004). Single nucleotide polymorphism (SNP) markers are mainly developed based on next-generation sequencing technology. The fast development of SNP markers through genotyping-by-sequencing (GBS) has paved the road to facilitating genomics-assisted breeding through quantitative trait loci (QTLs) and genome-wide association analysis (GWAS) in diverse crops (Basu et al. 2018). GWAS typically focuses on associations between SNPs and dominant traits. Moreover, GWAS is often utilized when we are interested in finding out all the genomic regions that may control a specific role. Association analysis based on linkage disequilibrium (LD) is an efficient way to dissect complex traits and to identify gene functions in rice (Zhang et al. 2016).

Some results of the previous mapping studies have shown that there are several genes or QTLs related to iron toxicity tolerant character. The genes or QTLs spread across multiple chromosomes of the rice genome, including on chromosome 1 that was detected on a physical map position of 25–30 Mb and in chromosome 3 at location of 0–5 Mb (Dufey et al. 2009; Wu et al. 2014). The case in this research is tolerance to iron toxicity. SNP markers that have been confirmed associated with the target character can be used as a tool for assisting in the selection process of molecular markers-based breeding for designing iron tolerant rice varieties. The study aimed to analyze candidate loci related to iron toxicity tolerance in rice by the GWAS approach using custom-designed 384-SNP markers.

MATERIALS AND METHODS

Genetic Materials

The genetic materials used were two subsets of different populations of rice. The first was 192 breeding lines (BL) subset population. The progeny lines used in the study came from diverse parents that were used for the crossing of iron toxicity breeding lines. The second was 50 rice landraces germplasm (LG) subset population. List of the two subset populations was presented in Appendix 1.

Designed Custom 384 SNP-Chips

Designed custom 384 SNP-chips were based on the genetic map of several genes associated with character of tolerance to Fe toxicity. A previous study has identified many SNPs (Utami and Hanarida 2014). The SNP primers designed were attached to BeadChip in a 2-micron bead that can hybridize with DNA samples at the PCR annealing time.

Field Assay for Iron Toxicity Evaluation

Phenotype characterization of rice landraces to iron toxicity tolerance was done in the acid soil of upland field in Taman Bogo Experimental Field, East Lampung. The geographic allocation of this field is 50 02” South Latitude and 1050 50” East Longitude, with an altitude of 300 masl. Taman Bogo rice field is a plain to rather plain landform (dominant slope of 0–3%). The soil properties at the experimental sites are shown in Table 1.

Table 1. Soil chemical properties of Taman Bogo experimental field, East Lampung.

Description	Control site	Iron-tox site	Exchangeable cation	
pH (H_2O)	5.4	4	Na (me 100^{-1} g)	0.01
C organic (%)	1.14	1.1	K (me 100^{-1} g)	0.02
N total (%)	0.09	0.09	Ca (me 100^{-1} g)	0.01
C/N	12.7	11	Mg (me 100^{-1} g)	0.02
PO_5 Bray 1 (ppm)	10.5	6.8	P (mg P_2O_5 100^{-1} g)	3.1
K_2O Morgan (ppm)	30	27.7	Al (me 100^{-1} g)	5.1
			Fe (ppm)	2030
			Pyrite as total Fe & S (%)	0.02

A field experiment was conducted under swampy inland with low soil pH and also suffered from iron toxicity (Fe soil of 2030 ppm). The soil texture consisted of 29% clay, 33% silt, and 39% sand. The soil macronutrients indicated in a low content of N total (%), Ca, and Mg.

Each rice genotype was planted on two rows of 2 m each, in each plot, with two replications. Tillage was done as a local recommendation by giving NPK 300 kg ha^{-1} and urea 100 kg ha^{-1}, at 4 and 7 weeks after planting. The performance of Fe toxicity stress was observed on bronzing assessment, which was scored at 1 month after planting. Mahsuri variety was used as a tolerant control and IR64 as a susceptible control.

Genomic DNA Preparation

Total DNA preparation followed the protocol recommended by Illumina, covering the extraction and purification of DNA from leaves of rice plants. Some 20–50 mg samples of fresh leaves were put into 2 ml microtube which already contains two pieces of stainless steel or tungsten carbide bead of 3 mm diameter and placed in Tissue Lyser Adapter Set 2 x 24. A total of 500 ml of lysis buffer (Thermo Kit) containing 0.25 mg ml^{-1} RNase was then added in the mixture. Samples were centrifuged 1500xg for 30 seconds and incubated at 56° C for 30 minutes. The samples were then centrifuged back at 6000xg for 20 minutes to separate the DNA from the debris and other contaminants. Purification of total DNA was performed by using Thermo King Fisher Scientific Flex (Thermo Scientific 2011). DNA concentration was standardized by dilution to 50 ng ml^{-1} as a final concentration.

GoldenGate Genotyping Assay

GoldenGate Genotyping Assay is divided into two main stages, namely the pre-amplification and post-amplification stages. Pre-amplification includes activation of biotinylated labeled DNA to prepare the DNA samples for the next post-amplification step. This process included on extension and ligation by the PCR process using the two primers labeled with a fluorescent dye (Primary 1 and Primary 2) and one biotinylated primer (Primary 3), where the Primary 3 allows for marking the PCR products and elute DNA thread containing a fluorescent signal. Post-amplification was finalized by visualizing the BeadChip-signal on the Iscan system. Data visualization was then analyzed to determine the genotype of SNP using Illumina's BeadStudio Gene Expression Module (Illumina 2009).

Data and Association Analyses

Phenotypic data were analyzed using two way ANOVA to test the effect of Fe toxicity stress and genotype factors. The data were analyzed using the XLSTAT 19.5 software program (www.xlstat.com). Association analysis was done on the whole population of 242 individual genotypes and subpopulation separately, consisting of 192 genotypes of Fe tolerant breeding lines and elite varieties and 50 accessions of rice landrace. Association between SNP markers and phenotypic data was tested using the General Linear Model (GLM) in the TASSEL (Trait Analysis by aSSociation, Evolution and Linkage) v. 3.0 software program (Bradbury et al. 2007). However, researchers must contend with the confounding effects of both population and family structure.

RESULTS AND DISCUSSION

Phenotypic and Genotypic Diversity for Association Analysis

The BL subset samples (A) have a different performance to LG subset samples. The BL was homogenized in each line, although they were diverse in agronomical characters, such as plant height. The overall responses of both populations varied on Fe toxicity tolerance based on bronzing score. This variation can be seen in each set of different populations. This is due to the influence of diverse genetic backgrounds (Figure 1).

The phenotypic performances of 50 accessions of LG population showed abundance on morphological variation. It was contrasted to 192 BL population, although they were developed from a broad genetic background (Figure 1A). It showed that the genetic diversity of BL had been reduced compared to LG.

The association analysis between phenotypic and genotypic data in the BL population detected three LD blocks spreading over in several SNPs markers locations. It was in contrast with the LG population which showed only one LD block (Figure 1B). These results were relevant to the LD map that showed three blocks and one block for BL and LG populations, respectively. LD block map has measured the strength of the correlation between markers caused by their shared genetic history (Bush and Moore 2012). Due to the BL population have the same genetic history on Fe toxicity tolerance breeding program, they have a lot of pairs of SNP that correlated with an allele of another SNP and associated with the Fe toxicity tolerance alleles. The different conditions were showed on LG population; they have breeding naturally as landraces

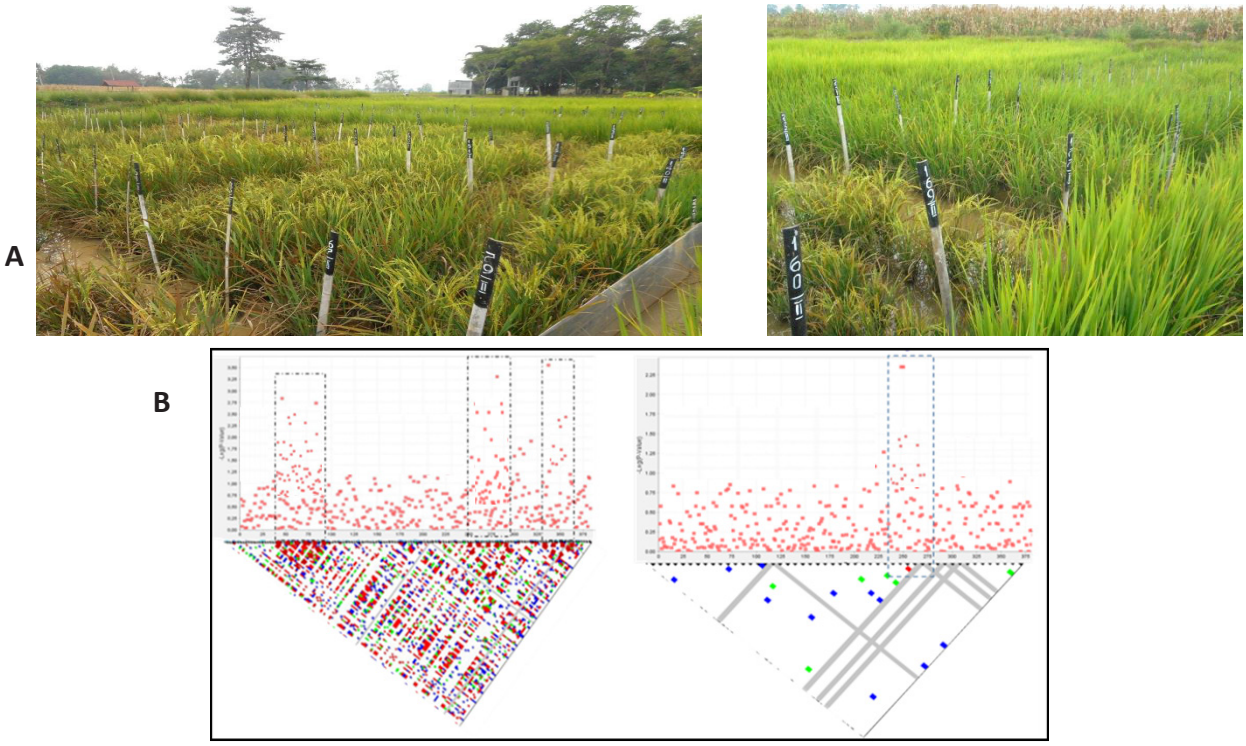

Figure 1. Linkage blocks of association analysis on two groups of rice populations (Fe breeding lines and landrace germplasm): (A) performance of rice plants in the field, (B) three LD blocks detected in the Fe breeding lines and one locus associated with Fe tolerance character identified in landrace germplasm.

originated from the swampyland, which only had one unsaturated LD block. These results are the critical thing in identifying the candidate loci associated with markers (Soto-Cerda and Cloutier 2012).

Analysis of Candidate Loci Associated with Iron Toxicity

Identification of some SNPs markers included in the set of 384 SNP-chip-2014 significantly related to the Fe tolerant character based on the field testing for two seasons in two different locations, namely Karang Agung, South Sumatra (2014) and Taman Bogo, Lampung (2015), which showed that some selected SNPs markers spread across on the 12 chromosomes of rice genomes (Table 1). These closely flanking markers based on the genetic position contained in several gene loci have been identified in earlier research. The association analysis results indicated there were several SNPs as candidate loci associated with Fe toxicity tolerant characters. The genetic position of the SNP markers is consistently significant on the set of the population and with different mapping association methods, i.e. Generalized Linear Models (GLM) and Linear Mixed Model (MLM).

Furthermore, the tracking analysis of gene functions following the genetic position of significant SNP markers at Rice Annotation Project (RAP) and The Institute for Genomic Research (TIGR) databases showed that the candidate loci were located on chromosomes 1, 2, 3, 4 and 7 of rice.

Chromosome 1

On chromosome 1, there were three positions of significant LD blocks, namely (1) *TBGI000823* (477.756)-*TBGI000824* (477 756); (2) *id1020828* (35,170,076); and (3) *TBGI066367* (39,606,044) -*TBGI067836* (40,320,704). Three blocks of LD significant SNP markers on chromosome 1 were sequentially mapped to the *AtIRT1* gene, *qFETOX1* (Dufey et al. 2009), and *qFETOX1-3* (Wu et al. 1997, 1998). Iron Regulation Transporters (*IRT1*) is a gene that plays a role in the partitioning of Fe^{2+} of the Fe tolerance mechanisms (Bennett et al. 2011; Rout et al. 2015). This gene plays a role in the transport of Fe^{2+} from root epidermal tissue through the plasma membrane into the cytosol (Rout et al. 2015). Vert et al. (2001) reported that the *IRT1* gene plays a role in the partitioning of Fe^{2+} mechanism to several plant so

the plant is more tolerant to Fe^{2+} excess conditions. *IRT* gene expression occurs in the leaves and stems (Ishimaru 2006).

The phenotypic performance of some test plants such as IR54, a tolerant variety, showed a bronzing score of 2–3, whereas IR64, a sensitive type, had a 9-bronzing score. IR54, a tolerant variety, had an excellent agronomic performance both during the vegetative or generative stages. It is different from IR64 that during the vegetative stage, high growth is hampered (Abu et al. 1989). IR64 also has a problem in tillering development (Cheema et al 1990). The effect of Fe toxicity was also seen when the plants entering the end of the vegetative stage or at initial of the generative phase. Fe toxicity inhibited the panicles formation and even the number of grains in each panicle (Singh et al. 1992). Fe toxicity also causes the plants to be sterile or disrupts the flowering (Virmani 1977).

In the high Fe conditions, root performance of IR54 and IR64 were not significantly different (Figure 2). This indicates that both varieties have Fe^{2+} transport activity by the same gene, *IRT*. However, IR54 can partition the Fe^{2+} absorbed into the tissue that is not done by IR64. Therefore, based on the candidate loci analysis, allele groups in LD blocks on chromosome 1 are thought to play a role in the Fe^{2+} partition activity as a part of Fe tolerance mechanisms in rice.

Chromosome 2 and 3

Based on association mapping analysis, chromosome 2 and 3 had two groups of significant alleles to bronzing levels of landrace samples tested (Figure 3A). The alleles group on chromosome 2 were mapped in position of 26.3 kb, in accordance with the genetic map of *QFE-TOX-2-1* (Shimizu 2009) and 31.8 kb, in accordance with *qFETOX-2* which mapped in the RIL (F8) population of IR29 (sensitive) and Pokkali (tolerant) (Wu et al. 2014). Observation of the cross section of the root showed that Pokkali had

Table 2. SNP markers selected from 384 SNPs-chip-2014 associated with iron tolerance character (bronzing score) in rice.

SNP ID	Chromosome	Genetic map	P-value	Reference
TBGI067836	1	40,320,704	0.01384305	Wu et al. (1997, 1998)
TBGI112858	2	29,286,111	0.003280955	Wan et al. (2003)
TBGI128877	3	3,247,991	0.0117282	Wan et al. (2003b); Dufrey et al. (2012)
TBGI129684		3,756,536	6.22428E-05	
id4010396	4	31,105,608	0.006359507	Dufrey et al. (2012)
TBGI187378	5	24,394,228	0.00312645	Utami (2017, unpublished)
TBGI272517	6	30,040,771	0.000195	Utami (2017, unpublished)
TBGI335079	7	27,546,143	0.0227899	Utami (2017, unpublished)
TBGI367853	8	17,135,276	0.000830558	Wu et al. (2014)
TBGI367853	9	15,013,927	0.00041	Wan et al. (2003a)
id10006100	10	19,833,428	0.004018	Utami (2017, unpublished)
id11000784	11	23,717,743	0.010649	Wan et al. (2003b); Dufrey et al. (2009)
id12001224	12	22,914,254	0.0024	Wu et al. (2014)

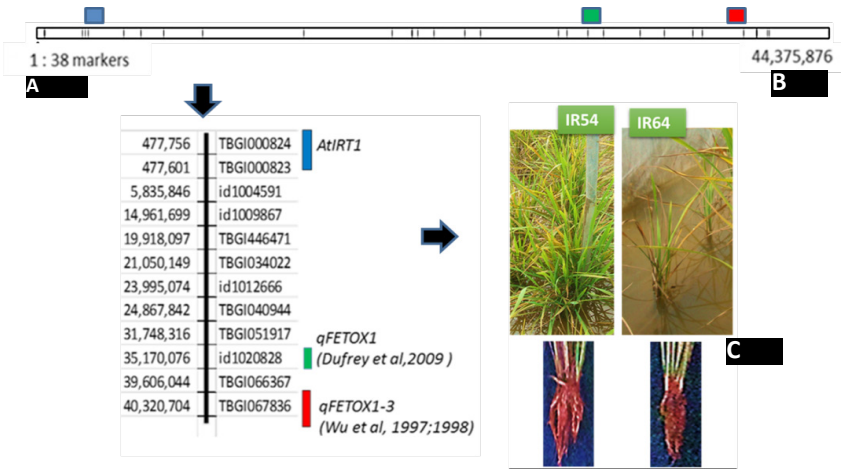

Figure 2. A. The three candidate loci on LD block accordance with the genetic map position of the significant SNP markers on the total 38 SNP markers spread in chromosome 1. B. Plant performance of IR54 (tolerant rice variety) and IR64 (sensitive rice variety) at two months after planting in high Fe conditions. C. Root performance of IR54 and IR64.

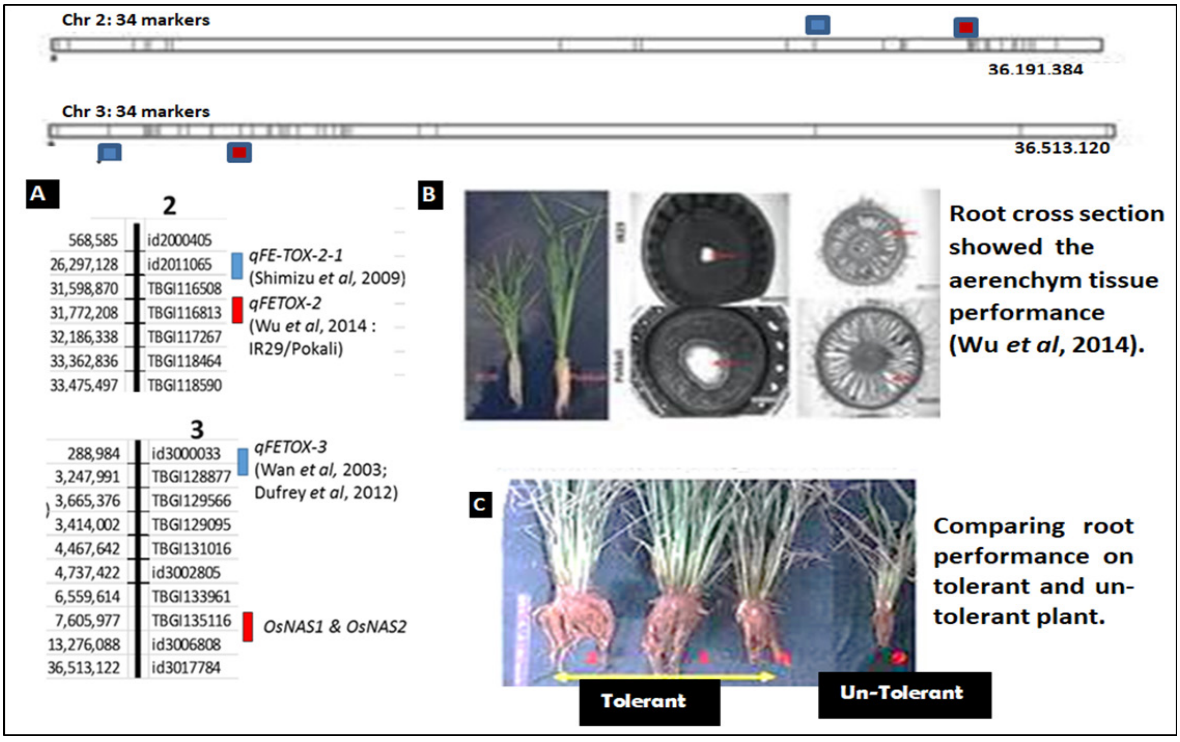

Figure 3. A. Two candidate loci on LD blocks accordance the genetic map position of the significant SNP markers on chromosome 2 and 3. B. Comparison of the cross-sectional root of tolerant variety, (Pokkali) and sensitive variety, (IR29) (Wu et al. 2014), C. Root performance of some plants with different bronzing scores in high Fe conditions in the inland field of Lampung.

aerenchymal tissue higher than IR29, both in the initial conditions or under Fe^{2+} stress conditions (Figure 3B). Aerenchyma is the parenchymal tissue that holds the air with the structure of an ample space between cells. These plant tissues contribute to the internal oxidation process in the plant (Colmer 2002). Great parenchyma will increase the oxidation of the roots, which will also further enhance the ability of Fe^{2+} exclusion on the roots of tolerant plant Pokkali (Wu et al. 2014). Thus, the plant can limit the absorption of Fe^{2+}.

Some rice landrace samples tested in the field (Figure 6C) showed the correlation between root and plant performance with a bronzing score, which is one of the Fe toxicity parameters. Tolerant plants (bronzing score 1–3) have longer and more robust roots than those of sensitive plants (bronzing score 9). This indicates the existence of Fe^{2+} prevention mechanisms into the roots to avoided damage to other plant parts.

As it is the case on chromosome 2, LD blocks on chromosome 3 were also detected in two genetic positions, on 3.2 kb (Wan et al. 2003; Dufrey et al. 2012) and 10.9 kb (Inoue et al. 2003). Based on the analysis of the candidate loci, allele groups in LD blocks on chromosome 2 and 3 presumed play a role in the Fe^{2+} exclusion activity, parts of tolerance mechanisms on inland contained high Fe.

Chromosome 4 and 7

Two groups of alleles were detected on chromosomes 4 and 7 respectively, on chromosome 4 the alleles were mapped in accordance with *OsFRO2* (Gross et al. 2003) and QTL *qFETOX-4* (Dufey et al. 2012) and on chromosome 7 mapped following *OsIRT2* (Gross et al. 2003) and *OsNAS3* (Inoue et al. 2003) (Figure 4A).

Some of the genes included in the LD blocks in chromosomes 4 and 7 have been known to play a role in the partitioning and exclusion of Fe^{2+} in Fe tolerance mechanism (Figure 3B) (Tsai and Schmidt 2017). The role of association results in this study was implicating to candidate loci detected, which contributed to Fe toxicity tolerance as shown in Table 3. The performance of the test plants was observed in the field on two varieties, i.e. IR64 (sensitive control) and Mahsuri (tolerant control) (Figure 3C). Mahsuri has strong roots and good shoot in Fe toxicity conditions. These varieties are expected to have tolerance mechanisms at the root level, i.e. the regulation of Fe^{2+} absorption and in the shoot level on Fe^{2+} partition capabilities. This is as proposed by Saikia and Baruah (2019), who reported that Mahsuri on 350 ppm of Fe^{2+} could control Fe^{2+} absorption and increase superoxide dismutase (SOD) accumulation.

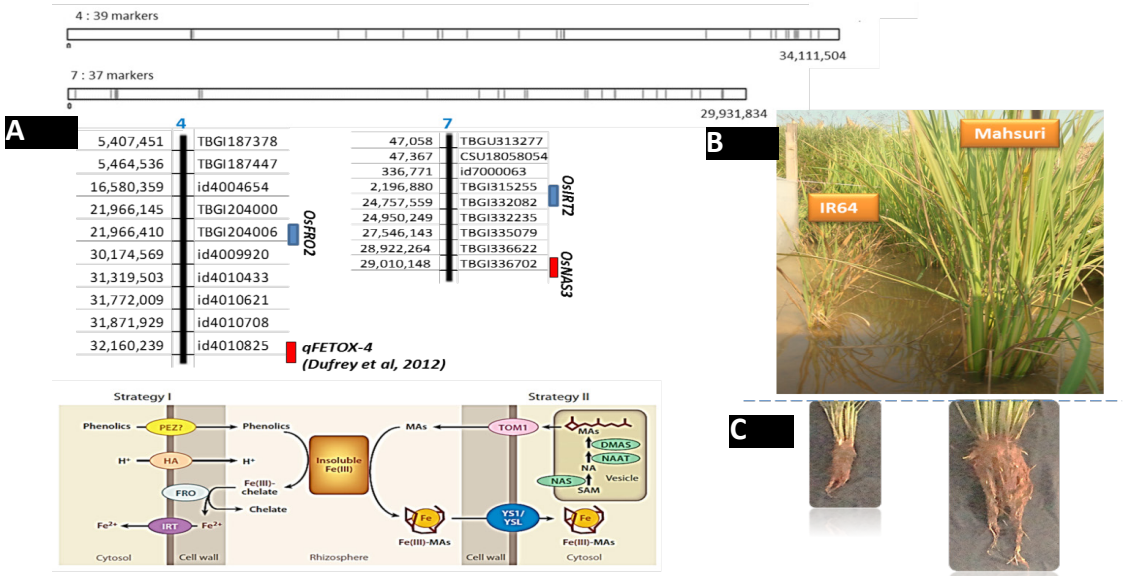

Figure 4. A. Two candidates loci on LD blocks mapped as on the genetic map position of significant SNP markers on chromosomes 4 and 7. B. Comparison of tolerant rice (Mahsuri) and un-tolerant plant (IR64) performance. C. Root performance of Mahsuri and IR64 (control plants) in the inland field of Lampung.

Table 3. The predicted role of candidate loci identified in this study referred to previous reports based on the association analysis.

LD block	Chromosome	Candidate loci	Proposed mechanism	Description of association
I	1	*AtIRT*	Partitioning of Fe^{2+}	Fe tolerance mechanism was contributed by partitioning activity and no absorption exclusion at the root level. The rice lines contained LD block were probably more adapted on the tidal swampland area because the toxic Fe^{2+} will decrease through the oxidation process.
		qFETOX1		
		qFETOX1-3		
II	2	*qFE-TOX2-1*	Exclusion of Fe^{2+}	Fe tolerance mechanism was contributed by exclusion of Fe^{2+} activity in root and no partitioning in tissue level.
		qFE-TOX2		
	3	*qFETOX-3*		The rice lines contained LD block II were probably more adapted in a lowland swamp area
		OsNAS1		
		OsNAS2		
III	4	*OsFRO2*	Partitioning and exclusion of Fe^{2+}	Fe tolerance mechanism was contributed to both Fe^{2+} partitioning and exclusion in root level activities.
		qFETOX4		
	7	*OsIRT2*		The rice lines contained LD block III will have broad tolerance in diverse swampland conditions.
		OsNAS3		

CONCLUSION

Iron tolerance response of 242 rice accessions based on phenotypic experiment in Taman Bogo, Lampung varied, both on BL and LG populations. Association mapping on the BL population detected three positions of LD blocks around significant SNPs. Based on the candidate loci analysis, there were three loci identified. The first LD block was in chromosome 1, mapped on the *AtIRT* gene locus, as well as QTL of *qFETOX1* and *qFETOX* 1-3. These loci assumed to play a role in Fe toxicity tolerance through the Fe partitioning mechanism. The second LD block located in chromosome 2, mapped on the *qFETOX-2-1* and *qFETOX-2* loci, as well as in chromosome 3, on *qFETOX-3* locus and *OsNAS1* and *OsNAS2* genes. These allele groups were predicted to contribute to the Fe exclusion mechanism.The third LD block is in chromosome 4, mapped on *OsFRO2* gene and *qFETOX-4* and chromosome 7 on *OsIRT2* and *NAS3* genes. These allele groups presumably contribute on both partitioning and exclusion of Fe. GWAS approach can probably detect allele groups that contribute to the

Fe tolerance mechanism, although the function of those candidate genes/QTLs should be verified. The study implies a valuable result for determining the strategy for developing Fe-toxicity tolerance rice for specific swamp land type through breeding programs.

ACKNOWLEDGEMENT

The research was funded by the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development. Thanks to the research team of the Indonesian Center for Rice Research (ICRR) and Taman Bogo Experimental Station, Lampung, for teamwork and collaboration.

AUTHORS CONTRIBUTIONS

DWU was the main contributor, responsible for designing the research, analyzing-interpretating data, and writing the manuscript. IR, LC, and SN assisted in data analysis. Sb and S supported genetic materials and conducted a field experiment.

REFERENCES

Abu, M.B., Tucker, E.S., Harding, S. S. & Sesay, J. S. (1989). Cultural practices to reduce iron toxicity in rice. *International Rice Research Newsletter*, 14, 19.

Basu, U., Srivastava, R., Bajaj, D., Thakro, V., Daware, A., Malik, N., Upadhyaya, H. D., & Parida, S. K. (2018). Genome-wide generation and genotyping of informative SNPs to scan molecular signatures for seed yield in chickpea. *Scientific Reports*, 8(1), 1–11. https://doi.org/10.1038/s41598-018-29926-1

Becker, M., & Asch, F. (2005). Iron toxicity in rice - Conditions and management concepts. *Journal of Plant Nutrition and Soil Science*, 168(4), 558–573. https://doi.org/10.1002/jpln.200520504

Bennett, S.A., R.L. Hansman, A.L. Sessions, K. Nakamura & K. J. Edwards. (2011). Tracing iron-fueled microbial carbon production within the hydrothermal plume at the Loihi Seamount. *Acta*., 75:5526–55.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: Software for association mapping of complex traits in diverse samples. *Bioinformatics*, 23(19), 2633–2635. https://doi.org/10.1093/bioinformatics/btm308

Cheema, S.S., U. Chaudhary, P. N. Takkar & B. D. Sharma. (1990). Effect of date of transplanting on uptake of micronutrients by rice cultivars of different growth stages. *Journal Research*, 27, 199–206.

Dobermann, A. & Fairhurst, T. (2000). *Rice: Nutrient Disorders & Nutrient Management.* Manila: The International Rice Research Institute.

Dufey, I., Hakizimana, P., Draye, X., Lutts, S., & Bertin, P. (2009). QTL mapping for biomass and physiological parameters linked to resistance mechanisms to ferrous iron toxicity in rice. *Euphytica*, 167(2), 143–160. https://doi.org/10.1007/s10681-008-9870-7

Gross, J., Stein, R. J., Fett-neto, A. G., & Fett, J. P. (2003). Iron homeostasis related genes in rice. *Genetica Molecular Biology*, 26, 477–497.

Illumina. (2009). GenomeStudio® Software 2009.2 Release Notes.

Inoue, H. K., Higuchi, H., Takahashi, S., Mori & Nishiawa, N.K. (2003). Three rice nicotian amine synthase genes OsNAS1, OsNAS2, and OsNAS3 are expressed in cells involved in long-distance transport of iron and differentially regulated by iron. *Plant Journal*, 36, 366–381.

Ishimaru, K. (2003). Identification of a locus increasing rice yield and physiological analysis of its function. *Plant Physiology*, 133, 1083–1090.

Matthus, E., Wu, L.B., Ueda, Y., Höller, S., Becker, M., Frei, M. (2015). Loci, genes, and mechanisms associated with tolerance to ferrous iron toxicity in rice (*Oryza sativa* L.). *Theoritical and Applied Genetics* , 128 (10), 2085–2098.

Moore, K.L. Chen,Y., van de Meene, A.M.L., Hughes, L., Liu W., Geraki, T., Mosselmans,F. McGrath, S.P., Groovenor, C. & Zhao, F. J. (2014). Combine Nano SIMS and synchrotron X-ray fluorescence reveal distinct cellular and subcellular distribution patterns of trace elements in rice tissues. *New Phytologist*, 201, 104–115.

Rout, G. R. & Sahoo, S. (2015). Role of iron in plant growth and metabolism. *Reviews in Agricultural Science*, 3, 1–24.

Saikia, T., & Baruah, K. K. (2019). Iron toxicity tolerance in rice (*Oryza sativa*) and its association with anti-oxidative enzyme activity. *Jounal of Crop Science,* 3 (3), 90–94. https://doi.org/10.9735/0976-8920.3.3.90-94.

Shimizu, A. (2009). QTL analysis of genetic tolerance to iron toxicity in rice (*Oryza sativa* L.) by quantification of the bronzing score. *Journal of New Seeds*, 10(3), 171–179.

Singh, B.P., Das, M., Prasad, R.N. & M. Ram. (1992). Characteristics of Fe toxic soils and affected plants and their correction in acid Haplaquents of Meghalaya. *International Rice Research, Newsletter*, 17, 18-19.

Soto-Cerda, B.J. & Cloutier, S. (2012). *Association Mapping in Plant Genomes*. In Caliskan, m. (ed.) Genetic Diversity in Plants, InTech.

Suhartini, T. (2004). Perbaikan variet 1as padi untuk lahan keracunan Fe. *Buletin Plasma Nutfah,* 10 (1), 1–11.

Technology, I. Life. (2011). *KingFisher Plant DNA Kit: Instruction Manual*. Thermo Fisher Scientific, Inc. Finland.

Tyagi, A., Khurana, J.P., Raghuvanski, S. Gaur,A., Kapur,A. Gupta,V.D., Ravi, V., Viji, S., Khurana, P. & Sharma, S. (2004). Structural & functional analysis of the rice genome. *Journal of Genetics*, 83, 79–99.

Utami, D. W. & Hanarida, I. (2014). Evaluasi lapang dan identifikasi molekuler plasma nutfah padi terhadap keracunan Fe. *Jurnal Agrobiogen*. 10(1), 9–17.

Vert, G., Briat, J.F & Curir, C. (2001). Arabidopsis IRT2 gene encodes a root-periphery iron transporter. *Plant Journal*, 26, 181–189.

Virmani, S. S. (1977). Varietal tolerance of rice to iron toxicity in Liberia. *International Rice Research Newsletter*, 2, 4–5.

Wan, J.L., Zhai, H.Q., Wan, J.M.,& Ikeshi, H.(2003). Detection and analysis of QTLs for ferrous iron toxicity tolerance in rice, *Oryza sativa* L. *Euphytica*, 131(2), 201–206.

William, S.B., Moore, J. H.. (2012). Genome-wide Association Studies. P*LoS Computational Biology*, 8 (12). e 1002822.

Wu, P., Luo, A., Zhu J.,Yang, J., Huang, N.& Senadhira, D. (1997). Molecular markers linked to genes underlying seedling tolerance for ferrous iron toxicity. *Plant Soil* 196(2), 317–320.

Wu, L., Shhadi, M. Y., Gregorio, G., Matthus, E., Becker, M., & Frei, M. (2014). Genetic and physiological analysis of the tolerance to acute iron toxicity in rice. *Rice* 7(1), 8.1–12. https://doi.org/10.1186/s12284-014-0008-3

Zhang, P., Zhong, K., Shahid, M. Q., & Tong, H. (2016). Association analysis in rice: From application to utilization. *Frontiers in Plant Science*, 7(AUG2016), 1–16. https://doi.org/10.3389/fpls.2016.01202.

Appendix 1. One hundred and two breeding and fifty local rice varieties used in the study

Breeding lines		Local varieties/ landraces		
No	Genotype	No.	Variety	Origin
1	B12810d-TB-1-11-2	1	Rendah Sanra	West Sumatera
2	B13630E-9MR-5	2	Kuning Samaso	West Sumatera
3	B11592F-MR-14-3-4-9 (A)	3	Lumaik Hitam	West Sumatera
4	B11216-4-PN-3-4-3-5-1-4 (A)	4	Lubuk Kenari	West Sumatera
5	B11592F-MR-16-1-5-4	5	Lumut	West Sumatera
6	B12803E-MR-29-17-1	6	Kuning Biaro	West Sumatera
7	B11592F-MR-14-3-4-9 (B)	7	Si Randah Darik	West Sumatera
8	B11216-4-PN-3-4-3-5-1-4 (B)	8	Kuning Padang	West Sumatera
9	IR60080-23	9	Randah Sasak	West Sumatera
10	B13630E-3MR-9 (A)	10	Gondokiah	West Sumatera
11	B13630E-1MR-4 (A)	11	Ampu Kunyit	West Sumatera
12	B11582F-MR-5-3-2 (A)	12	Bandang Sigadis	West Sumatera
13	B12810D-TB-1-11-1	13	Bandang Bujur	West Sumatera
14	B13630E-9MR-3-2	14	Si Randah Cogok	West Sumatera
15	B13630E-1MR-4 (B)	15	Empat	West Sumatera
16	B11582F-MR-5-3-2 (B)	16	Si Lunak	West Sumatera
17	B11949C-MR-1-1	17	Putut	South Sumatera
18	B11908F-TB-1-29-1 (A)	18	Dajang	Riau
19	B12498F-MR-1-2-5 (A)	19	Si Dollok	West Sumatera
20	B12165D-MR-33-1-3 (A)	20	Kalupah	West Sumatera
21	B13607E-9MR-3	21	Banjar Rodok	West Sumatera
22	B11908F-TB-1-29-1 (B)	22	Si Topas	West Sumatera
23	B12498F-MR-1-2-5 (B)	23	Si Hadap	West Sumatera
24	B12165D-MR-33-1-3 (B)	24	Kruet Sentang	Nanggroe Aceh Darussalam
25	B11923F-MR-35-5-2	25	Muda baru	Nanggroe Aceh Darussalam
26	B11592F-MR-16-1-5-1 (A)	26	Si Raja Bunga	Nanggroe Aceh Darussalam
27	B12489C-MR-49-1-4 (A)	27	Si Cantik/Si Gulan	Nanggroe Aceh Darussalam
28	33 IM (B13630E-9MR-3-2)	28	Si Heupah	Nanggroe Aceh Darussalam
29	KAL9118F-MR-2-1-2-1-6-1-1	29	Padi Sudara	Nanggroe Aceh Darussalam
30	B11592F-MR-16-1-5-1 (B)	30	Sidaek	Nanggroe Aceh Darussalam
31	B12489C-MR-49-1-4 (B)	31	Syair	Nanggroe Aceh Darussalam
32	Batutegi-SKI	32	Leukat Hitam	Nanggroe Aceh Darussalam
33	Inpago 4-SKI	33	Si Rendah	Nanggroe Aceh Darussalam
34	Awan Kuning	34	Irian	Nanggroe Aceh Darussalam
35	IPB 107	35	Rangkuh	Nanggroe Aceh Darussalam
36	Kencana Bali	36	Leukat Camprung	Nanggroe Aceh Darussalam
37	LTH	37	Leukat Pisang	Nanggroe Aceh Darussalam
38	Margasari	38	Cantik Keumala	Nanggroe Aceh Darussalam
39	Siam Saba	39	Merah	Nanggroe Aceh Darussalam
40	Situpatenggang	40	Padi Putih	Nanggroe Aceh Darussalam
41	IRBLta-CF2	41	Piaman Merah	Nanggroe Aceh Darussalam
42	IPB1 Dadahup	42	Piaman Gayo	Nanggroe Aceh Darussalam
43	Asahan	43	Si Aweuh	Nanggroe Aceh Darussalam
44	Dendang	44	Piaman Putih	Nanggroe Aceh Darussalam

Appendix 1. One hundred and two breeding and fifty local rice varieties ... (Continued)

Breeding lines		Local varieties/ landraces		
No	Genotype	No.	Variety	Origin
45	IRBLa-AA	45	Si Moa	Nanggroe Aceh Darussalam
46	B13100-2-3	46	Jambe Hasan	Nanggroe Aceh Darussalam
47	Cisokan	47	Si Beureuh	Nanggroe Aceh Darussalam
48	Inpago 9	48	Si Geupai	Nanggroe Aceh Darussalam
49	IRBLta2-Pi	49	Leukat Uno	Nanggroe Aceh Darussalam
50	Inpara 2	50	Seronang B	Nanggroe Aceh Darussalam
51	IR64			
52	Inpari 19			
53	Inpara 5			
54	Limboto			
55	Krueng Aceh			
56	Inpari 31			
57	Pokali			
58	IPB Kapuas			
59	Cisadane			
60	BMIP2			
61	Cilamaya Muntjul			
62	Indragiri			
63	Cisanggarung			
64	BMIP5			
65	BMIP9			
66	BMIP22			
67	BMIP39			
68	BMIP50			
69	BMIP10			
70	BMIP25			
71	BMIP40			
72	B14299E-KA-46			
73	BMIP12			
74	BMIP26			
75	BMIP44			
76	B14299E-KA-50			
77	BMIP16			
78	BMIP32			
79	BMIP45			
80	B14301E-KA-1			
81	BMIP17			
82	BMIP33			
83	BMIP46			
84	B14301E-KA-11			
85	BMIP19			
86	BMIP34			
87	BMIP47			
88	B14301E-KA-14			

Appendix 1. One hundred and two breeding and fifty local rice varieties ... (Continued)

Breeding lines		Local varieties/ landraces		
No	Genotype	No.	Variety	Origin
89	BMIP20			
90	BMIP35			
91	BMIP48			
92	B14301E-KA-17			
93	BMIP21			
94	BMIP37			
95	BMIP49			
96	B14301E-KA-22			
97	B14304E-KA-3			
98	B14311E-KA-1			
99	B14316E-KA-4			
100	B14333E-KA-29			
101	B14308E-KA-2			
102	B14311E-KA-34			
103	B14316E-KA-9			
104	B14333E-KA-35			
105	B14308E-KA-3			
106	B14315E-KA-1			
107	Inpara 3			
108	B14333E-KA-39			
109	B14308E-KA-4			
110	B14315E-KA-13			
111	B14332E-KA-10			
112	B14333E-KA-48			
113	IR42			
114	B14315E-KA-14			
115	B14332E-KA-12			
116	B14334E-KA-1			
117	B14308E-KA-35			
118	B14316E-KA-1			
119	B14332E-KA-15			
120	B14334E-KA-2			
121	B14308E-KA-37			
122	B14316E-KA-2			
123	B14332E-KA-22			
124	B14334E-KA-3			
125	B14308E-KA-38			
126	B14316E-KA-3			
127	B14332E-KA-25			
128	B14334E-KA-4			
129	B14334E-KA-5			
130	B14346E-KA-50			
131	B14354E-KA-7			
132	B14360E-KA-3			

Appendix 1. One hundred and two breeding and fifty local rice varieties ... (Continued)

Breeding lines		Local varieties/ landraces		
No	Genotype	No.	Variety	Origin
133	B14339E-KA-12			
134	B14351E-KA-19			
135	B14354E-KA-8			
136	B14360E-KA-17			
137	B14339E-KA-14			
138	B14354E-KA-1			
139	B14354E-KA-9			
140	B14360E-KA-38			
141	B14339E-KA-16			
142	B14354E-KA-2			
143	B14354E-KA-49			
144	B14361E-KA-15			
145	B14339E-KA-27			
146	B14354E-KA-3			
147	B14357E-KA-4			
148	B14366E-KA-19			
149	B14339E-KA-28			
150	B14354E-KA-4			
151	B14357E-KA-27			
152	B14366E-KA-48			
153	B14346E-KA-4			
154	B14354E-KA-5			
155	B14357E-KA-35			
156	B13925E-KA-1			
157	B14346E-KA-5			
158	B14354E-KA-6			
159	B14357E-KA-48			
160	B13926E-KA-23			
161	B13926E-KA-29			
162	B13988E-KA-40			
163	B11377F-M-34-2			
164	B13520E-KA-13-B			
165	B13926E-KA-43			
166	B13988E-KA-41			
167	IR70213-10-CPA-2-UBN-B-1-1-3			
168	B13522E-KA-5-B			
169	B13938E-KA-23			
170	B13989E-KA-8			
171	B13507E-MR-16			
172	B13531E-KA-1-B			
173	B13938E-KA-27			
174	B13989E-KA-31			
175	B13507E-MR-19			
176	B13545E-KA-1-B			

Appendix 1. One hundred and two breeding and fifty local rice varieties ... (Continued)

Breeding lines		Local varieties/ landraces		
No	Genotype	No.	Variety	Origin
177	B13957E-KA-40			
178	B13990E-KA-50			
179	B13136-6-MR-2-KA-2-1-7			
180	B13545E-KA-8-B			
181	B13957E-KA-50			
182	B13144-1-MR-2-KA-3-1			
183	B13100-2-MR-2-A-3-3-2			
184	B13578E-KA-1-B			
185	B13983E-KA-44			
186	B13100-3-MR-2-KA-2-4			
187	B13520E-KA-6-B			
188	B13578E-KA-3-B			
189	B13988E-KA-20			
190	B13134-4-MR-1-KA-1			
191	B13520E-KA-11-B			
192	B13578E-KA-5-B			