Comparative expression analysis of metal homeostasis-related genes in rice genotypes differing in grain micronutrient levels

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To understand the role of metal homeostasis-related genes in rice, micronutrient levels of different tissue types were analysed at mid grain-filling stage followed by the expression analysis of candidate genes in these tissues. Subsequently, the association between the gene expression pattern and micronutrient level in tissues as well as mature grains was analysed. Out of 11 candidate genes used for gene expression analysis utilizing bulked cDNA based RT-PCR, 8 genes showed high level of expression in flag leaf and second leaf tissues. Four genes showed poor level of expression in immature grains and low to negligible expression in stem tissues. Further, six candidate genes were selected based on differential response of cDNA bulk analysis for the expression studies of individual rice genotypes, including four high-zinc rice genotypes, namely R-RHZ-LI-25, IR92970-111-1-2, R-RHZ-SM-3, R-RHZ-SM-4 which showed higher level of expression for genes *OsVIT1*, *OsFER1*, *OsYSL2* and *OsYSL9*. Whereas low or negligible level gene expression in stem tissue of five genes, except *OsFER1* shows that rice stem tissue could be involved in the uptake of micronutrients. The thorough characterization of genes in this study provides deeper insight into the tightly regulated mechanism of metal homeostasis with respect to different tissue types and understanding of source–sink relationship of mineral acquisition and remobilization.

Keywords: Gene expression analysis, grain micronutrient level, metal homeostasis, rice genotypes.

MICRONUTRIENT malnutrition, particularly Fe and Zn deficiencies affect over three billion people worldwide, mostly in developing countries[1]. Poor grain micronutrient content in cereals is the primary cause of the prevalent nutritional deficiency-related disorders amongst people having cereals-based diet[2]. A diverse, complex diet is required to maintain health of an individual as well as a community[3]. To alleviate these food-based deficiencies, efforts are underway to develop new cultivars with elevated concentration of iron and other micronutrients[4]. However, to achieve this goal, a clear understanding of metal homeostasis at the molecular level, involving knowledge of the basic physiological processes of metal absorption, distribution and storage is essential[5,6].

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 and thus plants have adopted two distinct strategies to acquire metal ions from the soil[7,8]. 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[9]. Similarly, zinc is more readily available to plants as Zn–MA chelate than as Zn(II) ion[10]. Furthermore, little information is available about how metals are transported to rice seeds and which of the processes (uptake from the soil, transport from the roots to the shoots, phloem-loading, grain-filling) are the rate-limiting steps for metal ions to reach the grain. This information, together with the identification of the genes which actively participate in each one of these processes, is essential to develop rice cultivars with improved nutrient concentration. The present study focuses on assessment of iron and zinc content in different tissue types (flag leaf, second leaf, stem, immature grains) of a few selected rice genotypes and characterization of metal homeostasis-related genes. The study is advancement over our previously published work[11] in which expression analysis of 25 metal homeostasis genes was carried on a set of 12 rice genotypes belonging to different classes (cultivated and wild) through *in situ* and RT-PCR approach. Additionally, in this study a subset of genotypes, including those characterized with high grain Zn levels previously was used for Fe/Zn estimation in different tissue types like flag leaf, second leaf, stem, immature grains in addition to mature grains followed by gene expression analysis in their tissue types. The study also extends to working out grain partitioning among tissue types in a more comprehensive way compared to previous studies.

The plant material used for the study includes eight rice genotypes available at Indira Gandhi Agricultural University (IGKV), Raipur, selected on the basis of zinc content analysed previously. A panel of high and low zinc containing genotypes was made (see Table S1, Supplementary material online). Tissues, i.e. flag leaf, second leaf, stem and immature grains were collected from each plant at mid grain-filling stage and were frozen in liquid nitrogen for gene expression studies; the remaining half was dried in paper bags in an oven at 60°C for 48 h. The oven-dried samples were used for mineral analysis.

Total RNA was isolated from tissues, viz. flag leaf, second leaf, stem and immature grains of each cultivar using Trizol reagent (protocol developed at IOWA State University, Iowa, USA). The concentration of RNA was assessed using NanoDrop Spectrophotometer ND-1000® (NanoDrop Technologies, USA). Total RNA extracted

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was utilized for cDNA synthesis using Thermo Scientific Verso™ cDNA Synthesis Kit according to the manufacturer's instructions. On the basis of high and low grain zinc content of the rice genotypes, cDNA bulk of tissue type was prepared by making a pool of different genotypes of a particular tissue type. Semi-quantitative RT-PCR was first carried out on the bulk cDNA samples followed by expression analysis of genes selected on the basis of their significant change in expression levels (up- and down-regulation) in bulk tissue samples using cDNA-based primers on individual rice genotypes. Semi-quantitative RT-PCR reactions were carried out with 2 μg/g cDNA and final volume was adjusted to 20 μl. Genes were selected based on their strong association with grain Fe and Zn contents in previous experiments[12–14]. The temperature profiles for PCR reactions were set at annealing temperature of 55–60.5°C according to the requirement of metal gene-specific primers (see Table S2, Supplementary material online). Further quantification of expression data was performed using GelQuant.NET software version 1.8.2.

Forty-eight hours over-dried samples were used for the estimation of iron and zinc content. Next, 0.5 g of rice sample (mature grains, leaf, stem, immature grains) of each rice genotype under study was placed in a vessel, 11 ml of di-acid mixture (9 ml HNO_3, 2 ml HCl) was added and kept overnight at room temperature for pre-digestion. Digestion was carried out at 180°C in a microwave digestion chamber (CEM-MARS equipment) for 45 min. The digested samples were diluted to 50 ml with double-distilled water and utilized for iron and zinc estimation (standard method described under *HarvestPlus*, 2006 guidelines) using atomic absorption spectrophotometer (AAS200, Perkin Elmer) taking tomato leaf as standard. The individual tissue types were statistically analysed using completely randomized design. A summary of the statistics is presented in the Supplementary material Tables S3 and S4 (online) for iron and zinc contents respectively).

The elemental analysis results depict significant genotypic variation in mineral concentration ranging from 69.3 to 197.1 μg/g of Fe and 24.9 to 48.2 μg/g of Zn in flag leaf; 68.6 to 159.2 μg/g of Fe and 21.7 to 77.5 μg/g of Zn in second leaf; 30.4 to 156.1 μg/g of Fe and 50 to 75.5 μg/g in stem; 6.1 to 17.4 μg/g of Fe and 18.9 to 36.9 μg/g of Zn in immature seeds and 9.2 to 18.3 μg/g of Fe and 18.9 to 28.7 μg/g of Zn in mature seeds (see Figure S1, Supplementary material online). Rice cultivar R-RHZ-LI-25 (197.1 μg/g), showed higher Fe concentration in flag leaves followed by IR92970-111-1-2 (191.9 μg/g) and lower Fe concentration in IR 92937-189-1-2 (69.3 μg/g). In second leaf tissues, R-RHZ-IB-13 (159.3 μg/g) showed high Fe concentration followed by IR92937-189-1-2 (152.6 μg/g) and lower concentration in IR64 (68.6 μg/g). In stem and immature seeds, high concentration of Fe was found in IR64 (156.1 μg/g) and R-RHZ-IB-13 (17.4 μg/g) respectively. Seed iron concentration was higher in R-RHZ-LI-25 (16.5 μg/g) and R-RHZ-LI-25 (16.5 μg/g) and IR 92970-111-1-2 (16.0 μg/g) compared to the other five cultivars. Zinc content in flag leaf of cultivar R-RHZ-IB-13 (48.2 μg/g) showed higher concentration followed by IR 92937-189-1-2 (45.3 μg/g) and lower concentration in IR64 (24.9 μg/g). Rice cultivars R-RHZ-IB-13 (77.5 μg/g) and IR 92937-189-1-2 (61.6 μg/g) showed higher Zn concentration in second leaf tissue. R-RHZ-SM-4 (75.5 μg/g), R-RHZ-IB-13 (65.5 μg/g) and R-RHZ-LI-25 (63.6 μg/g) showed high Zn concentration in stem, while in immature seed higher Zn concentration was found in R-RHZ-IB-13 (36.9 μg/g) and lower concentration in IR 92937-189-1-2 (18.9 μg/g). Seed zinc concentration was found to be higher in IR 92970-111-1-2 (28.7 μg/g) and R-RHZ-IB-13 (27.9 μg/g) and R-RHZ-MI-32 (27.6 μg/g). Further, seed Fe and Zn concentration was compared with different tissue types and it was found that in majority of rice genotypes tissues, viz. flag leaf, second leaf tissue and immature grains were responsible for Fe/Zn concentration in mature grains (see Tables S3 and S4, Supplementary material online). Similarly, Narayanan *et al.*[12] performed elemental analysis on the flag leaves, non-flag leaves, mature seeds of each of four rice cultivars at mid grain-filling stage and found that iron and zinc contents in flag leaf were higher in IR68144 than the other three cultivars, with Cocodrie and IR58 being low and Taipei 309 being intermediate. Banerjee and Chandel[13] analysed wide variations in micronutrient levels for rice genotypes, which ranged from 4.82 to 22.69 μg/g and 13.95 to 41.73 μg/g for grain iron and zinc content respectively, after screening 46 rice lines, among which three genotypes, R-RF-31, Lalmati and R1033-968-21, were identified as high iron and zinc containing lines.

To understand the role of genes in metal ion uptake, transport and redistribution in plants, semi-quantitative RT-PCR was carried out to study the expression of metal homeostasis-related genes. The cDNAs generated from the total RNA isolated from tissues at mid grain-filling stage of eight rice genotypes were used to study the expression of metal uptake and transport-related genes. Strategy of bulk segregant analysis was performed by preparing cDNA bulk of tissue types by making a pool of different genotypes of a particular tissue type. Thus eight bulk cDNA samples were prepared representing four tissue types, viz. flag leaf, second leaf, stem and immature grains, including a panel of high and low zinc genotypes. In order to know about gene expression levels, RT-PCR was first carried out on the bulk cDNA samples and the genes showing significant change in expression levels (differential expression among tissue types and zinc content) were carried forward (Figure 1). Strategy for bulk segregant analysis for transcription profiling has been taken from the work of Pandit *et al.*[15]. In the present study, cDNA bulk analysis showed differential response

of metal homeostasis genes with respect to tissue types as well as Zn content. Out of 11 genes, 8 (*OsFER1*, *OsYSL2*, *OsYSL9*, *OsZIP8*, *OsZIP10*, *OsNRAMP4*, *OsNRAMP5*, *OsVIT1*) showed high level of expression in flag leaf and second leaf tissue, whereas four genes (*OsFER1*, *OsYSL2*, *OsNRAMP5*, *OsVIT1*) showed poor level of expression in immature grains and low to negligible expression in stem tissues. Based on differential expression level among tissue types and zinc content, six genes, i.e. *OsFER1*, *OsZIP8*, *OsZIP10*, *OsYSL2*, *OsYSL9*, *OsVIT1* were selected for expression analysis on individual rice genotypes for the study. The differential gene expression level implies the significant visible change observed in the form of up- and down-regulation of genes mentioned above with respect to tissue type among selected rice genotypes. Similar results were detected by Narayanan *et al.*[12], where high level of expression was shown by transcripts *OsFER1*, *OsZIP10*, *OsZIP8*, *NRAMP4* in flag leaf and non-flag leaf tissues. Banerjee and Chandel[13] reported high level of expression of transcripts *OsZIP8*, *OsZIP10*, *OsFER1*, *OsYSL2*, *OsYSL9*, *OsVIT1*, *OsNRAMP4*, *OsNRAMP5*. Short-listed genes on bulk RNA samples were then analysed on individual rice genotypes, including different tissue types (Figure 2). For all the genes detected by semi-quantitative PCR, transcripts were detected both in flag and second leaf tissues in most of the cases, and low to non-detectable transcripts were observed in stem and immature grains, although some differences were found in the expression levels of certain genes amongst the various cultivars and between tissues (see Tables S5 and S6, Supplementary material online). The expression pattern of six metal homeostasis-related candidate genes is reported below (Figure 3).

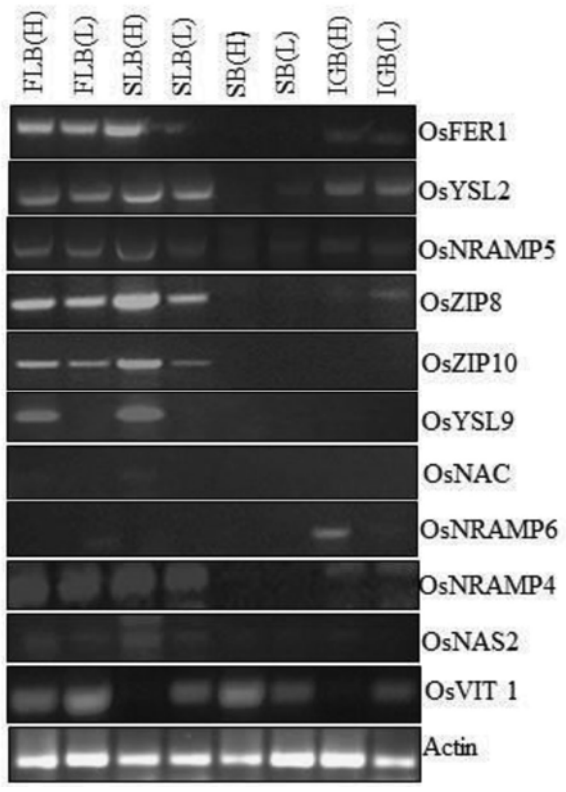

Figure 1. Semi-quantitative RT-PCR profile of metal homeostasis candidate genes on eight bulk cDNA samples representing four tissue types, viz. flag leaf, second leaf, stem and immature grains, including panel of high and low zinc genotypes. FLB(H), Flag leaf bulk high grain zinc; FLB(L), Flag leaf bulk low grain zinc; SLB(H), Second leaf bulk high grain zinc; SLB(L), Second leaf bulk low grain zinc; SB(H), Stem bulk high grain zinc; SB(L), Stem bulk low grain zinc; IGB(H), Immature grains bulk high grain zinc and IGB(L), Immature bulk low grain zinc.

OsFER gene family: *OsFER1* gene showed variable levels of expression in all tissues (flag leaf, second leaf, stem and immature seeds) of each of the eight rice genotypes, indicating that greater amount of *OsFER1* gene is synthesized in tissues during grain-filling stage. Narayanan *et al.*[12] also reported variable levels of expression in both flag leaf and non-flag tissues among four rice cultivars.

OsYSL gene family: The expression of two *OsYSLs* genes (*OsYSL2* and *OsYSL9*) was analysed in tissues of eight rice genotypes. *OsYSL2* transcript showed uniform level of expression in flag leaf and second leaf in the eight rice genotypes. Banerjee and Chandel[13] recorded high level of expression in flag leaf tissues of all tested genotypes at mid grain-filling stage. In immature seed, transcripts were observed in six genotypes, except R-RHZ-LI-25 and IR 92970-111-1-2, and showed low level to negligible expression in stem tissues. Similarly, *OsYSL9* gene showed expression in flag leaf tissue of rice genotypes R-RHZ-MI-32, IR 92970-111-1-2, R-RHZ-SM-3 and R-RHZ-SM-4. In second leaf tissues, high level of expression was observed in R-RHZ-IB-13, R-RHZ-MI-32 and R-RHZ-SM-3, and low level of expression in R-RHZ-LI-25, IR 92970-111-1-2 and R-RHZ-SM-4. In stem *OsYSL9* transcripts showed no expression. *OsYSL9* gene showed low level of expression in rice genotypes R-RHZ-IB-13 and R-RHZ-MI-32 in immature seeds.

OsZIP gene family: Expression of *OsZIP8* transcripts was observed in flag leaf tissues of rice genotypes R-RHZ-SM-3, R-RHZ-SM-4, IR 64 and IR 92937-189-1-2 at mid grain-filling stage. In second leaf tissues, *OsZIP8* genes showed expression in rice genotypes R-RHZ-IB-13, R-RHZ-SM-3, R-RHZ-SM-4, IR 64 and IR 92937-189-1-2. *OsZIP8* genes showed expression in immature seeds in genotypes R-RHZ-SM-4, IR 64 and IR 92937-189-1-2. However, no transcripts were depicted in stem tissues. Uniform level of expression of *OsZIP10* genes was observed in flag leaf tissue in rice genotypes R-RHZ-LI-25, IR 92970-111-1-2, R-RHZ-SM-3, R-RHZ-SM-4, IR 64 and IR 92937-189-1-2. In second leaf tissue, *OsZIP10* transcripts were detected in all rice genotypes, except IR 92970-111-1-2. *OsZIP10* showed poor to negligible expression in immature seeds and no expression was observed in stem. Similar results have been reported

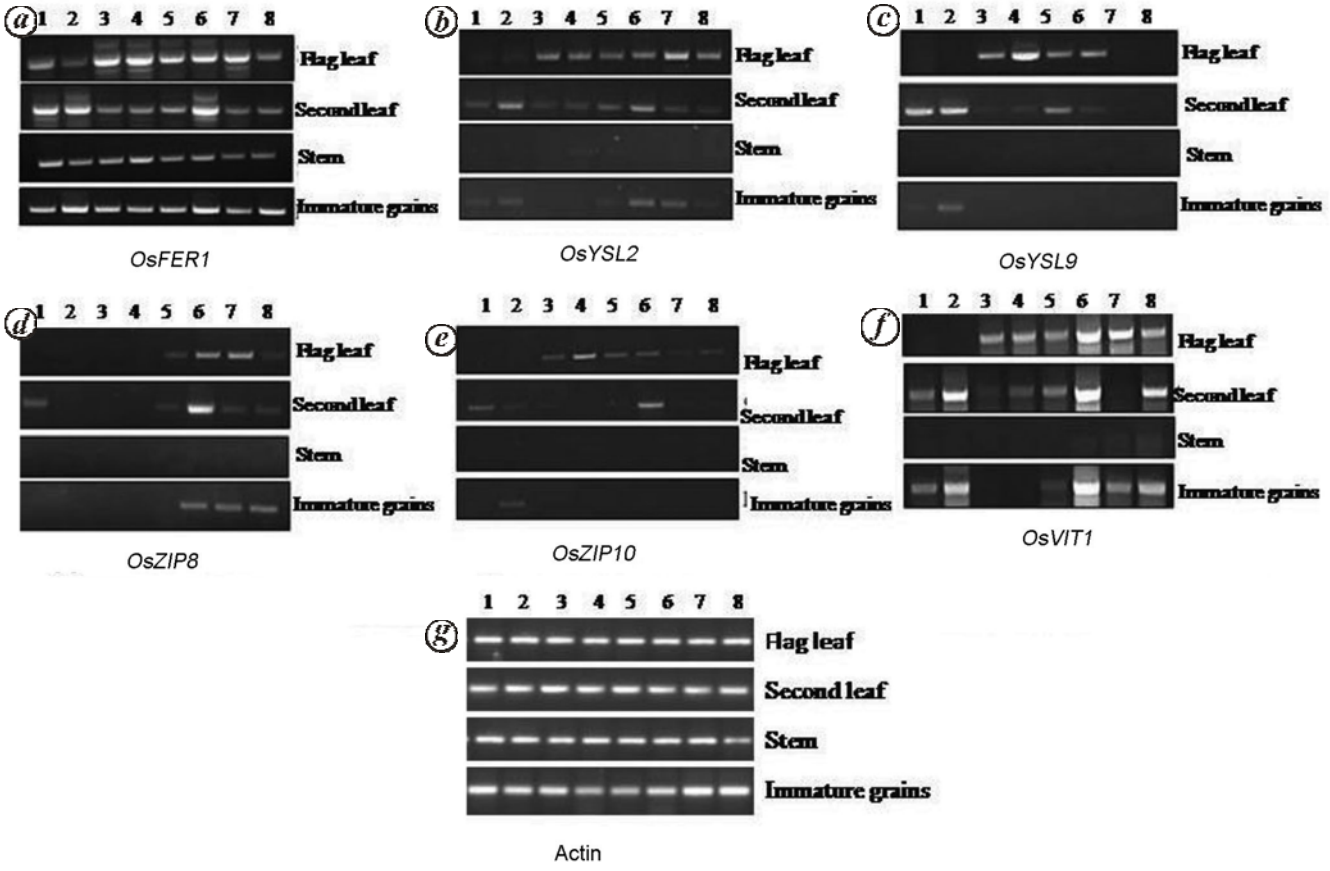

Figure 2 ***a*–*g***. Semi-quantitative RT-PCR profile of metal homeostasis related candidate genes in eight diverse rice genotypes at mid grain-filling stage in different tissues. Lane 1, R-RHZ-IB-13; lane 2, R-RHZ-MI-32; lane 3, R-RHZ-LI-25; lane 4, R92970-111-1-2; lane 5, R-RHZ-SM-3; lane 6, R-RHZ-SM-4; lane 7, IR64 and lane 8, R92937-189-1-2.

in expression analysis of *OsZIP8* and *OsZIP10* in both leaf and flag leaf tissues at mid grain-filling stage[12,14].

OsVIT gene family: Banerjee and Chandel[13] reported that *OsVIT1* gene expressed in flag leaf tissue showed genotypic variation in the level of expression. In the present study, we found that *OsVIT1* gene is expressed in flag leaf tissue of rice genotypes R-RHZ-LI-25, IR 92970-111-1-2, R-RHZ-SM-3, R-RHZ-SM-4, IR 64 and IR 92937-189-1-2 with high level of expression in R-RHZ-SM-4, IR 64 and moderate level of expression in rest of the genotypes. In second leaf tissue, *OsVIT1* gene showed expression in all genotypes, except IR64. Poor to negligible expression was observed in stem tissue. *OsVIT1* gene is expressed in immature seeds in all genotypes, except R-RHZ-LI-25 and IR 92970-111-1-2 (Figure 2).

Semi-quantitative RT-PCR was carried out to characterize the expression of genes among different tissue types with varied levels of iron and zinc concentration. It was observed that expression of most of the metal-related genes was well-correlated with tissue Fe and Zn concentrations (Figure 3). Metal-related genes (*OsVIT1*, *OsYSL2*, *OsYSL9*, *OsZIP10*, *OsFER1*) showing high level of expression in rice genotypes R-RHZ-LI-25, IR 92970-111-1-2, R-RHZ-SM-3 and R-RHZ-SM-4 are found to be correlated to variation in flag leaf tissue, and grain iron and zinc content (see Table S5, Supplementary material online). But variation in level of expression of *OsZIP8* was not correlated to difference in flag leaf tissue and grain iron or zinc content, and moderate level of expression of *OsZIP10* genes was observed in flag leaf tissues at mid grain-filling stage. In second leaf tissue, medium to high level of expression of majority of the genes was observed in rice genotypes R-RHZ-SM-4 followed by R-RH-BI-13 and R-RHZ-MI-32. High level of expression of *OsVIT1*, *OsFER1*, *OsYSL2* and *OsYSL9* was observed in R-RHZ-MI-32, which has high second leaf tissue iron concentration (140.37 μg/g Fe). *OsVIT1* gene expression in genotype R-RHZ-IB-13 (0.17) and IR 92937-189-1-2(0.09) was found to be correlated with high iron concentration (159.25 and 152.62 μg/g) and high zinc concentration (77.5 and 61.62 μg/g) in second leaf tissue. Expression of *OsYSL9* gene was observed in second leaf tissue of R-RHZ-IB-13 (0.14), which has higher second leaf Fe (159.25 μg/g) and Zn (77.5 μg/g) concentration. Furthermore, high expression of *OsVIT1*, *OsFER1*, *OsZIP8*, *OsZIP10* and *OsYSL2* genes in second leaf tissue was observed in R-RHZ-SM-4, which has high second

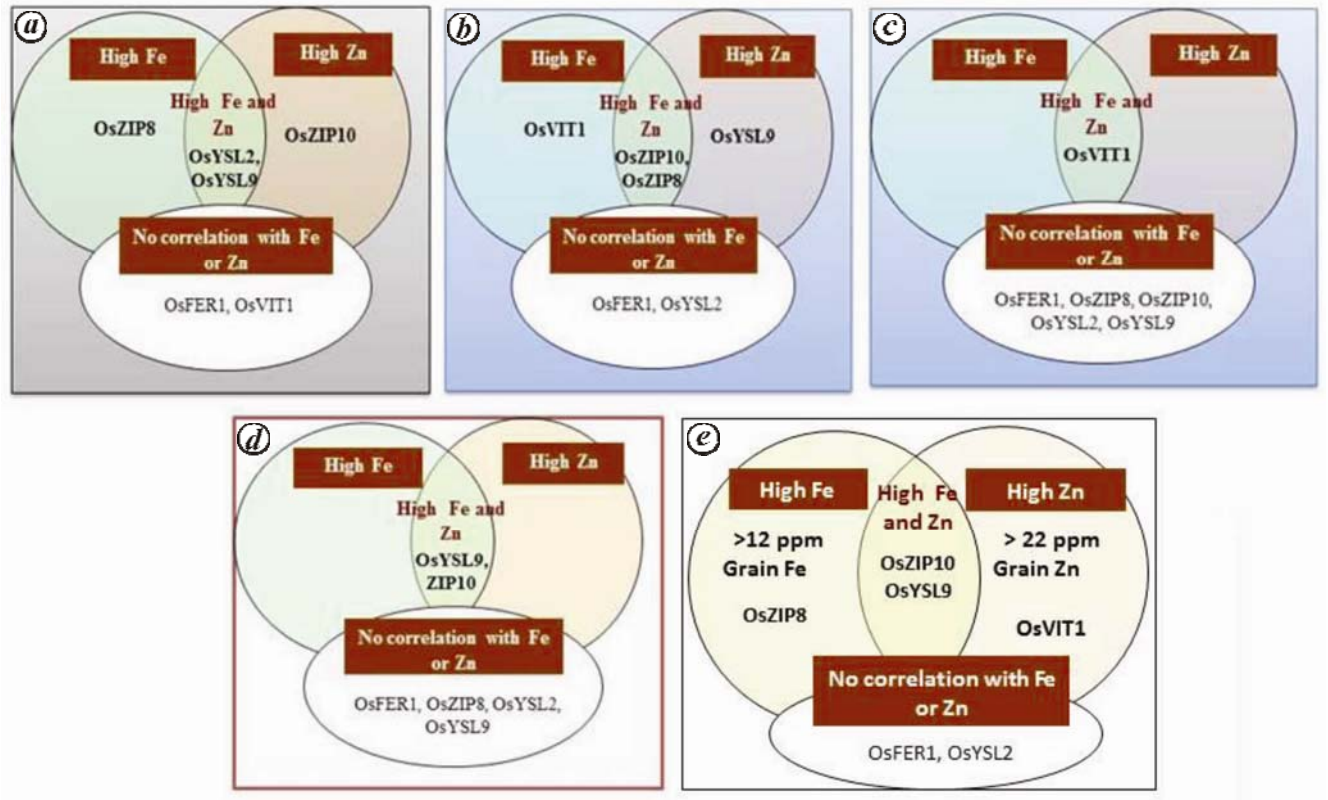

Figure 3 ***a–e.*** Schematic representation of candidate genes with tissues: (***a***) Flag leaf, (***b***) second leaf, (***c***) stem, (***d***) immature grains, and (***e***) micronutrient level of mature grains among the tested genotypes.

leaf tissue Fe (145.37 μg/g) concentration. In stem tissue, poor to negligible expression of candidate genes was observed in the present study excluding *OsFER1* gene which showed variable level of expression in all eight genotypes, but the difference in level of expression was not found to be correlated to difference in stem Fe/Zn concentration. Expression of *OsVIT1* genes was observed in stem tissues in genotypes R-RHZ-SM-4 (0.01) and IR64 (0.01), which have higher stem tissue Fe and Zn concentration. In immature seeds, high level of expression of *OsVIT1* gene was observed in R-RHZ-SM-4 (0.37), and R-RHZ-MI-32 (0.19). It was found to be correlated with immature seed Fe and Zn concentration in R-RHZ-SM-4 (15.15 μg/g Fe and 24.85 μg/g Zn) and in R-RHZ-MI-32 (15.16 μg/g Fe and 33.65 μg/g Zn). Expression level of *OsZIP8* gene was found to be correlated with iron and zinc concentration of immature seeds rice genotypes R-RHZ-SM-4 (0.04), IR64 (0.03) and IR 92937-189-1-2 (0.04). Further, in flag leaf tissue, expression of *OsZIP8* and *OsZIP10* was found to be correlated to high Fe and Zn concentration respectively, for the same tissue. *OsYSL2* and *OsYSL9* were found to correlated with Fe and Zn concentration of flag leaf tissue high, whereas *OsFER1* and *OsVIT1* were found to be weakly associated with Fe and Zn concentration of flag leaf tissue. In second leaf tissue, transcripts of *OsZIP10* and *OsZIP8* were found to be correlated with high Fe and Zn concentration of the same tissue. *OsFER1* and *OsYSL2* were found to be weakly associated with Fe and Zn concentration of second leaf tissue. Transcripts *OsVIT1* and *OsYSL9* were found to be in correlation with Fe and Zn concentration respectively, of second leaf tissue. *OsFER1* in stem tissue was found to be weakly associated with Fe and Zn concentration of stem tissue, whereas *OsVIT1* is found to be correlated to Zn concentration in the same tissue. In immature grains, *OsYSL9* and *OsZIP10* were found to be correlated to Fe and Zn concentration of immature grains, whereas *OsFER1*, *OsYSL2*, *OsZIP8* and *OsVIT1* were weakly associated. In mature grains, transcripts *OsZIP8* and *OsVIT1* were found to be correlated to Fe and Zn concentration respectively. *OsZIP10* and *OsYSL9* genes were found to be correlated to both iron and zinc concentration. *OsFER1* and *OsYSL2* were found to be weakly associated with Fe and Zn concentration. Sperotto *et al.*[16] reported expression levels of nine metal homeostasis-related genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) in flag leaves exhibiting significant correlation with Fe and/or Zn concentration in the seeds. Banerjee and Chandel[13] found the expression of nine (*OsFER1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsYSL6*, *OsYSL12*, *OsYSL4*, *OsZIP8* and

OsZIP10) and seven (*OsNAC*, *OsYSL2*, *OsYSL9*, *OsZIP4*, *OsVIT1*, *OsNAAT1* and *OsNRAMP7*) genes in correlation with high Fe and Zn contents respectively.

Elucidating the genotype-dependent response of the genes involved in metal homeostasis mechanism, particularly grain loading of micronutrients will be helpful in planning effective breeding strategies. The efforts made in the present study towards this concept reveal concordance of expression of related genes with the Fe/Zn content in tissues as well as mature grains for genotypes selected on the ground of their Zn content. Thus we have characterized the expression of metal-related genes in selected set of rice genotypes providing insight into the tightly regulated mechanism of metal homeostasis with respect to different tissue types, which is useful in understanding source–sink relationship of mineral acquisition and remobilization in the rice genome.

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Occurrence of the hispa *Asamangulia cuspidata* and its parasitoids in South India

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The occurrence of the leaf miner *Asamangulia cuspidata* Maulik (Coleoptera: Chrysomelidae: Cassidinae: Hispini) on sugarcane in Coimbatore, Tamil Nadu, India, is reported here with notes on pest biology and parasitoid activity. A minor pest in a few states of subtropical India, the miner was first noticed in May 2014 during routine surveys. Systematic observations in selected experimental and growers' plots revealed low levels of incidence and intensity, the highest mean attack rates being 4.18% on plant basis and 12.41% on leaf basis. Mean mined leaf area showed a high of 4.24 sq. cm and it constituted 1.28% of the total leaf area. Cross-sections of young and mature mines indicated feeding on softer tissues by the solitary grub in the early stages, but extensive mining by the grown-up grub leading to complete drying of the mined area. One apparently new *Bracon* sp. (Hymenoptera: Braconidae), two *Pediobius* spp. (Hymenoptera: Eulophidae) and one *Eurytoma* sp. (Hymenoptera: Eurytomidae) were recovered from the miner. While *Bracon* sp. contributed 70% to the overall parasitism rate of 39.3%, the remaining parasitoids accounted for 30% with likely hyperparasitism among them. The possible origin of the miner and the role of parasitoids in its natural control at the present study site are also discussed.

Keywords: Leaf miner, parasitoids, parasitism, pest biology, sugarcane.

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