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Identification of putative candidate gene markers for grain zinc content using recombinant inbred lines (RIL) population of IRRI38 X Jeerigesanna

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Nutrients in food crops can be enriched by adopting biofortification. Identifying the target quantitative trait loci (QTL) genes will help achieve biofortification with greater precision and accuracy. The objective of this experiment is to estimate grain zinc content, evaluation of candidate gene markers in recombinant inbred lines (RIL) derived from IRRI38 X Jeerigesanna and validation of putative candidate gene markers with rice accessions. Grain zinc content ranged from 16.1 to 35.5 ppm with an average of 23.7 ppm. Among twenty four candidate gene markers, eight showed polymorphism and out of three simple sequence repeats (SSR) markers, three showed polymorphism. Single marker analysis revealed that four (OsNAC, OsZIP8a, OsZIP8c and OsZIP4b) candidate gene markers showed significant variation among RIL population with a phenotypic variation of 4.5, 19.0, 5.1 and 10.2% respectively. Validation with 96 rice genotypes showed three markers (OsZIP8a, OsNAC and OsZIP4b) with phenotypic variation of 11.0, 5.8 and 4.8%, respectively.

Key words: Zinc, biofortification, single-marker analysis (SMS) and marker assisted selection (MAS).

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for most of the people across the world. About 50% world's populations depends on rice as their main source of nutrition (White, 1994). However, rice is a poor source of micronutrients (Bouis and Welch, 2010). Micronutrients deficiency is a global problem contributing to world's malnutrition. This in turn leads to high rate of mortality in women and children (WHO, 1996). Zinc is one of the essential nutrients for increasing the immunity and it works as a cofactor for over 300 enzymes. It also plays a critical role in synthesis of protein and DNA (Rosal et al., 2010). Fortification of zinc and food supplementation approaches are also can be selected but these inventions are not easy to implement in developing countries due to its cost effective (Bouis, 2003; Stein et al., 2007; Pfeiffer and McClafferty, 2007).

Biofortification of the zinc content using conventional breeding and biotechnological methods can enhance the nutrient content in grains of rice (Bouis, 2003; Pfeiffer and McClafferty, 2007; Howarth et al., 2010). Realizing the

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Abbreviations: QTL, Quantitative trait loci; **RIL,** recombinant inbred lines; XRF, X-ray florescence; **CTAB,** cethyltrimethyl ammoniac bromide; **PCR,** polymerase chain reaction; **SSR,** simple sequence repeats; **SMA,** single marker analysis.

importance of biofortification, several studies were undertaken for the evaluation of germplasm and advanced breeding lines for grain Zn content (Gregorio et al., 1999; Lu et al., 2008). Graham et al. (1999) reported the zinc concentration among 939 genotypes studied ranged from 13.5 to 58.4 ppm±1. Literature shows that 70% micronutrients are lost during polishing (Sellappan et al., 2009). Selection of varieties with trait for higher Zn content using marker assisted selection in rice grain is an effective strategy to address widespread dietary deficiency in human populations. The genetic basis of accumulation of micronutrients in the grains and mapping of the quantitative trait loci (QTL) will provide the basis for preparing the strategies and improving grain micronutrient content through marker assisted selection. DNA markers which are closely linked with desired traits allow the selection of plants possessing those traits prior to trait expression. Earlier reports have cited that grain zinc content in rice is governed by a number of QTL located on different regions of the chromosome with different phenotypic effects (Biradar et al., *2*007; Lu et al., 2008; Garcia-Olivera et al., 2009; Zhang et al., 2011). Chandel et al. (2011) reported three QTL (qZN-5, qZN-7 and qZN-11) for grain zinc content on chromosome 5, 7 and 11, respectively. To use this approach, preliminary steps are required to characterize molecular markers linked to QTL for grain Zn content and study their phenotypic variation. In rice, around 43 candidate genes were identified belonging to different gene families OsZIPs, OsNRAMPs, OsYSLs, OsFROs and Ferritin (Gross et al., 2003).

Markers identified through marker-trait association studies using one single mapping population has to be validated in different genetic backgrounds to determine its consistency (Miklas, 2007). Markers showing greater association and tighter linkage with the trait of interest can be used for marker assisted selection. The objective of this experiment was to evaluate candidate gene markers in recombinant inbred lines (RIL) population derived from IRRI38 X Jeerigesanna, grown under aerobic condition and validation of putative candidate gene markers with germplasms accessions.

MATERIALS AND METHODS

Plant material

Experiment was carried out during fall 2011 and 2012 using augmented experimental design as described by Federer (1960). One hundred sixty RILs derived from IRRI38 X Jeerigesanna were sown in 16 blocks using IRRI38 and Jeerigesanna as checks under aerobic condition.

Estimation of zinc

Seeds were harvested from RILs and a hand threshing was done to avoid contamination. Dehusking was done manually and seeds were washed immediately with 0.1 N HCl and with double distilled water to avoid surface contamination. Washed seeds were dried in an oven at 70°C for 72 h. Grain zinc content was estimated using X-ray florescence (XRF) (OXFORD Instruments X-Supreme 8000, Nicholas et al., 2012). Five gram (5 g) of dehusked rice grains of each sample was used for analysis. Measurement conditions were followed as recommended by the manufacture for analysis of Zn and Fe in a cellulose matrix.

Analysis time for each sample was 186 s, which included 60 s acquisition time for the separate Zn and Fe conditions as well as 66 s 'dead time' during which the XRF establishes each measurement condition. Scans was conducted in sample cups assembled from 21 mm diameter all cups combined with polypropylene inner cups sealed at one end with 4 µm Poly-4 XRF sample film. Calibration of instrument was done using known ICP-OES values of high, low zinc and iron containing genotypes.

Designing of candidate gene markers

For the designing of candidate gene primers, the gene sequence information was downloaded from National Centre for Biotechnology Information (NCBI) and primers were designed using primer-3 tool. Genes used for primer synthesis are shown in Table 1. Oligonucleotide synthesis was done by Eurofins genomics.

Molecular analysis of RILs using candidate gene and SSR markers

DNA isolation was done by cethyltrimethyl ammoniac bromide (CTAB) method from 21 day old leaves (Doyle and Doyle, 1990). Twenty four designed candidate gene primers and three SSR markers (Berhanu et al., 2013) (Table 2) were used for finding the association of zinc accumulation in the grains of rice. The polymerase chain reaction (PCR) mixture contained 50 ng of template DNA, 1 X PCR buffer (10 mM Tris, pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01 mg mL^{-1} gelatin), 2.5 mM of $Mgcl_2$, five picomoles of forward and reverse primer, 0.05 mM of dNTPs and 1 U of Taq polymerase in a 20 µl of reaction volume. Template DNA was initially denatured at 95°C for five minutes followed by 38 cycles of PCR (Applied Biosystems 2720) amplification with the following parameters. A 30 s of denaturation at 95°C, 1 min of annealing at 60°C and 1 min of elongation at 72°C. A final elongation was done at 72°C for 10 min. The amplified product was resolved electrophoretically on a 3% agarose gel for 2 – 3 h, visualized under UV trans-illuminator and documented (Alpha Innotech FluorochemFC2).

Single marker analysis (SMA) and validation

SMA was done with t-test and regression analysis using SPSS 16.0 (SPSS Inc.) to find the association between molecular markers and grain zinc content. Polymorphic candidate markers which showed significant association with grain zinc content were used for validation with different germplasm accessions.

RESULTS AND DISCUSSION

Estimation of zinc

RIL lines were developed by hybridization and advanced by continuous selfing up to F_7 generation without any trait selection by single seed descent method. During the developmental process RIL lines underwent continuous recombinations for stabilization of trait. The grain zinc content in brown rice of IRRI38 and Jeerigesanna was

Table 1. Genes used for designing the primer.

Name	Chromosome	Location	Reference
OsYSL2	2	High grain zn content	Chandel, 2011
OsNAAT1	2	High grain zn content	Chandel, 2011
OsNAC	3	High grain zn content	Chandel, 2011
OsZIP1	3	High grain zn content	Chandel, 2011
OsZIP3	4	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsZIP7	5	High grain zn content	Chandel, 2011
OsNRAMP4	5	Mid grain filling stage	Chandel 2011
OsNRAMP5	7	Mid grain filling stage	Chandel 2011
OsZIP8	7	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsZIP4	8	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsVIT1	NM	High grain zn content	Chandel, 2011
OsNRAMP7	11	High grain zn content	Chandel, 2011

NM, Not mapped.

Table 2. Candidate gene primers were designed using NCBI and Primer-3 tool.

Genes	Chr. No	Source	Forward primer	Reverse primer	Annealing temp	Exp Product size
OsYSL2a	2	NCBI	CCGCTCCCGAGATAGAGA	AAGGCCATCCCCCATGAT	NA	1010
OsYSL2b	2	NCBI	TCACTGCTAAGAGCCTGCAT	CTAGCTTCCGGGAGTGAACTT	60.0	980
OsVIT1	NA	Chandel et al 2011	AAGAGCGAGGCAGACCATTA	GGAATGGACGGTTTCCAGTA	63.0	980
OsNAAT1	2	Chandel et al 2011	CATCTTCCTAACCGCTGGAG	CCTTTGGCAGAAGGATTTGA	58.0	700
OsNAC	3	Chandel et al 2011	AGCGAGAAGCAAGCAAGAAG	ATGCCCTGGATATCGTCGTA	58.0	600
OsZIP1a	3	NCBI	GCTCTTGCTCGCTGCAATTC	CCAACCAGTACCCGTTCTCC	59.0	883
OsZIP1b	3	NCBI	GAACTGTTTCGGCCACGATT	TGAGATGAATTGCAGCGGAGC	59.5	561
OsZIP3a	4	NCBI	ACCCATCATTGCCTCCATCT	AGAACCTGCATGGCCAAA	59.0	1131
OSZIP3bII	4	NCBI	GGGAATCTTGGTGCATTCAGT	GATCACCTGAGATAAGCTTTGG	59.0	1104
OsZIP3b	4	NCBI	CCTGCTGAGGCTGAGTTGAA	CGAGAACAAAGTAACAGGCTGC	61.5	370
OsZIP7c	5	NCBI	GCATCGAATCCAATCCAATC	GCATTAATGAACTCAGCCTCCA	NA	940
OsZIP7d	5	NCBI	GTTTCTTGCAGATACTTGAGATGG	CTGGGAATATCAAAGTCCGATT	57.0	1032
OsZIP7e	5	NCBI	AGACTGCTATGCTTCTCATAACG	GGGAGTATACATCACATGATCACA	49.5	940
OsZIP8a	7	NCBI	ATGAGGACGAACACCACCAC	CGGAGGGAGGGAGTAGTAATG	67.0	880
OsZIP8b	7	NCBI	GGGAGTAGTACGTACGTTTCAAATA	GCTCCCTTTTCCCTCTTTACAT	52.0	1064
OsZIP8c	7	NCBI	TGTAAAGAGGGAAAAGGGAGCTA	GGCGAGTACATTCACTTCCATT	52.0	927
OsZIP4a	8	NCBI	GTGTTCTTCGCCGTCAAGG	GAAATGGATGGTGGCAAAGT	60.0	641
OsZIP4b	8	NCBI	TGAGCTCATCATCACCAACCGTC	CACCTCCACCATCAAGGACG	61.5	673
OsZIP4d	8	NCBI	TTGATGGGAGAAAGGCATGT	CCATTTCTGAAAACGCGGTA	60.0	963
OsZIP4c	8	NCBI	ACTGTATCCACTATCCAGTGCTGA	GCTGGGGATTATTTGATTCTTCAC	56.0	1000
OsNAC5a	11	NCBI	CGAAAGCTTCCATTAGCGACT	CCAATTTGGCACACCTTTCA	53.0	916
OsNAC5b	11	NCBI	TGGCTGTAGCCGCTAGGTAT	GATCGATCGAGCACGGTTA	55.0	885
OsNAC5c	11	NCBI	CTCCACCGGCAGATCAAAAT	CATGTCGCAATCACCCTTAC	53.0	600
OsNRAMP 7	12	Chandel et al 2011	CGGGGCAGACTAGTACCATAACG	CAGCAAGAGATAGCCATTGATCG	60.0	2000
RM263	2	Gramene	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	55.0	199
RM152	8	Gramene	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	56.0	151
RM21	11	Gramene	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	55.0	157

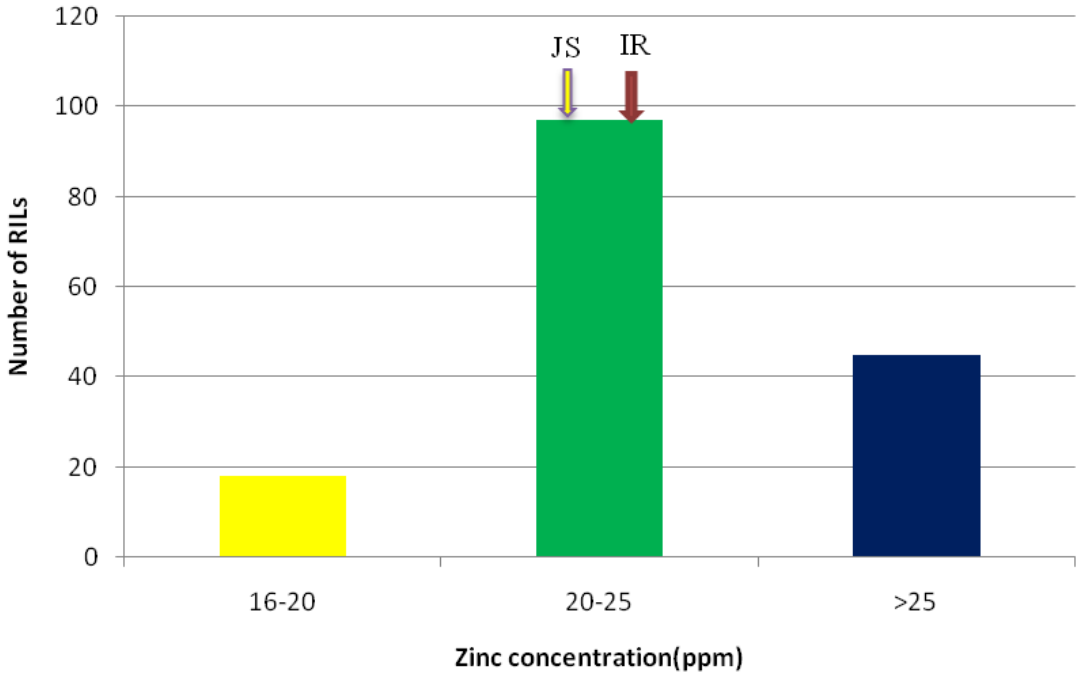

Figure 1. Distribution of grain zinc concentration (ppm) among RILs, arrow marks indicates for parents IR: IRRI38, JS: Jeerigesanna.

23.49 and 21.59 ppm, respectively. Among the RILs grain, zinc content in brown rice ranged from 16.1 to 35.5 ppm with an average zinc content of 23.7 ppm (Figure 1). Zinc estimation showed wide range of variability among RILs. This estimation revealed that the RIL lines had more zinc content than parents. 45 RILs had more zinc content (>25 ppm) than both the parents. Similar results were reported by Tiwari et al. (2009) on grain zinc content in wheat mapping population, ranging from 19.9 to 64.2 ppm. Grain zinc content ranged from 0.4 to 104 ppm in rice germplasm accessions (Anuradha et al., 2012) and Berhanu et al. (2013) reported 16.1 to 88.6 ppm for the rice RIL population. Depending on soil properties grain zinc content varies, pH, organic matter also showed effect on grain zinc content (Chandel et al., 2010; Pandian et al., 2011). Variation in zinc values in different samples of the same accession was observed due to absence or presence of embryo, time of harvest, sampling procedure such as seeds from only main panicle or all panicles or from the entire plant and different digestion or analytical methods (Chandel et al., 2010). Grain zinc content of brown rice was significantly influenced by native soil properties (Banerjee et al., 2010; Chandel et al., 2010; Suwarto, 2011).

Designing of candidate gene markers

Candidate gene markers for grain zinc content of different gene families are designed and synthesized as shown in Table 2.

Molecular analysis of RILs using candidate gene and SSR markers

Marker assisted technology hastens the breeding process and helps in more accurate QTL detection. Among 24 candidate gene markers used in the study eight markers (33.33%), (OsNAC, OsZIP3b, OsZIP3bII, OsZIP7e, OsZIP8a, OsZIP8c, OsVIT1 and OsZIP4b) showed polymorphism (Figure 2) among RIL population on the chromosome numbers 3, 4, 4, 5, 7, 7, not mapped and 8, respectively. Sixteen candidate gene markers showed monomorphism (Figure 3) among the RIL population, out of three SSR markers {RM263 (Figure 2), (RM152 and RM21)} three (100%), showed polymorphism among the RIL population on the chromosome numbers 2, 8 and 11 respectively. These polymorphic markers were used for single marker analysis to find the association.

Single marker analysis and validation

Single marker analysis revealed that out of 11 polymorphic markers, four (OsNAC, OsZIP8a, OsZIP8c and OsZIP4) showed association with a phenotypic variation of 4.5, 19.0, 5.1 and 10.2%, respectively (Table 3) among the RIL population. SSR markers did not show any significance difference among the RIL population. Similar results were reported for candidate markers by Sarala Neelamraju et al. (2012). They reported six QTL for grain zinc content showing >30% phenotypic variance. Anuradha et al. (2012) reported phenotypic variance in grain iron

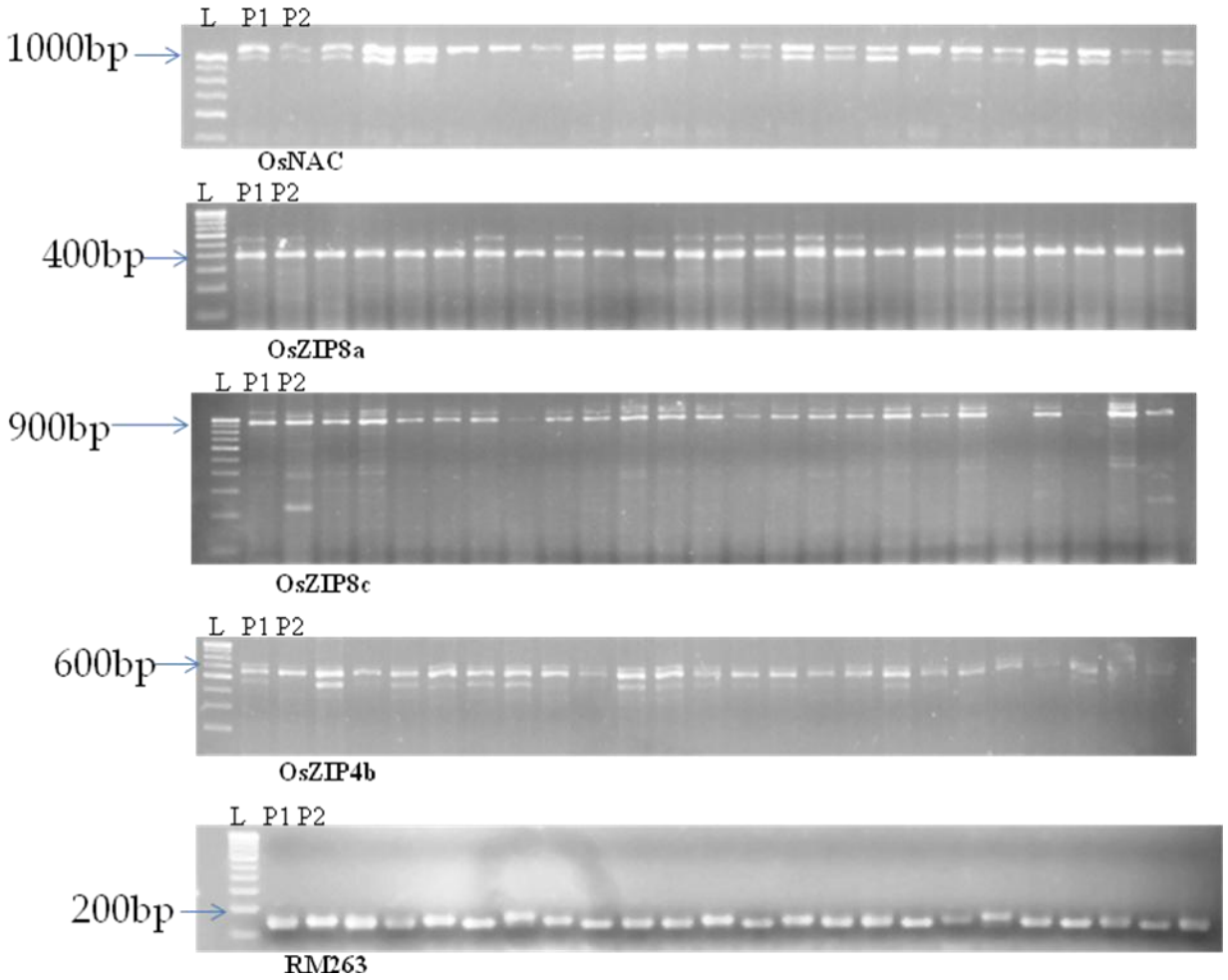

Figure 2. Polymorphic candidate gene and SSR markers.

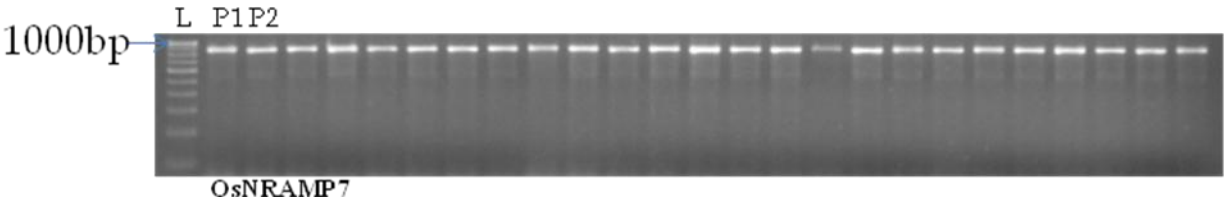

Figure 3. Monomorphic candidate gene markers. L, 100bp ladder; P1, IRRI38; P2, Jeerigesanna.

content with 69 to 71% variability (OsYSL1 and OsMTP1) and with zinc content of 29 to 35% variability (OsARD2, OsIRT1, OsNAS1 and OsNAS2). Grain zinc content for SSR markers (RM152, RM263 and RM21) with 6.1 to 11.7% phenotypic variability was reported by Berhanu et al. (2013). Nagesh et al. (2013) reported similar results from F_2 population of grain iron and zinc content (OsZIP1) with 13.09 and 19.51% variability, respectively.

Validation of putative markers is used to confirm the reproducibility of usefulness in marker aided breeding program. Validation of four candidate gene markers with 96 germplasm accessions showed significant association for three markers (OSZIP8a, OsNAC and OsZIP4b) with a phenotypic variation of 11.0, 5.8 and 4.8% respectively (Table 4). These markers can be further used in maker aided selection for zinc biofortification programs.

The present study revealed that RILs having high grain zinc content with high genetic variability. Single marker analysis showed four candidates gene markers with a significant phenotypic variation among the RIL population. Three putative candidate gene markers (OsZIP8a, OsNAC and OsZIP4b) with a phenotypic variation of 11.0,

Table 3. Single marker analysis (SMA) showing P and R^2 values of candidate gene and SSR markers in RILs of IRRI38 X Jeerigesanna for grain zinc content.

S/N	Marker	P	R^2 (%)	Mean Difference	Estimated effect
1	OsZIP3b	0.34	2.1	1.2	4.2
2	RM263	0.59	0.7	1.8	1.4
3	RM21	0.28	1.6	0.6	3.2
4	RM152	0.98	0	0.4	0.0
5	OsNAC	0.03*	4.5	1.7	9.0
6	OsZIP3bII	0.41	0.4	0.3	0.8
7	OsZIP8a	0.00**	19	3.9	38.0
8	OsZIP8c	0.02*	5.1	1.6	10.2
9	OsVIT1	0.73	0.4	0.2	0.8
10	OsZIP4b	0.00**	10.2	2.5	20.4
11	OsZIP7e	0.71	0.4	1.8	0.8
Mean	23.7ppm				
SD	3.37				

P, Significance; **R^2,** percentage of phenotype variability.

Table 4. Single marker analysis showing P and R^2 values of significant candidate gene markers with genotypes for grain zinc content.

S/N	Marker	P	R^2 (%)	Mean Difference	Estimated effect
1	OsNAC	0.02	5.8	4.2	22
2	OsZIP8a	0.01	11	2.6	2.8
3	OsZIP8c	0.51	1.4	1.8	11.6
4	OsZIP4b	0.03	4.8	3.2	9.6
Mean	29.35				
SD	6.26				

P, Significance; **R^2,** percentage of phenotype variability.

5.8 and 4.8% were found. These putative markers can be used in biofortification programs by breeders and biotechnologists.

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