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Identification and validation of SSR markers associated with tolerance to iron toxicity in rice

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Abstract
Iron toxicity is one of the main abiotic stresses affecting rice, usually seen in flooded rice environment. Locating out a closely linked marker that is linked with the trait is necessary so that it can be employed in Marker assisted selection for development of HYVs tolerant to iron toxicity. A number of markers linked to the trait have been reported, but these markers are needed to be validated for their further applications. In the present research, 150 germplasms which include land races and HYVs were grown in hotspots for phenotyping to assess tolerance to iron toxicity. Then, 30 germplasms were genotyped using 7 SSR markers earlier reported to be linked to Fe toxicity trait in rice for validation. After running of PCR, 3 SSR Primers were found to show bands of reported amplicon size in tolerant and moderately tolerant species and were hence found to be validated and can be further utilized for MAS.

Keywords: Iron toxicity, rice, SSR primers, germplasms

1. Introduction
Rice being a primary cereal crop has immense significance as a food crop as it serves as a staple diet of half of the worldwide population especially in Asia. Rice is affected by many biotic and abiotic stresses every year which has a huge impact on its yield. With growing population and increasing demand, decrease in productivity of rice is a major problem which is a threat to the food security of our country. Biotic stresses like insect pests and diseases lead to 37% loss in rice crop every year while abiotic stresses such as drought, salinity, high and low temperatures, submergence, oxidative stresses, toxicity & deficiency of certain nutrients e.g. B, Fe, Al, Mn has wide impact on rice plants which account for 50% crop damage worldwide. Iron has huge significance in growth and development of rice as it is an important component of many enzymes catalyzing crucial reactions of respiration, nitrogen assimilation and is involved in proper functioning of chlorophyll (Pawar *et al.*, 2021) [5]. However, high concentration of iron in soil is detrimental for growth and development of rice and leads to diminution of yield. Iron toxicity is one of the major abiotic stress affecting rice, usually seen in flooded rice environment which drastically reduces the yield by 15-30% and may cause entire crop failure especially if occurs in seedling stage (Becker and Ash, 2005; Audebert and Sahrawat, 2000) [4, 2]. High iron concentration promote formation of reactive oxygen species, cause leaf bronzing and other symptoms include tiny brown spots on lower leaves, interveinal spots, stunted growth and limited tillering. In case of extreme toxicity leaves appear purple brown. These symptoms can occur at different growth stages and may affect rice at the seedling stage, during the vegetative growth and at the early and late reproductive stages. Depending on the growth stage leaf bronzing occurs, other symptoms and growth effects may be associated. In the case of toxicity occurring during seedling stage, the rice plants remain stunted with extremely limited tillering (Abraham and Pandey, 1989) [3]. Though there are many approaches to control iron toxicity symptoms in rice crop, development of tolerant genotypes is the most promising approach. Development of tolerant varieties using transgenic approach is one of the advanced methods, however, is highly criticized because of biosafety concerns. Cis-genic breeding involving introgression of a desired trait followed by marker-assisted selection is, therefore, an advanced crop breeding of choice for development of HYVs tolerant to Fe-toxicity. So finding out a closely linked marker that is associated with the trait is required. A number of laboratories are involved in this research and hence a number of markers have been found and reported to be associated as revealed through traditional QTL mapping and association mapping. These markers have been generated with various constraints from size of mapping population, heterogeneity in soil and evaluation mis-match between *in-vivo* and lab condition of evaluation.

The linkage or association of the marker with the trait of interest needed to be validated so as to find out one of the most suitable one that would facilitate precision breeding through MAS.

In the present investigation, 150 germplasms which include land races and HYVs were grown in hotspots for phenotyping to assess tolerance to iron toxicity. Out of these, 30 germplasms were taken, 10 from each group of tolerant, moderately tolerant and susceptible plants and were genotyped using 7 SSR markers earlier reported to be linked to Fe toxicity trait in rice for validation.

2. Materials and Methods

A total of 150 germplasms lines used in the present investigation for phenotypic analysis were grown in Fe-toxicity plot in Regional Research and Technology Transfer Station (RRTTS), OUAT, Bhubaneswar in kharif season. The germplasms comprising of various landraces and cultivated varieties were planted in randomized block design in iron toxicity plot that as maintained under saturated anaerobic conditions.

2.1 Phenotyping of Germplasms under Fe-toxicity field

The seeds were sown in the nursery bed followed by transplanting to a Fe-toxicity hotspot field or a sick plot as well as a normal plot or a control plot at a spacing of 15cm and row to row spacing of 20 cm. The iron content of the soil in control plot was measured to be 103 ppm and sick plot was measured to be 456.6ppm. The experimental design followed was randomized block design with two replications and four blocks per replication.

Different parameters like days to 50% panicle initiation, days to 50% flowering, panicle length, plant height, no. of grains per panicle, 1000 grain weight, leaf bronzing score, no. of tillers/hill and yield in quintals/ha were used for phenotyping analysis of 150 genotypes. The observations were listed by following Standard Evaluation System of Rice (IRRI, 2013). LBI or leaf bronzing index was recorded for two replications. The genotypes were considered as susceptible at a score of 6 to 9; moderately resistant at 4–5; resistant at 1–3 and 0 as immune to Fe- toxicity tolerance.

An initial descriptive statistics, including mean, standard deviation, standard error and coefficient of variation (%) was performed by MS-Excel.

Table 1: Statistical analysis of phenotypic observations recorded for 150 genotypes

Sl.no	Genotype	50% DPI	50%DF	Tillers/hill	PH	PL	GN	GW	LBI	Yield	Response
1	Sankaribako	69.5	78.5	6.56	98	19.5	75.94	24.05	6	5.47	S
2	Kalakrushna	98.5	113	5.9	121.6	22.9	133.07	13.54	4	4.45	MR
3	Assamchudi	97	110.5	5.07	110.75	22.33	85.88	22.42	4	4.6	MR
4	Gelei	93.5	110.5	6.8	102.35	21.1	104.31	16.19	6	4.22	S
5	Kalamara	90.5	108	4.02	130.4	25.05	86.33	17.81	1	2.35	R
6	Nini	92.5	109.5	5.35	107.75	22.7	85.77	20.9	6	3.7	S
7	Gurumukhi	95	113.5	5.1	106.16	19.4	59.7	23.91	5	5.06	MR
8	Jubaraaj	96.5	114.5	5.65	103.25	33	80.8	18.03	1	4.3	R
9	Champa	101	112.5	6.65	112.25	28.1	101.76	21.96	6	3.72	S
10	Beleri	103.5	116.5	5.26	119.71	23.75	100.36	23.71	1	5.72	R
11	Dhinkisiali	96.5	110.5	6.91	114.46	28.3	104.77	15.92	6	4.45	S
12	Dhabalabhuta	94.5	112	6.04	118.75	20.3	81.27	19.79	7	6.57	S
13	Bayabhanda	100.5	116	5.37	119.95	18.2	96.26	17.76	5	4.05	R
14	Latamahu	95	111.5	5.77	114.98	19.6	72.55	16.68	3	6.57	R
15	Hatipanjara	93	110	6	125.3	23.8	75.16	21.93	1	4.62	R
16	Mugei	96	111.5	4.2	116.1	22.62	113.66	17.04	6	5.82	S
17	Sagiri	98	112	4.85	131.97	27.65	130.41	24.8	5	5.12	MR
18	Kakiri	97	110.5	5.65	113.96	24.1	100.65	24.19	2	5.22	R
19	Madia	95.5	111.5	4.81	122.54	18.5	99.83	21.96	5	4.3	MR
20	Dhusura	99.5	103.5	8.1	116.98	24.34	64.82	22.08	1.5	3.65	R
21	Bangali	95	101	5.57	122.99	21.43	82.95	19.98	1	4.3	R
22	Banda	95.5	105.5	4.1	142.19	28.02	90.4	20.87	1	3.64	R
23	Jalpaya	97	111.5	5.41	119.05	23.36	63.36	16.7	3.5	3.27	R
24	Chudi	102	111.5	5.86	118.4	21.4	124.86	21.88	3	4.57	R
25	Nilarpati	97.5	111.5	4.07	123.38	21.19	88.55	22.89	5	3.9	MR
26	Gelei	97	110.5	5.37	112.98	20.49	136.21	16.14	3.5	3.57	R
27	Ratanmali	94	106	6.02	97.45	18.93	119.37	15.83	1	3.92	R
28	Umarcudi	99.5	107	7	107.17	20.9	110.48	18.34	6	3.4	S
29	Jaiphula	94	109.5	6.05	116.48	19.14	127.88	11.66	4	2.97	MR
30	Karpurakranti	95	106	6.42	125.08	21.1	96.72	12.12	7	3.52	S
31	Ramakrushnabilash	95.5	104	6.46	127.13	24.75	120.53	8.62	1	3.42	R
33	Sunapani	93.5	104.5	6.38	99.1	22.75	94.12	21.12	4	7.99	MR
34	Anu	97	119	5.77	120.63	27.9	150.76	14.38	5	2.82	MR
35	Mayurkantha	100	120.5	5.23	129.65	22.8	97.21	23.04	5	4.35	MR
36	Champeisiali	100	120	5.3	122.63	23.1	92.76	24.97	3	4.3	R
37	Nalijagannath	102	115.5	5.09	121.44	24.7	114.39	20.03	1	5.72	R
38	Mahsuri	102	117.5	6.76	104.63	21.6	187.46	15.99	6	5.37	S
39	Ranisaheba	107.5	124	5.28	109.81	23.96	130.24	18.01	5	5.9	MR
40	Punjabniswarna	95	119	6.22	122.96	22.58	92.66	14.42	3.5	3.4	R

41	Kusuma	100.5	110	5.86	122.96	23.11	92.57	26.65	4	4.45	MR
42	Kendrajhali	96	111	7.44	106.94	23.4	144.31	24.68	3	4.91	R
43	Jaiphula	96	114.5	7.23	116.02	24.82	106.46	17.03	3.5	3.12	R
44	Jabaphula	98.5	113	5.56	110.78	25.53	129.01	12.92	3	3.81	R
45	Khandasagara	97.5	112.5	4.51	114.3	22.91	66.83	21.3	7	3.85	R
46	Pipalabasa	99.5	112	5.8	124.6	25.8	64.98	20.32	6	3.15	S
47	Budhidhana	107	113	5.97	113.12	18.25	174.71	15	6	3.3	S
48	Karpuragundi	101	112	5.16	115.51	22.55	143.88	12.36	6	2.57	S
49	Basapatri	99.5	110.5	5.65	115.05	21	88.23	11.63	2.5	3.2	R
50	Bagadachinamala	99.5	111.5	6.11	106.9	21.78	94.21	17.46	5	2.92	MR
51	Kalaheera	99	112.5	4.89	119.2	23.16	168.31	10.06	5	3.55	MR
52	Rasapanjari	101.5	111	4.23	103.84	21.8	122.98	18.5	3	4.65	R
53	Biridibankoi	97	113.5	6.9	113.59	22.9	76.8	20.27	3	3.95	R
54	Jagabalia	96	118	4.76	102.41	22.74	147.66	15.66	2.5	4.95	R
55	Dhoiamadhoi	102.5	115.5	7.21	114.47	23.35	143.82	20.11	5	7.19	MR
56	Kaniara	101	112.5	4.07	119.36	20.65	99.39	17.71	5	2.3	MR
57	Bishnupriya	97	113.5	5.48	72.88	16.86	108.38	11.8	4	3.7	MR
58	Madhabi	100.5	120.5	4.77	106.66	21.97	146.88	19.07	3	5.88	R
59	Jungajhata	105.5	114	5.44	114.97	18.1	93.05	17.77	5	4.42	MR
60	Rangasiuli	109.5	123.5	6.26	110.37	21.12	132.89	19.03	3	3.97	R
61	Sankarachini	103.5	127	5.98	107.39	23.7	83.05	19.2	6	3.8	S
62	Saluagaja	110	117.5	9.56	103.81	21.26	139.91	17.35	4	3.57	MR
63	Mayurachulia	99.5	113.5	4.38	103.85	22.01	187.81	13.81	8	4.37	S
64	Basudha	105	113	11.5	95.71	22.42	144.02	13.21	4	4.61	MR
65	Tikimahsuri	109	123	5.92	82.04	19.51	180.21	11.26	1.5	3.42	R
66	Tulasibasa	102.5	113	5.74	125.91	25.35	92.6	9.66	5	4.27	MR
67	Asinasita	102	113.5	6.12	112.34	20.3	109.19	12.06	1	3.5	R
68	Bhangar	110.5	114	5.21	78.68	19.35	108.82	11.93	5	2.6	MR
69	Kalajeera	101.5	115	6.7	124.8	21.28	137.98	8.07	1.5	3.3	R
70	Gobindabhog	106.5	112	6.31	103.33	19.21	157.65	14.65	5	2.99	MR
71	Bsudha	102	122.5	5.12	72.2	14.9	184.33	9.63	5	3.25	MR
72	Agnisar	107	114.5	3.13	112.99	19.79	92.2	13.63	3	3.32	R
73	Malata	108	109.5	5.66	106.38	19.05	110.86	17.97	3	2.55	R
74	Kabir	105.5	125.5	6.92	84.7	21.05	115.15	16.68	2	3.55	R
75	Nadalghanta	102.5	111.5	7.43	121.01	23.05	97.32	17.83	3	3.87	R
76	Latachaunri	102	126	5.77	126.18	22.63	163.93	12.85	2.5	4.59	R
77	Nalikalma	110	102	7.06	101.35	21.02	148.31	18.23	6	5.48	S
78	Sarubhajana	103.5	124	6.05	96.16	22.85	129.92	17.57	5	4.75	MR
79	Luna	104	117.5	6.7	149.4	20.74	98.32	19.03	5	4.48	MR
80	Abhiram	103	116	4.28	131.5	17.7	126.43	22.2	2	3.72	R
81	Sebati	98.5	118.5	6.28	67.45	19.88	72.6	9.72	7	3.07	S
82	Ahirman	101	113.5	5.54	121.8	19.26	84.21	17.37	4	3.99	MR
83	Bhutmundi	101.5	113.5	6.24	120.62	24.13	102.72	19.79	2.5	3.82	R
84	Makarkanda	104.5	113.5	7.86	116.03	20.73	90.16	16.7	2	4.32	R
85	Jata	101.5	121.5	5.36	123.27	18.7	102.21	18.24	5.5	4.62	MR
86	Khajurikandi	102.5	112.5	6.66	131.8	18.81	94.3	17.39	5	3.35	MR
87	Tulasimali	105	121.5	6.87	122.91	19.97	93.83	18.58	3	2.4	R
88	Nalibaunsagaja	102.5	120	5.6	122.83	20.05	65.71	20.12	3	2.57	R
89	Malabati	101	119.5	5.84	110.12	23.85	138.33	18.66	4	2.8	MR
90	Pateni	106	117.5	5.97	117.6	22.08	163.72	20.14	3	2.6	R
91	Nikipakhia	112.5	113	4.73	97.36	23.03	166.22	11.72	3	4.97	R
92	Maliphullajhuli	104.5	117.5	6.52	108.29	22.18	90.24	12.72	2	3.5	R
93	Jhilli	102.5	126.5	5.13	107.83	18.34	175.15	14.52	5	3.74	MR
94	Bharati	104.5	120.5	9.44	101.95	19.7	67.32	14.23	6	3.15	S
95	Hundar	98	112.5	3.46	111.63	15.84	111.51	15.71	6	3.46	S
96	Sapri	102.5	113	4.75	130.3	20.47	93.9	10.7	1	4.45	R
97	Dhoiabankoi	104.5	121.5	7.32	105.04	24.74	83.77	19.12	2	3.675	R
98	Korkaili	110	125.5	4.23	123.69	20.78	86.39	11.7	2	3.55	R
99	Kalamulia	111	126	7.5	110.1	20.76	65.9	17.49	3	2.97	R
100	Kusumakunda	106	115	5.81	102.32	17.91	103.26	15.71	3	3.27	R
101	Saraswati	110	122	6.3	135.95	16.26	101.71	17.92	4	3.67	MR
102	Budhamanda	110.5	128.5	5.2	95.46	19.36	116.71	26.16	3	4.66	R
103	Khajara	110	123.5	6.41	130.04	26.23	130.47	17.03	4	3.825	MR
104	Matiakhoja	111	124	5.57	111.85	21.76	89.27	10.12	5	6.236	MR

105	Haribhog	104	116	4.25	99.73	22.2	117.25	16.47	4	4.1	MR
106	Labangalata	103.5	119	4.34	96.28	22.29	223.35	18.09	5	5.32	MR
107	Dimapur	103.5	112.5	6.36	97.05	21.2	119.65	18.57	2	5.05	R
108	Padmakesari	98.5	107.5	5.88	84.91	22.56	103.62	13.38	4	5.15	MR
109	Mahipal	96.5	108.5	6.5	92.5	23.5	107.5	19.1	4.5	5.83	MR
110	Dhanasri	98	106.5	7.01	90.18	24.15	167.43	15.31	3	5.32	R
111	Khandiratnachudi	100.5	113.5	6.14	105.75	20.95	92.66	20.645	4	2.725	MR
112	Ruksal	107	116.5	5.43	129.9	19.96	130.65	15.33	2	3.55	R
113	Harisankar	106.5	114.5	5.31	94.57	22.86	98.93	15.02	3.5	3.275	R
114	Jagannath	103.5	114	5.25	77.5	21.95	140.4	15.13	2.5	3.8	R
115	Mahalaxmi	105	113.5	5.4	93.67	21	300.16	17.15	6	9.52	S
116	Manika	120	127.5	4.47	73.95	23.76	178.01	14.72	4	4.57	MR
117	Urbhasi	93.5	104	6.08	114.71	22.79	182.66	17.6	5	3.05	MR
118	Rambha	100.5	110.5	4.51	99.38	22.7	185.11	19.17	3	5.3	R
119	Salivahan	119.5	127.5	5.36	87.65	19.66	129.55	13.32	6	5.25	S
120	Kanchan	116	125	8.76	95.94	22.95	186.72	11.66	1.5	9.33	R
121	Savitri	118.5	127	7.22	86.6	21.95	127	16.56	3	6.17	R
122	Mahanadi	111	120	7.78	88.92	19.15	152.16	18.38	1.5	6.32	R
123	Ramachandi	121.5	130	6.49	73.9	21.01	89	14.26	3	6.06	R
124	Indrabati	120.5	129	5.91	96.8	20.9	145.1	20.18	3	5.7	R
125	Prachi	100.5	109.5	4.01	87.26	20.7	120.28	20.83	6	6.45	S
126	Jagabandhu	109.5	118	5.56	87.32	22.55	130.88	16.74	5.5	4.9	MR
127	Upahar	120.5	129.5	7.42	75.13	23.65	149.66	20.36	5	7.55	MR
128	Mrunalini	124.5	132.5	4.92	94.31	21.33	94.16	20.23	2	8.62	R
129	Tanmayi	111	120.5	6.61	115.68	21.4	131.62	16.05	1.5	5.97	R
130	Asutosh	115.5	124.5	8.35	92.5	21.75	143.86	16.83	5	6.25	MR
131	Hasanta	116.5	124	6.07	81.06	21.13	140.71	19.75	5.5	7.22	MR
132	Santepheap	116.5	125.5	9.33	78.31	22.1	271.22	21.85	3	7.02	R
133	OR2327-23	101.5	111	5.64	98.57	20.3	134.76	21.29	2	7.28	R
134	Ganjamgedi	100.5	110	7.21	76.61	21.84	209.21	18.6	2	8.29	R
135	Seulapana	103	113	5.86	113.03	20.2	115.56	15.71	2	4.55	R
136	Kadalipenda	98.5	108	8.11	107.15	23.92	104.11	15.77	6	6.9	S
137	Kakudimanji	101	110.5	5.55	97.365	21.14	103.82	15.26	5	5.17	MR
138	Habira	98.5	108.5	7.21	118.96	21.5	131.77	11.34	5	4.92	MR
139	Kanthakamal	97.5	106.5	4.62	124.21	22.06	131.9	15.27	3	3.75	R
140	Bankoi	100.5	110.5	4.68	98.47	21.28	117.48	16.54	4	4.27	MR
141	Laxmi	94	104.5	4.87	112.36	21.75	131.88	11.39	2	3.42	R
142	Pratikhya	99.5	109	7.22	90.35	22.88	116.65	18.25	3	7.3	R
143	Ranidhan	100.5	110.5	7	83.96	20.21	197.71	14.67	2	7.4	R
144	Swarna	105.5	113	6.31	89.9	20.88	175.67	18.67	2.5	8.32	R
145	Manaswini	99.5	108	5.12	90.1	22.86	176.77	17.23	2	7.67	R
146	MTU1010	100.5	110.5	5.12	91.21	22.6	102.66	16.56	3	7.56	R
147	Tejaswini	104	114	5.22	87.81	22.84	129.22	16.28	6	8.44	S
148	IR-64	102.5	111	11.4	85.84	22.72	98.1	9.66	5	7.56	MR
149	Hiranmayee	103.5	113	6.16	100.6	22.1	153	14.36	3	6.94	R
150	Lalat	100	110.5	9.6	85.9	24.2	198.15	16.5	2	6.02	R
CD(p=0.05)		2.05	5.48	1.48	6.87	3.6	9.75	1.98	3.05	5.47	
CV (%)		0.96	2.29	11.88	3.08	7.92	3.85	5.6	4.03	4.45	

DPI- Days to 50% Panicle Initiation, DF- Days to 50% Flowering, PH- Plant Height (cm), PL-Panicle Length (cm), GN- Number of grains/panicle, GW-1000 grain weight (g), LBI-Leaf bronzing Index, R-Resistant, MR-Moderately Resistant, S-Susceptible

2.2 Genotyping of Germplasms for Fe-Toxicity Tolerance

Rice genotypes, at least 10 each from three groups of resistant, moderately resistant and susceptible as evaluated previously in hotspots for Fe-toxicity and genotypes were selected for further evaluation for iron toxicity tolerance in the plot.

2.2.1 DNA Isolation and Molecular Characterization

The genomic DNA was isolated from the leaf samples of selected 30 rice germplasms employing CTAB protocol (Doyle *et al.* 1987) [1]. This was followed by purification and quantification of DNA and it was diluted so that the final concentration of DNA was optimum i.e. 25-50 ng for PCR reaction**.** A total of 7 SSR primers reported to be linked with Fe toxicity tolerance were run using DNA samples from 30 genotypes for validation of the markers. In PCR products obtained after running all primers, presence of band of reported amplicon size was observed in resistant varieties and scoring was done for further analysis. This was followed by Chi-square analysis which was done to find whether significant differences occur between expected and observed frequencies and on basis of this value it was found whether marker was linked to tolerance to iron toxicity trait.

Table 2: Selected genotypes which are used for genotyping with SSR primers

SL No.	Genotypes	Status
1	Assamchudi	MR
2	Kalakrushna	MR
3	Gurumukhi	MR
4	Jubaraj	R
5	Bavabhunda	MR
6	Latamahu	R
7	Sagiri	MR
8	Kakiri	R
9	Dhusura	R
10	Jalpaya	R
11	Nilarpati	MR
12	Basapatri	R
13	Anu	MR
14	Champeisiali	R
15	Ranisaheba	MR
16	Kusuma	MR
17	Kendrajhali	R
18	Jaiphula	MR
19	Jabaphula	R
20	Khandasagara	R
21	Pipalabasa	S
22	Budhidhan	S
23	Karpuragundi	S
24	Nini	S
25	Champa	S
26	Dhinkisiali	S
27	Dhabalbhuta	S
28	Mugei	S
29	Mayurkantha	S
30	Gelei	S

Table 3: List of SSR primers used for genotyping 30 germplasms

Sl. No	Marker name	Chromosome no.	Sequence (5'-3')	Expected product size (BP)
1	RM148	3	F-ATACAACATTAGGGATGAGGCTGG R- TCCTTAAAGGTGGTGCAATGCGAG	129
2	OsNRAMP5b	7	F-GATTGGACTCATCTTCGCACT R- TGCAACTGCTACACCACTGA	988
3	RM456	6	F-TTGTAGTCCGGGTCGTAACC R- GATAGAATAGGGAGGGGGGG	232
4	RM144	11	F-TGCCCTGGCGCAAATTTGATCC R-GCTAGAGGAGATCAGATGGTAGTGCAG	237
5	RM5473	4	F-ACACGGAGATAAGACACGAG R- CGAGATTAACGTCGTCCTC	105
6	RM217	6	F-ATCGCAGCAATGCCTCGT R- GGGTGTGAACAAAGACAC	133
7	RM7102	12	F-TTGAGAGCGTTTTTAGGATG R- TCGGTTTACTTGGTTACTCG	169

3. Results and Discussion

3.1 Phenotyping of Germplasms for Fe-Toxicity Tolerance

According to the observations, it was found that genotype Mrunalini has maximum no. of 50% days of panicle initiation and 50% flowering i.e. 124.5 days and 132.5 days respectively while genotype Sankaribako has minimum no. of 50% Days of panicle initiation and days of 50% flowering i.e.69.5 days and 78.5 days respectively. Genotype Luna has highest plant height of 149.40 cm while minimum plant height was found in genotype Sebati i.e. 67.45cm. Longest panicle was found in genotype Jubaraj i.e. 33cm while shortest panicle was found in Bsudha i.e. 14.9cm. No. of grains/panicle was found maximum in Mahalaxmi i.e. 300.16 and minimum grains/panicle was found in Kurumukhi i.e. 59.70. Genotype Basudha has maximum tillers/hill of 11.50 and genotype Agnisar has minimum tillers/hill of 3.13. 1000 grain weight of Kusuma is the maximum i.e. 26.65 and of Kalajeera is minimum i.e. 8.07. Mahalaxmi is the highest yielder with yield of 9.52 (Q/ha) while Kaniara is the lowest yielder among 150 varieties with yield of 2.30 (Q/ha). According to LBI Index, it was found that genotypes Veleri, Hatipaniara, Bangali, Banda, Ratanmali, Ramakrushnabilash, Nallijagannath, Asinasita and Sapri were found to be highly resistant with a scale of 1 while Mayurchulia was highly susceptible with a scale of 8.

3.2 Genotyping of Germplasms for Fe-Toxicity Tolerance

Four SSR primers i.e. OsNRAMP5b, RM 144, RM 148, RM 5473 showed reported amplicon size in all tolerant and moderately tolerant genotypes After running of primers along with DNA samples in PCR, Chi-square test was done.

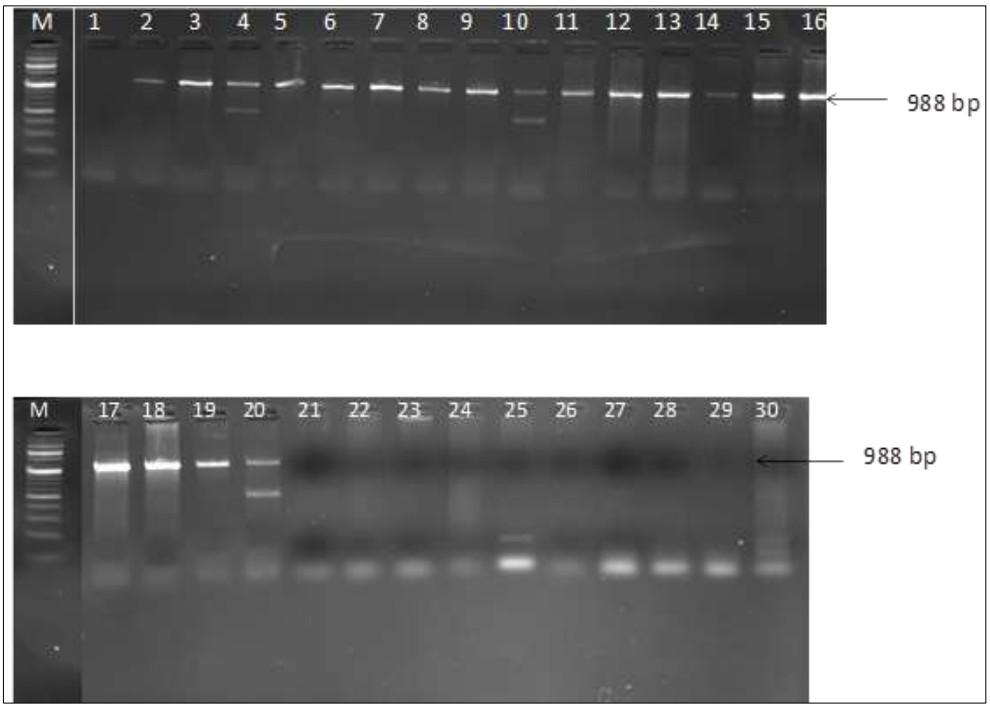

Fig 1: Representative amplification profiles of 30 genotypes generated using *OsNRAMP5b* Marker. The marker amplified around 988 BP of allele size in genotypes tolerant and moderately tolerant to iron toxicity. Lane M 100 BP DNA ladder. Lane 1-30 represents genotypes listed in Table 2 in the same order

After running of primers in PCR, a list of genotypes which showed amplification of reported amplicon size was noted and following table was made.

Table 4: Scoring of primers according to bands seen in gel photos. 1- Presence of band of reported amplicon size, 0- absence of band of reported amplicon size

SL. No	Status	*OsNRAMP5b*	RM 144	RM 148	RM 5473
1	S	0	0	0	0
2	MR	1	0	1	1
3	S	0	0	0	0
4	S	0	0	0	0
5	R	1	1	1	1
6	R	1	1	1	0
7	S	0	0	0	0
8	S	0	0	0	1
9	MR	1	0	1	1
10	R	1	1	1	0
11	S	0	0	0	1
12	MR	1	1	1	1
13	MR	1	0	1	0
14	MR	1	0	0	1
15	R	1	1	1	1
16	R	1	1	1	1
17	MR	1	0	0	0
18	R	1	1	1	1
19	MR	1	0	1	1
20	S	0	0	0	0
21	R	1	1	1	0
22	MR	0	1	1	0
23	MR	1	0	1	1
24	R	1	0	1	1
25	MR	1	0	1	1
26	R	1	1	1	1
27	R	1	1	1	1
28	S	0	0	0	0
29	S	0	0	0	0
30	S	0	0	0	0

Chi-square Test

Chi-square analysis was done for each SSR marker to find whether there is significant difference between expected frequencies i.e. each marker shows amplification in all resistant and moderately resistant genotypes and observed frequencies.

Here, null hypothesis states that the marker cannot distinguish between resistant and susceptible genotypes.

Expected ratio is 2:1 (based on phenotyping i.e. 20 resistant and moderately resistant and 10 susceptible).

Table 5: Chi-square analysis of *OsNRAMP5b*

Trait	O	ER	E	(O-E)	$(O-E)^2$	$(O-E)^2/E$
R	19	2	20	-1	1	0.05
S	11	1	10	1	1	0.1
0.15						

$\sum \chi 2 = (O-E)^2/E = 0.15$

Since DF = 1 at p=0.05, $\chi 2_{tab} = 3.84$, $\chi 2_{cal} < \chi 2_{tab}$

So, null hypothesis is rejected and marker *OsNRAMP5b*, was found to be linked to tolerance to iron toxicity trait and was hence validated.

Table 6: Chi-square analysis of RM144

Trait	O	ER	E	(O-E)	$(O-E)^2$	$(O-E)^2/E$
R	11	2	20	-9	81	4.05
S	19	1	10	9	81	8.1
12.15						

$\sum \chi 2 = (O-E)^2/E = 12.15$

Since DF = 1 at p =0.05, $\chi 2_{tab} = 3.84$

It was observed from the table that $\chi 2_{cal} > \chi 2_{tab}$

So, null hypothesis is accepted and marker RM144 was found not to be linked to tolerance to iron toxicity trait.

Table 7: Chi-square analysis of RM 148

Trait	O	ER	E	(O-E)	$(O-E)^2$	$(O-E)^2/E$
R	18	2	20	-2	4	0.2
S	12	1	10	2	4	0.4
0.6						

$\sum \chi 2 = (O-E)^2/E = 0.6$
Since DF = 1 at p=0.05, $\chi 2_{tab} = 3.84$

It was observed from the table that $\chi 2_{cal} < \chi 2_{tab}$
So, null hypothesis is rejected and marker RM 148, was found to be linked to tolerance to iron toxicity trait and was hence validated.

Table 8: Chi-square analysis of RM 5473

Trait	O	ER	E	(O-E)	$(O-E)^2$	$(O-E)^2/E$
R	16	2	20	-4	16	0.8
S	14	1	10	4	16	1.6
2.4						

$\sum \chi 2 = (O-E)^2/E = 2.4$
Since DF = 1 at p=0.05, $\chi 2_{tab} = 3.84$

It was observed from the table that $\chi 2_{cal} < \chi 2_{tab}$
So, null hypothesis is rejected and marker RM 5473, was found to be linked to tolerance to iron toxicity trait and was hence validated.
From Chi-square test, it was found that SSR markers, OsNRAMP5b, RM148 and RM 5473 were linked to tolerance to iron toxicity trait.

4. Conclusion
For development of rice genotype tolerant to iron toxicity, conventional breeding methods like backcrossing or molecular breeding is used out of which molecular breeding is a quicker and accurate method. Molecular markers especially SSR markers have a major role in marker assisted selection. Many laboratories are involved in discovery of markers linked to the tolerance to iron toxicity trait. These markers reported should be validated to find the precision of the marker or to found out whether they can be used in marker assisted selection. In the above investigation, RM 148, OsNRAMP5b and RM 5473 showed bands of reported amplicon size in case of tolerant and moderately tolerant genotypes and are hence are found to be linked to tolerance to iron toxicity trait. These markers can further be used for introgression of desired trait following the technique of marker assisted selection.

5. References

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