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A Metallochaperone HIPP33 Is Required for Rice Zinc and Iron Homeostasis and Productivity

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Abstract: Both zinc (Zn) and iron (Fe) are essential micro-nutrients for plant growth and development, yet their levels in plants are tightly regulated to prevent either deficiency or phytotoxicity. In agronomic reality, such an imbalance of metal bioavailability to crops occurs frequently. Thus, mining genetic resources to improve crop traits relevant to metal homeostasis is a great challenge to ensure crop yield and food quality. This study functionally identified an uncharacterized metallochaperone family HIPP protein gene *Heavy Metal Associated Isoprenylated Plant Proteins 33* (*OsHIPP33*) in rice (*Oryza sativa*). *OsHIPP33* resides in the nucleus and plasma membrane and constitutively expresses throughout the lifespan. Transcription of *OsHIPP33* is not induced by deprivation of Zn and Fe but upregulated under excessive Zn and Fe stress. In a short-term (one month) hydroponic study with the normal Zn and Fe supply, there were no significant changes in the growth and metal accumulation between the knockout (*OsHIPP33*) or knockdown (RNA interference) mutant lines and wild-type, while the long-term field trials (for two successive years) demonstrated that the mutation of *OsHIPP33* significantly compromised the rice growth and development (such as rice leave tissues, panicle length, spikelet fertility, seed weight per plant, 1000-grain weight, etc.), with the mature grain yield of *OsHIPP33* and RNAi lines reduced by 52% and 12–15% respectively, compared with wild-type. Furthermore, the accumulation of Zn and Fe in rice straw, husk and brown rice was also reduced. These results suggest that the disruption of *OsHIPP33* can dampen rice agronomic traits, signifying that *OsHIPP33* expression is required for Zn and Fe homeostasis and subsequent production of rice grains.

Keywords: metallochaperone; HIPP33; rice; zinc; iron; grain

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1. Introduction

Zinc is an essential micro-nutritional element for diverse biological functions in plants. One central role is as key cofactor for many metalloenzymes and other proteins actively participating in biological processes including conformation of structured proteins, activation of transcription factors or mediation of protein–protein interactions [1]. Despite its importance, Zn intake from staple food crops like cereals is inadequate in more than two billion people, accounting for 17.3–30% of the world population [2]. Most types of soil are unable to provide enough free Zn accessible to plants (Zn efficiency) for healthy plant growth and development [3]. As a consequence, zinc starvation symptoms appear with stunted plant growth, manifested by shorter internodes, smaller size of leaves, necrosis of root tips and young leaf yellowing [4]. Iron is another Zn-equivalent essential metal and also serves as a cofactor of diverse enzymes, playing an indispensable role in photosynthesis, respiration and many other metabolic processes [5]. Although most soils on Earth have sufficient iron elements, their real availability to plants is limited. Iron deficiency also causes the arrest of plant growth, leaf chlorosis, reduced reproductively, and low quality of seed [6].

Regulation of Zn and Fe homeostasis in plants largely depends on a group of metal transporters relevant to Zn-regulated transporter, Iron-regulated transporter-like Protein (ZIP), Metal Tolerance Protein (MTP), Natural Resistance-Associated Macrophage Protein (NRAMP), Heavy Metal ATPase (HMA) family members and others [7–9]. With regard to Fe transporters, two canonical uptake routines have been so far identified in dicots and graminaceous plants. The first routine in dicots such as *Arabidopsis* concern (1) the initial rhizosphere acidification by H^+ -adenosinetriphosphatases, followed by proton release to facilitate Fe solubility, and (2) the reduction of Fe^{3+} to Fe^{2+} catalyzed by a reductase, which is then absorbed into root cells by the iron transporter Iron-Regulated Transporter (IRT1) [10]. Graminaceous species such as rice and wheat utilize the second way of releasing phytosiderophores into the rhizosphere to chelate Fe^{3+} and develop a Fe^{3+} -phytosiderophores complex which is further transported into roots by some transporters such as YELLOW STRIPE 15-like (YSL15) [11]. OsZIP6, for example, functionally regulates a transition metal iron transport in rice [12]. To date, a number of metal transporters have been identified involving Zn and Fe absorption, transport and allocation [13].

In addition to the metal transporters, there are other types of metal-binding proteins involved in the regulation of metal accumulation, allocation and homeostasis. Recently, a unique protein family has emerged as a metallochaperone to coordinate metal transport [14–16]. Heavy metal-associated isoprenylated plant proteins (HIPPs) are one of the metallochaperone subfamily proteins and use diverse mechanisms to mediate metal homeostasis and promote growth, development and adaptation to abiotic or environmental stresses [17–19]. HIPPs share a conserved metal-binding core that interacts with metal ions and distinct isoprenylated motifs consisting of glycine- or proline-rich repeat domains [14]. Thus far, a large subset of HIPP genes has been identified from higher plant species; 45 in *Arabidopsis thaliana*, 59 in *Oryza sativa*, 74 in *Populus tomentosa*, 52 in *Setaria angustifolia* and five in *Selaginella officinalis*. These genes were divided into five different clusters based on their sequence identity [14,16]. Yeast (*Saccharomyces cerevisiae*) anti-oxidant1 (Atx1) is a copper (Cu) chaperone that delivers Cu to the copper-requiring oxidase Fet3p for metal uptake and metabolism [20]. Its homolog ATX1 in higher plants is also a Cu-binding protein that transfers Cu to some targets for physiological processes [21]. In rice, knocking out *OsATX1* enhances Cu accumulation in roots and may function with several P1B-ATPases for Cu homeostasis [22]. Despite the fact that many metallochaperone genes are identified in plants, only a few of them have been functionally characterized for metal uptake and accumulation during plant growth and development.

Rice is one of the most important food crops and requires multi-mineral nutrients to support its growth and reproduction. In this study, we identified a new HIPP family gene, *OsHIPP33*, which was isolated from our previous metal-stress responsive transcriptome [16]. Since its function has not been reported before, our studies show that expression of *OsHIPP33* is required for Zn and Fe accumulation, proper growth and seed development. The purpose of this paper is to examine the role of *OsHIPP33* in maintaining the steady-state of Zn and Fe in rice plants by hydroponic culture and field trial approaches.

2. Materials and Methods

2.1. Plant Materials and Metal Treatments

Rice (wild-type Nipponbare) seeds were sterilized with alcohol (75%) for 2 min and sodium hypochlorite (1.0%) solution for 10 min. After thoroughly bathing with distilled water, seeds were germinated at 28 °C for 72 h. For the hydroponic study, the germinating seedlings were transferred to a net floating on the half-strength Kimura B nutrient solution. Two-week-old plants continued to grow in the nutrient solution with (normal or excess levels) or without Zn, Fe, Cu or Mn. Cadmium (Cd) serves as a non-essential equivalent metal for the test. Plants were grown in a growth chamber under the condition of a 16 h luminous ($250 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 28 °C) and 8 h dark (26 °C) photoperiod with relative $60 \pm 10\%$ humidity [23].

For paddy field trials, three week-old rice seedlings prepared in the growth chamber (the condition is the same as hydroponic culture) were transferred and planted in the open paddy soil. The soil used in this study is the local realistic farmland, characterized as Nanjing yellow brown soil (117.02° east longitude, 28.23° north latitude) [24], with the following physical and chemical features: (1) textures (%): sand (67.2), clay (13.3) and silt (19.5); (2) total organic carbon: 2.13%, soil organic matter: 3.67%, cation exchange capacity: 21.40 cmol·kg⁻¹ and total nitrogen: 0.21%; and (3) major mineral elements: Ca: 2.04 g·kg⁻¹, Fe: 13.80 g·kg⁻¹, Zn: 43.78 mg·kg⁻¹, Mn: 241.94 g·kg⁻¹, and Cu: 12.11 mg·kg⁻¹. Biological triplicates with completely randomized blocks (each block contains 300 plants) were designed. Field trials for the rice varieties were conducted in Nanjing for two successive years (28 May–20 September 2020 and 20 May–1 September 2021). The rice varieties were grown under natural conditions. When harvested, the mature plants were separately sampled according to their special tissues or organs such as leaves, panicles or grains (husk and brown rice) and determined for agronomic traits and metal concentrations.

2.2. Analysis of Gene Structural Sequences

Putative *OsHIPP33* (LOC_Os02g30650) and related *HIPP* genes were obtained from the Rice genome (<http://rice.plantbiology.msu.edu>) and NCBI BLAST database (<http://ncbi.nlm.nih.gov/blast>). The HMA domain was predicted by the Simple Modular Architecture Research Tool (SMART, <http://smart.embl-heidelberg.de>). The amino acid sequences of *OsHIPP33* and its homologs in rice were loaded onto MEGA 6.0 to construct a phylogenetic neighbor-joining tree with 1000 replications. The protein sequence of *OsHIPP33* and its close homolog was aligned using DNAMAN.

2.3. Transcript Analysis by Quantitative RT-PCR

Rice tissues were sampled and total RNAs were isolated using Trizol reagent, with DNase I treatment to remove DNA in the sample. The desirable RNA samples were used as templates to synthesize cDNA using reverse transcriptase SuperMix (Transgene, Beijing, China) according to the manufacturers' instructions. Further, the cDNA was employed to isolate the full-length of *OsHIPP33*'s sequence using gene-specific primers (Table S1) and validated by sequencing. Quantitative RT-PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) [23].

2.4. Subcellular Localization of *OsHIPP33*

The fusion of *OsHIPP33* and green fluorescent protein (GFP) genes was constructed by inserting *OsHIPP33* CDS without the stop codon into a pCambia1300 vector harboring the CaMV35S promoter [25]. The open reading frame of *OsHIPP33* was amplified with primers adapted with KpnI and BamHI restriction sites (Table S1). The plasma membrane marker *OsMADS3* and nucleus biomarker *OsPIP2A* fused with a red fluorescent protein (RFP) were co-introduced with *OsHIPP33*-GFP into the epidermal cells of tobacco (*Nicotiana benthamiana*) and onions (*Allium cepa*) using *Agrobacterium tumefaciens* (GV3101). GFP and RFP signals were detected by a confocal laser scanning microscopy (LSM710, Zeiss).

2.5. Yeast Complementation Assay

Yeast (*Saccharomyces cerevisiae*) wild type strain BY4741 and its mutants *zrc1*, *ycf1*, *cup2*, *ccc1*, and *pmr1* displaying sensitive phenotypes under excess Zn, Cd, Cu, Fe, and Mn conditions were used for detoxification analysis [16]. The *OsHIPP33* ORF was cloned into the expression vector pYES2 and transformed into the yeast cells, which were grown overnight to OD₆₀₀ 0.5–0.8 in SD-Ura liquid medium containing 2% Gal and 0.67% yeast nitrogen base. The cell suspension with serial dilutions (1:10) was spotted onto SD-Ura agar supplemented with 2, 4 and 8 mM Zn; 5, 30 and 60 µM Cd; 6, 8 and 10 mM Fe; 2, 4 and 8 mM Mn; 30, 60 and 90 µM Cu in the presence of Gal, and incubated at 30 °C for 3 d. Agar plates without metals served as controls [18].

2.6. Generation and Analysis of RNAi and Mutant Lines

The sense and antisense 498-bp fragments of *OsHIPP33* were generated using two pairs of primers (Table S1) and cloned into pCAMBIA1390-RNAi to form pCAMBIA1390-*OsHIPP33*-RNAi construct under the control of the CaMV 35S promoter. The resultant constructs were transformed into rice callus via *Agrobacterium tumefaciens* to generate *OsHIPP33*-RNAi transgenic lines [23]. The suppression of *OsHIPP33* expression was confirmed by checking its transcripts in two randomly selected *OsHIPP33*-RNAi transgenic lines denoted i2 and i5.

The T-DNA insertion mutant *OsHIPP33* (Nipponbare background) was purchased from Kyung Hee University, Korea. Plants were confirmed for homozygosity for this allele by genotyping with specific primers (Table S1). The PCR cycling conditions were 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

2.7. Determination of Metals

Yeast cells were collected, centrifuged, rinsed in 10 µM EGTA solution (pH 5.0 under 4 °C) for seconds, then bathed with deionized water and freeze dried. For plant tissues, shoots and roots were separately harvested, soaked in 0.5 mM calcium chloride solution for 30 s, thoroughly rinsed with distilled water, and dried at 72 °C for 3 days. The dried yeast and plant samples were digested in nitric acid. The metal concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS) [16].

For soil metal determination, samples of nine replicates were randomly collected from the surface layer (20 cm) in the rice field. The collected soil samples were manually crumbled, air-dried, and sieved through a 2 mm mesh to remove root residues and small rocks. The dried soil samples were microwave-digested with nitric acid and hydrogen peroxide ($\text{HNO}_3:\text{HClO}_4 = 1:1, v/v$) at temperature of 160 °C [24].

2.8. Statistical Analysis

All experiments in the study were set up in triplicates, and each treatment contained at least 18 seedlings. Statistical analyses were performed using SPSS v.22.0. The significant disparity of the treatments or genotypes between wild-type (WT) and knockout or knockdown mutant lines was assessed through variance (ANOVA), followed by Tukey's test.

3. Results

3.1. Analysis of *OsHIPP33* Sequence and Structure

The whole genomic sequence of *OsHIPP33* is 3465 bp in length. Its coding sequence is 972 bp containing three introns and four exons which are predicted to encode a polypeptide of 324 amino acids (Figure S1A). There are two conserved heavy metal-associated (HMA) domains, with typical Cys-XX-Cys metal-binding motifs in the first ring similar to ferredoxin structural folding ($\beta\alpha\beta\beta\alpha$) (Figure S1B,C). Phylogenetic analysis of rice *OsHIPP33* homologous genes revealed that its close homolog is *OsHIPP32* (Figure S1D). Comparison of *OsHIPP33* and *OsHIPP32* sequences confirmed that both have highly conserved amino acids, particularly with two HMA domains and isoprenylated motif (CaaX) (Figure S1E).

3.2. Expression Pattern of *OsHIPP33*

The temporal and spatial expression pattern of *OsHIPP33* transcripts was analyzed throughout the lifespan using qRT-PCR. *OsHIPP33* showed the highest transcript abundance in Node II (Figure 1A). Root, node, basal stems and leaves show a moderate expression level at all growth and developmental stages. To understand whether *OsHIPP33* responds to metals, the rice plants were treated with several metals including Zn, Fe, Cu, Cd and Mn under deficient and oversupply conditions. Deprivation of Cu, Zn, Fe and Mn did not affect the expression of *OsHIPP33* (Figure 1B). However, when exposed to excessive metals, *OsHIPP33* expression changed. Treatment with Zn at 1, 5 and 10 mM led to significant upregulation of the gene in shoots and roots, of which the top expression

occurred at 5 mM (Figure 1C). *OsHIPP33* expression was also progressively induced under high levels of Fe (Figure 1D). With regard to Cu, Cd and Mn, only slight upregulation of *OsHIPP33* was detected in rice (Figure 1E–G). These results suggest that *OsHIPP33* most likely responds to Zn and Fe. Therefore, the following study only focused on studying the effects of Zn and Fe on rice growth and development.

Figure 1. Transcriptional expression of *OsHIPP33* in rice (Nipponbare) under normal and metal stress conditions. (A) *OsHIPP33* expression in diverse tissues at different development stages. (B) Response of *OsHIPP33* to metal deficiency. Ten-day-old rice was grown in a nutrient solution with (control) or without Zn, Fe, Mn or Cu for 2 weeks. (C–G) Two-week-old rice was grown in the nutrient solutions with 1, 5 and 10 mM Zn; 0.1, 0.5 and 1 mM Fe; 10, 150 and 300 μM Cu; 30, 120 and 300 μM Cd; and 0.5, 1 and 1.5 mM Mn for 6 h. Results indicate the means of biological triplicates. Asterisks indicate the significant difference between the treatments and control ($p < 0.05$).

3.3. *OsHIPP33* Is Localized to the Nucleus and Plasma Membrane

The subcellular localization of *OsHIPP33* was identified by the connection of GFP to *OsHIPP33* whose expression was driven by a 35S promoter. The construct *Pro35S:OsHIPP33:GFP* was transiently expressed in tobacco epidermal leaves. The green fluorescence of *OsHIPP33*

HIPP33:GFP was co-expressed with the nucleus (*OsMADS3-RFP*) and plasma membrane (*PIP2A-RFP*) markers (fused with mRFP). The overlapping and color changed fluorescence was visualized (Figure 2A–F). To double confirm the location, the *OsHIPP33-GFP* fusion vector was further transferred into the onion epidermal cells. The green fluorescence was also clearly detected in the nucleus and cell membrane of onion cells (Figure 2G–L). Together, these observations indicate that *OsHIPP33* is located in the nucleus and plasma membrane but cannot be avoided in the cytoplasm as well.

Figure 2. Subcellular localization of *OsHIPP33*. (A–F) Tobacco subcellular localization. (A,D) Green fluorescence of *OsHIPP33-GFP* fusion protein. (B) The red fluorescence of nuclear marker (*OsMADS3*). (C) The green fluorescence of the *OsHIPP33-GFP* fusion protein coincides with the red fluorescence of the nuclear marker (*OsMADS3*). (E) The red fluorescence of cell membrane marker (*PIP2A*). (F) The green fluorescence of *OsHIPP33-GFP* fusion protein coincides with the red fluorescence of cell membrane marker (*PIP2A*). (G–L) Detection of onion subcellular localization. (G,J) Bright field. (H) The fluorescence of GFP. (I) Merged between bright field and GFP fluorescence. (K) Fluorescence of *OsHIPP33-GFP* fusion protein. (L) The merger of *OsHIPP33-GFP* fusion protein fluorescence and bright field.

3.4. Expression of *OsHIPP33* Led to Zn Accumulation in Yeast Cells

To explore whether *OsHIPP33* has metal detoxification activity, we first identified the transformants by expressing *OsHIPP33* into the yeast strains defective in metal transport or detoxification. When *OsHIPP33* was transformed into a zinc-sensitive mutant (Δzrc) [16], its growth appeared better than that of pYES2 (empty vector as a control) mutants, especially under excessive zinc treatment (8 mM) (Figure S2A). We then transferred *OsHIPP33* into cadmium-sensitive ($\Delta ycf1$), copper-sensitive ($\Delta cup2$), iron-sensitive ($\Delta ccc1$) and manganese-sensitive ($\Delta pmr1$) mutant lines, and found that there was no significant difference in cell growth treated with the indicated concentrations of Cd, Cu, Fe and Mn (Figure S2B–E). The metal concentrations were determined in the cells expressing *OsHIPP33* but no significant disparity from controls was detected under Zn, Cd, Cu, Fe and Mn treatments (Figure S3).

3.5. *OsHIPP33* Did Not Functionally Regulate Rice Growth and Zn and Fe Accumulation during the Early Vegetative Stage

We attempted to identify the biological function of *OsHIPP33* in the mediation of rice growth and metal accumulation at the early stage. To perform the study, a T-DNA insertion mutant and RNAi lines were acquired. The T-DNA insertion into *OsHIPP33* was confirmed by PCR (Figure S4). Meanwhile, twenty-four independent *OsHIPP33* knockdown mutant lines (T3 generation) were generated by RNA interference (RNAi). Two of the homozygous lines RNAi-2 (i2) and RNAi-5 (i5) were randomly selected in use for the study. Their transcriptional expression was down-regulated by 47% and 55%, respectively (Figure S4C). Since the rice undergoes early vegetative growth for about one month, we thus performed a short term hydroponic experiment with rice growing under conditions of normal Zn, Fe, Cu and Mn supply. Following a four-week-long growth period, the rice seedlings did not show variations of shoot elongation and biomass between the wild-type and *OsHIPP33* mutant or RNAi lines, except the roots in which the knockdown and knockout of *OsHIPP33* led to a slightly significant reduction of root elongation (Figure S5). The same response was true for the accumulation of Zn, Fe, Cu and Mn in plants (Figure S6). These results indicate that *OsHIPP33* might not work at the early vegetative stage.

3.6. Mutation of *OsHIPP33* Compromised Growth and Reproductivity of Rice

To understand the functional role of *OsHIPP33* in rice, we performed a two-year field experiment on the knockout and knockdown mutant lines under natural conditions. The local agronomic practice was adopted to manage rice cropping throughout the lifespan. Dominant agronomic traits such as yield constituent and grain yield were surveyed. The significant disparity in plant height between *OsHIPP33* or RNAi2/5 and wild-type was identified at the mature stage (Figure 3A). The plant height of *OsHIPP33*, RNAi-2 and RNAi-5 decreased by 21%, 11% and 11% respectively, compared with WT (Figure 3B). We specified the measures relevant to the grain yield. All leaf sheaths of the plants were removed and the internodes were kept for analysis (Figure 3C). The length of internode III, IV, and V of the *OsHIPP33* plants decreased by 45%, 38%, and 41%, respectively, relative to the wild-type (Figure 3D). A similar trend was observed for the two RNAi lines, indicating that knockout or knockdown of *OsHIPP33* impaired the rice apical dominance.

We further counted the number of tillers and effective panicles and found that the tillers of *OsHIPP33* and RNAi-5 declined by 23% and 16%, respectively (Figure 3E). The number of effective panicles was reduced by 35% and 26%, respectively for *OsHIPP33* and RNAi-2 (Figure 3F). Although no significant variations in the tillers and effective panicles between RNAi-2 or RNAi-5 and wild-type were detected, knocking down *OsHIPP33* indeed repressed the agronomic phenotypes.

Figure 3. Morphological differences between *OsHIPP33* mutant, RNAi (i-2 and i-5) lines and wild-type (WT) at the maturity stage. The rice varieties were grown in the field under natural conditions for two successive year trials. (A) The phenotypes of WT, RNAi and *OsHIPP33* (scale bars: 10 cm). (B) Plant height comparison of WT, RNAi and *OsHIPP33*. (C) Stem structure of WT, RNAi and *OsHIPP33*. The interval between the two arrows shows the length of internode (scale bars: 3 cm). (D) Internode length comparison of WT, RNAi and *OsHIPP33*. (E) Comparison of tiller number per plant between WT, RNAi and *OsHIPP33*. (F) Effective panicle number per plant comparison between WT, RNAi and *OsHIPP33*. The vertical bars indicate the means of biological triplicates and asterisks show the significant difference between the mutant lines and WT ($p < 0.05$).

Further specific analysis of seed phenotypes and reproductivity revealed that the panicle length of *OsHIPP33* and two RNAi lines and the grain yield per plant were significantly lower than those of wild-type (Figure 4A). The panicle lengths of *OsHIPP33*, RNAi-2 and RNAi-5 were shortened by 20%, 13% and 12%, respectively compared with those of wild-type (Figure 4B). Analysis of the length and width of seeds showed no significant disparity of seed width between all mutant lines and wild-type, but only the length of *OsHIPP33* plants appeared significantly shorter than that of wild-type (Figure 4C–F). The

grain yields of *OsHIPP33*, RNAi-2 and RNAi-5 were 63%, 41% and 31% less than the wild-type (Figure 4G,H). Comparative analyses of seed fertility signified that the fertility rates of *OsHIPP33*, RNAi-2 and RNAi-5 lines declined by 47%, 22% and 28%, respectively (Figure 4I). For 1000-grain weight, there were 18%, 9% and 9% reductions for *OsHIPP33*, RNAi-2 and RNAi-5, respectively (Figure 4J). The yield per square meter had a downside by about 52%, 15% and 12% for *OsHIPP33*, RNAi-2 and RNAi-5 lines (Figure 4K). Overall, *OsHIPP33* knockout or knockdown indeed sabotaged the panicle elongation, seed fertility and grain development, which consequently reduced the grain yield.

Figure 4. *OsHIPP33* knockout (*OsHIPP33* mutant) and knockdown (RNAi, i-2 and i-5) lines developed the shortened panicle size and reduced grain yield. The rice varieties were grown in the field under natural conditions for two successive year trials. (A) Phenotypes of panicle (scale bars: 3 cm) and (B) panicle length. (C–F) Phenotypes of seed size (scale bars: 1 cm). (G) Total seeds collected (Scale bars: 3 cm) and (H) seed weight from one plant. (I) Spikelet fertility of one panicle and (J) 1000-grain weight. (K) Seed yield per square meter. The vertical bars indicate the means of biological triplicates and the asterisks show significant differences between the mutant lines and WT ($p < 0.05$).

3.7. Mutation of *OsHIPP33* Reduced Zn and Fe Concentrations in Rice Tissue

The metal concentrations were quantified using the rice tissues (the above-ground straw, husk and brown rice) sampled at the mature stage. The concentrations of Zn and Fe in the straw were significantly reduced. Zinc concentrations in husk and brown rice show no difference except for the husk in *OsHIPP33*, where the Zn concentration was slightly but significantly lower than that of wild-type (Figure 5A). Determination of Fe accumulation revealed a drastic reduction in the tissues, particularly in the husk and brown rice (Figure 5B). These results indicate that disruption of *OsHIPP33* led to reduced accumulation of Fe or Zn in the rice tissues.

Figure 5. Zn and Fe concentrations in different rice tissues at maturity stage of *OsHIPP33* mutant, RNAi lines and wild-type (WT). The rice varieties were grown in a field under natural conditions for two successive year trials. **(A)** Zn concentration. **(B)** Fe concentration. The vertical bars indicate the means of biological triplicates and the asterisks show the significant difference between the mutant lines and WT ($p < 0.05$).

3.8. *OsHIPP33* and RNAi Lines Are Sensitive to Excessive Zn and Fe Stress and Accumulated More Metals

Recent studies have shown that some rice HIPP genes can detoxify the metals at the higher noxious level [18,19]. This prompted us to investigate whether *OsHIPP33* plants had a similar effect on plant responses to excessive Zn and Fe. Two-week-old rice seedlings were treated with 0.4 μM (normal) and 500 μM Zn (excess) or 20 μM (normal) and 1000 μM Fe (excess) for two weeks. No significant difference was observed in the growth of the mutant lines and wild-type under normal conditions. However, the *OsHIPP33* and RNAi lines showed reduced growth phenotypes under the metal stress (Figure 6). For example, the shoot length of the *OsHIPP33* and two RNAi lines under Fe stress decreased by 19% and 15–17%, respectively, compared with the WT (Figure S7C). Further biomass assessment revealed that the shoot dry weight of the *OsHIPP33* line was 33% lower than that of the WT, and the RNAi lines also decreased by 28–30% (Figure 6E). These results suggest that the repression of *OsHIPP33* could be responsible for the reduced growth under Zn and Fe excess.

We further measured the Zn and Fe concentrations in the tissues in the plants. Analyses showed that accumulation of Zn and Fe in the root and shoot of the *OsHIPP33* mutant and RNAi lines were higher than WT with oversupply of Zn and Fe (Figure 7), except RNAi-2 where the increased Fe concentration in the root was not statistically evident. This result indicates that over-accumulation of Zn and Fe in the *OsHIPP33* and RNAi lines would be the cause of the stunted growth of rice.

Figure 6. Growth phenotypes of *OsHIPP33* mutant, RNAi lines (i-2 and i-5) and wild-type (WT) under excessive Zn and Fe stress. Two-week-old rice seedlings were exposed to the nutrient solution containing 0.4 μM Zn (normal) and 500 μM Zn (excess) (A–C) or 20 μM Fe (normal) and 1000 μM Fe (excess) (D–F) for two weeks. When harvested, the rice plants were photographed and phenotypically analyzed. The dry weight of shoot and root of the plants under excess Zn and Fe was estimated. Scale bars: 3 cm for (A,D). The vertical bars indicate the means of biological triplicates and the asterisks show the significant difference between the mutant lines and WT ($p < 0.05$).

Figure 7. Analysis of Zn and Fe concentrations in *OsHIPP33* mutant, RNAi lines and wild-type (WT) treated with excess Zn and Fe. (A) Zn concentrations in shoot and root with 500 µM Zn supply. (B) Fe concentrations in shoot and root with 1000 µM Fe supply. The vertical line indicates the error bar and the asterisks indicate a significant difference between the mutant lines and WT ($p < 0.05$).

4. Discussion

Zn and Fe are essential microelements for all organisms required for many biological processes; however, the abundance of Zn and Fe in plants must be under tight control [4]. Either deficiency or excess of the metal ions leads to dysfunctions of plant physiological, biochemical and molecular processes. For example, overload of Fe ions triggers oxidative stress response [26]. Previous studies have demonstrated that several HIPP metallochaperones respond to metal stress and in turn mediate the metal stress response [16,19,27,28], but how these HIPPs regulate the levels of Zn and Fe to attain homeostasis is not fully understood. This study characterized the rice locus encoding *OsHIPP33* and presented its critical role in regulating rice growth, grain yield, and Zn and Fe accumulation. These results allow us to conclude that *OsHIPP33* at least required for Zn and Fe accumulation and homeostasis in rice plants [29–32].

4.1. *OsHIPP33*-Mediated Zn and Fe Accumulation Is Responsible for Rice Grain Yield

Subcellular and tissue expression shows that *OsHIPP33* was targeted to the nucleus/cytoplasm and expressed in diverse organs throughout the development stages, especially at the flowering and mature phases (Figure 1), suggesting that *OsHIPP33* would play roles in fertility and seed development. Simultaneously, *OsHIPP33* expression in root, basal stem, nodes I–II as well as the above-ground lower leaves, sheath and grains implied that *OsHIPP33* is likely involved in Zn or Fe long-distance transport through the vascular bundles between root and shoot tissues. Many genes mediating rice architecture and reproductivity, particularly for those master genes controlling plant height, panicle phenotype and grain size and yield, have been identified [33–35]. The concentration of Zn in rice is associated with plant growth and development [1]. *OsmiR156*, for example, guides *OsSPL14* in maintaining an ideal plant architecture of rice under a suitable mineral supply [36]. The high concentration of Zn in rice vegetative organs or tissues is likely associated with the Zn concentration in grains, but depends more on the Zn distribution mediated by some Zn transporters such as *OsZIP4*, which can transport Zn to the phloem nodes and further to tiller buds and panicles [37]. However, knowledge of the functional role of HIPP proteins during rice development is stringently rare. Recently, a study with *HIPP24* in rice shows that *OsHIPP24* knockout or overexpression in rice resulted in suppressed growth response without any relevance to the concentration of Cu supply [38]. Different to this, the *OsHIPP33* knockout or knockdown led to attenuated growth phenotypes with decreased plant height, number of tillers and panicles, and spikelet fertility rate, all of

which determine the grain yield of rice. Furthermore, the *OsHIPP33* mutants recorded a lower degree of Zn and Fe accumulation in brown rice grains and husks. The reduction of grain yield in the mutant lines was most likely associated with the deficiency of Zn and Fe (Figure 5). The Zn deficiency was reported to compromise plant development with specific symptoms such as smaller leaf size and short internodes, which ultimately reduce grain production [7], while the enhanced level of Zn in rice contributes to the number and growth of tillers [39]. The possible mechanism for the physiological response can be linked to the phytohormone auxin [34]. Supportive evidence comes from the study with 2,4-D (an auxin-like plant regulator) and Zn treatment of rice that activated the elongation factor-1 β required for auxin- and zinc-induced root formation and as a result promoted the enhancement of plant growth and development [40]. A recent study demonstrates that cytokinins are actively involved in the grain filling of rice under alternate wetting and drying irrigation [41]. Moreover, the cytokinin metabolism was reported to be regulated by Zn availability in a complicated dynamic way, suggesting that the cytokinin-dependent signaling pathway covers the maintenance of zinc nutrition in rice [42]. Interestingly, a small group of Arabidopsis HIPP proteins (AtHIPP1 and AtHIPP6) was reported to regulate the abundance of cytokinin by a specific interaction with cytokinin-degrading CKX proteins which are subjected to endoplasmic reticulum-associated degradation for plant development. This demonstrates that manipulation of levels of the HIPP protein may be associated with cytokinin synthesis and morphological responses in the cytokinin signaling pathway [43]. These lines of evidence along with our observations in this study suggest that loss of *OsHIPP33* function could be the result of Zn and Fe deficiency or imbalance in rice plants.

4.2. *OsHIPP33* Maintains Homeostasis under Excessive Zn/Fe Stress

In this research, *OsHIPP33* was found to be induced by excessive Zn, Fe and Cu stress, but the yeast complementation experiment revealed that only Zn could be detoxified in the *OsHIPP33*-transformed cells (Figure S2). Previous studies show that when excess metals such as Cd enter plants, they can be chelated by HIPP proteins [18,28]. *OsHIPP29*, for example, maintains intracellular ion homeostasis under excessive Zn stress to prevent cellular toxicity [19]. In this study, *OsHIPP33* and RNAi lines were subjected to excessive Zn and Fe, the results of which show that these mutant lines were more vulnerable to the toxicity of overloaded Zn and Fe. Measurement of Zn and Fe concentrations revealed that the two metals in the *OsHIPP33* and RNAi lines were over-accumulated, suggesting that disruption of *OsHIPP33* could dampen the capacity of homeostasis of Zn and Fe. The cytosolic Zn and Fe ions should be maintained at relatively low optimal concentrations, while extra metal ions must be chelated or sequestered as non-toxic forms in the vacuole [44]. The tonoplast transporters OsOZT1 and OsMTP1 dispatch excess Zn inside the vacuoles for prevention of toxicity [45,46]. Ferritin stores about 4000 Fe atoms [47] and the rice ferritin genes *OsFER1* and *OsFER2* function against excess Fe accumulation [48]. Ferric reductase oxidase (FRO), an enzyme that reduces Fe³⁺ to Fe²⁺ for absorption in strategy I plants, is also shown to maintain Fe homeostasis in rice; knockdown of *OsFRO1* improves Fe resistance, whereas its overexpression enhances Fe availability in the cytoplasm, making it more sensitive to Fe toxicity in the transgenic lines [49]. Whether and how *OsHIPP33* would mediate Zn and Fe accumulation remains to be investigated. Taken together, this study has reported the identification of *OsHIPP33* as a novel component for Zn and Fe homeostasis in rice. Disrupting *OsHIPP33* by RNA interference and knockout compromised rice growth, biomass, and many other agronomic traits, with an overall lower level of Zn and Fe accumulation in plants. From these results, it can be concluded that *OsHIPP33* is required for proper Zn and Fe accumulation as well as healthy plant and seed development.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy12020488/s1>, Figure S1: Basic information of *OsHIPP33* gene and protein structures. (A) Diagram of the *OsHIPP33* gene structure. (B) Predicted HMA domains of *OsHIPP33* by Simple Modular Architecture Research Tool (SMART, <http://smart.embl-heidelberg.de>). (C) Three-dimensional structure of *OsHIPP33*. (D) Phylogenetic analysis of *OsHIPP33* homologous genes in rice. The numbers at branching sites indicate the posterior probability values for nodal support. The sequences of rice genes are from rice genome database (<http://rice.plantbiology.msu.edu>). The red triangles indicate *OsHIPP33*. (E) Alignment of amino acid sequences for *OsHIPP33* (LOC_Os02g30650.1) and *OsHIPP32* (LOC_Os07g20340.1). The red rectangle represents two HMA domains and CaaX motif. The neighbor-joining phylogenetic tree was constructed using MEGA 6.0. Sequence alignment was performed by DNAMAN; Figure S2: Transport activity of *OsHIPP33* in yeast. Yeast strains BY4741, $\Delta ycf1$, $\Delta cup2$, $\Delta ccc2$ and $\Delta pmr1$ transformed with empty vector pYES2 (Vector) or *OsHIPP33* were cultivated on varied concentrations of Cd, Cu, Fe and Mn as indicated. Eight microliters of cell suspension with an OD 600 nm of 0.5 and four serial 1:10 dilutions were spotted and incubated at 30 °C for 3 d; Figure S3: The metal concentrations in the yeast cells after *OsHIPP33* transformation. Zn, Cd, Cu, Fe, and Mn were absorbed by cells in the liquid medium. The yeast BY4741 and *zrc1*, *ycf1*, *cup2*, *ccc1* and *pmr1* carrying pYES2 or with pYES2 vectors containing the full *OsHIPP33* cDNA were cultured in a liquid medium containing 2% Gal in the presence of 200 μ M Zn, 5 μ M Cd, 10 μ M Cu, 2 mM Fe, and 2 mM Mn for 48 h, respectively. The metal concentration in the yeast was determined with ICP-MS after digestion. Data are means of three biological replicates; Figure S4: Analysis of *OsHIPP33* transcriptional expression in the mutant lines by qRT-PCR. (A) Schematic diagram of *OsHIPP33* mutant insertion sites. The triangle indicates the insertion site of the T-DNA. LP and RP are the primers on the genome near the insertion site. RB is the universal identification primer on the T-DNA. The white square boxes are the UTR region. The black square boxes are exons, and the black lines are introns. (B) Homogeneous identification of *OsHIPP33* mutants. (C) qRT-PCR analyses of *OsHIPP33* transcripts in the two RNAi lines. The vertical bars indicate the means of biological triplicates. Asterisks show a significant difference between the mutants and WT ($p < 0.05$); Figure S5: Growth phenotypes of *OsHIPP33* mutant, RNAi lines and wild-type under the normal Zn, Fe, Cu and Mn supply conditions. The germination rice seedlings were grown in the nutrient solution with the normal metals as mineral nutrients (0.4 μ M Zn, 20 μ M Fe, 0.2 μ M Cu and 0.5 μ M Mn) for four weeks. The rice plants were photographed. A: Growth phenotypes, B: the shoot and root length and C: dry weight of *OsHIPP33* mutant and RNAi lines (scale bars: 3 cm). The vertical bars indicate the means of biological triplicates. Asterisks show a significant difference between the mutants and WT ($p < 0.05$); Figure S6: The concentrations of Zn, Fe, Cu and Mn in shoots and roots of *OsHIPP33* mutant, RNAi (i2 and i5) lines and wild-type (WT) under the normal conditions of Zn, Fe, Cu and Mn supply. The germination rice seedlings were grown in the nutrient solution with the normal metals as mineral nutrients (0.4 μ M Zn, 20 μ M Fe, 0.2 μ M Cu and 0.5 μ M Mn) for four weeks. (A–D) Zn, Fe, Cu and Mn concentrations analyzed in WT and *OsHIPP33* mutants and RNAi lines. The vertical bars indicate the means of biological triplicates; Figure S7: Growth phenotypes of *OsHIPP33* mutant, RNAi lines and wild-type (WT) under excessive Zn and Fe stress. Two-week-old seedlings were exposed to the nutrient solution containing 0.4 μ M Zn (normal) and 500 μ M Zn (excess) (A, B) or 20 μ M Fe (normal) and 1000 μ M Fe (excess) (C, D) for two weeks. When harvested, the rice plants were photographed and phenotypically analyzed with shoot and root length. The vertical bars indicate the means of biological triplicates. Asterisks show a significant difference between the mutants and WT ($p < 0.05$); Table S1: Primer sequences used for this study.

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