

Improvement of Iron and Zinc in Rice Varieties Through Conventional and Molecular Approaches

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Abstract

Rice (Oryza sativa L.) is the 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. Scientific evidence shows that food fortification is technically feasible without compromising agronomic productivity. Identification of germplasm with high grain iron and zinc and understanding the genetic basis of their accumulation are the prerequisites for manipulation of the micronutrients. A line derived from the cross between Samba Mahsuri x Chittimuthyalu with short bold grains, semi-dwarf with high yield potential, medium duration, good quality characters with high iron and zinc in brown rice was identified through conventional breeding approaches and some more fixed lines with medium slender and long slender grain types with high iron and zinc are in the pipeline.

Keywords: Biofortification, iron, zinc, genetic diversity, mapping, QTL, candidate genes

Introduction

Rice (*Oryza sativa* L.) occupies the enviable prime place among the food crops cultivated around the world. It is a predominant staple food and a major source of dietary carbohydrate for more than half of the world's population (Zimmermann and Hurrell 2002). Unfortunately, it is a poor source of essential micronutrients such as iron, zinc and vitamin A. Modern agriculture has had reasonable success in meeting the energy needs of developing countries. In the past 40 years, agricultural research in developing countries has met Malthus' challenge by placing increased cereal production at its center. However, agriculture must focus on a new paradigm that will not only produce more food, but bring us better quality food as well. 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 (Welch and Graham 2004; Monasterio et al 2007).

Genetic variation for micronutrients in rice was reported to be narrow especially for iron and zinc (Gregorio et al 2000; Zhang et al 2004). With 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 (Vreugdenhil et al 2004). Using bioinformatics, several candidate genes associated with iron and zinc metabolism have been identified with availability of genome sequence in rice and their role in increased micronutrient content in grain is being explored through structural and expression analysis.

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 (RDA) for a range of vitamins, minerals and proteins. To overcome this, a genetic approach called bio-fortification (Bouis 2002) has been developed, which aims at biological and genetic enrichment of food stuffs with vital nutrients including vitamins, minerals and proteins. Ideally, once rice is bio-fortified with vital nutrient. 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 to reach and provide with essential nutrients to the malnourished population in rural India.

Using a plant breeding approach to address micronutrient malnutrition would provide a new tool in combating the problem. The micronutrient-density traits are stable across environments. It will be possible to improve the content of several limiting micronutrients together in the grains. High nutrient density in grains will give benefit to consumers and also produce more vigorous seedlings in the next generation. As staple foods are eaten in large quantities everyday by malnourished poor adding of even small quantities of micronutrients makes the difference. With the help of molecular markers the loci associated with nutrient content in grains can be identified and used for marker assisted selection in regular breeding programs.

Malnutrition is the most common cause of zinc deficiency (Ronaghy 1987) and 25% of the world's population is at the risk of zinc deficiency (Maret and Sandstead 2006). In Asia and Africa, it is estimated that 500-600 million people are at risk for low zinc intake (HarvestPlus 2010). Health problems caused by zinc deficiency include anorexia, dwarfism, weak immune system (Solomons 2003) skin lesions, hypogonadism, and diarrhea (McClain et al 1985). Males aged between 15-74 need about 12-15 mg of zinc daily while females aged between 12-74 need about 68 mg of zinc daily (Sandstead 1985). In the last two decades, new research findings generated by the nutritionists have brought to light the importance of vitamins, minerals (micronutrients) and proteins in maintaining good health, adequate growth and even acceptable levels of cognitive ability apart from the problem of protein energy malnutrition. In this context, the focus is on breeding for nutritional enhancement to overcome the problem of malnutrition. In brown rice, iron content ranges from 6.3 to 24.4 µg/g and zinc concentration from 13.5 to 28.4 µg/g. Such differences in iron and zinc concentrations, suggest the presence of a genetic potential to increase the levels of these micronutrients in rice grains (Gregorio 2002). Major nutritional problems in rice consuming countries comprise malnutrition and deficiencies of iron, zinc and vitamin A. Efforts have been made at Directorate of Rice Research (DRR) in collaboration with National Institute of Nutrition to evaluate Basmati, non-Basmati and high yielding rice cultivars collected from different parts of the country to study the iron and zinc in grains. This led to the identification of several varieties with relatively high iron and zinc in grains of rice germplasm and possibility of breeding to enhance iron and zinc contents in rice grains. Biofortification could be the best alternative to achieve adequate levels of minerals in the diets of poor

people around the globe. Besides developing improved crop varieties, another challenge is to obtain farmers willingness to grow them and acceptance by consumers. Therefore, biofortified crops need to perform as well as popular cultivars in field and maintain popular grain characteristics as form, taste and cooking properties.

Review of recent advances in research

Conventional breeding approach

Estimation of Fe and Zn content in grain was made by microwave digestion: Approx 0.8 g of the sample was taken in Teflon PFA digestion vessels to which the acid mixture (3 ml HNO₃:1 ml H₂O₂) was added and digested in Mars Express CEM microwave digestion system. Iron and zinc was quantified in Varian Techtron Atomic absorption spectrophotometer. Among the varieties iron (mg/100g) was ranged from 0.24 (PTB 51) to 3.44 (MSE 9) and zinc content varied from 1.01 (Karjat 3) to 3.27 (Ratna). Ten varieties each with high iron and zinc content (Table 1) were identified and some of these lines were used in the breeding programme to develop high nutritional genotypes. Selections were made in segregating populations and stabilized lines with high Fe and Zn content with good quality and yield were identified.

Forty-eight hybrids were used to study the association between the levels of both iron and zinc with grain yield. Grain yield had significant and positive correlations with productive tillers per plant, test-weight and number of grains per plant. The levels of iron and zinc in grains had no correlation with grain yield (Nagesh et al 2012). Therefore, simultaneous selection and breeding can be taken up to enhance iron and zinc levels in grains and grain yield. Path analysis revealed that selection of more number of productive tillers per plant, more number of grains per panicle and high test-weight will be useful in increasing the grain yield. 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 (Sperotto et al 2012).

Molecular studies and mapping

Several QTLs have been mapped for micronutrient content in rice grains using various germplasm sources including wild species. Three QTLs for iron on chromosomes 7, 8 and 9 in recombinant inbred lines (RIL) population have been reported by Gregorio et al (2000). Stangoulis et al (2007) identified three QTLs for iron on chromosomes 2, 8 and 12, and two for Zn on chromosomes 1 and 12 in doubled haploid mapping population.

Table 1. Rice varieties with high levels of iron and zinc mg/100g) in grains

Genotype	Grain type	Polished rice (mg/100g)		
		0%	5%	10%
Iron level in grains				
MSE-9	LB	3.44	1.24	1.08
Kalanamak	SB	3.4	1.21	1.09
Kanchana	MS	2.04	1.28	0.66
Karjat 4	MS	2.56	2.06	1.90
Chittimutyalu	SB	2.49	1.40	0.98
Udayagiri	SB	3.01	0.95	0.90
Jyothi	LB	1.98	1.49	0.40
VRM 7	SB	2.28	0.79	0.78
Matta Triveni	SB	2.61	0.70	0.70
Varsha	SB	3.75	1.12	0.81
Zinc level in grains				
Chittimutyalu	SB	3.05	2.57	2.44
Poornima	SS	3.13	2.78	2.70
ADT-43	MS	3.09	2.66	2.09
Ranbir Basmati	LS	3.09	2.83	2.74
Type-3	LS	3.03	2.83	2.65
Udayagiri	SB	3.01	1.95	1.13
Ratna	LS	3.27	2.52	2.30
Jyothi	LB	3.13	2.24	2.06
Pant Sugandh 17	LS	3.25	2.47	2.06
Kesari	MS	3.15	1.99	1.93

Two QTLs for iron on chromosomes 1 and 9 and three QTLs for zinc on chromosomes 5, 7 and 11 were reported in *indica* population (Lu et al. 2008). Using another RIL population derived from *indica* and *japonica*, four QTLs for iron on chromosomes 1, 3, 6 and 7 and three QTLs for zinc on chromosomes 6, 7 and 10 were identified (Norton et al 2009). Using introgression lines of *O. rufipogon*, Garcia-Oliveira et al (2009) identified QTLs for iron on chromosomes 2 and 9 and for Zn on chromosomes 5, 8 and 12 with a major QTL for zinc on chromosome 8. In another study, using backcross inbred lines (BILs) from Swarna and *O. nivara*, two QTLs for grain iron and zinc content on chromosomes 1 and 3 were identified (Swamy et al 2011). Based on EST and massively parallel signature sequence (MPSS) of candidate genes, Chandel et al (2011) reported QTLs governing iron and zinc concentration in rice grains. Seven QTLs for iron, and seven QTLs for zinc concentrations in unpolished rice were identified on chromosomes 1, 3, 5, 7 and 12 (Anuradha et al 2012). Using selective genotyping approach, three loci associated with high content of iron and zinc in grains were mapped on chromosomes 3, 4 and 8 in Chittimutyalu, a landrace and four loci on

chromosome 3, 4, 6 and 12 were mapped in Ranbir Basmati variety at DRR (Fig. 1 and Fig. 2). Two loci from chromosome 3 and one locus from chromosome 4 were found to be common between the two donors associated with iron and zinc metabolism. In the segregating population derived from Samba Mahsuri x Chittimutyalu, recombinants with *sd1* gene from Samba Mahsuri and aroma gene from Chittimutyalu were identified with maximum back ground genome of Chittimutyalu and high concentrations of iron and zinc in grains.

a) Transgenics

Using ferritin gene from soyabean, transgenic rice has been developed with two fold increase of iron in the endosperm (Goto et al 1999). Increased iron and zinc content after polishing has been demonstrated in transgenic rice lines with ferritin gene (Vasconcelos et al 2003). Activation tagging *OsNAS3* enhanced iron and zinc content in shoots and roots rice (Lee et al. 2009). Transgenic lines over-expressing *OsIRT1* showed enhanced iron and zinc content in shoots, roots and mature seeds with increased tolerance to iron deficiency at the seedling stage (Lee and An 2009).

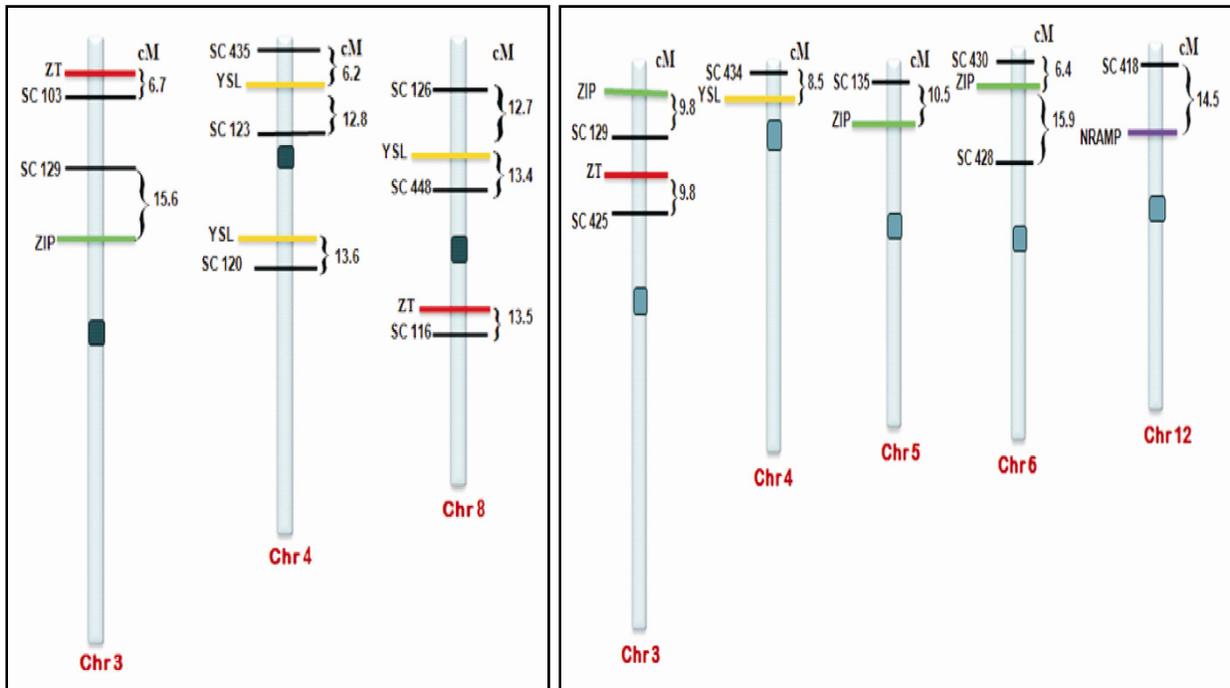


Figure 1. Tentative SSR markers based linkage maps for regions associated with enhanced micronutrient accumulation in grains of F_2 lines: with increased iron level from Samba Mahsuri x Chittimutyalu (bottom left) and lines with increased zinc level from of Samba Mahsuri x Ranbir Basmati (right)

Over-expression of *OsNAS1* gene in endosperm of rice grain showed that two fold increase of iron in polished rice (Zheng et al 2010). Transgenic rice lines with significant increase of iron and zinc in unpolished grains have been developed by over expression of *OsNAS1*, *OsNAS2* and *OsNAS3*. Overexpression of *OsNAS2* has resulted in four fold increase of iron and two fold increase of zinc in polished grain (Johnson et al 2011). In another study, over expression *OsNAS2* elevated levels of nicotianamine (NA) and zinc uptake, translocation and seed loading (Lee et al 2011).

Over-expression of nicotianamine synthase 2(*NAS2*), resulted in 3-fold rise in Fe content in mature seeds (Lee et al 2012). Recently three genes viz., ferritin from soybean, *OsYSL2* from rice and *HvNAS1* were incorporated in rice and fourfold increase of iron was observed in polished seeds (Masuda et al 2012). In addition, the antinutrients also play an important role influencing the bioavailability of dietary non-haem Fe, Zn and other nutrients to human. Phytate (phytic acid or phytin) is a major anti-nutrient present in legume seeds and cereal grains that reduces the bio-availability of dietary Zn and non-heme Fe. While breeding for low phytate genotypes has become a nutritional objective (Raboy 2001; 2002), the role of phytate in plant metabolism is very critical for general plant metabolism. Although there were significant phenotypic correlations between phytate and micronutrients, the QTLs of phytate were not located on the same chromosomal regions as those found for Fe, Zn and Mn, suggesting that they were genetically different and thus using molecular markers in breeding and selection would modify the level of phytate without affecting grain micronutrient density (Garcia-Oliveira et al 2009).

Multiple-transgene approach is used to elevate rice grain iron concentration (Wirth et al 2009). The *ferritin* gene from common bean and the *phytase* gene from *Aspergillus fumigatus* were expressed under the control of the *globulin (Gbl-1)* endosperm-specific promoter, while *AtNAS1* was over-expressed under the control of the 35S promoter. Upto 6.3 fold increase of iron concentration in white rice was seen, depending on the iron concentration present in the nutrient solution (Wirth et al 2009).

b) Bioinformatics and expression analysis

Several genes related to strategy micronutrient transport, phytosiderophore biosynthesis and mineral ion sequestration and grain portioning have been identified in rice (Eide et al 1996; Zhao and Eide 1996; Grotz et al 1998; Eckdard 2000; Vert et al 2001; Waters et al 2002). Forty three putative candidate genes belonging to five gene families viz. *OsYSLs*, *OsZIPs*, *OsNRAMPs*, *OsFROs* and Ferritin have been identified

in rice (Gross et al 2003). *OsYSL* genes are components of Strategy II of metal transport found in cereals, encoding oligopeptide phytosiderophore transporter proteins (Curie et al 2001). The rice genome contains 18 putative YSL family genes with listed nomenclature as *Oryza sativa Yellow Stripe-like* or *OsYSLs* (Gross et al 2003; Koike et al 2004; Schaaf et al 2004; Raymond et al 2004), among which only three members viz, *OsYSL2*, *OsYSL15* and *OsYSL18* have been characterized in detail (Aoyama et al 2009, Inoue et al 2009; Ishimaru et al 2006; Koike et al 2004). ZIP, Zinc transporter family (zinc-regulated transporter/iron regulated transporter proteins) plays a critical role as transporter protein and found to be correlated high grain iron concentration (Guerinot 2000). The involvement of plant NRAMP (Natural Resistance-Associated Macrophage) protein in iron transport in rice was demonstrated by Curie et al (2000). *OsNRAMP1* functions as a metal efflux transporter participating in the export of metals from the vacuolar compartment to the cytosol, resulting in increased metal concentration available to be transported to the seeds (Sperotto et al 2010). The rice *ferric chelate reductase oxidase* (*OsFRO*) gene family was found to be associated with metal transport along the concentration gradient (Banerjee and Chandel 2011). Ferritins are iron storage proteins and have been shown to increase iron content in rice through transgenics (Goto et al 1999). In addition, Nicotianamine synthase (*NAS*) is also found to be required for the biosynthesis of *nicotianamine* (NA), a non-peptidyl metal chelator that is believed to be a co-substrate of the YSL proteins (Schaaf et al 2004).

Expression analysis studies of 25 metal related genes in root and leaf tissues of twelve diverse genotypes at maximum tillering and mid grain filling stages showed expression of maximum number of genes in flag leaf with maximum variation. The differential expression at maximum tillering stage was found to be correlated with grain zinc content and at grain filling stage especially of root transcriptome was observed to be correlated with grain iron content. Root transcriptome profiling revealed *OsZIP4*, *OsZIP11*, *OsNRAMP5*, *OsNRAMP7*, *OsYSL2*, *OsYSL4*, *OsYSL6*, *OsYSL9*, *OsNAAT1* and *OsNAC* expressed in both developmental stages; *OsFER1*, *OsVIT1* and *OsFRO2* expressed in maximum tillering stage and *OsIRT1*, *OsFER2*, *OsZIP7*, *OsZIP8*, *OsZIP9*, *OsNRAMP4*, *OsNRAMP6* and *OsYSL12* expressed in grain filling stages (Banerjee and Chandel 2011).

Innovative studies underway

Considering the complexity of genetics controlling the iron and zinc content in the rice grains and their loss after processing for polished, the strategy of increasing

iron and zinc content in rice grains after polishing is mostly based on biotechnological approaches.

- Several transgenics with single genes showed enhanced iron and zinc content and are being evaluated at various regulatory systems.
- While the mapping of genomic regions for iron and zinc content is progressing, co-location of iron and zinc is also being studied simultaneously (Stangoulis et al 2007; Swamy et al 2011)
- Attempts are also being made to study co-location of QTLs for iron and zinc and other yield components (Ishimaru et al 2011; Anuradha et al 2012).
- Expression analysis using real time PCR to validate the identified genes and allele mining for efficient genes is also underway (Banerjee and Chandel 2011)
- Recently three genes viz., ferritin from soybean, OsYSL2 from rice and HvNAS1 were incorporated in rice and fourfold increase of iron was observed in polished seeds (Masuda et al 2012)
- Molecular characterization of the mutated genes which result in low grain phytic acid levels will provide insights into phytic acid biosynthesis in plants, and facilitate the development of *lpa* commercial lines with increased iron bioavailability (Sperotto et al 2012)
- Anti-nutrients like tannins and other extractable phenols are known to lower the iron bioavailability, further research is underway to confirm these initial observations and to indicate whether these traits should be addressed by breeding programs (Glahn et al 2002; Prom-u-thai et al 2006; Sperotto et al 2012)

Prospects of achieving the breeding goal

Biofortification is being projected as one of the sustainable and feasible key strategy for addressing the hidden malnutrition across the world. Concerted efforts of several laboratories leading to the identification of genes involved in micronutrient metabolism and their use to enhance micronutrient content especially iron and zinc in rice grain was taken up. Molecular analysis of much more populations and further generations would increase the stringency of the loci identified for the iron and zinc content in the rice grains. Several donors have been identified with high zinc in the endosperm after polishing and are being used to develop the breeding lines and varieties with high zinc content. For enhanced iron content in the polished rice, attempts are being made through transgenic strategy and mapping of QTLs

in brown rice. Several rice breeding lines developed through conventional and molecular approaches with enhanced zinc content in grains are under evaluation in multilocation trials across the world suggesting the possibility of zinc rich rice varieties in near future. Research efforts must be emphasized on producing biofortified foods in several other staple food crops to help the malnourished poor across the world. Biofortified crops, if successfully developed can have a large positive impact on micronutrient status. Rigorous cost-effective studies of biofortified crops will need to be undertaken, especially in the context of other food-based interventions.

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