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 BC₁F₁0 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 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 polymeromatic 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-105513 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 Teqing 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 RMI517, 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  | RM21132| 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   | RM7576 | 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  | RM28807| 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  | RM256  | 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  | RM25052| 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). Xue 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 |
|-------|---------|--------------------------------------------|-------------------------------------------------------------------------------------------------|------------|
| 1     | Swarna /O.nivara (IRGC81832) | 100 - | RM517, RM233, 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 Fe²⁺ to Fe³⁺ 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 \textit{O. rufipogon} in 3% agarose gel.

Numbers on top of bars refer to number of gene specific markers.

Expression of metal transporter genes \textit{AtNRAMP3}, \textit{AtNAS1} and \textit{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 \textit{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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