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Genetic variation and association of molecular markers for iron toxicity tolerance in rice

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ABSTRACT

Iron toxicity in rice field can cause abnormality in plant growth leading to yield loss of 35-45%. This is caused by microbial reduction under flooded conditions of insoluble iron-III (Fe^{3+}) into soluble iron-II (Fe^{2+}). The severity and symptoms of Fe toxicity depends on the growth stage of rice plant at which it is exposed to the stress. The plant developed various mechanisms to avoid/tolerate such stress which is a complex phenomenon governed by multiple genes/QTLs. Very few chromosomal loci are reported for Fe toxicity resistance in rice. But no locus has been fine mapped or cloned yet. Association mapping provides opportunity to have a greater coverage of genetic diversity in various germplasm lines so that large number of loci can be identified for Fe toxicity in rice. In the present investigation, 71 genotypes including landraces and released varieties were screened for their Fe toxicity resistance ability. Various agro-morphologic traits were observed to be affected by Fe stress. The genotypes Dhusura, Jalapaya, Gelei, Kendrajhali, Rasapanjari, Saluagaja and Asinasita were observed to be resistant under field stress condition and controlled condition in hydroponic culture. These genotypes can be used as donor lines for improvement of Fe tolerance in rice. The marker-trait association study could identify the markers namely RM243, RM234, RM248, RM501, RM594 and RM517 to be associated with leaf bronzing index which is considered to be indicator of Fe toxicity resistance. These markers individually showed phenotypic variance ranging from 6.0-10.5%. These markers can further be used for marker assisted breeding programs to incorporate the Fe resistance genes/QTLs into susceptible high yielding popular varieties.

Key words: *Iron toxicity, Fe toxicity tolerance, marker-trait association, genetic variation*

INTRODUCTION

Nutrients are essential for plant growth and development. Their excess, however, leads to toxicity and damage the plant. The major soil nutrient toxicity problem in rice is due to aluminium, iron, boron, hydrogen sulphide and manganese. Iron toxicity in rice has been reported in various countries such as Sri Lanka, India, Indonesia, Malaysia, Phillippines, Senegel, Sierra Leone, Liberia, Nigeria and Colombia (Ponnamporuma, 1976). In India, it has been reported from the young acid sulphate soil of Kerala (Elsy et al., 1994), poorly drained alluvial sandy soils of Tamilnadu (Ravichandran,1987), coastal and hilly zones of Karnataka, valley soils receiving inter-flow water from adjacent higher lands in Odisha (Sahu and Mitra,1992) and from the valleys of north east Himalayan region of Meghalaya. Iron toxicity is a condition caused by the microbial reduction under flooded conditions of insoluble iron-III (Fe^{3+}) into soluble iron-II (Fe^{2+}), which can be taken up by rice plants in excess amounts. The critical limit for iron toxicity for rice plants in lowland soil is 300 mg/kg (Benckiser et al.,1983).

Iron toxicity affects plant height, number of ear bearing tillers, panicle length, spikelet fertility, grain yield and duration of vegetative period depending upon the growth stage at which plants are exposed to toxicity.

The typical symptoms associated with iron toxicity in rice plants are leaf discoloration *i.e.,* yellowing or bronzing (due to accumulation of polyphenol oxides) and reddish spots. The whole leaf becomes orange to brown or purple brown when the toxicity is severe (Fairhurst and Witt, 2002). In the case of toxicity at the seedling stage, rice plant development stops, and tillering is extremely limited (Abraham and Pandey, 1989). Toxicity in the vegetative stages causes a reduction in height and dry matter (Abu et al., 1989). The aerial biomass can be more affected by the constraint than root biomass (Fageria et al., 1988). Tiller formation and the number of productive tillers can be drastically reduced (Cheema et al., 1990). When iron toxicity occurs at the end of the vegetative stage or at the beginning of the reproductive stage, the number of panicles drops (Singh et al., 1992), spikelet sterility increases (Virmani, 1977) and the flowering and maturity stages can be delayed by 20-25 days. Average yield losses due to iron toxicity are around 35-45% (Audebert and Sahrawat, 2000). In severe cases, this can cause plant death and could contribute to a 12- 100 % yield reduction depending on the intensity of the toxicity and the tolerance of the rice cultivar (Sahrawat 2004).

Some resistance mechanisms are developed by the plants for minimizing the effect of iron toxicity like modified root architectural traits facilitating the diffusion of oxygen into the rhizosphere, thereby increasing the redox potential above the threshold for Fe oxidation (Becker and Asch, 2005; Wu et al., 2014; Doran et al., 2006; Sahrawat, 2004; Briat, 1996), storage of excessive iron in the apoplasm and vacuole; adsorption of iron by ferritin in plastids (Briat, 1996) and detoxication of the active oxygen species by enzymes like catalases, peroxidases and superoxide dismutases (Becana et al., 1998; Fang et al., 2001; Becker and Asch, 2005; Briat and Vert, 2004). Alternatively, enzymatic Fe oxidation can be catalyzed by enzymes such as peroxidases (Becker and Asch, 2005). Various mechanisms have been proposed conferring 'shoot tolerance', i.e., the absence of stress symptoms despite high Fe2+ uptake. Fe partitioning both on the organ and the subcellular level may constitute such a mechanism (Engel et al., 2012).

The genetic architecture of tolerance to Fe toxicity in rice is a complex trait governed by many genes. Few quantitative trait loci (QTL) are reported for different phenotypes related to Fe toxicity (Dufey et al., 2015; Wu et al., 2014; Matthus et al., 2015). Although some common chromosomal regions were reported by independent studies, including chromosome 1 between around 25 and 30 Mb and on chromosome 3 between ~0 and 5 Mb (Dufey et al., 2015; Wu et al., 2014), no major locus has been identified, fine-mapped, or cloned yet. Using biparental population for identifying the QTL(s)/gene(s) responsible for Fe toxicity tolerance may not cover the huge genetic variability available in Asian rice, thereby constricting the coverage. Association mapping using large number of genotypes may help for identifying more number of loci responsible for Fe toxicity.

In the present study, we investigated 71 rice genotypes for Fe toxicity tolerance in field and controlled condition. These genotypes were screened with molecular markers in order to get associated markers for Fe toxicity that can be used in future crop improvement breeding program.

MATERIALS AND METHODS

Plant material, experimental site and design

A total of 71 rice (*Oryza sativa* L.) genotypes including landraces and released cultivars maintained at Regional research stations and sub-station, Orissa University of Agriculture and Technology, Bhubaneswar were used for the investigation (Table 1). The experiment was conducted in *kharif* season at RRTTS, OUAT, Bhubaneswar situated in 20^0 15' N latitude and $85^0$52' E longitude. The seeds were sown in nursery bed and were transplanted in an identified iron toxicity hotspot field with a spacing of 15 cm apart and 20 cm between rows in randomized block design (RBD). Fe content on the identified experimental site was observed to range between 200-250 ppm.Crop was raised with recommended fertilizers doses of 80 kg nitrogen ha, 40 kg phosphorus ha, and 40 kg potash ha. The initial Fe level in soil was 202.5ppm. The field was maintained under saturated anaerobic condition.

Phenotyping under Fe toxicity condition in field

Phenotyping of 71 rice lines was done by considering the parameters like days of 50% flowering, plant height, panicle length, number of grains per panicle, 1000 grain

weight, yield, leaf bronzing index (LBI) and numbers of tillers/hill. These observations were recorded following Standard Evaluation System of Rice (IRRI, 2013). The LBI was carried out for four replications of each genotype. The genotypes with score 6 to 9 were considered susceptible, 4-5 moderately resistant, 1-3 resistant and 0 as immune to Fe toxicity.

In vitro screening for Fe toxicity

In vitro screening of 71 genotypes was carried out in transgenic glass house, Dept. of Agricultural Biotechnology, OUAT, Bhubaneswar. Screening experiments were conducted in a hydroponic system. The seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min followed by heat treatment at 45º C for 6 hrs to minimize dormancy period. Then seeds were allowed to germinate in plastic cups for 4 days, after which the seedlings were transferred/transplanted to hydroponics container with Yoshida medium, pH 5.0 (Matthus et al., 2015). Plants were fixed with sponges on a styrofoam. Four replications of each genotype were taken for analysis. A 10 day Fe pulse stress of 1000 ppm Fe^{2+} (as $FeSO_4.7H_2O$) was imposed 4 weeks after the transplanting. As a measure of Fe stress, a leaf bronzing score (LBS) was assigned to the three youngest fully expanded leaves of each plant on day ten of pulse stress.

DNA isolation and molecular characterisation

The genotyping work was taken up at ICAR-National Rice Research Institute, Cuttack, Odisha. Total genomic DNA was extracted from five week old plants of the rice germplasm line and varieties following stepwise CTAB protocol (Doyle and Doyle, 1987). PCR amplification was performed in a Gradient Thermal Cycler (Veriti, Applied BioSciences) following the methods of Pradhan et al. 2016 and Pandit et al., 2016. The list of markers used in the study is presented in Table 2. The amplification products were loaded in 3% gel containing 0.8 g/ml Ethidium Bromide for electrophoresis in 1X TBE (pH 8.0). One lane was loaded with 50 bp DNA ladder. The gel was run at 2.5 V/cm for 4 hrs and photographed using a Gel Documentation System (SynGene).

Data scored were analysed on the basis of the presence or absence of amplified products for each genotype-primer combination. An unweighted neighbor joining un-rooted tree was constructed using the calculated dissimilarity index by using NEI coefficient (Nei, 1972) with bootstrap value of 1000 using FreeTree software (Hampl et al., 2001; Pavalicek et al., 1999) and the dendrograms were visualized by Treeview 32 software (Page, 1996). The genetic diversity parameters like number of alleles, allele frequency, gene diversity, heterozygosis and polymorphic information index (PIC) were estimated using the program PowerMarker Ver3.25 (Kejun and Spencer, 2005).

RESULTS AND DISCUSSION

Morphological diversity among rice genotypes under Fe-toxicity in field condition

The genotypes under study showed high variability for all the traits under study (Table 1). Significant differences among individuals were observed through ANOVA analysis for all measured traits (Table 1). The effect of Fe toxicity in terms of bronzing, stunted growth, increased spikelet sterility, reduced yield, plant height,

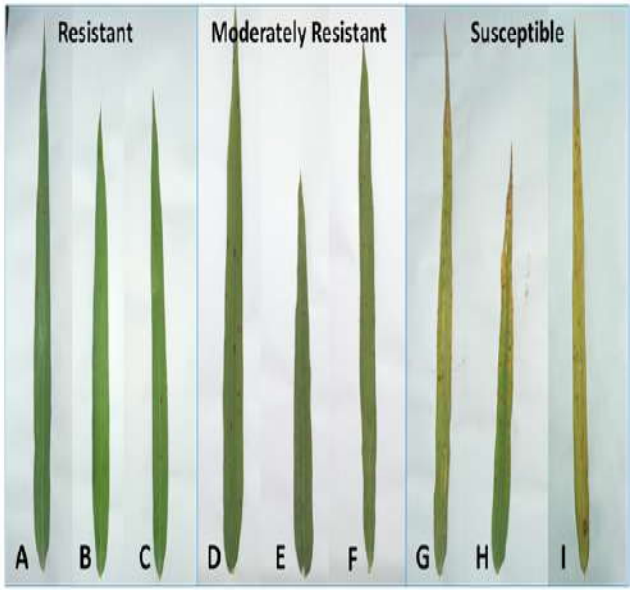

Fig. 1. Representative pictures of bronzing effect in the leaves of resistant, moderately resistant and susceptible rice varieties under field condition. The susceptible genotypes showed clear bronzing effect spreading upto leaf base (G, H, I) whereas the resistant ones with no bronzing effect (A, B, C) and moderately resistant ones were with very less bronzing character (D, E, F). A: Bayabhanda, B: Juiphula, C: Tikimahsuri, D: Jaiphula, E: Sagiri, F:Biridibankoi, G: Budidhan, H: Madhabi and I: Bagudi.

Table 1. Agro-morphologic characters and leaf bronzing index in response to Fe toxicity in field as well as controlled *in vitro* culture of 71 rice genotypes.

Sl. No.	Acce-ion No.	Genotype Name	Field condition (200-250ppm Fe)								Hydroponic culture (1000 ppm Fe)	Remark
			Days to 50% flowering	Plant height (cm)	Panicle length (cm)	Grain No./ panicle	1000 grain wt.	Yield (qtl/ha)	Tillers	LBI	LBI	
1	481	Sankaribako	106.50	123.30	22.00	80.00	25.96	16.98	6.40	4	4	Moderately Resistant
2	483	Kalakrushna	99.50	133.80	24.00	162.65	14.90	17.63	7.20	4.5	7	Susceptible
3	484	Assamchudi	99.00	114.40	24.35	130.00	24.86	18.15	6.40	5	5	Moderately Resistant
4	485	Gelei	97.00	106.00	24.00	142.65	15.43	17.44	5.80	5.5	6	Susceptible
5	487	Kalamara	95.00	121.60	22.15	50.85	19.06	5.68	5.80	4	4	Moderately Resistant
6	489	Nini	95.00	132.50	27.00	116.30	20.16	13.80	7.70	6	7	Susceptible
7	491	Gurumukhi	104.00	121.90	20.50	81.00	26.57	15.65	6.60	5	3	Resistant
8	495	Jubaraj	106.50	125.70	26.35	90.65	15.87	17.78	7.80	5.5	3	Resistant
9	496	Champa	104.00	122.00	20.00	141.65	21.97	27.59	6.60	6	8	Susceptible
10	500	Veleri	112.50	149.40	26.15	75.00	26.82	16.02	4.90	5	4	Moderately Resistant
11	501	Dhinkisiali	106.50	133.10	19.00	60.00	20.84	14.44	9.60	4	6	Susceptible
12	502	Dhabalabhuta	106.00	131.40	21.65	92.15	25.44	26.67	7.70	4	4	Moderately Resistant
13	505	Bayabhanda	108.00	136.90	21.50	67.55	19.83	12.10	9.50	3.5	3	Resistant
14	507	Latamahu	100.50	120.80	22.00	112.70	18.96	18.98	8.10	3	6	Susceptible
15	509	Hatipanjara	106.50	132.10	23.00	120.50	23.13	25.28	9.40	4	6	Susceptible
16	510	Mugei	106.50	119.60	22.00	59.15	19.85	9.42	6.30	5	4	Moderately Resistant
17	512	Sagiri	101.50	147.20	23.00	115.70	27.80	10.06	7.10	5	5	Moderately Resistant
18	514	Kakiri	103.50	109.30	23.50	116.50	24.44	21.36	5.70	4.5	5	Moderately Resistant
19	515	Madia	97.00	128.60	26.00	113.15	23.84	25.56	7.30	7	7	Susceptible
20	517	Dhusura	101.00	128.30	27.00	99.20	23.78	23.64	6.50	2.5	3	Resistant
21	518	Bangali	98.50	128.30	20.50	120.65	21.69	27.13	6.40	6	8	Susceptible
22	519	Banda	107.50	160.90	28.00	133.50	23.44	11.24	6.20	5	3	Resistant
23	520	Jalpaya	102.50	123.00	23.00	134.85	16.83	22.10	6.90	3	3	Resistant
24	521	Chudi	107.00	120.60	28.50	116.80	22.20	33.98	6.70	7	7	Susceptible
25	522	Nilarpati	107.00	118.30	24.85	122.80	28.39	28.95	6.30	4.5	5	Moderately Resistant
26	524	Gelei	106.50	109.10	19.50	126.20	16.83	27.59	7.50	3	3	Resistant
27	526	Ratanmali	106.00	109.90	26.00	125.85	18.79	22.91	8.30	2.5	4	Moderately Resistant
28	528	Umarcudi	100.00	109.20	26.00	109.65	20.79	20.62	7.40	4.5	3	Resistant
29	529	Juiphula	103.00	120.20	23.50	149.70	14.21	21.48	6.90	4.5	3	Resistant
30	530	Karpurakranti	104.00	128.90	23.15	113.15	13.54	15.96	7.10	6	7	Susceptible
31	535	Ramakrushanabilash	99.50	122.10	24.50	148.35	14.84	33.86	7.90	5	6	Susceptible
32	537	Bagudi	104.50	103.10	25.85	118.00	20.83	38.83	6.30	5	6	Susceptible
33	538	Sunapani	114.50	99.10	26.00	154.85	22.36	49.41	6.30	3.5	4	Moderately Resistant
34	539	Anu	98.00	137.30	21.00	136.65	12.20	23.06	6.80	5.5	5	Moderately Resistant
35	541	Mayurkantha	103.50	128.90	24.00	115.50	23.82	31.85	7.20	5	7	Susceptible
36	549	Champeisiali	107.00	101.50	23.50	93.50	27.53	16.30	6.10	5	3	Resistant
37	550	Nalijagannath	107.50	113.80	19.50	121.70	21.70	19.38	6.70	6	4	Moderately Resistant
38	554	Parbhatjeera	108.50	127.10	29.50	135.65	19.78	27.07	8.50	4	5	Moderately Resistant
39	555	Ranisaheba	103.50	122.10	21.00	111.80	18.91	28.99	8.10	3.5	4	Moderately Resistant
40	556	Punjab swarna	105.50	130.90	29.50	102.65	28.39	19.38	5.50	4	5	Moderately Resistant

Table 1. Continued....

41	557	Kusuma	98.00	107.50	21.50	105.30	19.84	18.18	5.70	3	4	Moderately Resistant
42	561	Kendrajhali	105.50	115.40	23.00	92.65	17.99	8.21	5.10	3	3	Resistant
43	562	Jaiphula	97.00	136.80	27.50	80.00	12.10	5.90	4.20	3.5	4	Moderately Resistant
44	564	Jabaphula	105.00	122.10	24.50	86.20	24.15	16.30	5.60	4.5	3	Resistant
45	566	Khandasagar	99.00	130.30	25.00	85.30	12.44	11.51	5.60	5.5	4	Moderately Resistant
46	567	Pipalbasa	102.50	119.80	22.15	68.50	24.80	9.54	5.30	7	5	Moderately Resistant
47	569	Budidhan	97.00	119.90	27.85	73.70	12.09	8.92	5.90	3	8	Susceptible
48	570	Karpuragundi	103.50	115.70	24.00	97.00	12.13	18.80	6.20	6	6	Susceptible
49	571	Basapatri	97.00	127.40	22.00	112.85	21.91	13.43	7.80	6	2	Resistant
50	573	Bagadachinamala	103.50	119.20	26.00	113.80	12.69	30.40	5.50	4	6	Moderately Resistant
51	578	Kalaheera	108.50	134.00	21.85	146.35	26.77	35.22	7.80	3	4	Moderately Resistant
52	579	Rasapanjari	99.00	132.20	26.00	119.00	29.54	28.12	5.20	4.5	3	Resistant
53	584	Biridibankoi	113.00	127.10	21.50	125.15	22.76	33.55	8.20	4	5	Moderately Resistant
54	589	Jagabalia	113.50	130.50	20.00	135.50	26.19	30.34	7.30	5	5	Moderately Resistant
55	592	Dhoiamadhoi	104.00	116.40	27.50	103.00	25.25	23.64	5.70	5.5	5	Moderately Resistant
56	594	Kaniara	104.00	90.50	21.00	112.00	13.25	19.01	6.60	3.5	5	Moderately Resistant
57	597	Bishnupriya	108.00	116.50	23.00	145.00	24.65	30.77	6.70	5	5	Moderately Resistant
58	599	Madhabi	107.00	119.50	22.00	103.00	26.49	20.96	5.60	5	6	Susceptible
59	601	Jungajhata	104.00	127.60	27.00	127.65	23.59	25.77	7.80	4	4	Moderately Resistant
60	602	Rangasiuli	104.00	129.90	28.50	118.00	22.30	19.79	5.30	4	5	Moderately Resistant
61	603	Sankarachini	101.00	126.90	23.50	116.00	26.86	18.80	6.70	3	5	Moderately Resistant
62	604	Saluagaja	102.50	113.30	25.00	133.65	15.06	16.73	5.50	6.5	3	R
63	606	Mayurachulia	106.00	99.00	19.00	157.65	14.36	31.36	6.00	4.5	7	Susceptible
64	607	Basudha	106.00	87.50	21.00	134.70	14.74	27.13	5.50	3.5	5	Moderately Resistant
65	608	Tikimahsuri	106.50	141.30	28.00	118.35	13.70	15.56	7.10	4	3	R
66	609	Tulasibasa	102.50	136.00	25.65	128.65	22.32	25.56	5.70	3	4	Moderately Resistant
67	610	Asinasita	103.00	90.20	19.00	116.50	12.96	26.42	8.00	4.5	3	R
68	611	Bhangar	106.00	126.50	22.50	135.85	12.51	20.96	7.80	4.5	5	Moderately Resistant
69	612	Kalajeera	108.00	128.60	22.00	141.20	14.66	26.36	8.80	3.5	4	Moderately Resistant
70	614	Gobindabhog	105.00	91.50	21.00	140.30	12.76	27.87	6.80	5	5	Moderately Resistant
71	616	Basudha	103.00	111.00	24.00	106.50	18.97	20.71	5.10	7.5	6	Susceptible
		C.D.	11.836	3.818	1.843	18.242	0.79543	8.566	0.626			
		SE(m)	4.231	1.365	0.659	6.52	0.28701	3.062	0.224			
		SE(d)	5.983	1.93	0.932	9.221	0.39420	4.33	0.316			
		C.V.	5.678	1.667	4.013	7.754	1.35870	15.611	4.723			

Fig. 2. Variation in root traits under different concentration of Fe treatment in hydroponic culture. A representative photograph showing the effects of higher Fe concentration on number of primary and secondary roots and root length in rice seedling.

dry matter and tiller number were also reported in earlier studies (Fairhurst and Witt, 2002; Abraham and Pandey, 1989; Abu et al., 1989; Fageria et al., 1988; Singh et al., 1992; Singh et al., 1992; Virmani, 1977; Cheema et al., 1990; Matthus et al., 2015).

Fe toxicity response of rice genotypes under field and hydroponics condition

The characteristic bronzing symptoms of leaves was used as an index of Fe toxicity tolerance. Such bronzing symptom on plant leaves due to higher Fe^{2+} concentration is also reported by Backer and Asch (2005). Representative comparative picture of leaf bronzing symptom of resistant, moderately resistant and susceptible varieties are shown in Fig. 1. A total of 10 genotypes were resistant, 45 moderately resistant and 16 were susceptible under field condition. The genotypes Dhinkisiali, Latamahu, Dhusura, Jalapaya, Gelei, Ratanmali, Kendrajhali, Rasapanjari, Saluagaja and Asinasita were found to be highly resistant with score of 2.5 whereas Bsudha was observed to be highly susceptible with score of 7.5 observed to be highly

Table 2. List of sixteen polymorphic primers used in the study.

Sl. No	Marker Name	Forward Sequence (5' – 3')	Reverse Sequence (5' – 3')	Repeat Motif	Expected Band size (bp)	Reference
1	RM488	CAGCTAGGGTTTTGAGGCTG	TAGCAACAACCAGCGTATGC	GA17	177	Anuradha et al., 2012
2	RM243	GATCTGCAGACTGCAGTTGC	AGCTGCAACGATGTTGTCC	CT18	116	Anuradha et al., 2012
3	RM490	ATCTGCACACTGCAAACACC	AGCAAGCAGTGCTTTCAGAG	CT13	101	Anuradha et al., 2012
4	RM7102	TTGAGAGCGTTTTTAGGATG	TCGGTTTACTTGGTTACTCG	(AGAT)8		Anuradha et al., 2012
5	RM17	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	GA)21	184	Anuradha et al., 2012
6	RM260	ACTCCACTATGACCCAGAG	GAACAATCCCTTCTACGATCG	(CT)34	111	Anuradha et al., 2012
7	RM234	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	CT25	156	Anuradha et al., 2012
8	RM248	TCCTTGTGAAATCTGGTCCC	GTAGCCTAGCATGGTGCATG	CT25	102	Anuradha et al., 2012
9	RM122	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	(GA)7A(GA) 2A(GA)11	227	Anuradha et al., 2012
10	RM517	GGCTTACTGGCTTCGATTTG	CGTCTCCTTTGGTTAGTGCC	(CT)15	266	Anuradha et al., 2012
11	RM8007	AATAGGATGGATCATGGATA	CATCTCATCAGGAACCTAAC	AT40	178	Anuradha et al., 2012
12	RM501	GCCCAATTAATGTACAGGCG	ATATCGTTTAGCCGTGCTGC	(TC)10(TA)21	179	Anuradha et al., 2012
13	RM574	GGCGAATTCTTTGCACTTGG	ACGGTTTGGTAGGGTGTCAC	GA11	155	Anuradha et al., 2012
14	RM594	GCCACCAGTAAAAGCAATAC	TTGATCTGCTAGTGAGACCC	(GA)n	300	Anuradha et al., 2012
15	RM7	TTCGCCATGAAGTCTCTCG	CCTCCCATCATTTCGTTGTT	(GA)19	180	Anuradha et al., 2012
16	OsIRT1	CGTCTTCTTCTTCTCCACCACGAC	GCAGCTGATGATCGAGTCTGACC	-	350	L Chrisnawati et al.(2016)

Table 3. Genetic diversity parameters obtained with 71 genotypes and 16 molecular markers.

Marker	Min. Allele size (bp)	Max Allele size (bp)	Major.Allele. Frquency	AlleleNo	GeneDiversity	Heterozygosity	PIC
RM488	170	200	0.5079	2.0000	0.4999	0.0317	0.3749
RM243	90	120	0.6232	2.0000	0.4696	0.1159	0.3594
RM490	100	120	0.7071	2.0000	0.4142	0.0143	0.3284
RM7102	170	200	0.9070	2.0000	0.1687	0.0465	0.1545
RM17	160	200	0.7324	2.0000	0.3920	0.0282	0.3152
RM260	40	70	0.8239	2.0000	0.2901	0.2958	0.2480
RM234	130	150	0.7786	2.0000	0.3448	0.0429	0.2854
RM248	70	100	0.5299	2.0000	0.4982	0.3134	0.3741
RM122	130	250	0.5227	2.0000	0.4990	0.8333	0.3745
RM517	260	280	0.6939	2.0000	0.4248	0.0816	0.3346
RM8007	140	160	0.6711	2.0000	0.4415	0.1842	0.3440
RM501	140	160	0.5250	2.0000	0.4988	0.5500	0.3744
RM574	150	170	0.9487	2.0000	0.0973	0.0513	0.0926
RM594	300	320	0.7899	2.0000	0.3320	0.0145	0.2769
RM7	200	250	0.5192	2.0000	0.4993	0.9615	0.3746
OsIRT1	350	380	0.8750	2.0000	0.2188	0.2500	0.1948
Mean	-	-	0.6972	2.0000	0.3806	0.2385	0.3004

resistant. The genotypes observed to be resistant under hydroponics were considered to be better than that of field condition as the stringency of selection stress was high under hydroponics condition. Variation was observed in some genotypes against different concentration of Fe (Fig. 2). Shoot and root growth was normal at control solution, whereas (Table 1). Under

Table 4. Marker trait association using generalised linear model and mixed linear model of TASSEL5 software.

Trait	Marker	GLM			MLM		
		F-Value	P-Value	R2	F-Value	P-Value	R2
Days to 50% flowering	RM7102	5.23208	0.02524	0.07048	4.10331	0.04667	0.05862
	RM234	8.64215	0.00447	0.11131	-	-	-
	RM235	5.56889	0.02112	0.07468	-	-	-
	RM501	4.52901	0.0369	0.06159	-	-	-
	RM574	5.67491	0.01997	0.07599	-	-	-
Plant height	RM17	-	-	-	4.24339	0.04318	0.06062
	RM122	4.26481	0.04267	0.05821	-	-	-
	RM517	-	-	-	4.54948	0.03649	0.06499
Panicle length	RM17	4.03939	0.04836	0.0553	-	-	-
	RM517	6.97985	0.01019	0.09186	5.84978	0.01822	0.08357
Grain Weight	RM490	20.20573	2.73E-05	0.22651	-	-	-
	RM17	4.65279	0.03449	0.06317	-	-	-
	RM234	15.97691	1.58E-04	0.18801	-	-	-
	RM248	6.48754	0.0131	0.08594	-	-	-
	RM517	20.63109	2.30E-05	0.23018	-	-	-
	RM8007	4.85743	0.03087	0.06577	-	-	-
	RM501	8.09449	0.00584	0.10499	4.81233	0.03163	0.06875
	RM594	6.9101	0.01056	0.09103	-	-	-
	RM7	4.9757	0.02896	0.06726	-	-	-
Yield	RM488	-	-	-	4.05494	0.04794	0.05793
LBI under field condition	RM517	4.39878	0.03963	0.05993	-	-	-
	RM243	8.0582	0.00595	0.10457	4.69967	0.03362	0.06714
	RM234	8.46549	0.00487	0.10928	-	-	-
LBI in hydroponics	RM248	5.49198	0.02199	0.07373	-	-	-
	RM501	5.29835	0.02437	0.07131	-	-	-
	RM594	5.18056	0.02595	0.06984	-	-	-

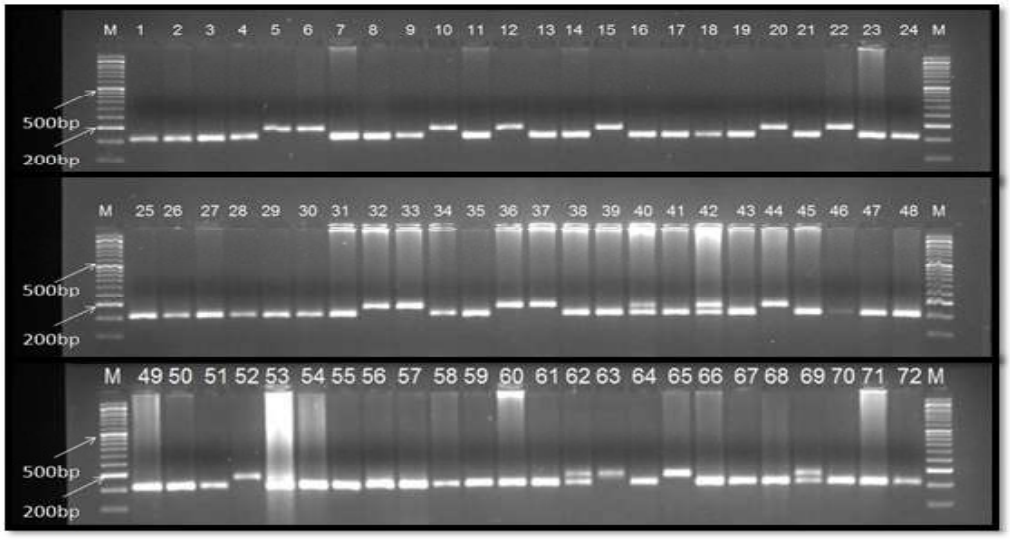

Fig. 3. Representative electrophoregram of the panel genotypes under study obtained with the marker RM17.

controlled condition *i.e.,* hydroponics condition the number of resistant, moderately resistant and susceptible changed to 17, 36 and 18 respectively (Table 1). The genotypes namely Gurumukhi, Jubaraj, Bayabhanda, Dhusura, Banda, Jalapaya, Gelei, Umarcudi, Juiphula, Champeisiali, Kendrajhali, Jabaphula, Basapatri, Rasapanjari, Saluagaja, Tikimahsuri and Asinasita were seedlings of 200ppm, 300ppm and 400ppm stress were observed to show the effect of Fe toxicity (Fig. 2). When the bronzing score of leaf under field situation and hydroponics condition were compared, most of the genotypes showed similar reaction status in both conditions. But some of the genotypes showed large variation in LBI. This may be due to high stringency of selection pressure in hydroponics condition (1000ppm Fe) as compared to 200-250ppm Fe in field condition. But six genotypes Dhusura, Jalapaya, Gelei, Kendrajhali, Rasapanjari, Saluagaja and Asina sita were observed to be consistently resistant under both field and hydroponics condition.

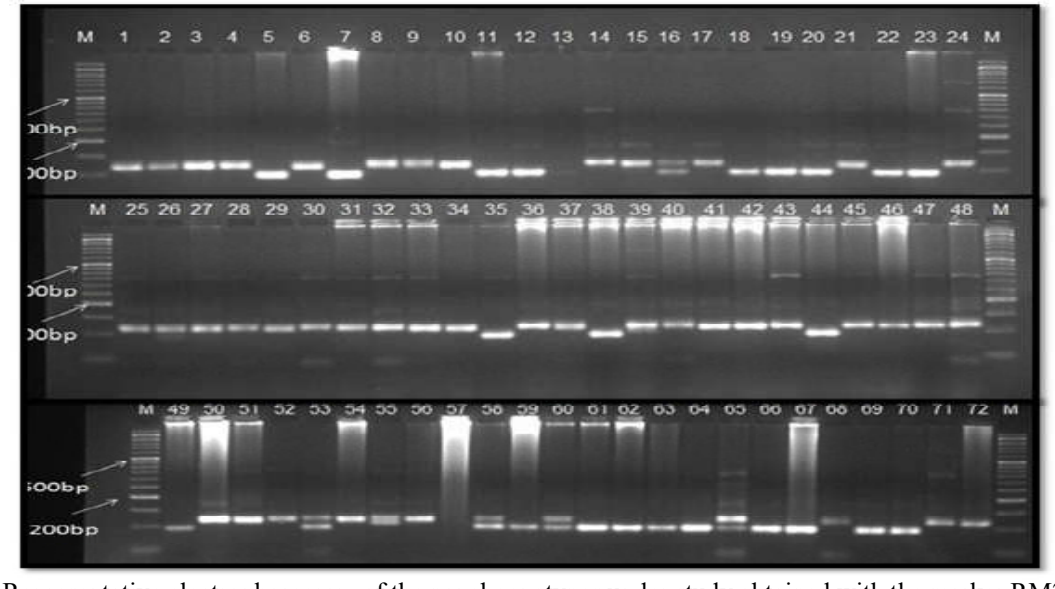

Fig. 4. Representative electrophoregram of the panel genotypes under study obtained with the marker RM243.

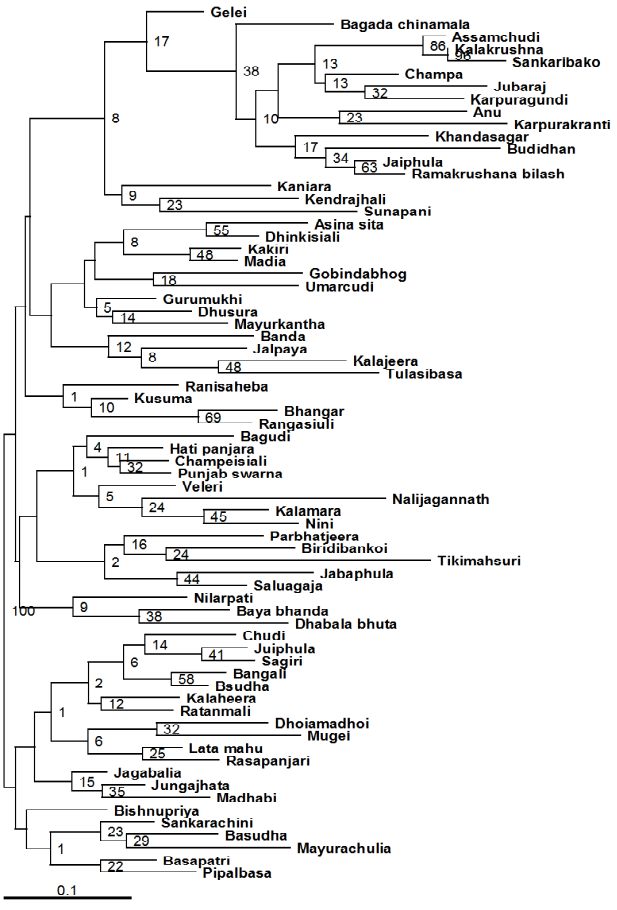

Fig. 5. Neighbour joining phylogram of the 71 genotypes using Nei's method.

Genetic diversity and clustering analysis

A total of 23 markers including 4 gene specific and 19 SSR markers were used of which only one gene specific and 15 SSR markers were observed to be polymorphic in the panel population under study. These 16 primers were considered for further analysis (Table 2). Representative electrophoregrams of polymorphic markers have been depicted in Fig. 3 and 4. The details of genetic diversity parameters obtained with these 16 polymorphic markers are shown in Table 3. Wide variations of alleles ranging from 70bp to 380bp were observed. The major allele frequency ranged from 0.949 (RM574) to 0.507 (RM488) with an average value of 0.0697. The average PIC value of 0.3 indicated moderate diversity in the population. The maximum PIC value of 0.3749 was observed in RM488 and minimum value of 0.093 was observed in RM574. The average

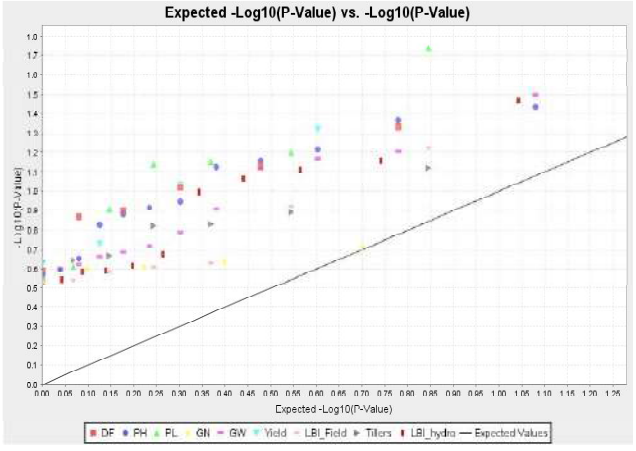

Fig. 6. Quantile-Quantile plot showing the significantly associated traits with the molecular markers. The traits plotted above the standard line are significantly associated with the markers.

of gene diversity among all the markers tested found to be 0.38. Among all markers, RM488, RM122, RM7 and RM248 showed maximum gene diversity whereas RM574 shown minimum gene diversity in the panel of 71 rice genotypes. A dendrogram was generated by using Nei dissimilarity matrix among the rice germplasms investigated to show their genetic relatedness (Fig. 5). The dissimilarity coefficients ranged from 0.037 to 0.83. The dendrogram grouped all the 71 genotypes into 2 major clusters and 5 sub clusters (Fig. 5). One sub cluster could separate out seven resistant genotypes Jalpaya, Dhusara, Banda, Gurumukhi, Asina sita, Umarcudi where accommodating only three susceptible genotypes Dhinkisiali, Madia and Mayurkantha.

Marker-trait association

The marker-trait association analysis was done using GLM and MLM (Q+K) model for leaf bronzing effect as well as the other agro-morphologic characters taken under Fe toxicity stress. A total of 15 markers were associated with different traits including LBI when p value of 0.05 was considered. Five markers namely RM243, RM234, RM248, RM501 and RM594 were associated with LBI under hydroponics condition with phenotypic variance ranging from 6.9 - 10.5% (Table 4). Only RM243 was associated in both GLM and MLM model. RM517 showed only 5.9% variance for LBI under field situation. Six markers namely RM17, RM517, RM234, RM248, RM501 and RM574 were

associated with multiple traits under study (Table 4). Matthus et al. (2015) also reported 20 SNP markers associated with Fe toxicity in rice. They also identified two genes namely LOC_Os01g49710 and LOC_Os01g49720 for Fe toxicity Lili et al. (2016) reported STS markers associated with Fe toxicity in rice. Chrisnawati et al. (2016) also revealed association between the genetic and phenotypic analysis showed that STS markers, i.e. OsIRT1 and OsIRT2 associated with iron tolerance trait in rice. But in the present association no significant association was observed for these markers in the panel population under study. The QQ-plot showed significant association of markers with leaf bronzing and other agro-morphologic traits (Fig. 6).

CONCLUSION

The markers RM243, RM234, RM248, RM501, RM594 and RM517 individually explaining 6-10% of phenotypic variance for Fe toxicity in rice can be used for selection of genotypes in marker assisted breeding programs for improvement of Fe toxicity in popular high yielding varieties. The tolerant genotypes Dhusura, Jalapaya, Gelei, Kendrajhali, Rasapanjari, Saluagaja and Asina sita identified in the present study can be used as donor lines in such breeding programs. These genotypes can also be used for bi-parental mapping for identification and cloning of the gene(s) responsible for Fe toxicity in rice. Further analysis with large number of molecular markers covering all the chromosomes can reveal new loci responsible for Fe toxicity.

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