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Research Article

SSR markers for grain iron zinc and yield-related traits polymorphic between Samba Mahsuri (BPT5204) and a wild rice *Oryza rufipogon*

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Abstract
Identification of molecular markers revealing polymorphism among the parental lines are prerequisite for mapping QTLs and genes for desired traits. The genomic regions which contributes to the accumulation of grain iron and zinc in rice could greatly help in rice bio fortification programs. A BC_4F_{10} mapping population was earlier developed from the cross between an elite fine-grain *Oryza sativa* indica cultivar, BPT5204 and a wild progenitor specie *O. rufipogon WR119*. A total of 800 randomly selected SSR markers distributed on all the 12 chromosomes of rice including 50 gene specific markers related to grain iron, zinc and yield traits were used to identify the polymorphic loci between the two genotypes. In all, 166 markers (20.75 %) showed distinct polymorphism. 149 SSR markers (19%) out of 750 SSRs and 17 out of 50 gene-specific markers (36%) were polymorphic. The 17 polymorphic gene-specific markers were related to gene families *OsZIP, OsYSL, OsNRAMP, OsNAAT, OsFRO, OsFDH, OsGSTU and OsPDR which are involved in metal transport and homeostasis in rice*. Among the markers reported to be significantly associated with QTLs for grain iron, zinc and yield related traits, RM517, RM81A, RM264, *OsYSL*-7, RM5460, RM3874 were polymorphic in this study.

Key words
BPT5204, wild rice, bio fortification, SSR markers, polymorphism, iron, zinc

INTRODUCTION

Rice (*Oryza sativa L.*), one of the most important staple food across the world, is being cultivated on approximately 167 million hectares of area under varied climatic conditions in tropical and subtropical regions. It occupies 23 per cent of the total area under cereal production in the world. The annual global milled rice production in 2018 was 487.35 million tonnes (Rice stat, 2019). In rice, while the carbohydrates and zinc are present in the endosperm, the important micronutrients such as iron are largely stored in the husk, aleurone layer, and embryo, a large proportion of which are lost during the milling and polishing processes. In polished form, rice cannot serve the required amount of micronutrients. The availability of large genetic variability in micronutrient concentration in rice grains and its huge preference as a staple food by large populations made it the best candidate for bio fortification of food grains to enrich with crucial micronutrients (Graham *et al.,* 1999). Efforts are being made to improve the micronutrient content in the existing cultivars with introgression of genes/QTLs responsible for their enhancement and there is a scope for increasing at least 8-10mg of Fe and Zn in rice grain. The rapid development of molecular technology provides greater opportunities to enhance the nutritive values of traditionally cultivated crops. Some wild relatives of rice were found to have higher grain Fe and Zn concentrations

compared with the cultivated rice germplasm (Garcia-Oliveira *et al.,* 2018). The AA-genome, wild progenitor of cultivated rice *O. rufipogon* is a rich source of natural allelic variation for several agronomic, grain quality and grain micronutrient trait. Wild accessions *O. nivara* and O. *rufipogon* have high concentrations of grain iron and zinc (Anuradha, *et al.,* 2012a). Breeding rice varieties with higher mineral densities can help in tackling hidden hunger in most of the Asian countries.

Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes. Microsatellites or SSRs are the most widely used markers over the last two decades for genotyping plants because they are co-dominant, efficient, reproducible, evenly distributed in the genome, requiring less quantity of DNA, cost-effective and the same markers are usable in related crops (Mason, 2015). The microsatellites are found in both coding and non-coding regions and have a lower level of mutation rate (10^{-2} and 10^{-4}) per generation. The studies of population structure, genetic mapping, and evolutionary processes in crop plants are easily conducted with SSR markers. These markers can be used for linkage map construction, gene mapping and MAS (Edwards and Batley, 2010; Gonzaga *et al.,* 2015). Enhancing the availability of grain iron and zinc in staple foods by bio fortification strategy involving molecular markers and breeding tools can help to reduce the problem of micronutrient malnutrition in mankind. The aim of this study was to identify informative polymorphic markers between Samba Mahsuri and *O. rufipogon* as a first step for QTL mapping in the advanced backcross mapping population.

MATERIALS AND METHODS

Two rice genotypes viz., BPT5204 (an elite fine-grain indica rice cultivar) and of wild species accession *O. rufipogon* WR119 available at Indian Institute of Rice Research, Hyderabad constituted the experimental material. The genomic DNA of these two rice genotypes were extracted by the CTAB method (Doyle and Doyle, 1987). Young leaves were selected as the ideal part for the extraction of the genomic DNA. 0.1 g of leaves was weighed and the genomic DNA was extracted with DNA extraction buffer (4% CTAB, 100 mM Tris HCl, 20 mM EDTA, 1.4 M NaCl, 2 % PVP and 0.2 % β-mercaptoethanol) preheated at 60 ˚C. DNA quantification and purity were checked by measuring the O.D. values at 260 and 280 nm using a NanoDrop ND100 spectrophotometer. The information regarding chromosomal location and sequences of primers were obtained from www.gramene.org. In all, 800 randomly selected microsatellite markers including 50 gene-specific markers from all 12 rice chromosomes were used to identify polymorphism between BPT5204 and *O. rufipogon* WR119. 166 markers including 17 gene-specific markers were polymorphic between the two genotypes. PCR was carried out in thermal cycler (Veriti Thermal cycler, Applied Bio systems, Singapore) with a final reaction volume of 10 µl containing 30ng of genomic DNA, 1X assay buffer, 200 µM of dNTPs, 1.5 mM MgCl2, 10 pmol of forward and reverse primers and 1 unit of Taq DNA polymerase (Thermo Scientific). PCR cycles were programmed as follows: initial denaturation at 94° C for 5 min followed by 35 cycles of 94° C for 30 s, 55° C (58°C for gene-specific markers) for 30 s, 72° C for 1 min, and a final extension of 10 min at 72° C. Amplified products were resolved in 3% agarose gel prepared in 0.5 × TBE buffer and electrophoresed at 120 V for 2 h. Gels were stained with ethidium bromide and documented using a gel documentation system (Syngene, Ingenious 3, U.S.A).

RESULTS AND DISCUSSION

The ratio of UV absorbance at OD260/OD280 ranged between 1.84 -1.92, and hence the DNA samples are rated as good and standard. The quantity of DNA in the isolated samples ranged from 1840.90 to 2052.46 ng/µl. The genomic DNA of the two parents BPT5204 and *O. rufipogon* WR119 were screened using 800 Rice Microsatellites (RM) markers distributed over the twelve chromosomes of rice. One or two amplicons were observed in the different RM markers of two parents. The size of amplicons resolved among the markers ranged from 70bp (RM21132) to 1131bp (*OsFDH*). Out of the 800 RM markers, 166 were polymorphic between BPT5204 and *O. rufipogon* WR119. The list of the polymorphic markers with their respective chromosome numbers are presented in **Table1**. 149 SSR markers (19%) out of 750 and 17 out of 50 gene-specific markers (34%) were polymorphic with an overall polymorphism of 20.75%. Among these, the highest number (36) of polymorphic markers was on chromosome 2 and the lowest numbers (5) of polymorphic markers were identified on chromosome 12. In a similar study, Yadav *et al.,* (2015) found 70 polymorphic markers between BPT-5204 and a landrace from Assam Rice Collection ARC-10531 out of 500 SSR markers. Anuradha *et al.,* (2012b), identified 22% polymorphism by using 101 SSRs between parents Madhukar and Swarna to map QTLs for grain Fe and Zn in the RIL mapping population. Swamy *et al.,* (2018) identified 100 polymorphic markers between Swarna and the wild rice *O.nivara accession IRGC81832* and mapped QTLs for grain iron and zinc. Ishikawa *et al.,* (2017) identified 164 polymorphic markers between Nipponbare and *O. meridionalis* and mapped QTLs for grain zinc. Garcia and Oliveira (2009) identified 179 polymorphic SSR markers between Tequing and *O. rufipogon* and mapped a major QTL for grain zinc. Ilango and Sarla, (2010) studied parental polymorphism between 5 genotypes Madhukar, Jalmagna, Swarna, BPT5204 and IR64 using 112 RM markers out of which 33 polymorphic markers were shortlisted. In all the 166 polymorphic markers were identified in the present work, 20 SSR markers were reported to be associated with QTLs and the 17 gene specific markers were associated with genes of grain iron, zinc and yield-related traits of rice from previous studies. Swamy *et al.,* (2018) reported that RM517, RM223, RM 81A, RM256, RM264, RM287, RM209 are polymorphic

Table 1. Chromosome wise list of 166 markers polymorphic between BPT5204 and O. rufipogon. Gene specific markers are shown in bold.

Sno.	Marker	Chr	Sno.	Marker	Chr	Sno.	Marker	Chr	Sno.	Marker	Chr
1	RM81A	1	53	RM213	2	105	RM19417	6	157	RM287	11
2	RM283	1	54	RM48	2	106	RM20866	7	158	RM209	11
3	RM522	1	55	RM60	3	107	RM21096	7	159	RM26826	11
4	RM272	1	56	RM14303	3	108	RM 21132	7	160	RM206	11
5	RM579	1	57	RM5474	3	109	RM21242	7	157	RM287	11
6	RM23	1	58	RM7576	3	110	RM21364	7	158	RM209	11
7	RM594	1	59	RM517	3	111	RM21596	7	159	RM26826	11
8	RM329	1	60	RM232	3	112	RM21622	7	160	RM206	11
9	RM129	1	61	RM3204	3	113	RM21632	7	157	RM287	11
10	RM5638	1	62	RM15203	3	114	RM11	7	158	RM209	11
11	RM7405	1	63	RM15204	3	115	RM3743	7	159	RM26826	11
12	**OsPDR-9**	1	64	RM15206	3	116	RM21794	7	160	RM206	11
13	**OsZIP-6**	1	65	RM6283	3	117	RM21970	7	161	RM26998	11
14	RM3324	1	66	RM3400	3	118	RM21976	7	162	RM7315	12
15	RM3738	1	67	**OsZIP-11**	3	119	RM429	7	163	RM3747	12
16	RM11969	1	68	**OsZIP 10**	3	120	RM2680	8	164	RM519	12
17	RM11997	1	69	RM168	3	121	RM3153	8	165	RM235	12
18	RM6831	1	70	RM14036	3	122	RM223	8	166	RM 28807	12
19	**OsYSL-7**	2	71	RM448	3	123	RM350	8			
20	**OsYSL-8**	2	72	RM85	3	124	RM210	8			
21	RM233	2	73	RM17585	4	125	RM5485	8			
22	RM12487	2	74	RM518	4	126	RM5353	8			
23	RM423	2	75	RM16443	4	127	RM 256	8			
24	RM555	2	76	RM16449	4	128	RM6948	8			
25	RM8080	2	77	RM16493	4	129	RM3840	8			
26	**OsNRAMP2**	2	78	RM273	4	130	RM264	8			
27	RM424	2	79	**OsYSL-12**	4	131	RM316	9			
28	**OsNAAT**	2	80	**OsFR01**	4	132	RM23814	9			
29	**OsYSL-6**	2	81	**OsFR02**	4	133	RM24382	9			
30	**OsYSL-19**	2	82	RM122	5	134	RM24383	9			
31	RM2634	2	83	**OsZIP3**	5	135	RM24423	9			
32	RM341	2	84	RM17804	5	136	RM5519	9			
33	RM5427	2	85	RM13	5	137	RM3164	9			
34	RM3688	2	86	RM3683	5	138	RM553	9			
35	RM13530	2	87	RM3695	5	139	RM160	9			
36	RM13603	2	88	RM3575	5	140	RM107	9			
37	RM13620	2	89	**OsZIP9**	5	141	RM24829	9			
38	RM13630	2	90	RM5968	5	142	RM205	9			
39	RM13637	2	91	RM19263	6	143	RM24941	10			
40	RM13659	2	92	RM190	6	144	RM 25052	10			
41	RM263	2	93	RM557	6	145	RM1126	10			
42	RM3874	2	94	RM8226	6	146	RM25328	10			
43	RM14008	2	95	RM19779	6	147	RM25453	10			
44	RM14014	2	96	RM19780	6	148	RM25474	10			
45	RM12500	2	97	RM19781	6	149	RM3229	10			
46	RM14029	2	98	RM20071	6	150	RM25771	10			
47	RM14031	2	99	**OsFDH**	6	151	RM25796	10			
48	RM14026	2	100	RM20118	6	152	RM484	10			
49	RM14037	2	101	RM340	6	153	**OsGSTU3**	10			
50	RM5607	2	102	RM19410	6	154	RM26423	11			
51	RM5404	2	103	RM19414	6	155	RM5590	11			
52	RM5460	2	104	RM19415	6	156	RM3428	11			

between Swarna and *O.nivara (IRGC81832)* and are also associated with grain iron and zinc QTLs. Kiranmayi *et al.,* (2014) identified 84 polymorphic markers between Jalmagna and Swarna and reported that RM264, RM223, RM3695 and RM24382 were significantly associated with grain iron and zinc respectively by single marker analysis. Two SSR markers, RM122, RM517 and a gene specific marker *OsYSL7* were polymorphic between Madhukar and Swarna and flank the QTLs associated with grain Fe and Zn concentration respectively (Anuradha, *et al.,* 2012b). Xu *et al.,* (2015) reported RM85 and RM340 associated with grain Fe and Zn. RM519, RM263, RM429 and RM235 were identified to be polymorphic and are associated with the QTLs associated with grain Fe and Zn respectively (Kumar *et al.,* 2014; 2019, Stangoulis *et al.,* 2007). RM5460 and RM3874 were associated with the QTLs related to grain size and yield (Surapaneni *et al.,* 2017). The details of the SSR markers from previous studies are presented in **Table 2.**

Table 2. Details of polymorphic markers identified in previous reports

S no.	Parents	Total no. of polymorphic markers identified		Markers reported linked with grain iron, zinc and yield traits and found polymorphic in this study	References
		SSR markers	Gene-specific markers		
1	Swarna /O.nivara (IRGC81832)	100	-	RM517, RM223, 81A, RM287, RM209 RM256, RM264	Swamy et al., 2018
2	Madhukar / Swarna	101	9	RM517, OsYSL7	Anuradha et al., 2012b
3	Jalmagna /Swarna	82	2	RM264, RM223, RM3695 RM24382	Kiranmayi et. al.,2014
4	Ce258 (Indica cultivar) / IR75862 (Japonica line)	129	-	RM340	Xu et. al., 2015
5	PAU 201(Indica rice)/ Palman 579 (Indica rice)	76	-	RM519, RM263, RM429	Kumar et al.,2014; 2015
6	IR64 (Indica variety) / Azucena (Japonica variety)	437	-	RM235	Stangoulis et al., 2009
7	Xieqingzao B (O.sativa) / O.rufipogon	108	-	RM11, RM340	Hu et al., 2016
8	Swarna / O.nivara (IRGC81848)	111	-	RM5460*, RM3874*	Surapaneni et al.,2017

Note: Markers in **bold** are associated with grain iron concentration; Markers in ***italics*** are associated with grain zinc concentration; Markers with * are associated with grain yield traits.

The 17 gene-specific primers polymorphic between BPT5204 and *O. rufipogon* include 5 markers of *OsZIP, 5 of OsYSL, 2 of OsFRO, 1 each of OsNRAMP, OsNAAT, OsFDH, OsGSTU and OsPDR gene families*. The gene families *OsZIP, OsYSL* and *OsNRAMP* are transporters of both iron and zinc along with other micronutrients such as manganese and cadmium while *OsNAAT, OsFRO, OsFDH, OsGSTU* and *OsPDR* are involved in metal homeostasis. These gene-specific markers showed significant association with grain Fe and Zn (Anuradha *et al.,* 2012b). The ZIP (ZRT or IRT like protein) family genes are important metal transporters involved in the transport of Zn within and between different parts of the rice plant, and their expression varied with different Zn conditions (Ishimaru *et al.,* 2011). *OsYSL* (*Oryza sativa* -Yellow Stripe Like) family proteins play an important role in phloem transport and long-distance transport of metals especially iron and zinc (Kakei *et al.,* 2012). *OsNRAMP* (Natural Resistance Associated Macrophage protein) is involved in Fe, Zn, Mn and Cd uptake from soil (Wang *et al.,* 2019). *OsNAAT* (*Nicotianamine aminotransferase*) is involved in biosynthesis, transport, and secretion of phytosiderophores in the root zone and thereby increases the uptake of iron from the rhizosphere and also helps in its internal translocation in rice (Li, Q, *et al*., 2020). The gene *OsFRO* (Ferric reductase oxidase) codes for the enzyme ferric chelate reductase oxidase which changes its oxidation state from Fe^{3+} to Fe^{2+} under iron deficiency conditions and helps in the uptake of more iron from the soil (Kar *et al.,*2020). The genes *OsFDH* (Formate dehydrogenase), *OsGSTU* (Glutathione –S-transferase), *OsPDR* (Pleotropic Drug Resistance) are involved in metal homeostasis especially in iron acquisition and also stress tolerance in rice plants (Narayanan *et al.,* 2007). These genes are involved in uptake, translocation, and storage of iron, zinc and other micronutrients in rice plants (Ludwig *et al.,* 2019). In a study the 9 metal related genes *OsYSL6, OsYSL8, OsYSL14, OSNRAMP1,OsNRAMP7, OsNRAMP8, OsNAS1, OsFRO1* and *OsNAC5* were specifically overexpressed in flag leaves of rice and showed significant correlation with grain iron and zinc concentrations in seeds (Sperotto *et al.,*2010).

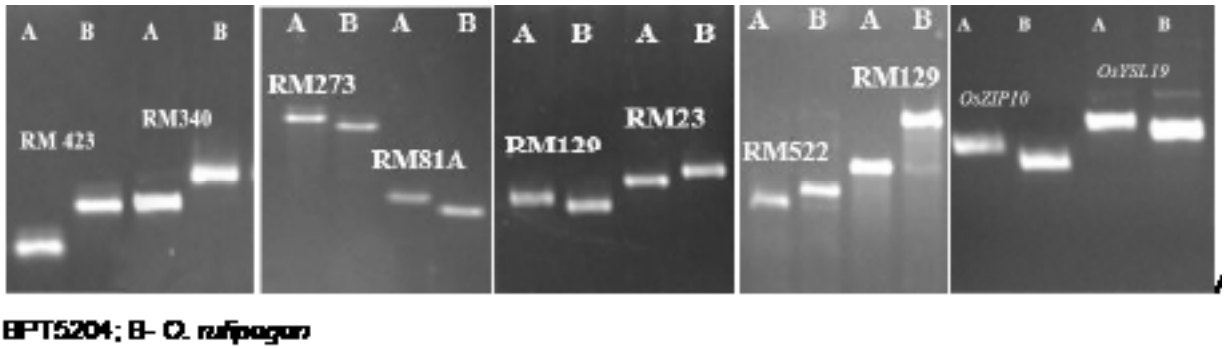
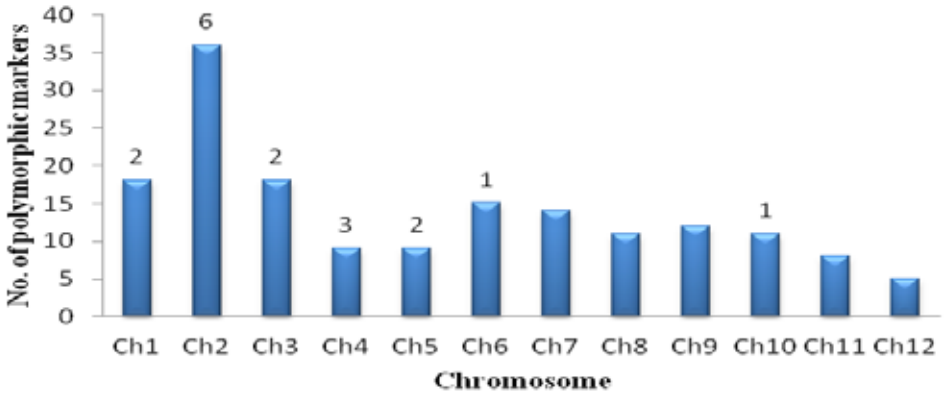


Fig. 1. 8 SSR markers and 2 gene specific markers showing polymorphism between BPT5204 and *O. rufipogon* in 3% agarose gel



Numbers on top of bars refer to number of gene specific markers
Fig. 2. Frequency distribution of 166 SSR markers polymorphic between BPT5204 and *O. rufipogon* chromosome wise.

Expression of metal transporter genes *AtNRAMP3*, *AtNAS1* and *PvFER* cassette increased the iron and zinc levels equalling more than 90% of the recommended iron increase in rice endosperm in transgenic indica IR64 lines (Wu *et al.,* 2019). Thus, it is very clear that these metal homeostasis genes play a major role in the uptake, distribution of iron and zinc from the soil to seeds in rice plants. Application of molecular marker technology in breeding programs will be useful for the efficient transfer of desirable genes among the varieties and to introgress novel genes from related wild species. Screening of molecular markers for parental polymorphism is the first step for assigning linkage and mapping of genomic region associated with iron, zinc and yield.

The 166 polymorphic markers identified between BPT5204 and *O. rufipogon* on all the 12 chromosomes can be used to map QTLs for grain iron, zinc, and yield-related traits in the advanced backcross mapping population derived from them and introgression lines tested for high Fe and Zn. Consistent major effect QTLs can be used to develop micronutrient dense rice varieties to overcome these deficiency disorders in the people depending on rice as their staple food.

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