Molecular Mapping of High Iron and Zinc Rich Regions in Rice (*Oryza sativa* L.) Grains Using Microsatellite Markers

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Abstract:

Rice (*Oryza sativa* L.) is a primary source of food for billions of people throughout the world, yet it contains insufficient levels of the key micronutrients iron, zinc and vitamin A to meet the daily dietary requirements. Biofortification of staple food crops has thus been considered a sustainable strategy to overcome the problem of micronutrient deficiencies prevalent in rice. The present investigation was conceptualized with the prime objective of mapping the chromosomal regions associated with high iron and zinc content involving the F_2 populations derived from the cross of Swarna with Madhukar for high iron and zinc content using microsatellite markers derived from the genomic regions associated with iron and zinc metabolism. Three polymorphic markers viz., SC 120, SC 128 and SC129 were identified which were unlinked and hence single marker analysis was done to check the association of the marker with the trait. SC129 showed highest significant variation with both iron and zinc at the tune of R^2=13.09% and R^2= 19.51%, respectively. The association could be made more stringent by further analysis of more number of lines and using more number of markers.

Keywords: *Biofortification, Iron, Mapping, Zinc*

Introduction:

Rice (*Oryza sativa* L.) occupies the enviable prime place among the food crops cultivated around the world. It is known as the grain of life and is synonymous with food for Asians as it supplies majority of starch, protein and micronutrient requirements [1], [2]. Poor grain micronutrient contents (iron, zinc and pro-vitamin A) in cereals is the primary cause of prevalent nutritional deficiency related disorders amongst population having cereals based diet, especially those dwelling in developing world[3]. Rice scientists have long recognized its micronutrient deficiencies, which are the basis of numerous human health problems worldwide. Mineral nutrient deficiencies have egregious societal costs including learning disabilities among children, increased morbidity and mortality rates, lower worker productivity and added high health care costs, all of which diminish human potential, felicity and national economic development[4], [5]. Malnutrition has been a serious problem in the developing world mostly in South and South East Asia and Sub Saharan Africa [6]. Over three billion people suffer from micronutrient malnutrition [7]. In the last two decades, new research findings generated by the nutritionists have brought to light the importance of vitamins, minerals and proteins in maintaining good health, adequate growth and even acceptable levels of cognitive ability apart from the problem of protein energy malnutrition. Rice is a predominant staple food and a major source of dietary carbohydrate for more than half of the world's population [8]. In order to enhance the micronutrient concentration in the rice grain, suitable breeding programmes should be followed. In recent years, attention has turned towards strategies for improving human vitamin and mineral nutrition, especially iron, zinc, selenium and iodine [9], [10]. Although, rice is not a major source of mineral in the diet, any increase in its mineral concentration could significantly help to reduce iron and zinc deficiency because of the high levels of rice consumption in Asia [11].

Even though the levels of carbohydrates are adequate in rice, parallel analysis of the levels and bioavailability of the other micronutrients in rice revealed that the levels are very low and consumption of rice alone cannot meet the recommended daily allowance for a range of vitamins, minerals and proteins. To overcome this, a genetic approach called Biofortification [12] has been developed, which aims at biological and genetic enrichment of food stuffs with vital nutrients. Biofortification of staple food crops for enhanced micronutrient content through genetic manipulation is the best option available to alleviate hidden hunger with little recurring costs [4], [13]. Ideally, once rice is biofortified with vital nutrients, the farmer can grow the variety indefinitely without any additional input to produce nutrient packed rice grains in a sustainable way. This is also the only feasible way of reaching the malnourished population in rural India.

Knowledge of the genes underlying the natural variation of iron and zinc composition in grains of crops is essential for the application of the molecular markers through plant breeding. Genetic variation for micronutrients in rice was reported to be narrow especially for iron and zinc [11], [14]. With the advent of several biotechnological approaches, increase of the iron and zinc concentrations in polished grain has become a possibility in rice. Initially the focus was the development of transgenics for enhanced iron and zinc content and recently attempts are being made to characterize the genomic regions associated with micronutrients in rice for iron and zinc using QTL mapping approach. Simultaneous improvement of iron and zinc has been found to be possible in rice suggesting a common molecular mechanism controlling the uptake and metabolism of these minerals in grains [15]. The genetic diversity available within existing germplasm collections sets the limit to the extent of iron content improvement that can be achieved through breeding. Therefore, transgenic approaches are necessary to enable effective and significant increase in iron content and bioavailability [16].

Rice has been at the forefront of plant genomics because of its small genome size, genetic relatedness to other major cereals, relatively low amount of repetitive DNA, its diploid nature and its ease of manipulation in tissue culture. In the 1990s, many advances occurred in the application of molecular markers in rice [17], [18], [19]. In addition to studying the relationship between iron, zinc, protein content and grain yield, nowadays molecular techniques go hand in hand with conventional breeding methods. For this Quantitative Trait Loci (QTL) analysis is a powerful technique to study complex traits and to unravel the genetic differences that are present within a species. QTL is an unbiased investigation of the genes affecting a certain trait, meaning that genes encoding structural as well as regulatory proteins involved in the process of investigation can be identified. Mineral accumulation is a typical quantitative trait and thus suitable for QTL analysis [20]. Considering the complex nature of polygenic controlled iron, QTL analysis will be applied to identify the chromosomal regions associated with iron and zinc content in the grain in the mapping population derived from the identified donors and popular varieties. Clearly, the potential exists for developing improved rice varieties with high iron and zinc content in the grain. Hence, agriculture must now focus on a new paradigm that will not only produce more food, but also bring us better quality food as well.

Materials and Methods:

Plant Material: The parental lines include Swarna and Madhukar which are involved in the development of 178 F_2 mapping population. Swarna is a popular high yielding semi-dwarf variety in Andhra Pradesh with parentage Vasistha/Mahsuri with low iron content of 2.93 mg per 100 g of brown rice grain and zinc content of 2.28 mg per 100 g of brown rice grain, while Madhukar is a selection from Gonda possessing high iron content of 4.72 mg per 100 g of brown rice grain and zinc content of 2.85 mg per 100 g of brown rice grain.

DNA Isolation and Polymerase Chain Reaction Analysis: The DNA was extracted from freshly germinated young seedlings of parental lines and F_2 population using the method of Zheng *et al*. (1991) [21]. 30 well spot test plate available from Thomas Scientific, USA was used for DNA isolations. The purity and concentration of the isolated genomic DNA samples were estimated by UV- absorption spectrophotometer (Beckman DU 650 model) as per the procedure described by Sambrook *et al*. (2001) [22]. Agarose gel electrophoresis (0.8%) was carried out for confirming the quality and quantity of the isolated DNA using a known concentration of λ DNA. The genomic DNA was subjected to PCR amplification as per the procedure described by Chen *et al*. (1997) [23]. DNA samples were amplified in 10µl reaction volumes containing 1X PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (v/v) gelatin] (Bangalore Genei, India), 0.2 mM of each dNTPs (Bangalore Genei, India), 10 pmol of each primer and 1 U of Taq polymerase (Bangalore Genei, India). PCR was carried out in a Thermal cycler (Perkin–Elmer-Gene Amp PCR System 9700, USA). A PCR profile consisting of 5 min initial denaturation at 94°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 2 min extension at 72°C and 7 min at 72°C final extension was followed. The amplified products were resolved on 3% agarose gels, stained with ethidium bromide and visualized under UV in a gel documentation system (Alpha Innotech, USA). Poly acrylamide gel electrophoresis was also employed to enhance the resolution of the polymorphism levels of the DNA bands.

Parental Polymorphism Survey: Parental polymorphism survey between Swarna and Madhukar was studied using 72 microsatellite markers mapped on rice chromosomes 3, 4, 5, 6, 8 and 12 derived from the genomic regions associated with iron and zinc metabolism. The details of the rice microsatellite markers employed, nature of the targeted gene and their chromosomal location is enlisted in Table 1.

Table 1. Details of rice microsatellite markers used, nature of targeted gene and their chromosomal locations

S. No.	Marker	Gene	Chr	S. No.	Marker	Gene	Chr
1.	SC 100	*Zntrp 1*	1	**37.**	SC 124	*OsYSL16*	5
2.	SC 101	*Zntrp 2*	2	**38.**	SC 125	*OsYSL17*	5
3.	SC 102	*Zntrp 3*	3	**39.**	SC 126	*OsYSL18*	8
4.	SC 103	*Zntrp 4*	3	**40.**	SC 448	*OsYSL18*	8
5.	SC 423	*Zntrp 4*	3	**41.**	SC 449	*OsYSL18*	8
6.	SC 424	*Zntrp 4*	3	**42.**	SC 450	*OsYSL18*	8
7.	SC 425	*Zntrp 4*	3	**43.**	SC 127	*OsFRO1*	4
8.	SC 104	*Zntrp 5*	3	**44.**	SC 128	*OsFRO2A*	4
9.	SC 105	*Zntrp 6*	4	**45.**	**SC 129**	***OsZIP1***	**3**
10.	SC 106	*Zntrp 7*	5	**46.**	SC 408	*OsZIP1*	3
11.	SC 107	*Zntrp 8*	5	**47.**	SC 409	*OsZIP1*	3
12.	SC 108	*Zntrp 10*	5	**48.**	SC 410	*OsZIP1*	3
13.	SC 109	*Zntrp 11*	6	**49.**	SC 130	*OsZIP2*	6
14.	SC 110	*Zntrp 12*	6	**50.**	SC 131	*OsZIP3*	4
15.	SC 111	*Zntrp 13*	6	**51.**	SC 428	*OsZIP3*	4
16.	SC 112	*Zntrp 14*	7	**52.**	SC 429	*OsZIP3*	4
17.	SC 113	*Zntrp 15*	8	**53.**	SC 430	*OsZIP3*	4
18.	SC 114	*Zntrp 16*	8	**54.**	SC 132	*OsZIP4A*	8
19.	SC 115	*Zntrp 17*	8	**55.**	SC 133	*OsZIP6*	3
20.	SC 116	*Zntrp 18*	8	**56.**	SC 134	*OsZIP7*	7
21.	SC 443	*Zntrp18*	8	**57.**	SC 135	*OsZIP8*	5
22.	SC 444	*Zntrp18*	8	**58.**	SC 413	*OsZIP8*	5
23.	SC 445	*Zntrp18*	8	**59.**	SC 414	*OsZIP8*	5
24.	SC 117	*OsYSL1*	1	**60.**	SC 136	*OsNRAMP2*	3
25.	SC 118	*OsYSL3 & 4*	2	**61.**	SC 137	*OsNRAMP3*	6
26.	SC 119	*OsYSL6*	2	**62.**	SC 138	*OsNRAMP4*	2
27.	SC 120	*OsYSL8 and 9*	4	**63.**	SC 139	*OsNRAMP5*	7
28.	SC 433	*OsYSL8 & 9*	4	**64.**	SC 140	*OsNRAMP6*	1
29.	SC 434	*OsYSL8 &9*	4	**65.**	SC 141	*OsNRAMP7*	12
30.	SC 435	*OsYSL8 & 9*	4	**66.**	SC 418	*OsNRAMP7*	12
31.	SC 121	*OsYSL10, 11 & 12*	4	**67.**	SC 419	*OsNRAMP7*	12
32.	SC 122	*OsYSL14*	4	**68.**	SC 420	*OsNRAMP7*	12
33.	SC 123	*OsYSL15*	4	**69.**	SC 142	*OsNRAMP8*	3
34.	SC 438	*OsYSL15*	4	**70.**	SC 143	*OsFER1A*	11
35.	SC 439	*OsYSL15*	4	**71.**	SC 144	*OsFER2*	12
36.	SC 440	*OsYSL15*	4	**72.**	SC 145	*OsTRSS*	2

SC indicates the microsatellite markers developed at Directorate of Rice Research

Zntrp -*Zinc transport*
OsYSL -*Oryza sativa Yellow Stripe protein Like*
OsFRO -*Oryza sativa Ferric Reductase Oxidase*
OsZIP -*Oryza sativa Zinc/Iron regulated transporter related Proteins*
OsNRAMP - *Oryza sativa Natural Resistance-Associated Macrophage Protein*
OsFER - *Oryza sativa FERritin gene*
OsTRSS - *Oryza sativa iron sTReSS and uptake related protein*

Selective Genotyping: Using selective genotyping, only individuals from the high and low phenotypic extremes are genotyped. It has been shown that the number of individuals genotyped to attain a given power can be

decreased significantly, at the expense of a moderate increase in the number of individuals phenotyped (Darvasi and Soller, 1992) [24]. The strategy of selective genotyping was assayed with the F_2 individual plants each showing high and low zinc content in grains individually with all polymorphic markers as suggested by Nandi *et al.* (1997) [25].

Mapping Analysis: The markers which were found to be associated in the selective genotyping were used for the analysis of all the 178 individuals. Each gel was scored for maternal, paternal and heterozygous banding pattern and scored accordingly. The maternal band was designated as 'A', paternal band 'B' and heterozygous band 'H'. Homozygotes were given a value of 0 or 1 based on their phenotype group. Heterozygotes were given a value of 0.5. Recombination frequency in percentage in relation to the total sample was calculated manually.

Recombination Frequency
= Number of Recombinant Progeny X 100%
Total Number of Progeny

Single Marker Analysis (SMA): Single marker analysis calculates whether phenotype values differ among genotypes for a given molecular marker. The null hypothesis tested is that genotypic classes do not differ in phenotype for a given molecular marker. Molecular marker genotypes can be classified into groups such that marker genotypes can be used as classifying variables for a t-test or ANOVA, or as variables for regression analysis. $Y = \mu + f(\text{marker}) + \text{error}$ where, Y is equal to the trait value, μ is equal to the population mean, f (marker) is a function of the molecular marker, Analysis of R^2 value was calculated by STATISTICA 4.5 software

Results and Discussion:

Parental Polymorphism Survey: Parental polymorphism survey with 72 markers between Swarna and Madhukar revealed that 22 markers (33.56%) were polymorphic, 29 markers were monomorphic and 21 were not amplified. Details of markers involved in parental polymorphism survey are shown in Table 2.

Table 2. Parental polymorphism survey between Swarna and Madhukar

Polymorphism	No. of markers	Markers
Polymorphic	22	SC-102, SC-103, SC-104, SC-106, SC-116, SC-120, SC128, SC-129, SC-132, SC-143, SC-145, SC-408, SC-409, SC-409, SC-413, SC-420, SC-425, SC-429, SC-433, SC-435, SC-439, SC-444, SC-450
Monomorphic	29	SC-101, SC-105, SC-107, SC-108, SC-109, SC-112, SC-113, SC-114, SC-117, SC-118, SC-121, SC-123, SC-124, SC-126, SC-131, SC-133, SC-135, SC-136, SC-138, SC-142, SC-144, SC-410, SC-418, SC-424, SC-428, SC-430, SC-434, SC-438, SC-445
Not amplified	21	SC-100, SC-110, SC-111, SC-115, SC-119, SC-122, SC-125, SC-127, SC-130, SC-134, SC-137, SC-139, SC-140, SC-141, SC-414, SC-419, SC-423, SC-440, SC-443, SC-448, SC-449

Selective Genotyping: The strategy of selective genotyping was attempted and was shown to be effective to identify specific associated regions of the chromosomes as suggested by Nandi *et al*. (1997) [25]. The methodology of selective genotyping could be successfully used to identify the chromosomal regions associated with high iron and zinc content in rice grains. In the present study, only three markers were polymorphic. The details of selective genotyping for rapid identification of regions associated with iron and zinc content are shown in Table 3.

Table 3. Details of selective genotyping for rapid identification of regions associated with iron and zinc content in cross Swarna × Madhukar and parents

F_2 population	Nutrients	Concentration (mg/100g)	No. of individuals
Lowest	Fe	1.20 to 1.62	10
	Zn	1.68 to 2.12	
Highest	Fe	3.24 to7.09	12
	Zn	3.89 to 5.07	
Parents			
Swarna	Fe	2.93	1
	Zn	2.28	
Madhukar	Fe	4.72	1
	Zn	2.85	

Single Marker Analysis (SMA): To identify associated regions of the chromosomes with iron and zinc metabolism, 178 F_2 plants were assayed individually with three polymorphic markers which are associated with cation uptake *i.e.* SC120 marker based on *Yellow Stripe protein Like* transporters (*YSL*) (Fig. 1), SC128 marker based on *Oryza sativa Ferric Reductase Oxidase 2A* gene (*OsFRO2A*) (Fig. 2) and SC 129 marker based on *Zinc/Iron regulated transporter related Protein* (*ZIP*) (Fig. 3). The results showed that these three markers were unlinked among themselves. This result is expected because three of the markers represent three different genes. Though three markers per locus were surveyed for parental polymorphism, polymorphism was detected only for one marker per locus in the two parents. Since for mapping, a minimum of two markers per locus are prerequisite, the present data was analyzed using Single Marker Analysis and the R^2 values for each trait were presented in the Table 4. From the three markers, two markers (SC120 and SC128) were located on chromosome-4 and these were unlinked and one (SC129) on chromosome-3. Due to this SMA is the feasible way to check the amount of variation by the marker.

SMA revealed the association of markers, SC120 (Chromosome-4) with grain iron content (R^2= 2.91%) and grain zinc content (R^2=7.76%), SC128 (Chromosome-4) with grain iron content (R^2=0.67%) and grain zinc content (R^2=7.12%) and SC129 (Chromosome-3) with grain iron content (R^2=13.09%) and grain zinc content (R^2=19.51%). These results are in conformity with Kanatti (2009) [26] for grain iron content and Luisa *et al.* (2009) [27] and Kanatti (2009) [26] for grain zinc content. SC120, SC128 and SC129 were identified to be associated with grain iron, zinc content. As only few markers were analyzed with few mapping population lines, the obtained values indeed suggest a close association. This is expected because these markers were selected based on their proximity to the candidate genes involved in the iron and zinc metabolism. The microsatellite markers used in the present study were selected based on their proximity to the candidate gene(s) involved in iron and zinc metabolism. Normally the repeat numbers of microsatellite markers is closely related to the level of polymorphism. Since, the available microsatellite markers were used irrespective of their repeat number, the observed polymorphism levels could be low.

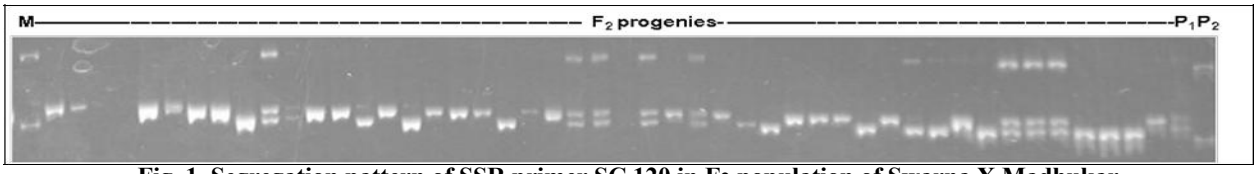

Fig. 1. Segregation pattern of SSR primer SC 120 in F_2 population of Swarna X Madhukar

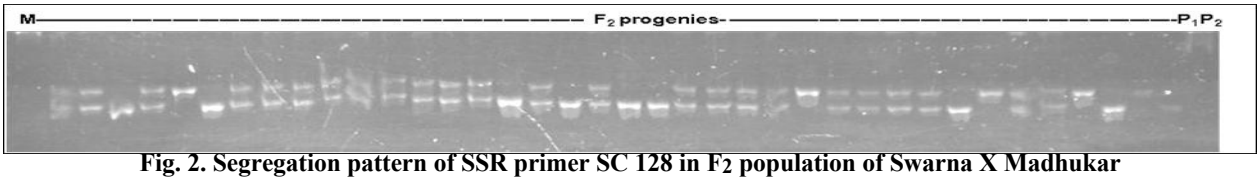

Fig. 2. Segregation pattern of SSR primer SC 128 in F_2 population of Swarna X Madhukar

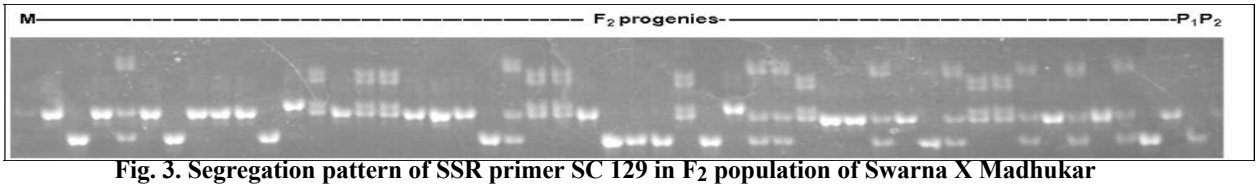

Fig. 3. Segregation pattern of SSR primer SC 129 in F_2 population of Swarna X Madhukar

Legend: M-100bp DNA marker, P_1 – Swarna, P_2 – Madhukar

Table 4. Single marker analysis for iron and zinc with SC120, SC128 and SC129

Trait	Marker	R^2 %
Iron content in the grain	SC120	2.91
	SC128	0.67
	SC129	13.09
Zinc content in the grain	SC120	7.76
	SC128	7.12
	SC129	19.51

Conclusion: The genetic modifications offer good opportunities to increase the iron and zinc content in rice grains. Biofortification is being projected as one of the sustainable and feasible key strategy for addressing the hidden malnutrition across the world. The markers identified could be used as a tool for identifying and mapping of new high iron and zinc content genes. The association could be made more stringent by further analysis of more number of lines and using more number of markers. The knowledge of QTL analysis and the information of DNA in identified genes on mineral accumulation is helpful for the identification of interesting alleles of relevant genes. Further studies have to be conducted to know the genetics involved in the inheritance of high iron and zinc density in rice grains and pertinently the breeding strategies are to be formulated.

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