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INVITED ARTICLE

Biofortification of Rice

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. Several biofortified rice varieties with high zinc in polished grains have been released under All India Coordinated Rice Improvement Project (AICRIP).

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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. 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. 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 Biofortification has been developed, which aims at biological and genetic enrichment of food stuffs with vital nutrients. 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.

Using the 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. High nutrient density not only can benefit the consumer but also produce more vigorous seedlings in the next generation. Because of staple foods 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. 25% of the world's population is at risk of zinc deficiency. In Asia and Africa, it is estimated that 500-600 million people are at risk for low zinc intake. Health problems caused by zinc deficiency include anorexia, dwarfism, weak immune system skin lesions, hypogonadism and diarrhea. 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. 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. In this context, breeders are now focusing on breeding for nutritional enhancement to overcome the problem of malnutrition. The range of iron and zinc concentrations in brown rice is 6.3 – 24.4 mg g^{-1} and 13.5 – 28.4 mg g^{-1} respectively. There was approximately a fourfold difference in iron and zinc concentrations, suggesting some genetic potential to increase the concentration of these micronutrients in rice grains. Major nutritional problems in rice consuming countries comprise malnutrition and deficiencies of iron, zinc and vitamin A. Realizing the importance of crop biofortification in achieving the nutritional security of the nation, ICAR has sanctioned an Consortia Research Platform (CRP) to ICAR-Indian Institute of Rice Research (IIRR), during the plan period of 2014-2017 for enhancing the nutritional status of major food crops of the country through biofortification approach targeting rice, wheat, maize, sorghum, pearl millet, small millets, and centers encompassing institutes of ICAR, ICMR, state agricultural universities (SAU) and traditional universities. With the availability of the genotypes and genes as proof of concept for biofortification and considering the significant progress witnessed in several crops with respect to biofortification, the CRP aims at development of cereals biofortified with enhanced â-carotene, quality protein, iron, zinc and their bioavailability studies.

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 in this direction is to obtain farmers willingness to grow them and acceptance by final consumers. For that, researchers must keep in mind that biofortified crops need to perform as well as popular cultivars in the field and to maintain popular grain characteristics, as form, taste and cooking properties.

Conventional breeding has traditionally been used to improve rice grain quality, milling characteristics, cooking and eating qualities. By using conventional breeding and selection techniques, cultivars with the highest iron content and the lowest inhibitors content might be bred into high-producing lines. Attempts to increase rice grain protein had negative impacts on yield. This lack of success with protein delayed for many years new attempts to enhance other nutritive aspects of rice grains through breeding. However, research is revealing that higher grain iron and zinc content does not necessarily impair agronomic productivity. High iron and zinc traits can be combined with improved agronomic traits. Additionally, several reports indicate a positive correlation between iron and zinc grain concentrations, raising the possibility of simultaneously biofortifying crops with more than one micronutrient. Conventional breeding is made even more efficient by integrating tools like marker assisted selection for key agronomic and nutritional traits, molecular biology for gene discovery of relevant traits and transgenics to increase existing levels or provide opportunities that are lacking within the respective crop species.

A strategy to enhance iron bioavailability in cereal grains was taken up wherein the most explored path is the reduction of phytic acid content. The grain phytic acid content was evaluated in different cultivars and mutants, leading to the conclusions that there is a significant interaction between genotype and growth location in terms of phytic acid content, indicating the importance of using suitable cultivars for a given location and that *lpa (low phytic acid)* mutants had consistently lower phytic acid content across locations and seasons. There seems to be sufficient genotypic variation in grain phytic acid content to be used in breeding efforts (Liu et al 2005 Frank et al 2009. Additionally, at least four non-allelic recessive mutations generated by gamma radiation have produced plants with lower grain phytic acid content and these mutations could be introgressed into elite rice lines by breeding, similar to what is already being done for maize and barley.

a)Genetic Studies

Lines contrasting for high iron and zinc content were taken up at Directorate of Rice Research for studying the combining ability and heterosis and various other genetic aspects. Six lines *viz.,* RP Bio 226, Swarna, MTU 1010, IR 64, PR 116 and Mandya Vijaya were crossed (Line × Tester design) with eight testers *viz.,* Chittimutyalu, Ranbir Basmati, Madhukar, Jalmagna, Type 3, Suraksha, Jalpriya and BR 2655 and the resultant 48 F_1 s along with parents were evaluated. The analysis of variance for combining ability revealed significant differences in parents and hybrids indicating the existence of wider variability in the material studied. The ratio of GCA to SCA variances revealed that non-additive gene action was predominant in inheritance of all characters studied. The *gca* effects of the parents revealed that the lines *viz.,* MTU 1010 and PR 116 and testers *viz.,* Madhukar, Jalmagna, Jalpriya and BR 2655 were found to be promising general combiners for grain yield and its components. None of the parents recorded significant positive *gca* effects, hence no parent was found to be general combiner for grain iron content. Chittimutyalu, Madhukar, PR 116, IR 64 and RP Bio 226 were found to be good general combiners for grain zinc content. Based on significant *sca* effects, ten hybrids were identified as promising specific combiners for grain yield and other characters. Mandya Vijaya × Jalmagna, PR 116 × Chittimutyalu, PR 116 × Suraksha, Swarna × Ranbir Basmati and Mandya Vijaya × Type 3 were had good specific combiners for grain zinc content. Based on heterosis studies MTU-1010 × Jalmagna, PR116 × BR-2655, MTU-1010 × Madhukar hybrids were found highly heterotic for grain yield. MTU-1010 × Jalmagna hybrid exhibited significant positive heterosis for panicle length, number of tillers, number of productive tillers per plant, test-weight, number of grains per panicle, grain length, grain breadth and negative significant heterosis in desirable direction for days to 50 per cent flowering and days to maturity. IR64 × Chittimutyalu and PR 116 × Chittimutyalu were found to be good heterotic hybrids for grain iron and zinc content. The association studies among 48 hybrids during *kharif,* 2010 revealed that grain yield had significant positive correlation with productive tillers per plant, test-weight and number of grains per panicle. Grain iron content and zinc content had no correlation with grain yield. Simultaneous selection / breeding can be taken up to enhance grain iron and zinc and grain yield because of no correlation (Nagesh et al 2012). Path analysis revealed 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 .

b)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). Three QTLs were identified for iron on chromosomes 2, 8 and 12, and two for Zn on chromosomes 1 and 12 in doubled haploid mapping population. 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 . 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. Using introgession lines of *Oryza 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 *Oryza nivara,* two QTLs for grain iron and zinc content on chromosomes 1 and 3 were identified. Based on EST and MPSS of candidate genes, Chandel et al (2011) reported QTLs governing iron and zinc concentration in rice grains. Seven QTLs for iron concentration and seven QTLs for zinc concentration in unpolished rice were

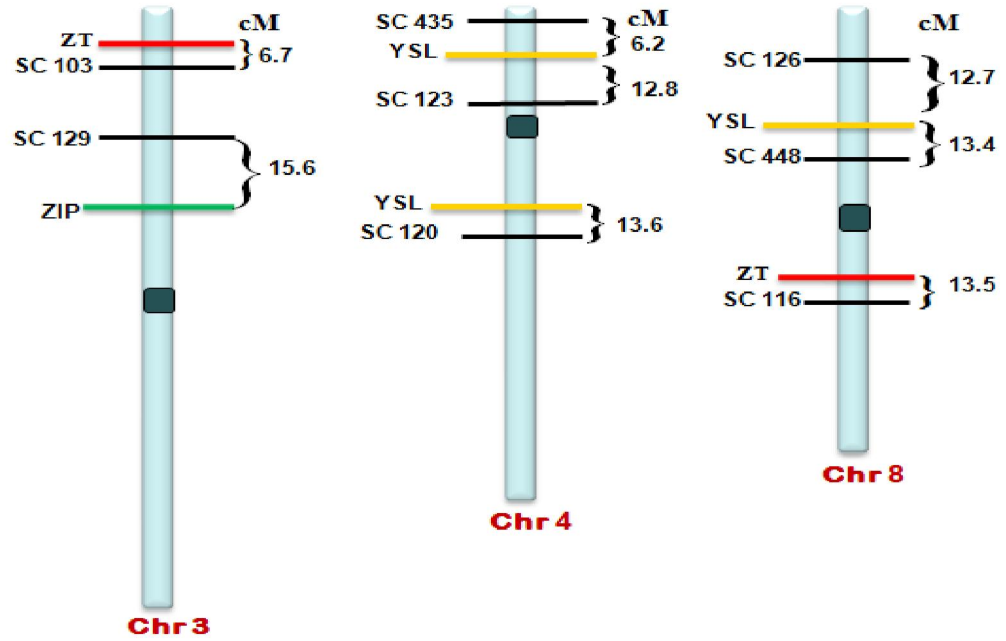

Fig.1. Tentative SSR based linkage maps for regions associated with enhanced iron accumulation in F_2 lines from Samba Mahsuri/Chittimuthyalu

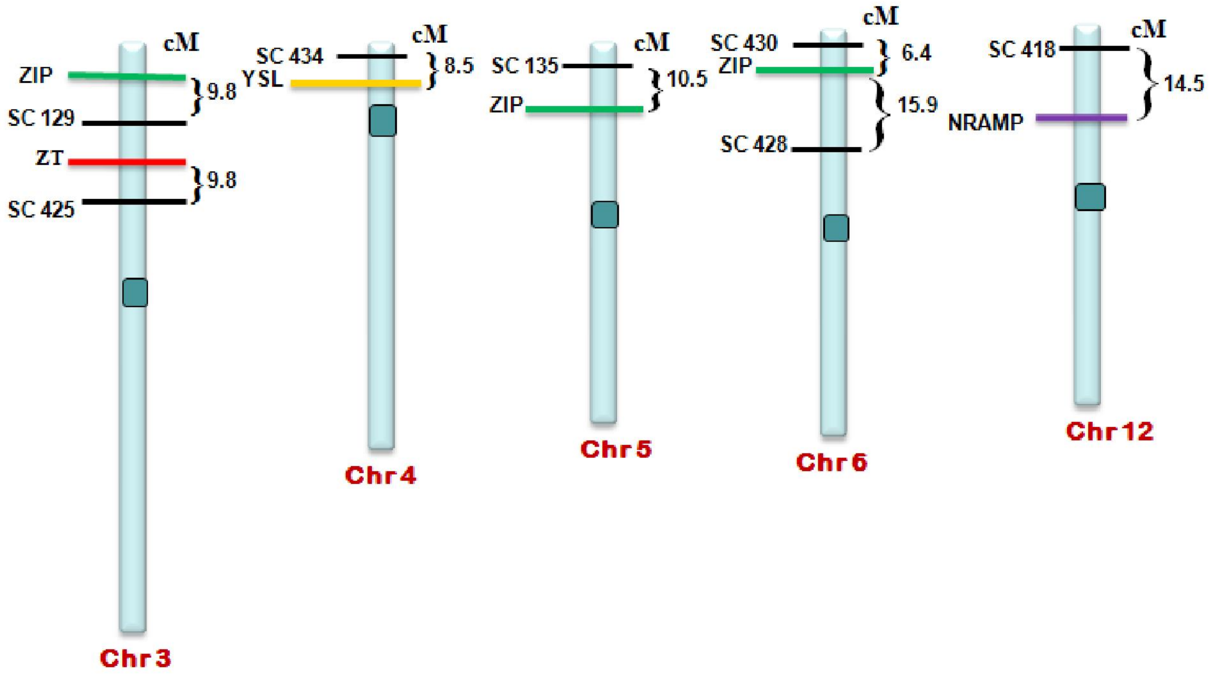

Fig.2. Tentative SSR based linkage maps for regions associated with enhanced zinc accumulation in F_2 lines from Samba Mahsuri/Ranbir Basmati

identified on chromosomes 1, 3, 5, 7 and 12 . Using selective genotyping approach, three loci associated with high iron and zinc content in grains were mapped on chromosomes 3, 4 and 8 in Chittimuthyalu, a landrace and four loci on chromosome 3, 4, 6 and 12 were mapped in Ranbir Basmati variety at Directorate of Rice Research (Fig 1 and Fig 2). Two loci from chromosome 3 and one locus from chromosome 4 found to be common between the two donors associated with iron and zinc metabolism. Recombinants with *sd1* gene from BPT5204 and aroma gene from Chittimuthyalu were identified from BPT 5204 and Chittimuthyalu segregating population with maximum back ground genome of Chittimuthyalu and with high iron and zinc content.

a) Transgenics

Using ferritin gene from soyabean, transgenic rice has been developed with two fold increase of iron in the endosperm . Increased iron and zinc content after polishing has been demonstrated in transgenic rice lines with ferritin gene. Activation tagging *OsNAS3* enhanced iron and zinc content in shoots and roots rice. 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. Over-expression of *OsNAS1* gene in endosperm of rice grain showed that two fold increase of iron in polished rice. 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. Recently three genes *viz*., ferritin from soybean, OsYSL2 from rice andHvNAS1 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 humans Phytate (phytic acid or phytin) as a major anti-nutrient present in legume seeds and cereal grains, reducing the bio-availability of dietary Zn and non-heme Fe. While breeding for low phytate genotypes has become a nutritional objective, 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 .

Multiple-transgene approach is used to elevate rice grain iron concentration. 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.

Thousands of rice germplasm lines have been screened for iron and zinc content in brown and polished grain across the world, and many promising donors were identified. However, 90 % of iron and 40 % of zinc is lost during polishing[6,7]. Several QTL for grain zinc concentration and genes associated with zinc metabolism have been reported in rice[8]. Using donor from the HarvestPlus program, 'DRRDhan 45' was released by IIRR. Two more varieties with high nutrient content in polished rice were released as 'Chattisgarh zinc rice-1' for the state of Chattisgarh by Indira Gandhi Krishi Viswavidyalaya (IGKV) and as 'Mukul' (CRR Dhan 311) for the state of Odisha by ICAR-National Rice Research Insitute. Transgenics in rice have been developed with 3-4 times as much iron than wild-type using different sources of ferritin gene[9,10].

Protein

Several landraces and released varieties have been characterized for their protein and amino acid profiles in rice and 'Heera' an old variety of rice found to have >10% protein. The mean crude protein content of the varieties as estimated using Kjeldhal method was in the range of 6 – 8 %[11]. 'CRRDhan 310' with >10% protein in polished rice developed by ICAR-NRRI has also been nationally released. Genomic regions and genes associated with protein in rice are being deciphered[12].

Provitamin A

Golden rice was developed with three genes for biosynthesis of â-carotene in the rice grain and latest version is GR2R with > 20 ppm of total carotenoids. Three research groups in India viz., ICAR-Indian Agricultural Research Institute (IARI), IIRR and Tamil Nadu Agricultural University (TNAU) have been involved in development of Indian versions of Golden rice from the original prototype in collaboration with the International Rice Research Institute (IRRI) supported by Department of Biotechnology (DBT).

Prospects

Biofortification is being projected as one of the sustainable and feasible key strategy for addressing the hidden malnutrition across the world. Initially research efforts in the agriculture were prioritized for achieving the self sufficiency for food grains, and now the scope is also extended to the biofortification of major food crops as a strategy to ensure nutritional security to address the malnutrition. In India, a variety with high zinc (DRR Dhan 45) and a variety with high protein (CRR Dhan 310) in polished rice were developed through conventional breeding without compromising yield, and later were nationally released through AICRP-rice. 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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