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Association of OsYSL9 gene with high iron and zinc content in rice grains and identification of elite rice lines

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ABSTRACT: *Rice with significant mineral content would be great advantage to half of the world population. Identification of mineral enhancing genomic regions in rice could help to further increase the content of iron / zinc or both in grain. Considering the advantages of BPT 5204 and Chittimutyalu, RIL (Recombinant Inbreed Line) population (300) was developed and grain iron and zinc content (brown and polished) was estimated in F8 generation. The iron content in brown rice was ranged from 4.4ppm to 32.6ppm and in polished rice 1.3-12.6ppm, while the zinc content was 8.1-57.5ppm in brown rice and 5.8-37.4ppm in polished rice. Loss of iron during polishing is many times more than zinc. The OsYSL9 putative gene showed significant association with iron and zinc in both brown and polished rice grains. Out of 300 populations six lines have high zinc content in endosperm with good yield.*

Key words: *iron and zinc, rice biofortification, Yellow stripe-like (YSL), single-marker analysis (SMS).*

INTRODUCTION

Minerals play important role in various physiological processes of plant and animals. In the earth's crust Fe is 4th most element and insoluble ferric oxide form at neutral pH in aerobic environment (Guerinot and Yi, 1994). Zinc is the twenty fourth most abundant element on earth's crust with a content of 75 mg kg^{-1}. Soil contains 5-770 mg kg^{-1} of Zn with an average of 64 mg kg^{-1}, sea water has 30 µg Zn L^{-1}, and the atmosphere contains 0.1 - 4 µg Zn m^{-3} (Emsley, 2001). Rice is the primary staple food for 50% of the world's population and it is generally considered as poor source of essential micronutrients such as Iron (Fe) and Zinc (Zn) (Bouis and Welch, 2010). In contradiction, several studies on rice germplasm having high iron and zinc in rice grains have been reported and they were used as donors through traditional breeding approach and developed lines possessing comparatively high Fe and Zn content in grains (Brar *et al.*, 2011; Gregorio *et al.*, 2000; Sanjeeva Rao *et al.*, 2014; Martínez *et al.*, 2010). However, almost 80% of iron and around 30% of zinc was lost during polishing of the brown rice that contains 10–11 ppm Fe and 20–25 ppm Zn (Sellappan *et al.*, 2009). The average daily intake of rice is around 220g and thus polished rice having 45.5 ppm (mg/Kg) iron and 54.5 to 68.2 ppm (mg/Kg) zinc can only meet the RDA (FAO/WHO, 2000) without considering bioavailability (Sanjeeva rao *et al.*, 2014). Hence, there is a need to further enhance the grain mineral content by traditional or marker assisted breeding methods.

Iron and zinc in plants is influenced by several families of putative iron transporters, including YSL (Yellow stripe-like), ZIP (ZRT, IRT-like proteins), OsNAS2 (nicotianamine synthase 2) and NRAMP (natural resistance associated macrophage protein) while ferritin gene is specific for iron. Recently, over-expression of single rice gene (OsNAS2) was reported to enhance the concentration of both iron (Johnson *et al.*, 2011) and zinc (Lee *et al.*, 2011) in rice grain. Around 18 OsYSL genes have been reported on different chromosomes of rice and they are involved in the uptake and long-distance transport of minerals by producing ferric-chelating phytosiderophore complexes (Gross *et al.*, 2003; Garcia-Oliveira *et al.*, 2009; Lu *et al.*, 2008; Norton *et al.*, 2009; Chandel *et al.*, 2011). It was opined that activation of OsYSL16 gene could enhance the Iron efficiency in rice grain (Sichul Lee *et al.*, 2012). RNAi line of OsYSL2 exhibited decreased Fe-translocation to seeds, lower Fe

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concentrations in shoots and seeds and greater accumulation of Fe in the roots (Ishimaru *et al.*, 2010) which indicates that OsYSL genes might be useful in further enhancement of grain iron and zinc content.

Hence, the objectives of this study are to evaluate iron and zinc concentrations in 300 recombinant inbred line (RIL) population derived from the cross BPT 5204×Chittimutyalu, to identify the OsYSL 9 gene contribution for both iron and zinc in rice grains of RIL population and to identify elite lines that contain promising grain mineral content as well as yield.

MATERIAL AND METHODS

Phenotyping

Chittimutyalu is a land race that is being cultivated in some regions of southern India. Its grain is short bold, possess aroma and comparatively contains higher zinc and iron among the cultivars. BPT 5204 was released in 1986 from Andhra Pradesh. It has superior grain quality and eventually emerged as a leading variety of southern states, Tamil Nadu, Andhra Pradesh and Telangana, of India (AICRIP, 2015). Hence, a population of 300 recombinant inbred lines (RILs) derived from the cross BPT 5204 X Chittimutyalu using single seed descent method was used for RIL multiplication.

Dried seeds of F7 were sown at 2 to 5 cm depth on wet bed and the germinated seedlings were transferred to IIRR field after 25 days. The 60 plants of two parents were planted in 3 rows of 20 plants each adopting a uniform spacing of 20 cm between rows and 15 cm between plants. Standard agronomic practices and need based plant protection measures were adopted uniformly to raise the crop. All panicles from five plants in the middle row were collected and seed was bulked for Fe and Zn analysis in 300 RILs. Dried F8 seed of each line was dehusked and polished separately using non-ferrous and non-zinc de-husker and polisher (*krishi international*) to avoid metal contamination as described in Ravindra babu *et al.* (2014 biofortification bulletin). Five grams of brown and polished rice samples from each line were separately subjected to energy dispersive X-ray fluorescent spectrophotometer (ED-XRF) for Fe and Zn was estimation (Sanjeeva rao *et al.,* 2014). This equipment gives 4 ppm less than the ICP which is the standard equipment for elemental analysis.

Genotyping

Targeted OsYSL9 gene (LOC_Os04g32050) (Gross *et al.,* 2003) sequence was downloaded from Rice Genome Annotation Project (*http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice*) and subjected to nucleotide blast tool in NCBI. Ten primers were designed (Table 1) using primer3 online software (*http://primer3plus.com.*) and these oligo-nucleotides were synthesized by Eurofins genomics.

Paddy of each line was separately sown in petridish on blotting paper. Tissue from 15 days seedlings was used for DNA isolation Zheng *et al.* (1995). Quantification of DNA was done with Nono-drop (*Thermo scientific*). The designed primers were used to study polymorphism in the RIL population along with parents by setting up polymerase chain (PCR) reaction reaction following;

The PCR mixture contained 30 ng/ µl of template DNA, 1 X PCR buffer (10 mM Tris, pH8.4, 50 mM KCl, 1.5 mM MgCl2 and 0.01 mg mL-1 gelatin), 2.5 mM of Mgcl2, five picomoles of forward and reverse primer, 0.05 mM of dNTPs and 1 U of Taq polymerase in a 15 µl of reaction volume. Template DNA was initially denatured at 95°C for five minutes followed by 35 cycles of PCR (Applied Biosystems 2720) amplification with the following parameters. A 60 s of denaturation at 95°C, 1 min of annealing at 55°C and 1 min of elongation at 72°C. A final elongation was done at 72°C for 10 min. Amplicons were separated in 2.5% Agarose gel having ethidium bromide in submarine electrophoresis at 120 V for 2 hour and the gel was processed in gel documentation unit.

Trait correlations between iron and zinc concentrations with marker were analyzed using R statistical software version 3.2.0.

RESULTS AND DISCUSSION

Grain Fe and Zn content

Among the RIL population, iron content in brown rice was in the range of 4.4 to 32.6 ppm and in polished rice 1.3 to 12.6 ppm, while zinc content ranged from 8.1 to 57.5 ppm in brown rice and 5.8 to 37.4 ppm in polished rice. The means and ranges of various yield parameters are presented in Table 2. The mean iron content was 11.4 ppm in brown rice, 5.4 ppm in polished rice. Mean zinc content was 29.8 ppm in brown rice and 20.5 ppm in polished rice.

The distribution of Fe and Zn concentrations in brown and polished rice among the 300 RILs population was shown in Fig. 1. Different levels of iron and zinc content in rice grain were noticed in earlier studies. Anuradha *et al.*, (2014) reported iron and zinc in the ranges of 0.2 to 224 ppm and 0.4 to 104 ppm respectively the brown rice of

Table 1
Primer sequences for OsYSL 9 gene

Sl No	*ID*	*Forwarded primer*	*Reverse primer*	*Aprox. amplicon size*
1	SC3319	AATATCGTTGGGCAGGTTAG	GGTTGTTTCTTTTCTCGACC	600
2	SC3320	CTACGTGTCAGACTCGTATC	AAAATCTCTTGGTTCTTCGC	637
3	SC3321	CTAGTATCCGAAGCCACTTC	TCCTCCACCTAGTAGACAAG	665
4	SC3322	TTGTCTACTAGGTGGAGGAG	TTTTCTGATCCATTGCAAGC	678
5	SC3323	GCTTGCAATGGATCAGAAAA	TTCGGAGATGCCACTAAAAA	693
6	SC3324	TGCTATGTGCTTGTACTGTT	TACCAGTTGAAGAGACAAGC	696
7	SC3325	TGACAATTTCCCATCTCTGG	AACAGTTTTTCAAAGCTCGG	735
8	SC3326	CCTTTTACCTCTCCATGCTT	GAAGATCATTGGCACAGTTG	632
9	SC3327	ACTGTATGGTGCACATCTAC	AATAGCTGCGACAAAGAAGA	706
10	SC3328	TCCATGTTTGTGTCACAGTT	CACTTTTGCGCAGTCTAATT	624

Madhukar×Swarna RIL population. In IRRI38 X Jeerigesanna RIL population, 16.1 to 35.5 ppm zinc content in brown rice with an average of 23.7 ppm was reported (Gande *et al.*, 2014).

Polymorphic study and Single Marker Analysis

Among the ten gene specific markers used to identify polymorphic loci between the two parents BPT 5204 X Chittimutyalu one marker (SC3322) exhibited polymorphism between parents. Therefore, all 300 RILs were genotyped using this marker. Considering overall mean of the RIL population, 53% lines showed donor (Chittimutyalu) OsYSL 9 gene.

SC3322 marker exhibited significant association with both iron and zinc contents of brown and polished rice grains. However, less correlation value observed for Fe content in polished rice is due to more loss of this micronutrient during polishing. There is a strong correlation between zinc content in brown and polished rice which indicates that zinc is more available in endosperm than iron. Though positive association was observed between the marker and days to 50% flowering higher value of micronutrient content was associated with donor gene more than the days to 50% flowering (Table 3 and Fig. 3).

Table 2
Mean values of yield parameters

	Days to 50% flowering	*Plant height in (cm)*	*Productive tillers per plant*	*Five plant yield (gr.)*
Mean	114	107.7	11	29.5
Maximum	130	157.7	20	87.5
Minimum	87	65.3	4	2.7

Grain yield Vs Fe and Zn content

Apart from genotypic potential, yield is also influenced by soil health, nutritional supplements, agronomical practices and environmental conditions. Since all the lines were cultivated under similar conditions, an attempt was made to understand the genotypic relationship between yield and grain iron as well as zinc contents within the population. Among these, two lines, Z-262 and Z-239, contain high zinc as well as iron, however, their yield is less than the mean yield (29.5 grams) of this population (Table 2). The ranges of yield in top ten lines with high iron and zinc were 9.3 to 37.5 and 6.6 to 49.4 grams respectively. Two lines, Z-158 and Z-261, of iron and four lines, Z-69, Z-269, Z-81 and Z-58, of zinc had shown yield above the mean yield. Since various biofortification programmes were initiated to alleviate the hidden hunger among the poor who are mostly dependent on staple foods like wheat, rice, etc. and hence, the developed varieties under these projects should target on yield at least on par with cultivars along with grain micronutrient content. Therefore, the top ten lines of iron or zinc and the two lines that contain high zinc and iron are not promising to recommend as varieties, but they can be used as donors in various breeding programmes targeted to enhance iron or zinc.

Though the correlation between micronutrients and yield was nearer to zero (Fig. 3), it appears as negative in the top ten iron or zinc containing lines. Nonetheless, among the top ten high yielding lines of this population (Table 3), majority of them possess grain zinc content in polished rice $\geq$ 16.0 ppm which is considered as the threshold value of Biofortification trial of the AICRIP. Since, the harvest plus target value of zinc is 24 ppm (Harvest plus, 2013) in polished rice and the ED-XRF used in this study gives 4 ppm less than ICP (AICRIP, 2015), eight of the top ten yielding lines contain zinc content $\geq$ 20.0 ppm (adding plus 4 ppm to the values given in the study) which can be

Table 3
Top ten lines with high iron/ zinc content /five plant yield with other parameters.

Genotypes	*SC 3322 marker*	*DFF*	*PH in cm*	*NT*	*PT*	*FPY gr.*	*Iron in ppm*		*Zinc in ppm*	
							Brown	*Polished*	*Brown*	*Polished*
Genotypes with high iron										
Z-266	2	105	98.7	9	9	19.7	15.0	12.6	31.9	28.7
Z-268	2	105	99.7	12	11	13.1	12.9	10.9	32.5	29.8
Z-262	2	109	105.0	9	9	16.7	11.7	10.4	36.4	31.0
Z-239	2	109	86.7	13	12	9.3	12.2	10.0	35.3	32.3
Z-236	2	109	96.3	9	8	16.2	11.6	9.4	28.9	26.6
Z-158	2	117	105.7	14	12	37.5	16.3	9.3	34.7	26.5
Z-261	2	121	114.0	13	12	33.4	17.4	9.3	29.6	24.8
Z-263	2	105	103.3	16	15	28.1	13.3	9.3	29.4	23.6
Z-46	2	98	133.7	13	12	28.8	18.5	9.2	32.6	23.1
Z-233	1	105	122.0	15	14	17	12.0	9.1	27.4	22.1
Genotypes with high zinc										
Z-269	2	124	103.0	13	13	47.1	10.6	6.1	42.1	37.4
Z-239	2	109	86.7	13	12	9.3	12.2	10.0	35.3	32.3
Z-184	2	105	96.7	16	10	6.6	15.5	7.1	39.8	31.3
Z-90	2	124	101.7	8	8	9.6	11.9	5.6	43.1	31.2
Z-81	0	121	118.7	14	12	30.5	13.7	5.4	40.9	31.2
Z-262	2	109	105.0	9	9	16.7	11.7	10.4	36.4	31.0
Z-69	2	124	105.7	10	10	49.4	12.3	5.3	48.3	30.8
Z-145	0	124	117.7	12	10	26.8	14.7	7.0	57.5	30.2
Z-59	2	124	96.7	9	9	10.6	13.7	5.8	42.5	30.2
Z-58	0	105	101.3	15	14	33.7	14.2	6.8	41.3	29.9
Genotypes with high five plant yield										
Z-210	2	89	108.0	13	12	87.5	12.1	3.6	32.1	24.1
Z-209	1	89	104.0	15	13	85.7	8.4	4.6	24.7	17.2
Z-4	2	92	91.7	10	9	78.8	13.2	5.6	36.4	22.3
Z-185	2	89	100.0	18	14	73.6	11.6	4.9	27.9	20.5
Z-234	1	89	105.3	15	13	73.4	9.7	7.3	21.8	19.2
Z-289	0	128	102.0	15	15	71.8	8.9	3.2	21.3	14.6
Z-205	2	124	99.7	11	11	70.7	11.2	5.0	38.7	21.2
Z-72	2	128	103.7	9	8	69.9	12.7	6.3	40.0	27.4
Z-255	2	105	117.0	11	11	69.4	10.4	5.0	37.8	24.3
Z-1	2	124	95.3	14	14	68.7	17.3	6.0	33.2	23.2

Note: In the SC 3322 marker column, '2', '1' and '0' indicates the presence of donor gene, recipient gene and absence of genotypic data respectively. DFF= Days to 50% flowering, PH= Plant height (cm.), PT= Productive tillers per plant, FPY= Five plant yield (gr.), B-Fe= Iron in brown rice, P-Fe= Iron in polished rice, B-Zn= Zn in brown rice and P-Zn= Zn in polished rice (Fe & Zn content in ppm)

considered promising in terms of yield and zinc content. Therefore, they are promising and can be further developed as pure lines. Among these, Z-4, Z-234, Z-1 and Z-72 also contain iron content around 7.0 ppm (which is desired) and the first two come under medium duration while the remaining two fall in long duration categories.

CONCLUSION

There is a strong correlation between iron and zinc content in rice grain both brown and polished rice. One of the designed markers (SC3322) of *OsYSL9 gene has* showed significant association with iron and zinc in both brown and polished rice and therefore, this can be used for further screening programmes. Among this RIL population, four promising lines were identified with zinc and iron content equal to or above the targets of various biofortification programmes and also on par with cultivated varieties in terms of yield. This study also confirms the scope of developing elite varieties combining conventional breeding with marker assisted breeding.

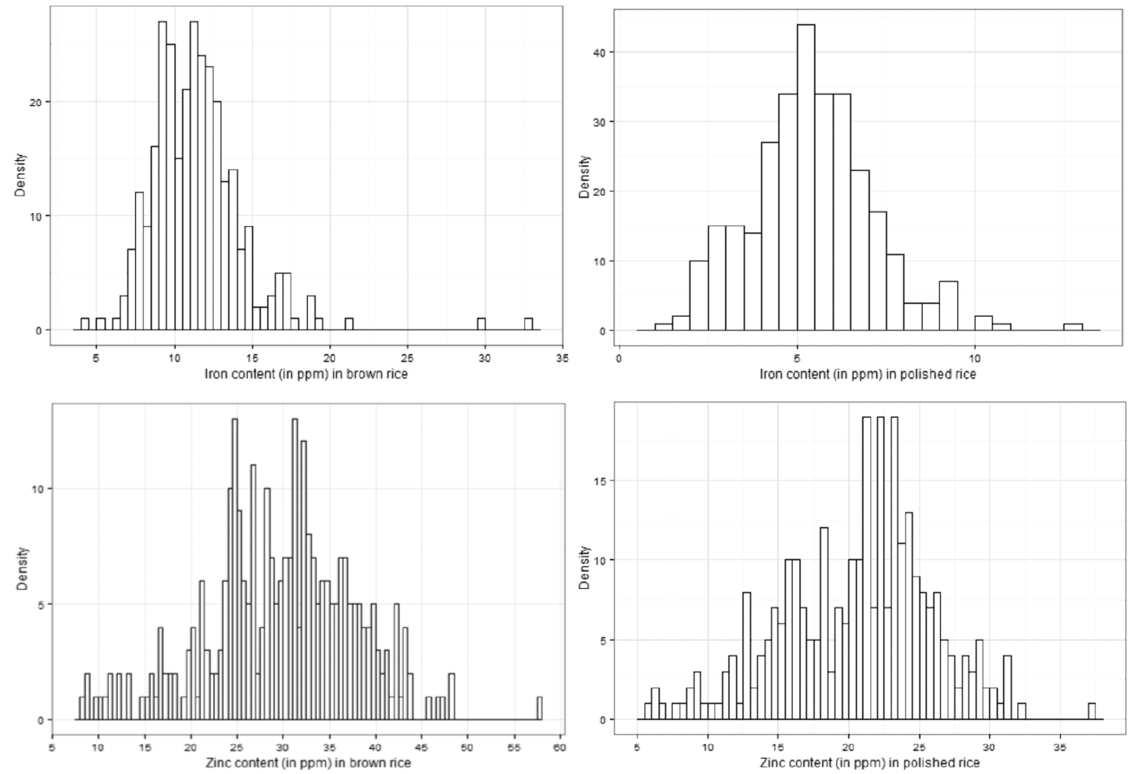

Figure 1: Frequency of Fe and Zn contents in 300 RILs

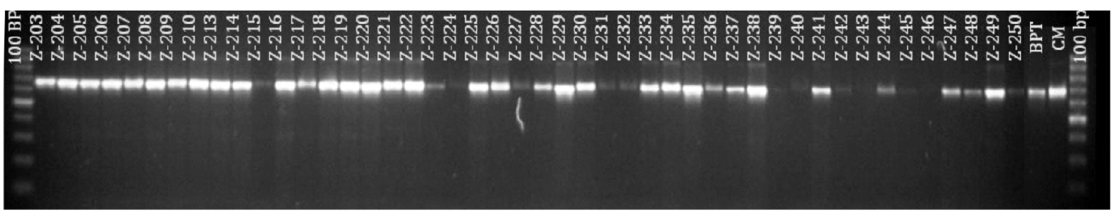

Figure 2: Agarose gel image showing Polymorphic profile of parents and population

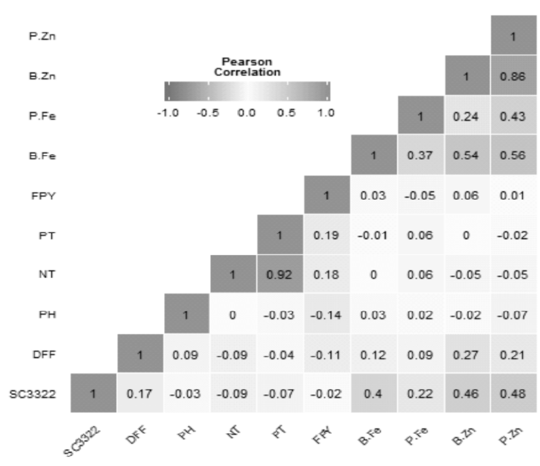

Figure 3: Correlation table of BPT 5204 X Chittimutyalu 300RIL population. DFF= Days to 50% flowering, PH= Plant height (cm.), PT= Productive tillers per plant, FPY= Five plant yield (gr.), B-Fe= Iron in brown rice, P-Fe= Iron in polished rice, B-Zn= Zn in brown rice and P-Zn= Zn in polished rice (Fe & Zn content in ppm). DF(299) and P (0.01)

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REFERENCES

Anuradha K., Surekha Agarwal, Y. Venkateswara Rao, K.V. Rao, B.C. Viraktamath, N. Sarla. (2012), Mapping QTLs and candidate genes for iron and zinc concentrations in unpolished rice of Madhukar×Swarna RILs Gene 508 (2012) 233–240.

Bari, G., G. Mustafa, A. M. Soomro and A. W. Baloch, (1984), Effect of plant density on grain yield and yield components of different varieties and mutant strains of rice. Pak. J. Bot., 16: 169-174.

Bisht, P. S., P. C. Pandey and P. Lal, (1999), Plant population requirement of hybrid rice in the Taria region of Utter Pardesh, India. Int. Rice Research Newsletter, 24: 38.

Bouis, H.E., Welch, R.M., (2010), Biofortification—a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. Crop. Sci. 50, S20–S32.

Brar, B., Jain, S., Singh, R., Jain, R.K., (2011), Genetic diversity for iron and zinc content in a collection of 220 rice (Oryza sativa L.) genotypes. Ind. J. Genet. Plant Breed. 71 (1), 67–73.

Chandel, G., Samuel, P., Dubey,M., Meena, R., (2011), Insilico expression analysis of QTL specific candidate genes for grain micronutrient (Fe/Zn) content using ESTs and MPSS signature analysis in rice (Oryza sativa L.). J. Plant Genet. Transgenics. 2, 11–22.

Emsley, J. (2001), Nature's Builiding Blocks: An A-Z Guide to the Elements. Oxford, England: Oxford University Press.

FAO/WHO (2000), Preliminary report on recommended nutrient intakes. Joint FAO/WHO Expert Consultation on Human Vitamin and Mineral Requirements.

Gande Naveen Kumar, Pavan J Kundur, Rakhi Soman, Rajeswari Ambati, Ashwathanarayana R, Berhanu Dagnaw Bekele and Shashidhar H. E. (2014), Identification of putative candidate gene markers for grain zinc content using recombinant inbred lines (RIL) population of IRRI38 X Jeerigesanna. Afr. J. Biotech Vol. 13(5), pp. 657-663.

Garcia-Oliveira, A.L., Tan, L., Fu, Y., Sun, C., (2009), Genetic identification of quantitative trait loci for contents of mineral nutrients in rice grain. J. Integr. Plant Biol. 51, 84–92.

Gravois, K. A. and R. S. Helms, (1992), Path analysis of rice yield and yield components as affected by seedings rate. Agron. J., 84: 1-4.

Gregorio, G.B., Senadhira, D., Htut, H., Graham, R.D., (2000), Breeding for trace mineral density in rice. Food Nutr. Bull. 21, 382–386.

Gross, J., Stein, R.J., Fett-Neto, A.G., Fett, J.P., (2003), Iron homeostasis related genes in rice. Genet. Mol. Biol. 26, 477–497.

Guerinot, M.L., and Yi, Y. (1994), Iron: nutritious, noxious and not readily available. Plant Physiol. 104, 815-820.

Harvest plus. (2013), Zinc Rice. Visited website on 08-12-2015. *http://www.unscn.org/layout/modules/resources/files/HarvestPlus_Rice_Strategy_EN.pdf.*

Ishimaru, Y., Bashir, K., Nishizawa, N.K., (2011), Zn uptake and translocation in rice plants. Rice 4, 21–27.

Johnson, A.A.T., Kyriacou, B., Callahan, D.L., Carruthers, L., Stangoulis, J., (2011), Constitutive overexpression of the OsNAS gene family reveals single gene strategies for effective iron- and zinc-biofortification of rice endosperm. PLoS One 6 (9), e24476.

Lee, S., *et al.*, (2011), Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. Plant Biotechnol. J. 9, 865–873.

Lee, S., Kim, Y.S., Jeon, U.S., Kim, Y.K., Schjoerring, J.K., An, G., (2012), Activation of rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for bio fortification. Mol. Cells 33 (3), 269–275.

Lu, K., Li, L., Zheng, X., Zhang, Z., Mou, Y., Hu, Z., (2008), Quantitative trait loci controlling Cu, Ca, Zn, Mn and Fe content in rice grains. J. Genet. 87, 305–310.

Martínez, C.P., *et al.*, (2010), Rice cultivars with enhanced iron and zinc content to improve human nutrition. 28th International Rice Research Conference, 8–12 November 2010, Hanoi, Vietnam, p. OP 10.

Miller, B. C., J. E. Hill and S. R. Roberts, (1991), Plant population effects on growth and yield in water seeded rice. Agron. J., 83: 291-297.

Norton, G.J., Deacon, C.M., Xiong, L., Huang, S., Meharg, A.A., Price, A.H., (2009), Genetic mapping of the rice ionome in leaves and grain: identification of QTLs for 17 elements including arsenic, cadmium, iron and selenium. Plant Soil 329, 139–153.

Ravindra Babu V., Neeraja, C.N., Sanjeeva Rao, D., Sundaram, R.M., Longvah, T., Usharani, G., Padmavathi, G., Balachandran, S.M., Nirmala Devi, G., Bhadana, V.P., Suneetha, K., Rao, K.V., Surekha, K., Sarla, N., Brajendra, P., Raghuveer Rao, P., Girish, C., Shashidhar, H.E., Bijan, A. and Viraktamath, B.C. 2014. *Biofortification in rice, Bulletin*, Directorate of Rice Research, 1-98. ISBN: 978-81-928249-5-6.

Sanjeeva Rao D, P. Madhu Babu, P. Swarnalatha, Suneetha Kota, V.P. Bhadana, G.S. Varaprasad, K. Surekha, C.N. Neeraja and V. Ravindra Babu, (2014), Assessment of Grain Zinc and Iron Variability in Rice Germplasm using Energy Dispersive X-ray Fluorescence Spectrophotometer (ED - XRF), JRR. 7 1&2, 45-52.

Sellappan, K., Datta, K., Parkhi, V., Datta, S.K., (2009), Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, b-carotene) of rice cultivars including transgenic indica rice. Plant Sci. 177, 557–562.

Volume 1 All India Coordinated Rice Improvement Project, 2015.

Zheng, K., Huang, N., Bennett, J., Khush, G.S., (1995), PCR-based marker assisted selection in rice breeding. IRRI Discussion Paper Series, No.12. International Rice Research Institute, Manila, the Philippines.