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Research paper



Role of Zn-Regulated Transporterand Iron (Fe)-Regulated Transporter-Like Protein (ZIP) Gene Family in Rice(*Oryzasativa.L*) in Foliar Application of Zinc as Bio Fortification Strategy to Enhance Grain Zinc Content

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Abstract

Cereal crops especially rice (*Oryza sativa L.*) play an important role in satisfying daily calorie intake in developing world, but they are inherently very low in zinc concentrations, more so when grown on zinc deficient soils. Biofortification is one among various immediate strategies for overcoming Zn deficiency in humans. Availability of soil applied zinc to the plant is limited by chemical and physical soil properties and hence foliar application of Zn is an effective strategy. Mobilization of foliar applied zinc and remobilization of plant accumulated zinc to grain is dependent on Zinc(Zn)-regulated transporter and iron(Fe)-regulated transporter-like protein(ZIP) gene family. Here we discuss the expression pattern of OsZIP1, OsZIP2, OsZIP3, OsZIP4, OsZIP5 and OsZIP7 transportersin two different seed zinc genotypes(high grain zinc content types and low grain zinc content types) at vegetative stage(65DAS) and at reproductive stage(50% panicle initiation)in stem, leaves and reproductive parts in response to Zn foliar applicationin the form of $ZnSO_4.7H_2O$as an essential advance for understanding and manipulating the Zn absorption and translocation in rice. RT-PCR analysis at 65DAS showed OsZIP1, OsZIP2, OsZIP7 expression to be very minimal in leaves. OsZIP3 andOsZIP4 expression was medium to highindicatingOsZIP3 and OsZIP4 to be actively involved in mobilization of foliar applied zinc from leaves.OsZIP1, OsZIP3 and OsZIP4 had higher activity at reproductive stage in leaf compared to vegetative stage suggesting their involvement in zinc remobilization/transport during reproductive stage.OsZIP4 and OsZIP7 hadhigher expression activity in developing grains.There was no difference observed in expression levels of transporters among zinc types. There was large variability observed in zinc mobilization due to transporter function specificity and it is possible to enhance Zn content in grains under zinc sufficient conditions by direct foliar application at grain filling stage as transporters would facilitate this mobilization.

Keywords: *Biofortification, Foliar Zn application, Zn deficiency, Zn-regulated transporters and iron(Fe)-regulated transporter-like protein(ZIP), Oryzasativa.L.*

1. Introduction

Micronutrients are essential for balanced nutrition in plants and animals [1,2]. Zinc deficiency ranks fifth among the most important health risk factors in developing countries and eleventh worldwide, and is as important as iron(Fe) and vitamin A deficiencies. There are many possible strategies to improve micronutrients intake in the human diet including dietary diversification, mineral supplementation and post-harvest food fortification.

Rice is an important cereal as staple food. Nutrition provided by rice are particularly important in Asia, where it accounts for 50-80% of daily caloric consumption. Thus, increasing the Zn content of rice has a great potential to mitigate wide spread Zn deficiency problems in humans [3]. Agronomic zinc biofortification through fertilizer application is a complementary approach to increase zinc concentration in genotypes to enable them to reach the target zinc even in zinc deficiencies. Work on agronomic biofortification of rice through soil applied zinc fertilization has shown it to be inconsistent [4]. Several micronutrients, including calcium, magnesium, potassium and sodium are known to inhibit the absorption of zinc by plant roots in solution culture experiments.

Foliar zincapplication is more economically efficient as foliar Zn fertilization rates are less than soil Zn fertilization rate and it avoids the complex soil interactions that limit plant zinc uptake through roots. When compared to soil applications, foliar application is more effective in increasing grain zinc [5,6]. Mobilization of soil applied zinc into aerial parts is through Zn-regulated transporters and iron(Fe)-regulated tranporter-like protein(ZIP) gene family.[7]These proteins vary considerably in overall length, due to variable region intrans membrane domain providing a potential metal-binding domain rich in histidine residues involved in transport [7].

Although the understanding of Zn uptake and translocation in rice is increasing, information regarding expression of Zn transporters during different developmental stages is scarce. There has been no studies on Zn transporters expression pattern in response to foliar applied zinc in terms of mobilization of foliar Zn application.

In rice there are around 21 ZIP family of transporters reported for zinc. We investigated fiveof the zinc transporters that were reported earlier by various researchers to be involved in Zn transport

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from leaf, stem and developing grains. It is reported that OsZIP1 and OsZIP3 transport Zn but not Fe or Mn [8]. OsZIP4 was also reported to be highly selective for Zn, but not for other metals. Hence understanding these 3 transporters which are very specific to zinc would provide us understanding on Zn bio fortification and also for any genetic engineering approach as manipulating these transporters would not jeopardize the levels of other metals like Fe, Mnetc in plants. In addition, ZIP2 and ZIP7 werealso investigated as ZIP2 and ZIP7 were reported to be enhanced in leaves with the application of zinc

2. Material and Methods

2.1 Plant material selection

Plant material was selected based on previous study where 320 entries of diverse group of rice germplasm lines were assessed for variability in leaf and seed Zn levels [9]. From these entries, four genotypes having high seed zinc content (Rasi, BR-82655, BPT-5204, RathnaChudi with seed zinc ranging from 3.9-5.08mg/100g DW)and four genotypes with low seed Zinc content (CTH-1, CTH-3, IR30864, MTU1001 with seed zinc ranging from 2.11-2.48mg/100g DW) were selected for the studies on foliar zinc application on grain zinc content.

2.2 Experimental design

Rice seedlings were raised in nursery bed, transplanted 2 seedlings of 21 days old into pots of 10 kgs soil holding capacity containing red sandy loam soil with recommended dose of N:P:K:Zn.

2.3 Zinc treatment

Three pots per treatment was allotted. Treatments were:Recommended dose (Control: 5g/lt of zinc), 10% below recommended practice (T1: 4.5g/lt) and 10% above recommended practice (T2: 5.5g/lt).
Zinc in the form of $ZnSO_4.7H_2O$ spray was taken up at 2 times. 1) 65 days of sowing (vegetative phase) 2) 50% panicle emergencestage (reproductive phase).

2.4 Sample collection

Samples for zinc analysis was collected 10 days after imposing the treatment in three replications. Total leaf (Leaf+sheath) and stem zinc were analysed at 65 days and total leaf (Leaf+sheath), stem and developing grain zinc were estimated at panicle initiation stage. All the sampling collected were frozen immediately in liquid nitrogen and stored in -80^0 C freezer and used of RNA extraction and CDNA synthesis.

2.5 Zinc estimation

Zinc was estimated in the grains, leaf and stem samples using Polarized Zeeman Atomic Absorption Spectrophotometer (AAS-2-6100) [10].

2.6 Expression analysis

2.6.1 Extraction of total RNA

RNA was extracted following the protocol with some modifications [11].

2.7 Qualitative and quantitative estimation of RNA

Extracted RNA was qualitatively monitored in 1% agarose gel electrophoresis. Concentration of the nucleic acids was spectrophotometrically determined at OD of 260 and 280 nm. The quality was checked by running 1 ug of RNA for all the samples on a 1.0% formaldehyde agarose gel.

2.8 RT- PCR analysis

Total RNA was treated with RNase-free DNaseI to remove contaminating genomic DNA. First strand cDNA was synthesized using M-MuLV reverse transcriptase. The resulting fragment was amplified by PCR.There was no genomic contamination and no differences in the internal control in each sample.Expression levels of transporters were scored based on figure I reference for cDNA expression intensity

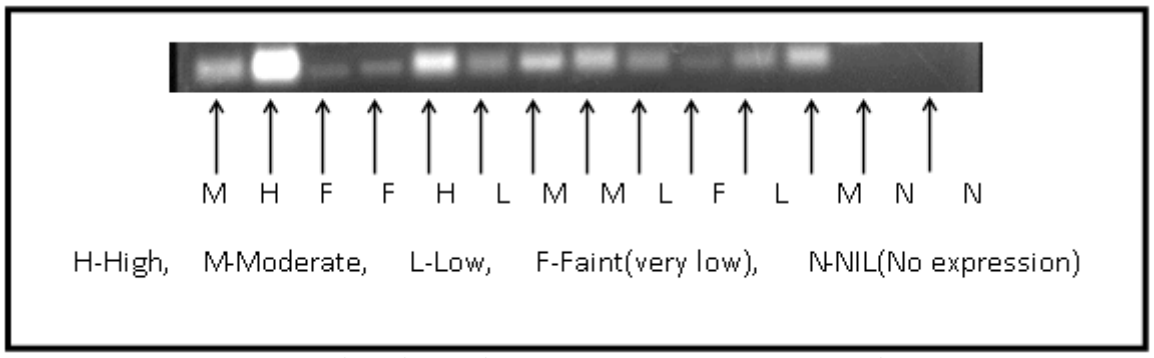

Figure1: Criteria of classification of cDNA expression intensity based on levels of expression

2.9 Multiple sequence alignment of ZIP transporter proteins

ZIP protein sequences ranged from 349 to 402 amino acids in length. NCBI reference sequence ID was obtained in NCBI protein search and protein sequences alignment was done in multalinhttp://multalin.toulouse.inra.fr/multalin/.We used the program MEMEfor protein domain identification [12].

3. Results

3.1 Zinc uptake and mobilization at 65 DAS

Though individual genotypes responded differently to zinc foliar treatment, there was higher levels of zinc in stem compared to leaves(figure II).Treatment T1 had higher leaf and stem zinc compared to control and T2.

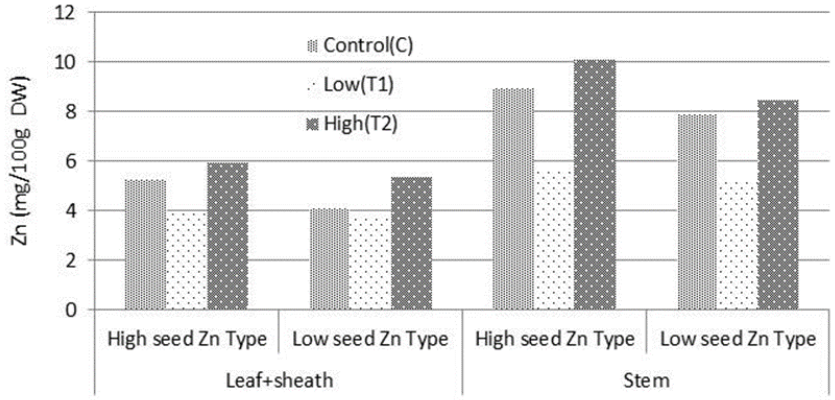

Figure 2: Zinc content in different plant parts after 15 days of foliar Zn application at vegetative stage(65DAS).

3.2 Zinc uptake and mobilization at panicle initiation stage

Zinc content of leaves(leaf+sheath), stem and grain that had started to develop were analysed for zinc content. (figure III)

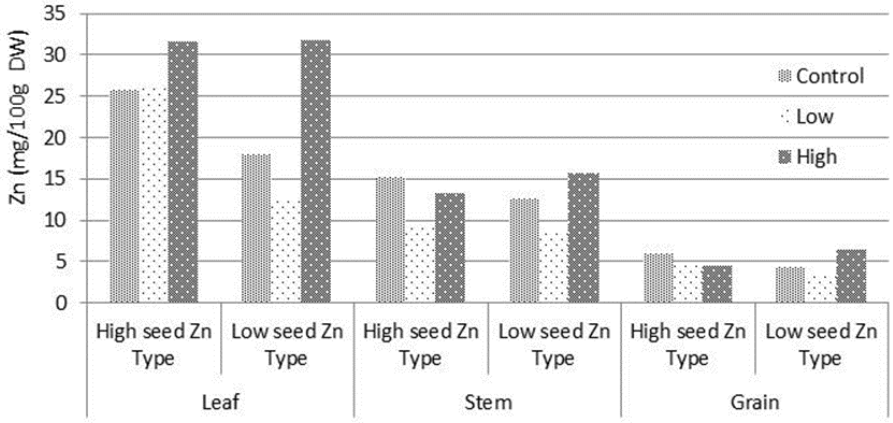

Figure 3: Zinc content in leaf, stem and grain after 15 days of foliar Zn application at Panicle initiation stage

Mean zinc concentration of genotypes in leaves was high in T1 treatment, followed by control and T2 treatment.In stem, control had highest zinc content followed by T1 treatment. T2 treatment had higher zinc in low zinc type compared to high zinc type. Similar trend was observed in developing seed. Differences were significant in developing seed.

3.4 Expression analysis of zinc transporters at 65DAS

When semi quantitative zinc transporter expression analysis was done 15 days after imposing the treatment at 65DAS, all the ZIP transporters investigated had higher levels of expression in leaves compared to stem.(Figure IV)

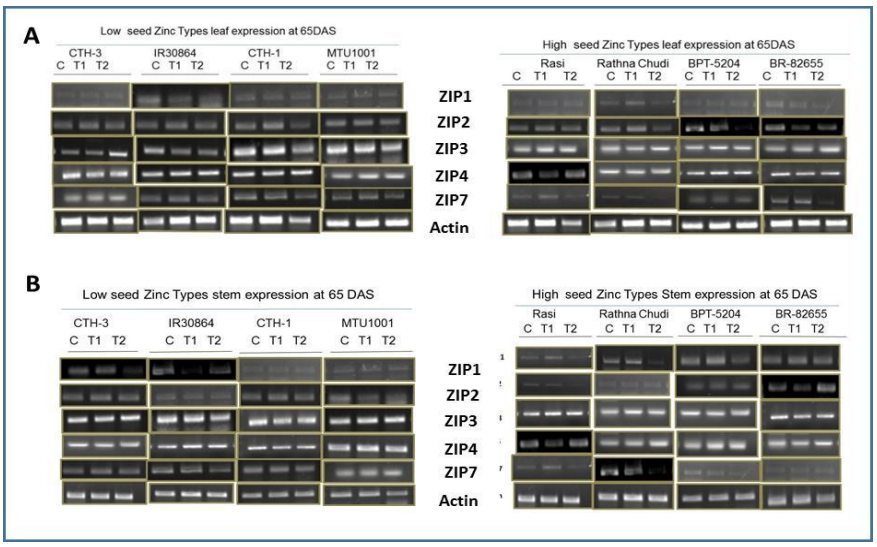

Figure 4: Expression pattern of Zinc transporters at 65DAS in leaf and stem of high seed zinc types and low seed zinc types across all the treatments of Zn- RT PCR analysis

ZIP1 expression levels were very minimal in leaves(A, Figure IV) for all three treatments. ZIP2 expression was found to be at low levels in low zinc type and medium expression in high zinc type. ZIP3 expression level was medium in low zinc type and higher in high zinc type. In both zinc types, T2 had higher expression compared to Control and T1. Expression higher than ZIP3 was noticed in ZIP4 expression across treatments and 2 zinc types. ZIP7 had low levels of expression in leaves.

Stem expression at this stage(B, Figure IV) was same as leaf for ZIP1, lower for ZIP2. Stem expression was high for ZIP3 and ZIP4 compared to leaves. ZIP7 expression was low.

3.5 Expression analysis of zinc transporters at Panicle initiation stage

Semi quantitative zinc transporter expression analysis results at panicle initiation showed all the ZIP transporters with higher levels of expression in leaves compared to stem and developing seed(Figure V).

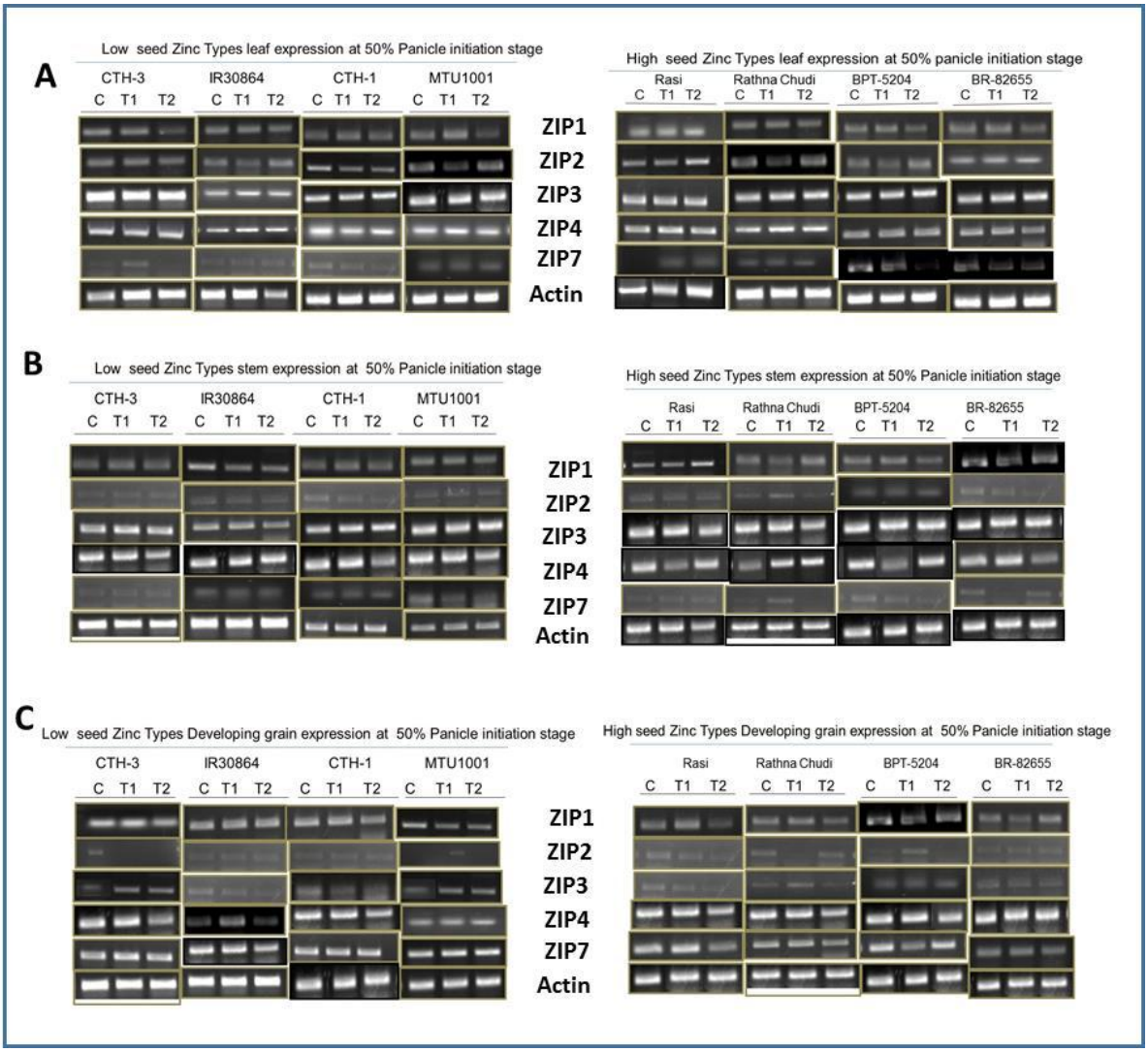

Figure 5: Expression pattern of Zinc transporters at 50% of Panicle initiation in leaf, stem and developing grains of high seed zinc types and low seed zinc types across all the treatments of Zn- RT PCR analysis

ZIP1 and ZIP2 leaf expression was similar and was at low levels for all three treatments and for two zinc types. ZIP3and ZIP4 had higher expression for both zinc types but very faint to no expression for ZIP7. Leaf expression for all ZIP transporters was similar to 65 days stage samples except for drop in ZIP7 expression.

ZIP1 expression in stem was on par with leaves. Expression levels of ZIP2 reduced in stem compared to leaves. ZIP3 and ZIP4 expression was higher in stem compared to ZIP1 and ZIP2. ZIP7 stem expression was very faint or undetectable.

Expression analysis of developing seeds revealed transporter ZIP4 levels slightly higher in seeds than stem but ZIP2 levels were minimal to no expression. For ZIP1 and ZIP3, expression levels of developing seed was slightly lower compared to stem. ZIP7 expression was found to be higher in both genotypes in developing seed compared to stem and leaf.

3.6 Multiple sequence alignment of ZIP transporter proteins

ZIP transporters under investigation were analyzedfor sequence similarity to correlate with expression pattern(Figure VI).

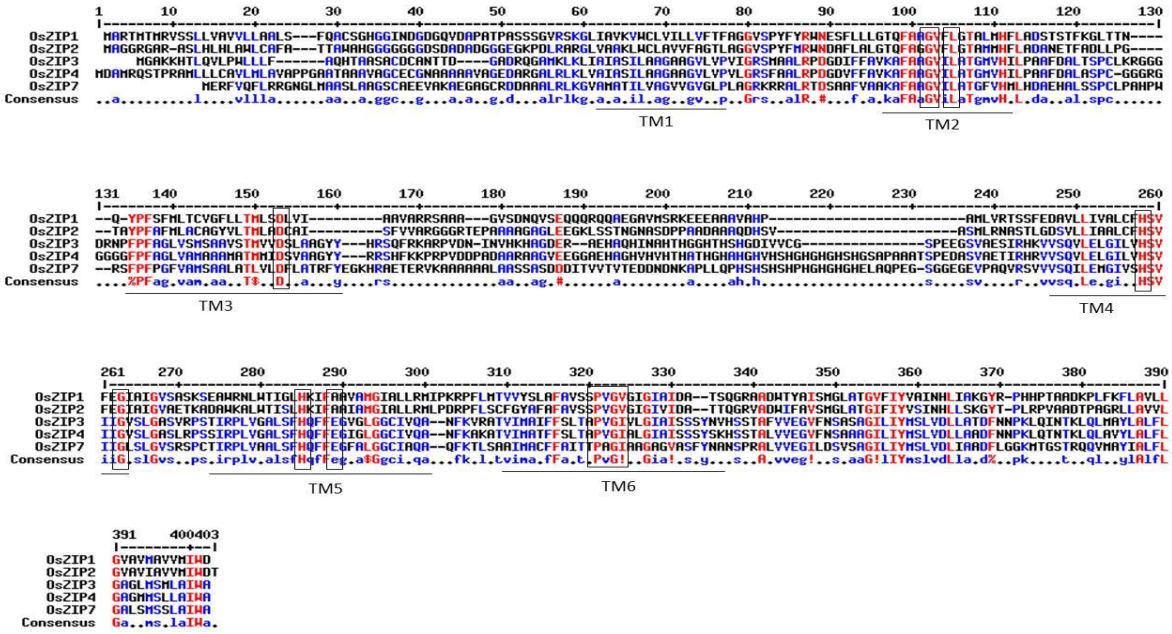

Figure 6: Amino acid alignment of ZIP proteins of rice genome. Sequences were aligned using multalinhttp://multalin.toulouse.inra.fr/multalin/. Conserved Residues are marked in black boxes whereas similar amino acid residues were highlighted as red colour text. The membrane spanning domains are indicated as line below the sequence and numbered TM1 to 6 respectively.

The amino acids in the extracellular region between trans membrane domain TM3 and TM4 of ZIP proteins were highly variable in size and content, yet rich in histidine. OsZIP4 and OsZIP7 has four histidine residues in this area. Interestingly, OsZIP1, OsZIP2 and OsZIP3 possessed the same variable region with a lack of general sequence conservation.

10 distinct motives were identified in these genes (Figure VII). All of the 5 ZIP transporters had 4 motifs in common indicating conserved motives contributing to similar function. On the other hand, the divergence in motif composition among different ZIP proteins may indicate that they are functionally diversified.

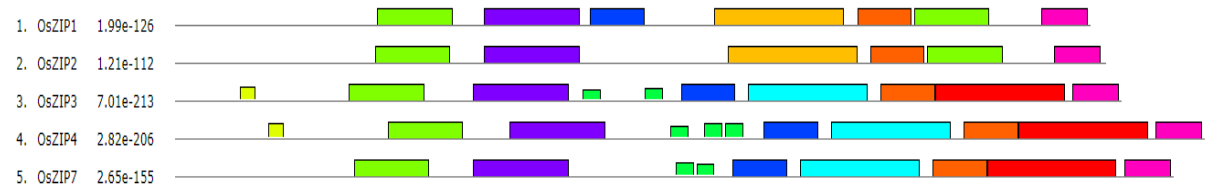

Figure 7: A schematic representation of conserved motifs(obtained using MEME) in OsZIP proteins. Different motifs are represented by different color boxes.

4. Discussion

4.1 The relevance of zinc transporters in zinc uptake and translocation

4.1.1 Expression analysis of zinc responsive genes

ZIP family Fe and Zn transporter proteins play an important role in improving micronutrient density in plants [13-17]. In most cases, the genes encoding the Zn transporter proteins are expressed in response to Zn deficiencies. Similarly several homologs of ZIP transporters have been shown to be highly expressed in rice and several other species under zinc starvation. However, the role of these transporter proteins for grain Zn accumulation in response to Zn foliar application under Zn sufficiency condition is not clear.

4.2 Zn transport at vegetative stage(65DAS)

Previous studies suggest that OsZIP1 is primarily involved in metal transport when rice is deprived of zinc and that OsZIP3 is constitutively active and involved in overall cell zinc homeostasis, particularly in leaves [8].

Expression pattern of ZIP1, 2, 4, 7 proteins at this stage for stem samples were lower than leaf but ZIP3 levels were higher compared to leaves in stem. This indicates role of ZIP3 in active mobilization of zinc into stem or to other organs.OsZIP3 expression was reported in the stem vascular bundles and epidermal cells while OsZIP4 expressed mainly in the phloem [8, 18].

4.3 Zn transport at reproductive stage(Panicle initiation stage)

Since there was no difference in expression levels of transporters among high seed zinc types and low seed zinc typesin the current studies, it can be concluded that zinc transporters investigated were actively transporting available Zn from direct foliar application.

Expression studies have reported stem Os.ZIP3 expression in vascular bundles and epidermal cells [8]. OsZIP1, OsZIP3, OsZIP4, OsZIP5 and OsZIP8 were also expressed in stem at 83 and 90 DAT. Zn (and Fe) in the rice grains may be actively supplied via the phloem after mobilization from the leaf blades.Information about the expression of OsZIP genes during different stages of flowering and seed development is scarce. In embryo and endosperm, the expression of OsZIP4 was reported to be significantly high compared to other OsZIPs [18]. The expression of OsZIP1, OsZIP3, OsZIP4, and OsZIP5 increased slowly from 7 to 42 DAF.

As OsZIPs are expressed during seed developmental stage, it can be concluded that these genes play an important role in Zn transport to developing seed. The role of OsZIP4 and OsZIP7

seems particularly important in transporting the Zn to the developing seed.
It can also be concluded that under zinc sufficient conditions, transporters actively mobilise foliar applied zinc to developing grains though a larger amount may have been mobilized to stem and root as reported in 65Zn studies [19].

4.4 Bioinformatic analysis of ZIP transporter proteins

Alignment results revealed that all the investigatedZIP proteins processed the same variable region with lack of general sequence conservation between TM3 and TM4 trans membrane domains. OsZIP4 and OsZIP7 had rich histidine sequence similarity in this extra cellular region which we could probably correlate to expression activity in developing grains which is different compared to other ZIPs investigated. Previous research study in AtIRT1 mutant yeast strains has demonstrated the effects of point mutations on transport activity, and validated the importance of specific residues in this extra cellular loop between transmembrane domains TM2 and TM3 [20].
All the above studiesnamely zinc content analysis in various tissues, expression analysis of zinc transporters(ZIPs) and sequence alignment correlate with one another and with experimental evidence from previous researchers. It can be concluded that there is large variability in zinc mobilization due to transporter function specificity and it is possible to enhance Zn content in grains by direct foliar application at grain filling stage as transporters would facilitate this mobilization.

5. Conclusion

This foliar Zn application strategy could be a rapid solution to the low grain zinc problem and can be considered an important complementary approach to the on-going breeding programs. Findings of this investigation on expression pattern of zinc transporters can be utilized further for transgenic approach of improving grain zinc content.

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Conflict of Interest

Conflict of interest declared none.

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