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Use of *in silico* and semiquantitative RT-PCR approaches to develop nutrient rich rice (*Oryza sativa* L.)

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Inspite of striking agricultural progress and adequate food grain production, protein energy and micronutrient malnutrition are widespread among rural and poor population. The pharmaceutical and diet diversification based approaches have achieved little success due to poverty and educational unawareness. Biofortification of staple food crop rice, which is consumed in large amounts daily, will serve as an important vector to combat malnutrition. The possible approach to improve nutritive value of rice involves exploitation of available genetic variability for grain protein and Fe/Zn contents with conventional and modern biotechnological tools. We have analysed variability in grain protein and Fe/Zn levels in rice, and factors affecting them for identification of rice genotypes with higher nutritive value. Wide variation for grain protein and micronutrient levels were recorded among the tested rice genotypes, which ranged from 6.19 to 10.75% for grain protein content, 4.82 to 22.69 µg/g for grain Fe and 13.95 to 41.73 µg/g for grain Zn content. Significant effect of nitrogenous fertilizer dose and native soil properties were observed on grain protein content, whereas grain Fe and Zn levels were more significantly affected by native soil properties and showed non-significant effect of nitrogen applied. Three genotypes, R-RF-31, Lalmati and R 1033-968-2-1 were identified as high protein and Fe/Zn containing rice lines. In view of our findings and previous studies, where significant Gene × Environment (G × E) effect has been reported on grain nutritive traits, molecular markers are of great use for intensive screening of large populations and identification of environmentally stable outperforming genotypes. The genomic sequence of candidate genes governing micronutrient content in rice were, thus, analysed *in silico* for identification of novel gene specific markers (SSRs and SNPs) and expression sequence tags (ESTs and MPSS) to understand putative expression pattern. Further, to confirm *in silico* expression results and functionally characterized Fe and Zn homeostasis related genes, root and shoot transcriptome analysis of a set of 12 diverse rice genotypes was carried out for expression of 21 metal homeostasis related genes belonging to OsYSLs, OsFROs, OsZIPs, OsNRAMPs and OsFERs families, and OsNAAT1, OsVIT1, OsNAC and OsNAS2 genes. A total of 176 novel SNPs and 39 novel SSRs were identified within metal related genes, which can be used for developing gene specific markers. The ESTs and MPSS tag based *in silico* expression analysis results were in consistency to the semi-quantitative RT-PCR based transcriptome analysis. Expression profiling of rice root and shoot transcriptome at maximum tillering and mid-grain filling stages revealed high level of expression of most of the Fe and Zn uptake and transport related genes in genotypes showing higher grain Fe and Zn concentrations. The rice genotypes with high grain protein and micronutrients, identified in this study, will provide the basis of bioavailability assay and will also serve as potential genetic material for molecular breeding of nutrient rich rice.

Keywords: Expression analysis, *in silico*, iron zinc protein rich rice, *MGS*, *MTS*, RT-PCR,

Introduction

Food security, once a problem in India, was overcome with introduction of semidwarf and dwarf varieties of cereal crops during green revolution that ensured adequate food production in the country with about 30 million tons rice and wheat stored in government godowns as buffer stock[1]. Yet, malnourishment is widespread in Asian and African countries—International and national media refer to this as the co-existence of "Grain mountains and hungry millions". India stands even poorer than Sub-Saharan countries with about 47% children suffering from protein energy malnutrition (PEM); 74% children under three, 43% preschool children, 90% adolescent girls and 50% of women are severely suffering from iron (Fe) deficiency; and 27% of total population is affected by zinc (Zn) deficiency related disorder, such as, poor immune system, diarrhoea,

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etc[2]. The prevalence of PEM and Fe/Zn deficiency is more common among populations having cereal (rice, wheat) based diets, which lack essential minerals and vitamins. Dietary diversification (egg, fruits and vegetables), food fortification (iron and protein rich flour) and pharmaceutical supplementation (Fe/Zn pills, protein powders) are commonly practiced to combat malnutrition but have achieved little success due to educational unawareness and poverty[3]. A recent agriculture based approach called "crop biofortification", aimed to increase concentration of bioavailable nutrients (protein, Fe, Zn and Vit A) of food crops, has gained attention worldwide. Staple food crops (rice, maize, wheat, beans and cassava) are consumed in large amounts daily and are, therefore, targeted under biofortification to ensure adequate attainments of essential nutrients and reducing protein energy and micronutrient malnutrition[4].

Rice is the part of 50-85% of daily intake in South Asian population and a little increase of its grain nutritive value can produce a significant cumulative effect, and in turn may become an important vehicle for improving nutrient intake of common people[5]. The efficacy of nutrient rich rice has been proved by long-term feeding trials in children's institutions in India and Philippines, where replacement of average-protein (6 to 7 per cent) milled rice with an equal weight of high-protein (10 per cent) milled rice led to improvement in children's health status[6]. Similarly, feeding trial involving non-anaemic Filipino woman with high iron rice led to significant increase in blood serum iron levels. However, they reported higher increase serum iron levels of non-anaemic subjects with comparatively lower base iron level than those having higher base line iron level. Therefore, a target level of 9-11% grain protein, $\geq$ 8 ppm grain Fe and $\geq$ 22 ppm grain Zn concentrations have been decided, on the basis of Recommended Dietary Allowance (RDA) and bio-availability values, to ensure adequate attainment of these nutrients daily. The possible approaches to improve grain nutritive value in rice includes increase in protein and Fe/Zn concentration, reducing anti-nutritional factors like phytic acid, and increase in the concentrations of sulphur-containing amino acids that increases bioavailability of minerals. Grain accumulation of protein and Fe/Zn is governed by two distinct phenomena one related to protein biosynthesis in source tissues (leaves & roots) and its re-assimilation into the developing grains (sink tissue), while second phenomena related to metal ions homeostasis includes their absorption from soil, transport to other plant parts (serve as source) and then redistribution into the developing grains (sink tissue)[7]. The grain protein and Fe/Zn values are determined by variables, such as, rate of biosynthesis for proteins and absorption for Fe/Zn, and rate of translocation of photo-assimilates and nutrients to source tissues and their remobilization to developing grains. And, thus, these are governed by a large number of genes[8]. Each of these processes is influenced by environmental factors, such as, soil type, drought, fertilizers application and interaction of genotype with the environment[9]. Several Fe and Zn homeostasis related genes, belonging to ZIPs (Zrt/Irt-related proteins), NRAMPs (Natural resistance-associated macrophage protein), FROs (Ferric reductase oxidase), FERs (Ferrtin), YSLs (Yellow stripe like) NAATs (Nicotinamine aminoransferase), NACs (Transcription factor encoding NAM, ATAF and CUC proteins), NAS (Nicotinamine synthase) and VITs (Vacuolar iron trasnporters)[10-16] have been identified in rice, wheat, barley and Arabidopsis.

Wide variations exists in gross protein content of milled rice ranging from 5.5-11.4%, whereas lysine content ranged from 0.25 to 0.65 gm/100 gm protein[17, 18]. Quantitative trait loci (QTLs) controlling grain protein content has been identified in rice and efforts have been made to improve grain protein content in rice. Krishnan *et al*[19] developed an interspecific rice hybrid by crossing *Oryza nivara* and *O. sativa* (cultivated species) that showed 12.4% grain protein. Rice grain protein is considered to be high quality owing to low prolamine content, higher proportion of sulphur-containing amino acids (cysteine and methionine) and relatively higher proportion of lysine[20,21]. The favourable amino acid composition makes rice protein an easily digestible protein component of food and its low phytic acid content increases bioavailability of mineral nutrients (Fe & Zn). Efforts made to assess genetic variability in rice reported a variation of about 2 to 24 μg/g and 4.45 to 33 μg/g, respectively in grain Fe and Zn concentrations[18,22-27]. Genetic variation for grain protein and Fe/Zn content present in landraces, distant relatives and cultivated rice can be exploited by conventional and modern biotechnological tools that involves marker assisted breeding, and identification and functional characterization of genes governing grain protein and micronutrient

contents in rice. Modern biotechnological tools including computational and functional genomics approaches will help to decipher the molecular components of proteins and metal assimilation in developing grains in plants in general and rice in particular, which form the basis to design strategies for development of nutrient rich rice. The availability of the complete genome sequence of *japonica* and *indica* rice subspecies have paved way for genomics based approaches for discovery of novel genes, molecular markers underlying QTLs and genes[28-30]. A new generation markers, called expressed sequence tag (EST) derived SSRs (single sequence repeats) and SNPs (single nucleotide polymor phigms) are the markers of choice for molecular breeders and are convenient to use in intensive breeding programmes. Moreover, computational transcriptome analysis with expression tags, such as, ESTs, SAGE (serial analysis of gene expression) and MPSS (mssive parallel signature sequence), provides unprecedented opportunity for identification of novel genes and predicting pattern of expression of known genes in a genome[31].

In the present study, authors have assessed the variability in grain protein and Fe/Zn contents in rice and analysed the effect of soil and nitrogenous fertilizer dose on grain protein and Fe/Zn levels. Structural and functional characterization of genes related to metal ion uptake and transport was carried out using *in silico* tools, which provided valuable information for development of novel genetic markers directly related to this trait and putative temporal and spatial expression pattern of these genes. cDNA based expression analysis on single gene basis was performed in root and leaf tissues at maximum tillering and mid-grain fill stages to study role of metal related genes in Fe/Zn uptake, transport and remobilization in rice. The expression pattern of these genes were also analysed for any correlation with grain Fe and Zn concentrations in order to identify potential candidate genes affecting grain micronutrient levels. The novel putative markers generated along with functional analysis of genes governing grain Fe/Zn concentrations will serve as valuable source for molecular markers assisted breeding for exploitation of available genetic variation and development of nutritive rich rice cultivar.

Materials and Methods

Plant Material

Forty six rice lines (Fig. 1) including established cultivated *indica* and *japonica* rice cultivars, germplasm accessions, advanced breeding lines and wild rice genotypes were grown under two nitrogen fertilizer doses, viz., 80 and 120 kg/ha, in three locations. A set of 12 genotypes (Table 1) selected on the basis of classification of cultivated rice described by Garris *et al*[32] were grown under controlled condition on coco-peat substrate with macro and micro nutrients supplied in solution as described by Grusak[7] having 10 μ*M* Fe as Fe(III)-HEDTA.

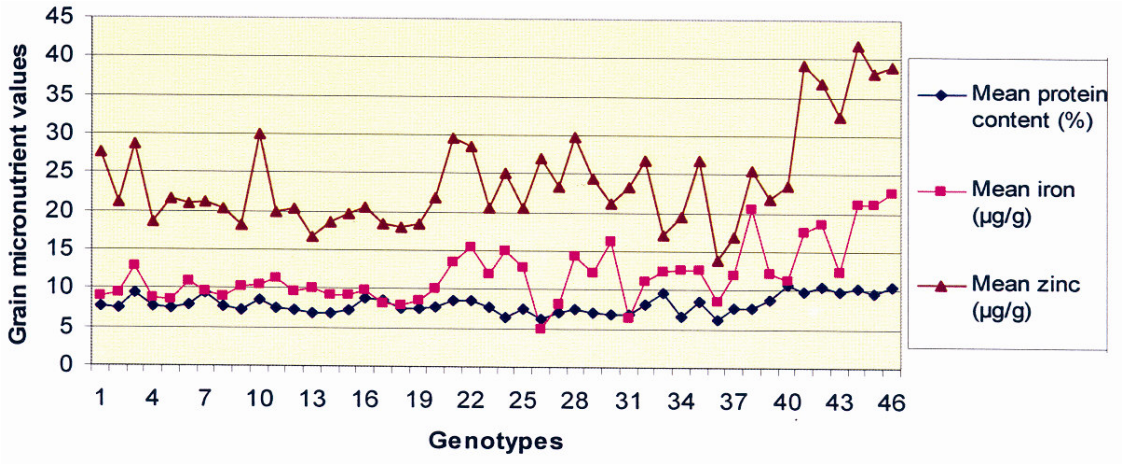

Fig. 1—Variation in grain protein and Fe/Zn concentrations of diverse rice genotypes studied: (1-46) as R-RF-31, R-Rl-29, Lalmati, SL-100, R-RF-21, ARB-6, R-RF-25, R-RF-22, R-RF-23, R 1033-968-2-1, R-RF-32, R-RF-28, R-RF-34, R-RF-35, R-RF-36, Poornima, MTU1010, IR64, R 1027, R-RF-27, Wild rice 224, Early 2035, Medium 2417, BD 823, Early 2119, Medium 2877, Extra early 44, Medium 332, Medium 301, RG173, Medium 1488, BD 162, Nipponbare, Moroberekan, Nagina22, Swarna, Kranti, BAS370, IR68144-3B, Jaldoobi, WR-1, WR-2, WR-3, WR-8, WR-171 and WR-175.

Grain Protein and Micronutrient Estimation

Grain protein and micronutrients were estimated in dehusked, brown rice grains carrying intact embryo. Grain protein content was estimated by modified micro-Kjeldahl method by Johri *et al*[33] and Fe and Zn concentration were estimated as per HarvestPlus[34] guidelines using Atomic absorption spectrophotometer (AAS200) and tomato leaf powder as standard. The grain protein and micronutrient data were statistically analysed using factorial RBD (randomized block design) to test significance of effect of nitrogenous fertilizer and location over grain protein and Fe/Zn contents.

Total RNA Isolation and cDNA Synthesis

Root and leaf tissue were collected from 12 rice genotypes (Table 1) at maximum tillering and mid-grain fill stages and used for isolation of total RNA using Plant RNeasy kit from Qiagen[35] according to the manufacturer's instructions. The isolated RNA was quantified with NanoDrop Spectrophotometer ND-1000® (NanoDrop Technologies, USA) and then used for cDNA synthesis using oligo-dT 18 mer primer and Omniscript First strand cDNA synthesis kit as per manufacturer's instructions and stored at −20ºC for further use. Semi-quantitative RT-PCR analysis of 25 metal homeostasis related genes was carried out using cDNA based primers along with α tubulin gene primer as an internal control. The cDNA amplicons were resolved on 1% agarose gel at 100 V. The presence of amplicons and their respective intensity were recorded under gel documentation system. The relative intensity of amplicons provided basis for quantification of level of expression of gene as high, moderate, low and negligible.

***In silico* Structural and Functional Characterization of Genes**

Genomic sequences underlying metal homeostasis related genes obtained from Gramene, NCBI and TIGR rice genome browsers (http://www.gramen.org, www.ncbi.org.in & www.tigr.org.in) were analyzed for identification of candidate SNPs loci, repetitive sequences, ESTs and MPSS tags. The public databases and search tools, such, Oryza SNP database (http://www.oryzasnp.org/cgi-bin/gbrowse/osa_snp_tigr), SSRIT (single sequence repeat identification tool) available at Gramene (http://www.gramene.org/db/searches/ssrtool), PASA (Program to Assemble Spliced Alignments) program tool (http://www.tigr.org/tdb/e2k/osa1/dnav/), EST database at RGP website (http://rgp.dna.aafrc.go.jp/E/publicdata/estmap2001/) and rice MPSS database (http://mpss.udel.edu/rice). TIGR locus identifier for each gene was used as 'query' to obtain all annotated or non-annotated MPSS tags using 'Query by chromosome position tools' available at TIGR genome browser (http://www.tigr.org/tdb/e2k/osa1/dnav/) were used. The output of each search was recorded and analysed to obtain a meaningful result. Gene specific primers were designed for 25 metal homeostases related candidate genes using Primer-3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Results

Estimation of Grain Protein and Fe/Zn Concentrations

Whole grain Fe, Zn and protein content estimation revealed that grain protein content ranged from 6.19 to 10.75% with mean value 8.07% (SE±0.045) (Fig. 1). Wild rice genotypes showed higher grain protein values than cultivated rice genotypes. Among

Table 1—Characteristic features of twelve diverse rice genotypes used in expression analysis

Category	Genotypes	Characteristic features
Temperate japonica	Nipponbare	Reference sequence in gene identification, *in silico* and expression analysis
Tropical japonica	Moroberekan	Drought tolerant, tall cultivar comparatively higher grain iron concentration
Aus	Nagina22	Mutant line
Aromatic	Bas370	Aromatic indica cultivar
Indica	Swarna	Popular cultivar, drought susceptible, low grain iron and zinc concentration.
	IR681444-3B	Cross of IR72 (IRRI) and Zawa Bonday (India), used as check
	R-RF-31	Homozygous breeding line, identified for high grain zinc concentration
	R 1033-968-2-1	Homozygous breeding line, identified for high grain zinc concentration
Wild rice	*O. officinalis* (WR1)	Wild rice, small grains, grain shattering while maturity, poor milling quality
	O. latifolia (WR2)	Wild rice, small grains, grain shattering while maturity, poor milling quality
	O. nivara (WR3)	Wild rice, small grains, grain shattering while maturity, poor milling quality
Submerged	Jaldoobi	Submerged indica cultivar

cultivated rice genotypes, R-RF-25 (9.40%) and Lalmati (9.03) were found to have ~30% more protein content than popular *indica* cultivar Swarna (6.29%) (Table 2). Wild rice genotypes showed higher grain protein and Fe/Zn levels than cultivated rice genotypes. Of all *indica* rice genotypes analysed, R-RF-31, Lalmati and R 1033-968-2-1 were identified as high zinc lines. Our estimation revealed that these 3 rice genotypes have significantly higher grain micronutrient and protein level than established cultivars like Swarna, IR64 and Kranti. Level of nitrogenous fertilizer significantly affected grain protein content in all the locations as depicted by higher grain protein values under 120 kg/ha N application. Grain Fe and Zn concentrations were found to be in the range of 4.82-22.69 µg/g and 13.95-41.73 µg/g, respectively (Fig. 1). Native soil properties significantly affected grain micronutrient content, whereas non-significant effect of nitrogen level was observed (Fig. 2).

Table 2—Mean grain protein and micronutrient concentrations of selected rice genotypes

No.	Genotypes	Protein* (%)	Zn* (µg/g)	Fe* (µg/g)
1	R-RF-31	7.80	27.636	8.957
2	Lalmati	9.03	28.795	12.797
3	R 1033-968-2-1	8.47	30.057	10.600
4	R-RF-25	9.40	21.254	9.652
4	Nagina22	8.81	26.837	12.652
5	Swarna	6.29	13.954	8.499
6	IR68144-3B	7.62	21.791	12.232

* = Mean grain micronutrient values over the location, treatments and replications

Identification of Novel SNPs and SSR Markers *In Silico*

A total of 176 SNPs were identified within eleven metal homeostasis related genes (OsFRO2, OsYSL1, OsYSL2, OsNAS2, OsNAC, OSZIP2, OsZIP4, OsZIP6, OsZIP7, OsNRAMP6 & OsVIT1) with 2-44 SNPs per gene (Fig. 3). The identified SNPs, called candidate, were categorised into exonic, intronic, 5′ UTR and 3′ UTR on the basis of their position in the gene. Novel SSR sequences belonging to class I and II SSRs were identified within 11 genes (OsIRT1, OsFER1, OsFRO2, OsNAAT1, OsNAC, OsZIP1, OsZIP2, OsZIP4, OsZIP5, OsZIP6 and OsYSL2) ranging from 1 to 3 SSRs per gene. The SSRs consisted of mostly 2 and 3 nucleotide repeat units with 5-16 numbers of repeat. These novel SSRs and SNPs, especially those identified in 3′ and 5′ UTR (un-translated region), will serve as basis for developing gene specific markers, which will be applicable across the genotypes.

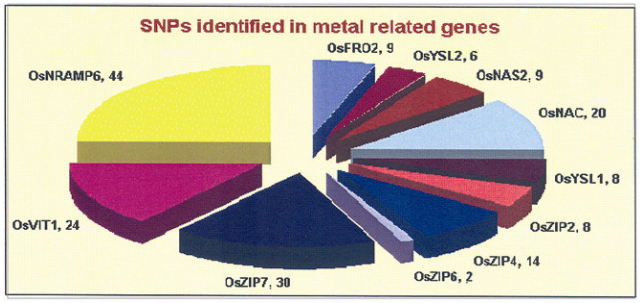

Fig. 3—Total no. of candidate SNPs identified within eleven Fe and Zn homeostasis related genes.

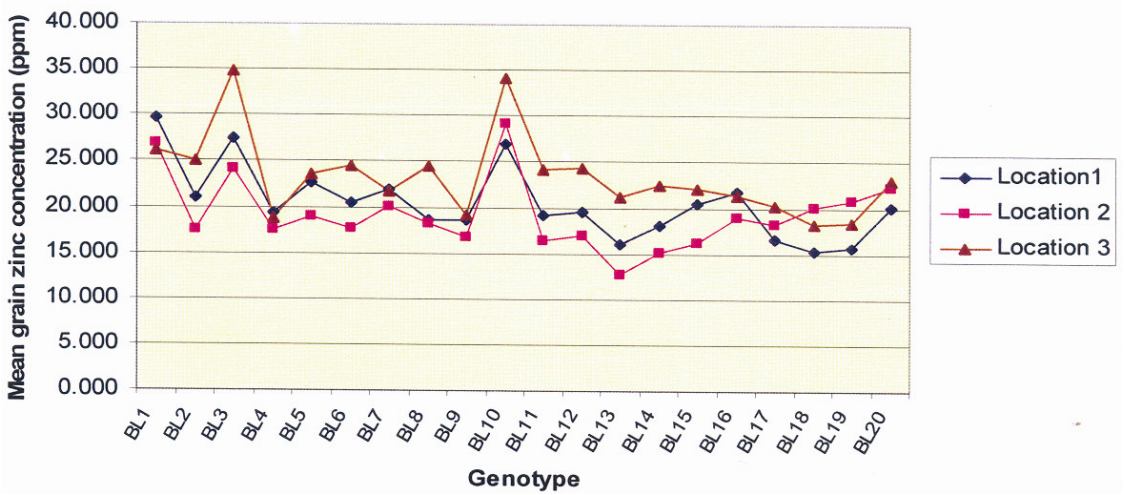

Fig. 2—Effect of native soil properties on grain Zn concentration of homozygous breeding *indica* rice genotypes grown in three locations (R-RF-31, R-Rl-29, Lalmati, SL-100, R-RF-21, ARB-6, R-RF-25, R-RF-22, R-RF-23, R 1033-968-2-1, R-RF-32, R-RF-28, R-RF-34, R-RF-35, R-RF-36, Poornima, MTU1010, IR64, R 1027, R-RF-27).

***In Silico* Study of Temporal and Spatial Expression Pattern of Metal Homeostasis Related Candidate Genes**

Co-localized ESTs and MPSS tags were identified for analysis of temporal and spatial expression pattern of metal homeostasis related genes (Table 3). Total 349 ESTs were identified in genomic region encompassing OsFRO2, OsFER1, OsIRT1, OsZIP1, OsZIP2, OsZIP3, OsZIP4, OsZIP5, OsZIP6, OsZIP7, OsZIP8, OsZIP9, OsNAAT1, OsNAC, OsYSL1 and OsNRAMP6 genes with 3-60 ESTs per gene. The EST sequences were then analysed for corresponding expression libraries developed from tissues, such as, flower, panicle, seed, leaves, roots, stem, etc. The analysis revealed that OsFRO2, OsFER1, OsZIP2, OsZIP3, OsZIP5, OsNRAMP5 and OsNRAMP6 expressed more in flower, panicle and mature shoot tissues, i.e., in reproductive growth phase (Table 3).

Co-alignment of MPSS tags with putative gene sequence, under question, enables quantitative estimation of level of expression of the gene based on its TPM (transcript per million) value. The MPSS tags sequences are derived from specific tissue expression library and abundance of these tags in a library is depicted in terms of its TPM values, therefore, TPM value of co-aligned MPSS tags in corresponding expression library will provide an indirect estimate of the levels of expression of that putative gene. Higher abundance of MPSS tags identified in 7 genes (OsFRO2, OsFER1, OsZIP2, OsZIP3, OsZIP5, OsNRAMP5 & OsNRAMP6) in flower, panicle and mature shoot tissues suggested that these genes express preferably in mature tissues during reproductive growth phase. MPSS tag identified in OsFRO2, OsFER1, OsIRT1, OsZIP8, OsYSL2 and OsZIP4 genes showed higher abundance in mature leaves, ovary, stigma and immature panicle, whereas MPSS tags corresponding to OsZIP6, OsZIP7, OsNAC and OsVIT1 gene showed abundance in both root and shoot tissues.

Semi-quantitative RT-PCR Analysis

The semi-quantitative PCR analysis of 25 Fe and Zn homeostasis related genes, including 21 genes belonging to OsZIPs, OsNRAMPs, OsFROs, OsFERs and OsYSLs families and 4 non-rice genes (OsNAAT1, OSNAC, OSNAS2 and OsVIT1), was performed in root and leaf transcriptome at maximum tillering and mid-grain filling stages. Differential expression of genes was observed between tissue types, developmental stages and among genotypes (Fig. 4). Expression profiling of metal homeostasis related candidate genes indicated that OsFER2, OsNAS2, OsYSL2, OsYSL7, OsNRAMP4, OsNRAMP6 and OsZIP5 showed mid-grain filling stage specific expression. Seven of eight OsZIP family (OsIRT1, OsZIP4, OsZIP5, OsZIP7, OsZIP8, OsZIP9 & OsZIP10), and OsNAC and OsYSL9 genes showed poor expression in root tissue at maximum tillering stage. Higher expression of 8 genes (OsNAAT1, OsNAC, OsVIT1, OsNAS2, OsFRO2, OsYSL6, OsZIP4 & OsZIP7) in leaf transcriptome and 3 genes (OsNRAMP5, OsNRAMP7 & OsFRO2) in root transcriptome of high Fe and Zn rice lines suggested involvement of these genes in iron and zinc uptake and translocation in rice. Four genes (OsFER1, OsIRT1, OsVIT1 & OsFRO2) showed higher expression at maximum tillering stage and 8 genes (OsFER2, OsZIP7, OsZIP8, OsZIP9, OsNRAMP4, OsNRAMP6 and OsYSL12) expressed at higher level at mid-grain filling stage, while 10 genes (OsZIP4, OsZIP11, OsNRAMP5, OsNRAMP7, OsYSL2, OsYSL4, OsYSL6, OsYSL9, OsNAAT1 & OsNAC genes) expressed in root tissues at both the

Table 3—Putative spatial expression pattern of metal homeostasis related genes on the basis of co-localized ESTs identified *in silico*

Gene	ESTs	Library	Flower	Leaf	Shoot	Panicle	Anther	Root	Seed	Callus	Mixed	Unknown	Whole plant
OsFRO2	18	6	√	√	√	-	-	-	-	-	√	√	-
OsFER1	13	9	√	-	√ -	√	-	-	√	√	√	-	-
OsZIP1	6												
OsZIP5	27	11	√	-	√	√	-	-	-	√	√	-	-
OsZIP4	5	3	-	-	-	-	-	√	-	√	-	√	-
OsZIP3	11	2	√	-	-	-	-	-	-	-	√	-	-
OsZIP7	10	5	-	√	-	√	-	√	-	√	-	√	-
OsZIP6	15	5	-	√	√	-	-	√	-	√	√	-	
OsZIP8	8	7	-	-	-	√	-	√	-	√	√	-	-
OsZIP9	22												
OsZIP2	32	11	-	-	√	√	√	-	-	√	√	-	-
OsIRT1	50		-	-	√	√	-	√	-	-	√	-	-
OsNAAT1	35		-	√	-	-	-	√	-	-	-	√	-
OsNAC	34	11	-	√	-	√	-	√	√	√	√	√	-
OsYSL1	3	2	√	-	-	-	-	-	-	-	√	-	-
OsNRAMP6	60		√	√	√	√	√	-	√	√	√	√	√

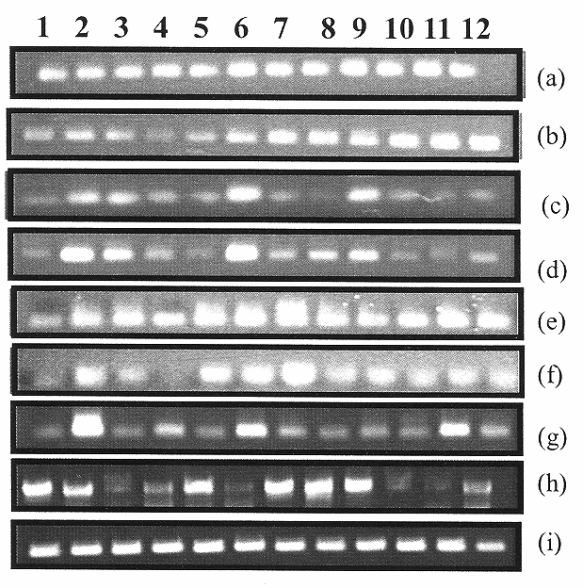

Fig. 4—Differential expression of metal homeostasis related genes in leaf and root tissues at maximum tillering (MTS) and mid-grain filling (MGF) stages as obtained by semi quantitative PCR analysis of 12 rice genotypes: (a) OsFER1 root transcriptome MTS, (b) OsFRO2 root transcriptome MTS, (c) OsYSL4 root transcriptome MGF, (d) OsYSL6 root transcriptome MGF, (e) OsVIT1 leaf transcriptome MTS, (f) OsNAC leaf transcriptome MTS, (g) OsNRAMP5 Flag leaf transcriptome MGF, (h) OsNAAT1 flag leaf transcriptome MG

stages but at different levels in different genotypes. In leaf transcriptome, 5 genes (OsFER2, OsNAS2, OsZIP10, OsYSL7 & OsYSL12) did not express and 4 genes (OsNRAMP4, OsNRAMP6, OsZIP5 & OsZIP9) expressed at very low level, whereas remaining 16 genes showed variable level of expression in all the 12 rice lines at maximum tillering stage. Of all 25 metal related candidate genes expressed in flag leaf transcriptome, 5 genes (OsFER1, OsZIP5, OsYSL2, OsYSL6 & OsYSL12) were expressed in all the 12 rice genotypes at uniform high level, while remaining 21 genes showed variable level of expression among the genotypes.

Correlation of Level of Expression with Grain Fe / Zn Levels

Characterization of leaf and root transcriptome of high Zn lines (R-RF-31, R1033-968-2-1, three wild rice genotypes & Jaldoobi) revealed a higher expression of OsFER1, OsIRT1, OsZIP9, OsNRAMP7, OsYSL6, OsYSL9 and OsVIT1 genes in leaf transcriptome, and OsNRAMP5 and OsFRO2 genes in root transcriptome at maximum tillering stage. High level of expression of OsZIP7 and OsNRAMP5 genes was observed in leaf tissue, and OsNAS2 and OsNRAMP7 genes in flag leaf tissue of Nagina22, which has higher grain Fe (12.652 μg/g) concentration. On the other hand, poor expression of OsIRT1, OsZIP7 and OsZIP5 genes was observed in all types of tissue in Swarna, which has lower grain Fe (8.499 μg/g) as well as Zn (13.95 μg/g) concentration. Further, high expression of OsFRO2, OsNAAT1, OsNAC, OsVIT1, OsZIP7 and OsNRAMP7 genes in leaf transcriptome, OsNAAT1 and OsNAS2 in flag leaf, and OsNRAMP5 and OsNRAMP7 in root transcriptome was observed in BAS370, which has high grain Fe (20.506 μg/g) concentration.

Discussion

Considering the prevalence of protein energy and micronutrient malnutrition all over the world and its acuteness in India, especially among rural and tribal parts of the country, biofortification of rice for higher grain nutritive value seems to be a promising approach to combat malnutrition. Our results of estimation of grain protein and micronutrient contents indicated that 3 homozygous breeding *indica* genotypes, R-RF-31, Lalmati and R 1033-968-2-1, possess high grains Zn concentration as well as relatively higher protein content than Swarna, Kranti and IR64 (Figs 1 & 2; Table 2). The low prolamine content, greater proportion of sulphur-containin amino acids and low phytic acid are responsible for increasing effective protein and increased bioavailability of grain micronutrients in rice[7,20,21,23]. The three rice genotypes also showed higher yield and drought tolerance characters and, hence, have potential to be developed as nutrient rich rice cultivar after necessary field trials. Several studies carried out in Philippines, Bangladesh, Korea and Vietnam have reported a significant G × E interaction effect on grain nutritive value related traits in rice, including factors, such as, wet and dry season, inherent soil properties like saline, acidic, neutral soils, nitrogen supply and period of water logging during crop growth[23,26,27]. Present results also indicated that both grain protein and micronutrient (Fe & Zn) content is affected by native soil properties. The level of nitrogenous fertilizer significantly affected grain protein content, while showed non-significant effect on grain Fe and Zn concentrations. Comparative analysis of grain nutrient contents (protein, Fe & Zn) of genotypes grown in three locations showed significant difference, thus indicating a strong influence of native soil properties on Fe and Zn levels in grain. Despite of significant G × E interaction observed on grain micronutrient level, the ranking of genotypes for grain Zn concentration remained largely unchanged in all locations (Fig. 2).

The polygenic complex inheritance of grain nutritive traits necessitates use of molecular marker assisted breeding (MAB) approach to develop nutritionally rich rice. Microsatellites (SSRs) and SNPs are the markers of choice[28] for most plant breeders and availability of complete genome sequence of *japonica* and *indica* rice subspecies have paved way for computational genomic studies for discovery of new markers as well as to provide basic framework for experiments involving expression analysis of candidate genes[29,30,36]. The technical simplicity, reliability as well as high number of polymorphic loci has increased applicability of genomic SSRs, EST-SSRs and SNP markers in MAB[31]. The putative SSR and SNP markers were identified within metal related gene sequences. It was observed that out of the total SNPs identified in each gene, maximum numbers of SNPs were present in coding region followed by intron, while least number of SNPs was present in 3′ and 5′ UTR region. After validation under wet lab and analysis for association with grain Fe and Zn values, the novel candidate SNPs located in UTR (un-translated regions) regions and hyper variable class I SSRs (more than 20 nt repeat length) identified within metal homeostasis related genes will allow faster genotyping and screening of large population for association mapping of grain micronutrient trait as well as cross transferability across related cereal genomes.

Aligning genome sequences with ESTs and identification of expression tags, such as, SAGE and MPSS, more recently, has arisen as a reliable strategy for structural as well as functional annotation of genes[37]. The ESTs and MPSS libraries may serve as a source for identification, analysis and functional characterization of genes controlling agronomically and commercially important traits[37]. In the present study, the co-localized ESTs and MPSS tags identified within metal related genes were used to predict their putative site and stage of expression. Both MPSS and EST based analysis revealed that OsFRO2, OsFER1, OsZIP2, OsZIP3, OsZIP5, OsNRAMP5 and OsNRAMP6 expressed more in flower, panicle and mature shoot tissues, i.e., reproductive growth phase; whereas OsZIP6, OsZIP7, OsNAC and OsVIT1 gene showed putative expression in both root and shoot tissues. Similar pattern of expression of these genes was observed in transcriptome profiling or root and shoot tissues at maximum tillering and mid-grain filling stages.

The transcriptome analysis of root, leaf and flag leaf tissue revealed differential expression of metal homeostasis related genes among tissues and genotypes that may explain variation in grain Fe and Zn concentration among rice genotypes. The genes related to iron transport through membrane bound proteins, such as, OsNAS2, OsNAAT1, OsNAC and OsVIT1, expressed at higher level in leaf transcriptome, while 24 of 25 genes (except OsNRAMP4) expressed at high level in flag leaf tissue of Moroberekan that has 12.721 μg/g grain iron concentration. High level of expression was observed for OsNAC, OsNAAT1, OsZIP7 and OsNRAMP5 genes in leaf tissue and OsNAS2 and OsNRAMP7 genes in flag leaf tissue of Nagina22 that has 12.652 μg/g grain iron concentration. While poor expression of OsVIT1, OsNAAT1, OsNAS2, OsIRT1, OsZIP7 and OsZIP5 genes related to Fe/Zn uptake, transport and sequestration in all tissue types in Swarna may account for lesser grain accumulation of Fe and Zn in this cultivar. High expression of OsFRO2, OsNAAT1, OsNAC, OsVIT1, OsZIP7 and OsNRAMP7 genes in leaf transcriptome; OsNAAT1 and OsNAS2 in flag leaf; and OsNRAMP5 and OsNRAMP7 in root transcriptome of BAS370 was observed, which has 20.506 μg/g grain iron concentration. Characterization of leaf and root transcriptome of R-RF-31 and R 1033-968-2-1, the identified high zinc lines, revealed a higher expression of 11 genes (OsFER1, OsIRT1, OsZIP7, OsZIP9, OsZIP11, OsNRAMP5, OsNRAMP7, OsYSL6, OsYSL9, OsVIT1 & OsNAAT1) in leaf transcriptome, and OsYSL4, OsNRAMP5 and OsFRO2 genes in root transcriptome at maximum tillering stage; while OsYSL4, OsYSL9, OsYSL12 and OsNAAT1 genes showed high expression at mid-grain filling stage. A similar expression pattern was observed in three wild rice lines (*O. officinalis, O.ninara* & *O. latifolia*) and Jaldoobi genotypes, all of which showed higher grain Fe and Zn concentrations. The analysis also revealed that OsNAAT1, OsNAC, OsVIT1, OsNAS2, OsFRO2, OsYSL6, OsZIP4 and OsZIP7 genes in leaf transcriptome and OsNRAMP5, OsNRAMP7 and OsFRO2 genes in root transcriptome expressed at lower level in Swarna and Nipponbare that have lower grain zinc concentration (~13 μg/g). Largely, grain Fe and Zn levels were in correlation with level of expression of metal homeostasis related candidate genes in one or both tissues at maximum tillering and mid-grain fill stages.

The study revealed that grain protein and Fe/Zn values are polygenic traits, which show significant G × E interaction and, hence, needed to be dissected at molecular levels. The identification of molecular players governing these traits using modern biotechnological approaches, such as, computational and functional genomics tools, will provide valuable information regarding inheritance of these complex traits and, further, in designing breeding strategies to develop rice variety with high nutritive value. Understanding of molecular biology behind grain protein accumulation and factors affecting rate of Fe/Zn absorption, transport and their remobilization into grains will be instrumental in development of high grain protein and Fe/Zn containing rice varieties without compromising with yield. Overall, our findings suggested that R-RF-31 and R1033-968-2-1 are two promising genotypes with comparatively higher grain protein (almost 30%) and Fe (20-30%) and Zn (150-200%) levels than Swarna, Kranti and IR64, the established rice cultivars. These two rice cultivars will not only serve as healthier rice to combat micronutrient malnutrition but also can be used in further molecular marker mining and gene validation studies.

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