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Characterization of the Root Transcriptome for Iron and Zinc Homeostasis-related Genes in *Indica* rice (*Oryza sativa* L)

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Micronutrient malnutrition is the most common form of nutrient deficiency among populations having a cereal-based diet. Rice is the staple food for one third of the world's population but is poor source of iron and zinc content. We have characterized root transcriptome of diverse *indica* rice cultivars for expression of ten known metal homeostasis related genes in plants grown under controlled condition [with Fe(III)-HEDTA iron source]. Fe/Zn contents of root and shoot tissues were also determined. Expression analysis showed expression of *OsFRO2, OsZIP9, OsYSL3, OsIRT-1* and *OsZip5* in most of the cultivars. The cDNA amplicons of *OsFRO2* and *OsZIP9* from different cultivars were analysed for sequence homology and several variation in their nucleotide sequences among rice genotypes. More than 94% sequence homology was observed for both the genes. We analysed the genomic region underlying these genes to obtain information about possible spatial localization based on overlapping ESTs and MPSS tags. Also, putative SNPs were identified within *OsFRO2* and *OsZIP9* genes that need to be validated. The sequence based information may be useful in further development of gene specific markers for screening and breeding of high iron and zinc lines.

***Key words*:** transcriptome, metal homeostasis, candidate genes, sequence homology, *in-silico,* RT-PCR.

Rice is the dominant cereal crop and staple food in developing countries. It constitutes the largest proportion of daily dietary calories but is a poor source of micronutrients like iron and zinc. There are an estimated 800 million undernourished people in the developing world, with about 4-5 billion people being affected by iron deficiency and 2.7 billion being zinc deficient. Iron and zinc malnutrition is the most common deficiency problem that has affected the lives of about 500 million children all over the world (1, 2). Increasing the nutritive value of rice by enhancing iron and zinc concentration seems to be the most suitable strategy to combat micronutrient malnutrition. Rice, like other plants, obtains metal ions such as iron and zinc from the soil. These metal ions though abundant in the soil are not readily available to plants owing to their poor solubility in alkaline pH. Plants thus have adopted two distinct strategies to acquire metal ions from the soil (3, 4). Graminaceous species release mugenic acid (MA) derived phytosiderophore in the rhizosphere to bind insoluble Fe(III). The so formed Fe(III)–MA chelates are then reabsorbed by the roots *via* Fe(III) specific transporters (5, 6). On the other hand, non-graminaceous monocots and dicots acidify the rhizosphere (membrane H^+ ATPase pump) to solubilise Fe(III) and use a ferric reductase to reduce Fe(III) to Fe(II). The reduced Fe(II) is then absorbed by Fe (II) transporter(s) across the root plasma membrane (7, 8). Similarly, zinc is more readily available to plants as Zn-MA chelate than as Zn(II) ion (9).

To increase iron and zinc concentration in edible grains there must be an increase in absorption of these metal ions from the soil and also the transfer of stored iron from the source tissues (leaf and root) to the sink tissues (seeds). The molecular understanding of metal homeostasis in plants in general and rice in particular, with the knowledge of the physiology of metal uptake, translocation and movement across the cell membranes will provide a basis to design strategies for development of micronutrient rich staple foods. This can be achieved by identification and critical functional characterization of

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Abbreviations: EST- expressed sequence tag, Fe- Iron, FRO- ferric reductase oxidase, HEDTA- N (2- hydroxyl ethyl) ethylene diamine – N, N′, N′ – triacetic acid trisodium salt hydrate. IRT- iron deficiency responsive transporters, MPSS- massive parallel signature sequences, Os-*Oryza sativa*, SNP- single nucleotide polymorphism, YSL- yellow stripe protein like, ZIP- Zrt/Irt-related proteins, Zn- Zinc.

genes involved in metal uptake and transport in rice. Several molecular players have been identified with speculated functions in transporting minerals into the plants such as those belonging to *ZIP, NRAMP* and *YSL* family of transporters (10-13) and shown to participate in metal uptake and transport in plants. Rice genes orhologous to *NAS* and *NAAT* genes of barley (*Hordeum vulgare* L) *viz OsNAS1*, *OsNAS2* and *OsNAS3* have also been isolated (14), and characterized for functions in metal uptake and translocation. These genes, which are related to the phytosiderophore biosynthetic pathway, have shown to be involved in iron acquisition during germination (15).

Most of the iron and zinc homeostasis-related genes isolated so far showed inducible expression in iron deficient roots while few genes like Fe (III) - MA transporters encoding *YSLs* and Fe (II) transporters like *OsIRT1* express in leaves as well as roots. The functional analysis of *OsYSL2* gene governing Fe, Mn transport into grain and *OsZIP4* gene encoding the functional zinc transporters revealed that these genes are expressed in roots as well as shoots (16, 17). These expression studies, although indicating a relationship between metal transporters coding genes and uptake and distribution of iron and zinc in the plants (18-20) are not sufficient to provide enough evidence on the specific pathways related to iron uptake, transport and partitioning to the grain (21).

Since an increased absorption of iron and zinc by roots from rhizosphere may lead to a proportionate increase in their concentration in sink tissues, functional characterization of metal uptake genes in roots and identification of genomic DNA based functional markers will be helpful in breeding iron/zinc dense rice. The present study was undertaken to analyze expression of ten known metal related genes including *OsYSLs, OsZIPs, OsFROs, OsFERs, OsIRTs* and *OsNRAMPs* in root tissue of 8 rice genotypes. cDNA amplicons of two genes (*OsFRO2* and *OsZIP9*) were sequenced and analyzed using ClustalW to generate sequence similarity information. At the same time *in silico* characterization of *OsFRO2* and *OsZIP9* genes was done to identify candidate single nucleotide polymorphism (SNPs) within these genes and study putative temporal and spatial expression pattern.

Materials and Methods

Plant Material — Twenty *indica* rice genotypes obtained from the USDA, ARS National Small Grains Germplasm Research Facility (Aberdeen, Idaho, USA) were used for the study (Table 1). Plants were grown in the hydroponics solutions (22) with 20 µM Fe (III)-HEDTA. Root and shoot tissues were collected from the four weeks old plants. Half of the shoot and root tissues were frozen in liquid nitrogen for gene expression studies and the remaining half were dried in paper bags in a 60°C oven for 48 h. The oven dried samples were used for the mineral analysis.

RNA isolation — Total RNA was isolated from root tissues of each cultivar using RNA-easy kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. To remove contaminating genomic DNA, RNAs were treated with the TURBO DNA-free kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions. The concentrations of RNAs were assessed using NanoDrop Spectrophotometer ND-1000® (NanoDrop Technologies, USA) according to the manufacturer's instructions.

RT-PCR — RT-PCR was carried out using Qiagen RT-PCR kit (http://www1.qiagen.com/literature/handbooks/PDF/RTPCR_Omniscript/) and 0.2 ml thin walled PCR tubes/strips. The cDNAs were made from root RNA samples of twenty rice genotypes using Omni Script first strand cDNA synthesis kit (http://www1.qiagen.com/literature/handbooks/PDF/RTPCR_Omniscript/) as per manufacturer's protocol**.** Reactions were carried out with 1.0 µg of cDNA and final volume made up to 10 µl. The temperature profiles for PCR reactions were set at annealing temperature of 54 to 56 °C as per the requirement of metal gene specific primers (Table 3). Ten genes used in the study were *OsFRO2* (BK000590), *OsFER1* (AF519570), *OsFER2* (AF519571), *OsIRT1* (AB070226), *OsZIP3* (BK000616), *OsZIP6* (13810566), *OsZIP9* (BK000620), *OsNRAMP8* (BK000596), *OsYSL12* (BK000606) and *OsNAS2* (AB023819).

Elemental analysis — Forty eight hours oven dried shoot and root samples were taken for mineral analysis. The samples were pre-digested overnight in borosilicate glass tubes with 4 ml of redistilled 98.8% HNO_3. One ml of concentrated trace metal grade $HClO_4$ was added to the predigested samples and heated at 100°C for 1 h, 150°C for 1h, 180°C for 1h and then at 210°C to dryness (2-3 h). Digestions were performed using a heating block (Model 1016, Tecator, Höganäs, Sweden) with an exhaust-collecting manifold. Digests were resuspended in 5 ml of redistilled 2% HNO_3. Elemental analysis was performed using inductively coupled plasma-optical emission

spectroscopy (CIROS ICP Model FCE12; Spectro, Kleve, Germany). Rice flour (of certified elemental analysis) was digested and analyzed along with the rice samples to ensure accuracy of the instrument calibration.

Identification of co-localized ESTs — Genomic sequences underlying *OsFRO2* and *OsZIP9* genes were analyzed for co-localization of identified ESTs. PASA (Program to Assemble Spliced Alignments) program tool available at TIGR rice genome browser (http://www.tigr.org/tdb/e2k/osa1/dnav/) and EST database at RGP website (http://rgp.dna.aafrc.go.jp/E/publicdata/estmap2001/) were used to identify co-localized ESTs. It resulted in incorporation of high quality ESTs by transcript alignment in a genomic and full length cDNA (fl-cDNA). ESTs identified for each gene were further characterized for respective expression tissue library using digital northern and anatomy viewer search tools. ESTs corresponding to a tissue library provided information about putative site of expression of the metal related genes in which it was identified. The minimal alignment allowed by the PASA program is 95% identity over 90% length of the transcript.

Identification of MPSS tags — MPSS tags corresponding to metal homeostasis related genes in rice were identified. The rice MPSS database includes a comprehensive set of libraries which can be accessed at 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/). The search resulted in 17 and 20 nt long MPSS tags, tag sequence, chromosome coordinate position, tissue library information and transcript abundance values such as TPM (transcripts per million) value, normalized abundance in different steps and 'p' value displayed in table format. The transcript number under "Norm Abund" category was considered mainly to draw a conclusion about abundance of transcript and henceforth level of expression of gene in the particular tissue type. It has been demonstrated in the previous study using MPSS tag based characterization of expression pattern in *Arabidopsis* (23) that TPM<5 corresponds to normalized housekeeping genes and TPM<15 indicated very weak expression hence only those tissue were considered that showed TPM>15.

Identification of candidate SNPs — *Oryza* SNP database freely available for public use at *Oryza* SNP consortium (http://www.oryzasnp.org/cgi-bin/gbrowse/osa_snp_tigr) containing Perlgen model based SNPs derived from sequence comparison of multiple rice genomes with Nipponbare genome sequence as the reference (24, 25) was used to identify candidate SNPs within metal homeostasis related genes. "*Oryza* SNP search pages" tool was used for TIGR locus id and chromosome coordinate as query. The SNP resource data set, carrying SNPs identified by comparison of *indica* (93-11) and *japonica* (Nipponbare) genome (26), available on http://www.plantgenome.uga.edu/snp website was used to identify SNPs in OsZIP9 gene.

ClustalW analysis of OsFRO2 and OsZIP9 Sequences — The cDNA PCR products of two metal related genes, *OsFRO2* (1000bp) from four rice cultivars and *OsZIP9* (133bp) for five rice cultivars were gel eluted and sent for sequencing. The cDNA sequences of these two genes for each of the cultivar were analysed for sequence similarity and variations using ClustalW (27).

Results and Discussion

Grain iron and zinc concentration in rice is largely associated with their absorption by roots therefore, in order to understand role of metal homeostasis related genes in iron/zinc uptake and transport in rice, root transcriptome of 20 rice genotypes were characterized for expression of ten genes (*OsFRO2, OsFER1, OsFER2, OsIRT1, OsZIP5, OsZIP6, OsZIP9, OsNRAMP8, OsYSL12* and *OsNAS2*) belonging to OsYSLs, OsZIPs, OsNRAMPs, OsFRO2, OsFERs families of candidate genes identified for iron and zinc homeostasis in rice (12, 28) and non-rice genes such as NAS, NAC, NAAT, IDEs etc. related to phytosiderophore biosynthesis as well as its regulation pathway (11, 8). Here we have discussed in detail expression results of only those genes that showed expression in all genotypes (Fig. 1). Elemental analysis of shoot and root tissues was also performed to estimate iron and zinc levels in these tissues and study any correlation between mineral

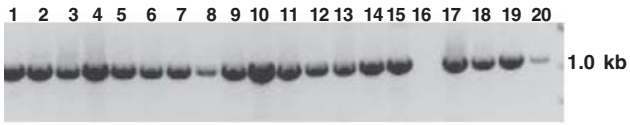

Fig. 1. Amplification of *OsFRO2* gene in twenty diverse rice genotypes. 1-Csa He Jao 1, 2- Kyzyl shala, 3- IR68144, 4- Nigro Apiculata, 5- Kitaake, 6- Gum Nisiki, 7- Kulu, 8-560 A, 9- Nucleoryza, 10- Rodjolele, 11- Nipponbare, 12- Sugdasi type, 13- Bluebelle, 14- Italica M1, 15- Bozu, 16- GPNO 19011, 17- Cocodrie, 18- Ostiglia, 19- Szaniszlo 1, and 20- Norin 19 Selection

concentrations and levels of gene expression. Several studies conducted so far to analyze expression of metal homeostasis related genes in different tissues and developmental stages in rice, maize and Arabidopsis have revealed high level of expression of most of the metal related genes in roots tissue as compared to shoot tissues (13, 16, 20, 22, 29). These studies suggest that metal homeostasis related genes play a role in the absorption and distribution of iron and zinc throughout the plant, thereby allowing the maintenance of sufficient levels in the plant, while at the same time preventing metal toxicity (30). Thus, higher expression of metal related genes in roots, which are the primary plant organ to absorb and transport metals ions, might be related to ensure adequate production of metal chelators and transporters proteins.

Expression analysis — Semi-quantitative RT-PCR was carried out to characterize the rice root transcriptome for expression of ten putative metal related genes (*OsFRO2, OsFER1, OsFER2, OsIRT1, OsZIP5, OsZIP6, OsZIP9, OsNRAMP8, OsYSL12* and *OsNAS2)*. The cDNAs generated from the total RNA isolated from root tissues of twenty rice genotypes were used to analyze expression of these metal uptake and transport related genes. Preliminary results, using semi quantitative RT-PCR revealed that five among the 10 metal related genes studied *(OsFER2, OsIRT*1, *OsZIP5*, *OsZIP9* and *OsFRO2)* expressed in root tissues of rice cultivars Csa He Jao1, Kyza shala, IR68144 and Cocordie only, (Fig.2) and not others which may be due to a non functional gene in other genotypes or due to occurrence of SNPs in the target sequence of the gene specific primer. The *OsFRO2* gene was found to express in roots of all tested rice genotypes with variations in level of expression but showed negligible expression in GPNO 19011 (Fig.1). The *OsFRO2* gene orthologous to Arabidopsis *AtFRO2* genes belongs to the strategy I of Fe uptake and encodes a trans-membrane protein which is known to be induced under Fe deficiency

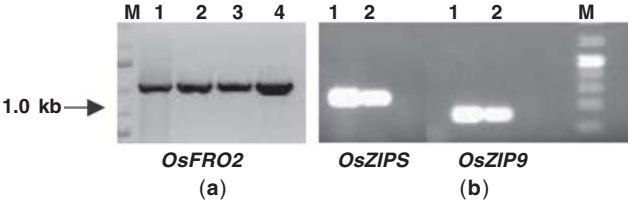

Fig. 2. RT-PCR analysis of metal homeostasis related candidate genes in root tissues of rice cultivars. (**a**) *OsFRO2*: M: 1 kb ladder, 1-Csa He Jao 1, 2- Kyzyl shala, 3- IR68144, and 4- Nigro Apiculata; and (**b**) *OsZIP5* and *OsZIP9*: 1-Csa He Jao 1, and 2- Kyzyl shala

in roots (8). A relatively higher level of expression of *OsFRO2* gene in root tissues of most of the genotypes is in agreement to the fact that *OsFRO2* gene plays role in absorption of iron from soil.

Estimation of micronutrient concentration in shoot and root tissue — Elemental analysis of all 20 rice genotypes was performed to determine iron and zinc concentration of the root and shoot tissues but reproducible and consistent results were obtained for eight rice genotypes only and hence root and shoot Fe/Zn concentrations of these genotypes was included in the study (Table 1). The elemental analysis results depicted significant genotypic variation in mineral concentrations ranging from 59.66 to 114.85 µg/g of Fe and 38.87 to 137.13 µg/g of Zn in shoot and 1605.37 to 2470.05 µg/g of Fe and 62.00 to 135.67 µg/g of Zn in root tissues. The average iron concentration in root tissues (2052.60 µg/g) was significantly higher than that of shoot tissues (78.80 µg/g) which may be because of metal ion contamination that was adsorbed over the root surface. Still the variation among genotypes remains the same as all genotypes were grown under identical growth conditions. The iron concentrations in roots of cultivars IR68144 (2470 µg/g), Cocodrie (2240 µg/g) and ItalicaM1 (2119 ug/g) were much higher than corresponding shoot iron concentration. Genotypes Norin19 selection and Kyzyl Shala showed higher zinc concentration in roots as well as shoots.

Correlation of semi-quantitative RT-PCR results with shoot and root tissue Fe/Zn concentration indicated that the variations observed in shoot and root tissue mineral contents were in accordance to the variation in level of expression of metal related genes. Similar results have been reported in expression analysis of *OsIRT1, OsZIP1, OsZIP5, OsZIP8, OsYS15, OsYS16, OsYS17, OsYS18, OsNRAMP2, OsNRAMP4* and *OsNRAMP7* where higher level of expression was observed in non-flag and flag leaves of cultivar IR68144 having higher grain iron (~21µg/g) concentration (21). Although variations in level of tissue Fe and Zn levels was found to be associated to level of expression of some of the metal related genes, studies suggesting their role in both Fe and Zn homeostasis yet none of them were found to have a distinct functional relation to significant difference in iron/zinc concentration. The possible explanation to the fact lay in the physiology of transport of these metal ions from source roots to leaves and their re-distribution to developing grains (31). The physiology as well as molecular biology of both of these

Table 1. Iron and zinc concentration (in µg/g) of shoot and root tissues of one-month-old eight rice genotypes grown on 20µM iron supplied as Fe (III)-HEDTA

S. No.	Genotypes	PI number	Fe Concentration (µg/g)*		Zn Concentration (µg/g)*	
			Shoot	Root	Shoot	Root
			Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
1	Csa He Jao 1	393176	-	-	-	-
2	Kyzyl shala	439686	59.66±0.48	1962.45±2.10	147.13±0.87	111.85±0.83
3	IR68144	-	60.33±0.43	2470.05±1.70	38.87±0.49	90.65±1.34
4	Nigro Apiculata	439616	-	-	-	-
5	Kitaake	-	64.58±0.46	1605.37±4.05	50.30±1.33	62.50±0.67
6	Gum Nisiki	162205				
7	Kulu	388596	65.89±0.60	2066.66±2.36	49.70±0.55	109.07±1.04
8	560 A	402702	-	-	-	-
9	Nucleoryza	435977	-	-	-	-
10	Rodjolele	560295	-	-	-	-
11	Nipponbare	514663	-	-	-	-
12	Sugdasi type	430967	-	-	-	-
13	Bluebelle	9544	78.91±0.48	1991.57±1.49	44.05±0.52	62.00±1.08
14	Italica M1	265111	85.21 ± 0.43	2119.0±3.04	45.65±0.61	69.25±0.59
15	Bozu	291657	-	-	-	-
16	GPNO 19011	510463	-	-	-	-
17	Cocodrie	-	100.99±0.74	2240.38±0.41	52.87±0.760	73.62±1.11
18	Ostiglia	179169	-	-	-	-
19	Szaniszlo 1	271889	-	-	-	-
20	Norin 19 Selection	12455	114.85±0.85	1960.9±1.87	73.38±0.440	135.67±0.95
	SEm		0.85	1.87	0.44	0.95

*Mean of three replications

Table 2. Candidate SNPs identified within *OsFRO2* and *OsZIP9* genes

SNP id	Coordinate	Variation	SNP classification
OsFRO2 gene[1]			
TBGI203991	21964450	A->G	resides in gene
TBGI203991	21964450	A->N	resides in intron
TBGI203998	21965907	G->C	resides in gene
TBGI203998	21965907	G->C	non-synonymous SNP
TBGI203998	21965907	G->N	resides in gene
TBGI204000	21966145	C->T	synonymous SNP
TBGI204002	21966277	A->G	resides in gene
TBGI204002	21966277	G->N	synonymous SNP
TBGI204006	21966410	G->C	resides in gene
TBGI204006	21966410	G->C	resides in intron
OsZIP9 gene[2]			
SNP id	Contig id	Variation	SNP classification
Contig000483	bgi_contig_449312	G->A	-
Contig000483	bgi_contig_449339	T->A	-

[1] candidate SNPs identified in *OsFRO2* gene sequence belonged to SNP database available at Oryza SNP consortium (http://www.oryzasnp.org/cgi-bin/gbrowse/osa_snp_tigr (23, 24).

[2] candidate SNPs identified in *OsZIP9* gene sequence belonged to SNPs databse available at Oryza SNP consortium (23, 24) and SNP data set available on http://www.plantgenome.uga.edu/snp website (26).

phenomena is complex, poorly understood and largely unknown therefore, more information about gene products that may contribute to iron and zinc concentration in edible sink tissues with precise elemental data at different sub-stages of rice grain filling is required. Analysis of micronutrient concentrations in flag leaves which is considered a major source for nutrient loading in developing grains (32), secondary leaves, roots and developing grains in diverse rice genotypes at grain development stages will be useful in drawing meaningful conclusion about critical molecular players of Fe/Zn uptake and transport. Many transformation based experiments and expression analysis studies have revealed the role of genes belonging to different families in iron acquisition and distribution however, little differences has been observed in grain iron/zinc concentrations (33-35). These findings suggest a need for more accurate methods of precise quantitative estimation of metal ions in living tissues and a better understanding of micronutrient demand and supply equilibrium between soil and roots as well as between leaves and the grain part.

***In silico* characterization of *OsFRO2* and *OsZIP9* genes —** The cDNA amplicon sequences of *OsFRO2* (1000bp) and *OsZIP 9* (133bp) obtained in different genotypes were analyzed using *ClustalW* (27). The rice genotypes *Nigro apiculata* and *Kyzyl Shala* showed 96% sequence homology for *OsFRO2* gene and 94% homology for *OsZIP9* gene. The conserved regions in all *OsFRO2* and *OsZIP9* alleles of different rice genotypes and variations were observed among the sequences obtained from different rice genotypes for two genes analyzed. The variations in the cDNA sequences among the rice genotypes may have generated due to insertion, inversion or deletion in the genomes of the different rice genotypes. The information inferred from sequence homology search between gene specific amplicons from different rice cultivars will then be used to design gene specific functional markers and also to probe expression of these genes in different tissues. The results will therefore be more useful in screening additional genotypes and also assay of expression of these genes in other tissues at various growth stages. Further the genomic sequences underlying *OsFRO2* and *OsZIP9,* obtained from TIGR rice genome browser (http://www.tigr.org/) were analyzed for identification of co-localized SNPs and expression tag sequences such as ESTs and MPSS.

Expression analysis of OsFRO2 and OsZIP9 genes using ESTs and MPSS tags — In order to predict putative temporal and spatial pattern of expression co-localized ESTs and MPSS tags were identified in the genomic regions underlying *OsFRO2* and *OsZIP9* genes using ESTs and MPSS databases available at http://www.tigr.org/tdb/e2k1/osa1/dnav.shtml and http://www.systemsbiology.org/technology/Data_Generation/MPSS, respectively. The analysis revealed that a total of 49 ESTs showed high sequence homology to genomic sequence of *OsFRO2* gene, of which 17 ESTs were found to express in 6 expression libraries belonged to floral, shoot and leaf tissues. The tissue expression libraries corresponding to *OsFRO2* gene specific ESTs suggests the gene putatively expresses in shoot tissues. Similarly, a total of 22 ESTs were mapped over two alternate spliced sequences corresponding to *OsZIP9* gene. Eight of these 22 ESTs were found to correspond to 7 tissue expression libraries, ranging from 1-3 ESTs pre tissue library, i.e. floral tissue (panicle, stigma and ovary) and developing grains thus suggesting putative expression of *OsZIP9* gene in reproductive plant parts. In order to quantitatively characterize the expression of these genes Massive Parallel Signature Sequence (MPSS) tag based analysis was performed. The results of MPSS analysis of *OsFRO2* gene sequence resulted in alignement of a 17 nucleotide long (GATCCGAACCGGAGGCC) and three 20 nucleotide long (GATCCGAACCGGAGGCCAAT, GATCGAGGAA GGCAAACCAT and GATCGGAAACGTGAGCATAC) MPSS tags with *OsFRO2* gene sequence. Two of the four MPSS tags belonged to an expression library derived from 60 days old mature leaf while the other two MPSS tags belonged to 14 days old leaves, 60 and 90 days old ovary, stigma and panicle tissues. The aligned MPSS tags and their corresponding expression libraries indicated that it expresses at moderate level in different young as well as mature shoot tissues such as leaves, ovary with highest expression in 2-week-old leaves and particularly in floral tissue at reproductive stage of development The MPSS tag based data set also indicated that the *OsFRO2* gene may express under cold and salinity stress.

MPSS tag alignment for *OsZIP9* gene sequence resulted in identification of two 17 nucleotide long MPSS tags (GATCCTCATCTACATGG, GATCGAGGGTGCACCTG) corresponding to 60 days old mature leaf and 10 days old etiolated seedling tissue and one 20 nucleotide long MPSS tag (GATCGAGGGTGCACCTG) corresponding to 35 days old callus tissue. *OsZIP9* gene is thus supposed to express in shoot tissues at different developmental stages as well as under interaction with biotic stress factors like *Xanthomonas oryzae.* The putative expression pattern obtained by tag based computational analysis also indicated a cross talk between pathways involved in metal homeostasis and biotic-abiotic stress related genes in rice. The results of MPSS tag based characterization of expression of *OsFRO2* and *OsZIP9* genes indicated its potential application in predicting putative spatial and temporal pattern of expression of a sequence or putative genes in question and designing wet lab expression profiling experiments.

Identification of SNPs — SNP resource data set available on http://www.plantgenome.uga.edu/snp (25) generated from whole genome alignment of *indica* (93-11) and *japonica* (Nipponbare) genomes and Rice SNP Consortium database ((http://www.oryzasnp.org/cgi-bin/gbrowse/osa_snp_tigr) generated from alignment of partial genome sequences of Nipponbare, Swarna, Moroberekan and N22 (24) were used to identify candidate SNPs loci

present in the genomic region corresponding to *OsFRO2* and *OsZIP9* genes. The SNP database available at *Oryza* rice SNP consortium resulted in identification of thirteen SNPs within *OsFRO2* gene and no SNPs was found in the genomic region underlying *OsZIP9* gene sequence. While the SNP database available in Plant genome database (http://www.plantgenome.uga.edu/snp) resulted in identification of two candidate SNPs within *OsZIP9* gene sequence. The candidate SNPs identified in *OsFRO2* were further characterised on the basis of their position in candidate gene sequence as on intron, 5′ and 3′ Un-translated regions (UTRs), coding sequences or anonymous regions (Table 3). Of all the SNPs identified least number of SNPs was found to be located in UTR regions and these candidate SNPs are most suitable to develop SNP based genetic markers. Since the candidate SNPs are located within metal related genes *OsFRO2* and *OsZIP9* genes they may be validated in mapping population derived from a cross between *indica* × *japonica* rice genotypes and study linkage with grain micronutrient trait. The candidate SNPs provide an opportunity to critically characterize these genes for expression and function as well as Linkage disequilibrium (LD) based trait specific association studies and also screening of larger mapping population.

Table 3. Forward and reverse primers used of genes used for the semi-quantitative RT-PCR amplifications

Genes	Primer (5′ to 3′) Forward / Reverse	Annealing temperature (°C)
OsFRO2	GATCCATGTCAAGCCTGTCGA AGGGACGAGATCGTCTCGTACA	56
OsFER1	TAGGCAAAAGTTCGTCGACGA TCTTCTCAGCTGGGCGACATAC	54
OsFER2	CTTGCTAGGCAAAAGTTCGTCG CATTCACTCCATTGCTACTGCGT	56
OsNAS2	CTCAAGTGCTCGTGCAACATT GATGTCCTCGAGATCGACGAT	54
OsIRT1	TTGTAGTAGGTGAGCATGAGCG CAGAGTGTGATGCATCGTCAG	54
OsZIP3	AGGTTCTACGAGGGCAATCAC ATTGGGGTTGTAGAATGACGC	54
OsZIP6	GATGAGGGTTTCTTCGCTCCTT AGCTCTCGTTCCACCGGTAGA	56
OsZIP9	TTCGATCTCAAGCTAACCGCA GAGGATGCAGAAGATGGCGAT	54
OsNRAMP8	CGGGGCAGACTAGTACCATAACG CAGCAAGAGATAGCCATTGATCG	56
OsYSL12	GCCCCCACATTGTGAACGTAT CTGTTCTTCTTCATCATCACGACG	54

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