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# Identification of markers associated with iron and zinc concentration in recombinant inbred lines of brown rice

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**Abstract**

**Association of marker and traits were studied using single marker analysis for iron and zinc concentration in rice grains. Grain iron and zinc concentration of rice at two locations was estimated using Atomic Absorption Spectrometer. Across the locations. Iron concentration ranged from 4 to 270 ppm, while that of zinc ranged from 14 to 31 ppm. Parental polymorphism studies identified 82 (11.2%) polymorphic markers from 735 rice microsatellites across 12 chromosomes, and 2 polymorphic markers, ZIPRM 1067 and ZIPRM 5511 out of 36 gene specific primers. The 84 markers with 126 recombinant inbred lines ($F_6$) were used for simple linear regression analysis. A total of 25 markers were significantly associated with iron and/or zinc concentration at one or both locations. Out of these, RM3322 and RM7488 were associated with both iron and zinc.The markers identified may be useful in future breeding programmes aimed at biofortification in rice.**

**Key words:** Iron, zinc, polymorphism, recombinant inbred lines

## Introduction

Rice is one of the most important staple food crop for more than half of the world's population. However, rice is a poor source of essential micronutrients such as iron (Fe) and zinc (Zn) [1]. Micronutrient deficiency is a global health problem contributing to high rate of children and women's mortality. It is estimated that more than 3.5 billion people in the world are deficient in vitamin A, I, Fe or Zn [2]. Fe serves as an important cofactor for various enzymes performing basic functions in humans. Fe deficiency results in anemia and is also reported to have pathological consequences [3]. Fe is also essential for various cellular events in plants, such as cellular respiration, chlorophyll synthesis, photosynthetic electron transport [4].

Zinc is a vital micronutrient for all organisms. This element is involved in many reactions of the cellular metabolism, including biological processes, such as antioxidative defense, protein synthesis, carbohydrate metabolism, auxin metabolism, and stability of genetic materials [5]. They are expected to increase the crop production in areas with low-Zn bioavailability and alleviate human malnutrition problems due to zinc deficiency [6]. Zinc deficiency in plants causes biochemical changes in membranes, which modify the permeability and architecture of biological membranes. Zinc deficiency in humans is a nutritional problem worldwide. It is estimated that one-third of the world population (around 2 billion people) suffers from mild Zn deficiency and over 450,000 children die each year due such deficiency [6].

Attempts have been made to increase Fe and Zn concentrations in rice grains with the aid of molecular breeding program. Breeding for higher concentrations of essential micronutrients like Fe and Zn in the grains is possible as there is ample genotypic variation in the germplasm of major cereal crops like rice, wheat etc. Earlier, studies mostly confined to production of high yielding varieties, but now the focus has been shifted to enrichment of micronutrients in staple food crops that helps in ameliorating the problems of micronutrient deficiency in the form of hidden hunger in the human population. Also, in the past 2 decades, the major effort in breeding has

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changed from traditional phenotypic-pedigree based selection systems to molecular genetics with emphasis on quantitative trait loci (QTL) identification and marker assisted selection (MAS). The identification of QTLs for rice grain Fe and Zn content in cultivated species could be helpful for improvement of rice grain nutritional quality by marker-assisted selection. Molecular markers augment conventional plant breeding for efficient and precise identification or selection of a trait of interest linked to them.

Association between a quantitative trait and genetic markers can be evaluated using single markers or multiple markers. The statistical power of different methods for quantitative trait locus (QTL) mapping has been studied by many researchers [7] earlier stating that two marker models more powerful than single marker models of detection. But simulation studies for comparing the general behavior of an intersection test for single marker analysis and a two marker test under a variety of conditions revealed that there are cases when an intersection test may outperform the two marker approach [8]. Thus considering the importance of the single marker analysis approach, the present study has been applied to Recombinant Inbred Line (RILs) population of unpolished rice with 84 polymorphic microsatellite markers to detect QTL associated with Fe and Zn concentration in rice grains.

## Material and methods

### *Plant material*

In the present investigation, 126 RILs were developed from a cross between two cultivars Jalmagna, a deep water rice cultivar and Swarna (MTU7029), a popular rainfed lowland high yielding cultivar. The population was grown in augmented block design at two locations and in two replications each at Directorate of Rice Research (DRR), Hyderabad and at Regional Agricultural Research Station (RARS), Warangal during wet season *kharif*, 2010. Standard agronomic practices were followed to raise the crop. In each replication, each line was raised in two rows of 30 plants with a spacing of 20 cm between the rows and 15 cm between the plants. Young leaves were collected for DNA extraction from each line separately. Harvested seed was bulked for each line separately and used for Fe and Zn analysis.

### *Atomic absorption spectrometry for Fe and Zn estimation*

This analysis was conducted in two replications of each sample at Quality Control Laboratory, ANGRAU, Rajendranagar with Atomic Absorption Spectrometer (model A Analyst 700, Perkin Elmer, USA). Bulked seed taken separately from each line of RIL population at two locations and with two replicates was subjected to de-husking with the aid of palm de-husker. 1g of de-husked rice sample was weighed and subjected to microwave digestion upon addition of 7 ml of concentrated Nitric Acid ($HNO_3$-65 %) and 1 ml of $H_2O_2$. The microwave program was run for 40 minutes at 200ºC up to 1000 Watt. Finally the vessel was opened and the solution was transferred to a 25-ml volumetric flask and final volume was made up to 25ml with MilliQ water. Each sample was stored in polypropylene flasks to avoid possible contamination.

### *Correlation analysis and mean performance for grain Fe and Zn in RIL population*

Standard Excel program of Microsoft Office was used to calculate mean, range and standard deviation for grain Fe and Zn concentration. Pearson's Correlation analysis between character pairs were computed at $p < 0.05$, $p < 0.01$, $p < 0.005$, $p < 0.001$ in Microsoft Excel using trait averages for Fe and Zn concentration in the mapping population at both environments.

### *Parental polymorphism survey*

Parental polymorphism survey between Jalmagna and Swarna was studied using 735 microsatellite markers and 36 gene specific markers. 82 polymorphic markers from 735 Rice Microsatellites (RM) across 12 chromosomes and 2 polymorphic markers, ZIPRM 1067 and ZIPRM 5511 out of 36 gene specific primers were identified. These polymorphic markers were used for screening the mapping population.

### *Genotyping of RIL population*

Genomic DNA of rice was extracted from fresh leaf samples by following the methods of Doyle and Doyle [9] with few modifications. Parental polymorphism survey was done using 735 RM primers and 36 gene specific markers from the genomic regions associated with Fe and Zn metabolism with the aim of identifying markers closely linked with high micronutrient content in rice grains. The gene specific primers designed from genes YS (Yellow Stripe), FRO (Fe3+-chelate reductase oxidase), ZIP (Zinc regulated transporter/ Iron regulated transporter Protein), NRAMP (Natural Resistance-Associated Macrophage Protein), PDR (Pleiotropic drug resistance), NAAT (Nicotianamine Amino Transferase), GSTU (Glutathione *S*-transferase-

U type), FDH (Formaldehyde Dehyrogenase), FER (Ferritin) and NAS (Nicotianamine Synthase) reported to be associated with Fe and Zn homeostasis in plants were used for screening the parents.

PCR reactions were performed with the reaction mixture of 50 ng template DNA, 10 pmol of both forward and reverse primers, 1 µl of 10X PCR buffer (10 mM Tris-HCl (pH-8.0); 50 mM KCl, 1.5 mM $MgCl_2$), 1.0U of *Taq* DNA polymerase (Genei Taq), 2 mM dNTPs. The thermal profile used for PCR amplification comprised an initial denaturation step at 94$^oC$ for 2 minutes; denaturation step at 94$^oC$ for 30 seconds followed by primer annealing at 55$^oC$ for 30 seconds and elongation at 72$^oC$ for 1minute. After 34 cycles, the profile was terminated with final extension step at 72$^oC$ for 7 minutes. The temperature profile was same for all primers used under this study including primer annealing temperatures. The amplified products were resolved on 3% agarose gels stained with ethidium bromide and visualized under UV in a gel documentation system (BIO-RAD, USA) and 8% Polyacrylamide gel electrophoresis (CBS-Scientific MGV-202-33) gels was also employed to enhance the resolution of the polymorphism levels of DNA bands.

### *Data scoring*

126 RILs/$F_6$ mapping population DNA was extracted and segregation pattern of 84 polymorphic microsatellite markers obtained was studied. Clear and unambiguous bands were scored for their presence or absence with the score of homozygous Jalmagna loci as A, homozygous Swarna as B and heterozygous loci as H.

### *QTL analysis*

Segregation data of 84 polymorphic microsatellite markers in 126 Recombinant inbred lines was used for analysis. QTL analysis was performed using single-marker analysis. Single marker analysis was done using the software QTL Cartographer 2.5 version and Map-Disto version 1.7.7.0.1. The analysis followed simple linear regression model; $y = b0 + b1\ x + e$, (if b1 is significantly different from 0 then that marker is associated with the trait of interest) on excel work sheet which involved comparing traits for each marker where 'y' is the phenotypic value of a line, 'b0' is the population mean, 'b1' is the additive effect of the locus on the trait and 'e' is the residual error term. 'x' is directly related to the genotypic code at the locus being tested for the line considered, it is -1 (for female parent) or 1 (for donor or male parent). The difference between the phenotypic means of iron and zinc traits at two locations was used to estimate the phenotypic effect of the markers genotypes. The null hypothesis is tested in the way that the mean of the trait value is independent of the genotype at a particular marker.

## Results and discussion

### *Fe and Zn estimation*

Micronutrient analysis in parents revealed higher concentration of Fe and Zn in the grains of Jalmagna than Swarna (Table 1). Atomic Absorption Spectrometry of 126 RILs obtained from the cross revealed wide variations in grain Fe and Zn concentration. The overall mean, range and standard deviation of both the traits measured in the grains of 126 RILs grown at two locations with two replications each in *Kharif* 2010 are presented in Table 1. Transgressive seggregants were observed for both grain Fe and Zn concentrations in the population. In the mapping population, 4 lines showed higher concentration (44.2-187 ppm) of grain Fe concentration than Jalmagna and 8 lines showed more Fe concentration (34.2-187 ppm) than Swarna parent at Hyderabad, while at Warangal 3 lines recorded more Fe concentration (59.7-270 ppm) than Jalmagna and 9 lines had higher Fe concentration (28.08-270 ppm) than Swarna. Among the RILs grown at Hyderabad, 7 lines had higher concentration (24.43-28.13 ppm) of Zn than Jalmagna and 121 lines had more Zn concentration (14.9-28.13 ppm) than Swarna while at Warangal 69 lines recorded more Zn concentration (22.7-31 ppm) than Jalmagna and all the lines except one exhibited higher concentration of Zn (15-31 ppm)

**Table 1.** Mean performance for Fe and Zn in brown rice grains among 126 RILs studied at both the locations

| S.No. | Trait (ppm) | Range | Mean ± SD | Jalamagna | Swarna |
|---|---|---|---|---|---|
| 1. | Iron at DRR, Hyderabad | 4-187 | 19.26 ± 20 | 40.05 | 32.05 |
| 2. | Zinc at DRR, Hyderabad | 14-28 | 19.7 ± 2.76 | 24.28 | 14.88 |
| 3. | Iron at RARS, Warangal | 6-270 | 18.1 ± 30.1 | 38.95 | 28.05 |
| 4. | Zinc at RARS, Warangal | 14-31 | 22.9 ± 3.43 | 22.53 | 13.73 |

than Swarna. The transgressive segregation in the RIL population is an indication of the presence of different sets of genes in the parental lines for the target traits [10]. Wider variability was observed the traits, and this provides the breeder with more opportunities to select plants with different combinations of desirable traits. These transgressive segregants might have resulted due to the accumulation of favourable genes controlling grain characters or development of new combinations of genes controlling grain traits derived from the parents. Frequency distribution curves for Fe and Zn concentration at both locations were plotted (Figs. 1 and 2). Grain Zn concentration showed normal distribution, while grain Fe concentration did not. This clearly indicated that Zn concentration was probably governed by a QTL, while few genes might be involved in controlling Fe concentration. The parental lines used by Anuradha *et al.* [11] had grain Zn concentration of 53.7 ppm (Madhukar) and 27.2 ppm (Swarna) and the RIL population generated from this cross showed Fe concentration in the range of 0.2 to 224 ppm and Zn in the range of 0.4 to 104 ppm. The study conducted by Bekele and coworkers [12] on the estimation of genetic variability and correlation studies for grain Zn concentration in 64 rice genotypes revealed the range of variation for grain Zn concentration as 18.90 ppm to 36.90 ppm with an average value of 26.74 ppm. Wide variation was observed in Fe concentration among the population than Zn at both locations and similar results were obtained earlier [11, 13]. There could be several reasons for these variations that may include effect of environment, genotype and environment interactions [14], soil properties like pH, organic matter content, Fe and Zn levels in the soil etc. [15, 13].

### *Statistical analysis*

Pearson correlation coefficient was calculated for two environments to test the correspondence of Fe and Zn data in different environments as well as to study correlation between Fe and Zn accumulation in the grains of the RIL population. Fe and Zn showed significant positive correlation (r = 0.256) at Warangal and negative correlation (r = –0.193) at Hyderabad. Zn at Warangal was weakly correlated with Fe at Hyderabad (r = 0.118), Zn at Hyderabad showed negative correlation with Fe at Warangal (r = –0.182). This might be because of G x E interactions or due to the influence of environment or soil factors like soil Fe and Zn content at both locations. Gregorio [16] reported the presence of the significant positive correlation for Fe and Zn accumulation in the grains of

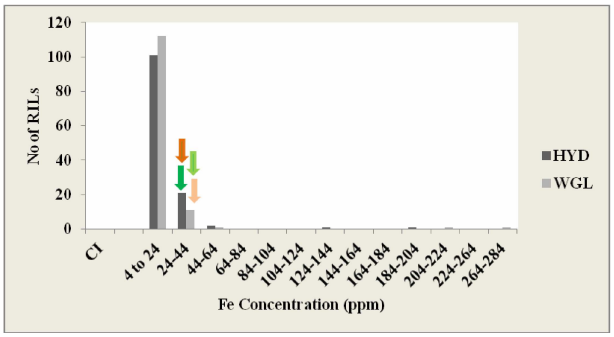


**Fig. 1. Frequency distribution of grain iron concentration of 126 RILs of rice at two locations. Dark green arrow-Grain Fe concentration of Jalmagna (40.05 ppm) at Hyderabad; Red arrow-Grain Fe concentration of Swarna (32.05 ppm) at Hyderabad; Green arrow-Grain Fe concentration of Jalmagna (38.95 ppm) at Warangal; Light red arrow-Grain Fe concentration of Swarna (28.05 ppm) at Warangal**

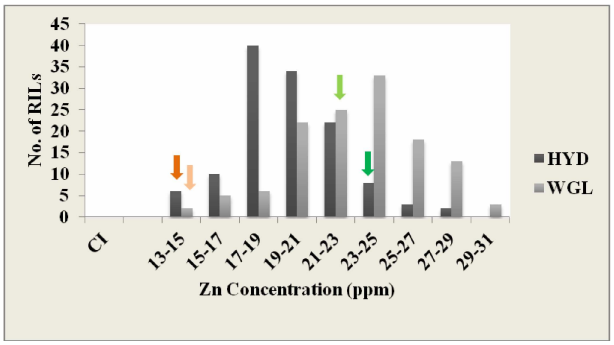


**Fig. 2. Frequency distribution of grain Zn concentration of 126 RILs of rice at two locations. Dark green arrow-Grain Zn concentration of Jalmagna (24.28 ppm) at Hyderabad; Red arrow-Grain Zn concentration of Swarna (14.88 ppm) at Hyderabad; Green arrow-Grain Zn concentration of Jalmagna (22.53 ppm) at Warangal; Light red-Grain Fe concentration of Swarna (13.73 ppm) at Warangal**

rice among 1,138 genotypes studied. Some reports showed positive correlation between Fe and Zn concentration in rice grains [11] and some showed negative or even independent or weak correlation [17]. The significant positive correlation of gain Fe and Zn concentrations at Warangal suggested the simultaneous improvement of these two traits in brown rice but the negative correlation between these traits at Hyderabad indicated the absence of association between these traits, for; therefore, the selection should be executed only after conducting more number of multi-location trails.

### *Parental polymorphism and genotyping of mapping population*

Parental polymorphism survey with 735 RM primers revealed that 82 (11.2 %) markers were polymorphic that covered all the chromosomes. Among the 36 gene specific markers used in the present investigation two markers, ZIPRM 1067 and ZIPRM 5511 located on chromosome 1 and 4 respectively of the 36 gene specific primers were found to be polymorphic between the parents Jalmagna and Swarna. Mapping population was genotyped with the 84 polymorphic markers. Segregation pattern of 126 RILs with a representative primer RM 1 is depicted in Fig. 3. In a study on microsatellite polymorphism in rice by Shankar and Sarla [18], out of 112 markers selected for screening, 33 (29.4%) were found to be polymorphic. Several reports indicated the narrow genetic variability for mineral elements in cultivated rice, whereas a higher level of mineral elements was observed in wild rice *O. rufipogon* [19].

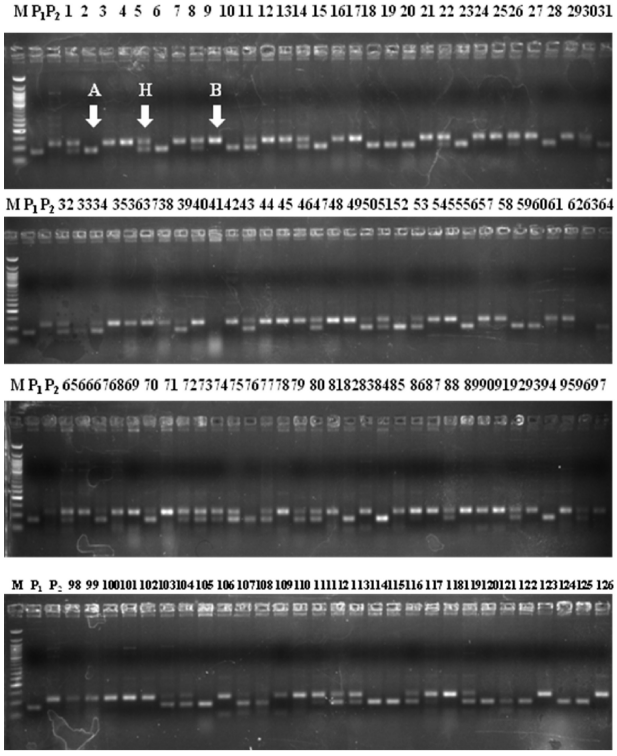


**Fig. 3. Segregation pattern of 126 RIL population with RM1 Rice Microsatellite marker. M-represents 100 bp ladder; $P_1$-Jalamagna parent; $P_2$-Swarna parent; 1 to 126-RIL population; A-Jalmagna allele homozygote B- Swarna allele homozygote; H-Heterozygote**

**Table 2**. Pearson's Correlation coefficients between grain Fe and Zn concentrations in RIL population of rice obtained from a cross between Jalmagna and Swarna grown at two locations

| | Zn, WGL | Zn, HYD | Fe, WGL | Fe, HYD |
|---|---|---|---|---|
| Zn, WGL | 1 | -0.093 | 0.256*** | 0.118 |
| Zn, HYD | | 1 | -0.182 | -0.193 |
| Fe, WGL | | | 1 | 0.126 |
| Fe, HYD | | | | 1 |

*=Significant at 0.05 level; **=Significant at 0.01 level; ***=Significant at 0.005 level; **** = Significant at 0.001 level; (P>0.05, 0.17499) (P>0.01, 0.22870) (P>0.005, 0.24860) (P>0.001, 0.28970); Fe: Iron concentration, Zn: Zinc concentration, HYD: Directorate of Rice Research, Hyderabad; WGL: Regional Agricultural Research Station, Warangal

### *Single marker analysis*

Single marker analysis involving simple linear regression model among 126 RILs showed significant association of 25 markers with either of the traits (grain Fe and Zn concentration) on all the chromosomes except chromosomes 10 and 11 (Table 3). Among these markers 17 were significantly associated with grain Zn concentration while 10 were significantly linked to grain Fe concentration. The range of phenotypic variance ($R^2$ %) for these 25 linked markers was 0.01 to 6.1%. $R^2$ value is phenotypic variance which indicates the relative importance of a QTL in influencing a trait. Out of the 10 markers associated with Fe, RM 6999 on chromosome 8 was highly significant (0.1 % level of significance) (P value 0.0001) with highest $R^2$ (6.1 %). Out of the 17 markers that were found linked to Zn concentration, RM 501 on chromosome 7 and RM 3481, and RM 3395 on chromosome 8 were found to be highly significant with the phenotypic variances 1.8, 2.42 and 0.3 % respectively. RM 3322 and RM 7488 markers located on chromosomes 5 and 6 respectively were associated with both Fe and Zn concentration. RM 7488 marker was earlier reported to be linked with a QTL for plant height [20]. RM 501 which showed highest % of Jalmagna allele in the RIL population was also found to be significantly (at 1 % level) associated with Zn concentration at Warangal (Table 3). RM488 on chromosome 1 linked to zinc concentration in the present study was previously reported to be associated with a meta-QTL for yield under drought conditions [21]. RM 223 on chromosome 8 which was found to be associated with grain Fe concentration was also found to be linked to salt tolerance in rice [22]. Among the two polymorphic gene

**Table 3.** Single marker approach for Fe and Zn concentration in RILs obtained from a cross between Jalamagna and Swarna

| S.No. | Character | Location | Markers associated | Chromosome | P-value | $R^2$ |
|---|---|---|---|---|---|---|
| 1. | Zn | HYD | RM 217 | 6 | 0.048* | 0.4 |
| | | | RM488 | 1 | 0.026* | 0.01 |
| | | | RM586 | 6 | 0.037* | 0.2 |
| | | | RM3322 | 5 | 0.031* | 6.0 |
| | | | RM7488 | 6 | 0.016* | 0.8 |
| | | | RM21975 | 7 | 0.027* | 3.9 |
| | | | RM24382 | 9 | 0.046* | 1.51 |
| | | | ZIPRM1067 | 1 | 0.024* | 1.5 |
| 2. | Zn | WGL | RM106 | 2 | 0.046* | 0.2 |
| | | | RM247 | 12 | 0.026* | 3.55 |
| | | | RM335 | 4 | 0.012* | 2.3 |
| | | | RM501 | 7 | 0.008* | 1.8 |
| | | | RM514 | 3 | 0.016* | 2.14 |
| | | | RM3395 | 8 | 0.005** | 0.3 |
| | | | RM3481 | 8 | 0.004** | 2.42 |
| | | | RM3644 | 8 | 0.042* | 0.71 |
| | | | RM8039 | 5 | 0.039* | 1.0 |
| 3. | Fe | HYD | RM264 | 8 | 0.045* | 2.83 |
| | | | RM1111 | 8 | 0.012* | 0.6 |
| | | | RM3322 | 5 | 0.045* | 0.03 |
| | | | RM3666 | 2 | 0.040* | 1.3 |
| | | | RM3695 | 5 | 0.010* | 0.8 |
| | | | RM6843 | 2 | 0.028* | 2.6 |
| | | | RM6999 | 8 | 0.0001*** | 6.1 |
| 4. | Fe | WGL | RM223 | 8 | 0.048* | 2.3 |
| | | | RM6999 | 8 | 0.044* | 0.02 |
| | | | RM7488 | 6 | 0.001** | 0.8 |
| | | | RM12486 | 2 | 0.023* | 1.8 |

HYD-Directorate of Rice Research, Hyderabad; WGL- ANGRAU, Regional Agricultural Research Station, Warangal. Significance at the 5%, 1%, 0.1% and 0.01% levels are indicated by *, **, *** and ****, respectively.

specific markers, only ZIPRM 1067 located on chromosome 1 was found linked to Zn concentration only at Hyderabad.

The present study revealed higher genetic variability for grain iron concentration than grain zinc concentration among RILs. Single marker analysis identified 25 markers associated with grain Fe and Zn concentration among which RM 3322 and RM 7488 markers located on chromosomes 5 and 6 respectively were associated with both Fe and Zn concentration.

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