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# Genome-Wide Association Study Using a Multiparent Advanced Generation Intercross (MAGIC) Population Identified QTLs and Candidate Genes to Predict Shoot and Grain Zinc Contents in Rice

**Shilei Liu [1,2,†], Wenli Zou [1,2,†], Xiang Lu [2], Jianmin Bian [1], Haohua He [1,*], Jingguang Chen [2,3,*] and Guoyou Ye [2,4]**

1 Group of Crop Genetics and Breeding, School of Agriculture Science, Jiangxi Agricultural University, Nanchang 330045, China; Liujindong@caas.cn (S.L.); yangwenna@caas.cn (W.Z.); jmbian81@126.com (J.B.)
2 CAAS-IRRI Joint Laboratory for Genomics-Assisted Germplasm Enhancement, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518116, China; luxiang@caas.cn (X.L.); g.ye@irri.org (G.Y.)
3 School of Agriculture, Sun Yat-sen University, Guangzhou 510275, China
4 Rice Breeding Innovation Platform, International Rice Research Institute, DAPO Box 7777, Metro Manila 1301, Philippines
* Correspondence: hhhua64@jxau.edu.cn (H.H.); chenjg28@mail.sysu.edu.cn (J.C.)
† These authors contributed equally to this work.

**Abstract:** Zinc (Zn) is an essential trace element for the growth and development of both humans and plants. Increasing the accumulation of Zn in rice grains is important for the world's nutrition and health. In this study, we used a multiparent advanced generation intercross (MAGIC) population constructed using four parental lines and genotyped using a 55 K rice SNP array to identify QTLs related to $Zn^{2+}$ concentrations in shoots at the seedling stage and grains at the mature stage. Five QTLs were detected as being associated with shoot $Zn^{2+}$ concentration at the seedling stage, which explained 3.7–5.7% of the phenotypic variation. Six QTLs were detected as associated with grain $Zn^{2+}$ concentration at the mature stage, which explained 5.5–8.9% of the phenotypic variation. Among the QTLs, *qSZn2-1/qGZn2* and *qSZn3/qGZn3* were identified as being associated with both the shoot and grain contents. Based on gene annotation and literature information, 16 candidate genes were chosen in the regions of *qSZn1*, *qSZn2-1/qGZn2*, *qSZn3/qGZn3*, *qGZn7*, and *qGZn8*. Analysis of candidate genes through qRT-PCR, complementation assay using the yeast Zn-uptake-deficient double-mutant ZHY3, and sequencing of the four parental lines suggested that *LOC_Os02g06010* may play an important role in $Zn^{2+}$ accumulation in *indica* rice.

**Keywords:** $Zn^{2+}$ content; genome-wide association analysis; quantitative trait loci (QTL); MAGIC population; rice

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## 1. Introduction

Zinc (Zn) is an essential micronutrient for plant growth and development [1]. Zn is also a component of numerous enzymes [2,3]. For instance, alcohol dehydrogenase (ADH), carbonic anhydrase (CA), copper/zinc superoxide dismutase (CSD), and zinc finger domain-containing proteins (ZFPs) are related to Zn in plants [1]. Furthermore, Zn is an essential component of the zinc finger motif found in many DNA-binding transcription regulators (TRs) [4]. Therefore, Zn is necessary for many biochemical processes to function properly, and adequate Zn supply is critical to crop productivity [5]. However, Zn deficiency is the most widely occurring soil micronutrient deficiency worldwide and adversely affects crop production on millions of hectares of arable land, especially in alkaline soil [6].

Zn deficiency in the edible parts of crops is one of the main reasons for Zn deficiency in humans, since the primary source of Zn intake is through food and vegetables. Zn deficiency is related to health problems such as growth retardation, impaired immune function,

loss of appetite, diarrhea, hair loss, weight loss, eye and skin lesions, mental lethargy and delayed healing of wounds [7]. It has been estimated that about one-third of the human population, particularly women and children, suffer from Zn-deficiency-related health problems. Rice is one of the major food crops in the world and the main food source for more than half of the world's population [8]. Most rice-dependent regions are underdeveloped and cannot afford to diversify their meals by adding mineral-rich fruits, vegetables, and meat [9]. The zinc content of polished rice, one of the rice varieties currently popular in the world, is only 12 mg/kg [10]. However, the international recommendation for a human's daily intake of zinc is 15 mg [11]. Therefore, increasing the zinc content in rice could play an important role in the improvement of human health. Breeding for high grain zinc content is now one of the key objectives of rice breeding programs worldwide. Projects dedicated to crop biofortification all have Zn content as a key component.

To date, many zinc-related absorptions and transport genes in rice have been cloned. These mainly include the P1B-type Heavy Metal ATPase (HMA) and Zinc-regulated transporter iron regulated transporter-like (ZIP)-family proteins. *OsHMA2* is mostly expressed in the roots of rice [12] and mediates the transport of zinc from roots to stems [13]. OsHMA3 has been reported to be a transporter of $Cd^{2+}$ located on the vacuolar membrane [14]. Recently, Cai et al. [15] showed that *OsHMA3* plays an important role in rice roots in both Zn detoxification and storage by sequestration into the vacuoles, depending on Zn concentration in the environment. *OsZIP1* is involved in the detoxification of the poison of zinc ions in rice [16–18]. *OsZIP3* is mainly expressed in the nodes and participates in the transport of zinc ions in the shoots of rice [18,19]. OsZIP4 has zinc ion transport activity, and overexpression of the *OsZIP4* zinc transporter produces disarrangement of zinc distribution in rice plants [20–22]. OsZIP5 is a plasma-membrane zinc transporter in rice [5], and OsZIP5 functions redundantly to OsZIP9 but has a relatively weaker effect [23]. *OsZIP7* encodes a plasma-membrane-localized protein with influx transport activity for both Zn and Cd, which plays an integral role in xylem loading in roots and intervascular transfer in nodes to preferentially deliver Zn and Cd to developing tissues and rice grains [24–26]. OsZIP9 is localized at the root exodermis and endodermis, and functions as an influx transporter of Zn and contributes to Zn uptake under Zn-limited conditions in rice [23,27,28].

Quantitative trait locus (QTL) identification via linkage mapping and association mapping has been widely used to discover genetic factors controlling agronomic traits, including absorption and transport of metal ions [29–31]. Linkage mapping using different biparental populations has been used to identify a series of QTLs for shoot and grain Zn contents [32–34]. Association mapping, or genome-wide association study (GWAS), has been used to identify QTLs for grain Fe, Zn, and several other mineral elements [33,35]. Yang et al. [29] identified three QTLs associated with Zn concentration in shoots at the mature stage using a panel of 529 varieties. To date, no QTLs have been fine-mapped or cloned for $Zn^{2+}$ accumulation in rice. In recent years, multiparent advanced generation intercross (MAGIC) populations have become popular population types for mapping and developing breeding lines with multiple desirable traits [36–39]. MAGIC populations have a relatively wide genetic background without significant population structure, which is a major constraint in association mapping using diversity panels. In this study, we aimed to use a highly diverse MAGIC population, genotyped with a 55 K single nucleotide polymorphism (SNP) array, to illuminate the genetic basis of Zn accumulation.

## 2. Materials and Methods

### *2.1. Plant Materials*

The MAGIC population used in this study consisted of 215 lines of the DC1 population developed with four parental lines, A, B, C, and D, from different countries [36]. The sources and agronomic characters of the four parental lines are given in Supplementary Table S1. The rice varieties for haplotype classification of *LOC_Os02g06010* were from Liu et al. [40].

### 2.2. Plant Growth Condition

A pot experiment was carried out in the greenhouse of the Agricultural Genomics Institute in Shenzhen, Chinese Academy of Agricultural Sciences. The soil from a field site was naturally dried, and then crushed and sieved through a 3 mm screen mesh. After mixing evenly, the soil was put in a pot, with 15 kg per pot. Additionally, 1.3 g urea and 0.37 g $KH_2PO_4$ were added to each pot as basal fertilizer for N, P, and K supply, respectively. Each pot was then saturated with distilled water and drained to equilibrium for 7 days under natural conditions.

The rice seeds of the MAGIC population lines were disinfected in a 1% sodium hypochlorite solution for 15 min, followed by thorough washing with deionized water. Seeds were then germinated in the dark in water at 30 °C for three days, and the germinated seeds were cultivated in the seedling trays for four weeks. Healthy seedlings were then selected and transferred to the pots with the same culture condition. Six seedlings from a single line were planted in each pot. Throughout the entire growth period, all pots were irrigated with distilled water every day to maintain a water level of 3 cm above the soil surface. Three seedlings were used to collect shoot samples three weeks after transplanting, and grain samples were collected from the remaining three plants at the mature stage. Brown rice samples were taken and peeled. An augmented randomized complete block design with two replicates of 25 lines was used as the experimental layout.

### 2.3. Measurement of Zinc Concentration

The shoot and grain samples were dried at 65 °C for 3 days. The dried samples were crushed, wet-digested in concentrated $HNO_3$ at 120 °C for 30 min, and further digested with $HClO_4$ at 180 °C until the samples became transparent. The samples were then diluted with ultrapure water. The zinc concentrations were determined by ICP-MS (inductively coupled plasma mass spectrometry).

### 2.4. SNP Genotyping and Association Analysis

Meng et al. [37] genotyped the MAGIC population with a 55 K SNP array. A three-step filtering strategy was used to select high-quality SNPs for QTL mapping. First, monomorphic markers among the four parents were removed. Second, markers with missing values higher than 10% were deleted. Finally, markers with a minor allele frequency of less than 3% were filtered out. After filtering, 22,160 high-quality markers were kept to be used for analysis.

A mixed linear model (MLM) implemented in TASSEL version 5.2.3 [41] was used to analyze the associations between SNP markers and traits. A value of $p < 10^{-2.5}$ was used as the threshold to declare the significance of marker–trait associations. $R^2$ was used to evaluate the percentage of phenotypic variance explained.

### 2.5. RNA Extraction and Real-Time PCR

To examine the expression response of the candidate genes to $Zn^{2+}$ deficiency, root samples were taken at the three-leaf stage from seedlings of rice variety cv. Nipponbare or the four parental lines grown in 1/4 strength IRRI (International Rice Research Institute) solution with 0.77 μM $Zn^{2+}$ or without $Zn^{2+}$. A randomized complete block design with three replicates and a plot size of 24 seedlings were used as the experimental layout. Samples were taken from all 24 seedlings in each plot and mixed for RNA extraction. The composition of the full-strength IRRI solution was as follows: 1.0 mM $MgSO_4 \cdot 7H_2O$, 1.25 mM $NH_4NO_3$, 0.3 mM $KH_2PO_4$, 1.0 mM $CaCl_2$, 0.35 mM $K_2SO_4$, 0.5 mM $Na_2SiO_3$, 20.0 μM Fe-EDTA, 20.0 μM $H_3BO_3$, 9.0 μM $MnCl_2$, 0.32 μM $CuSO_4$, and 0.39 μM $(NH_4)_6Mo_7O_{24}$, pH 5.5. All plants were grown in a greenhouse under 14 h light (30 °C)/10 h dark (25 °C), at a humidity of 60%.

To investigate the expression pattern of *LOC_Os02g06010* in different organs at different growth stages, the 3 week old seedlings of cv. Nipponbare precultured hydroponically were transplanted to the paddy field in the Shenzhen Experimental Farm of the Agricultural

Genomics Institute in Shenzhen, Chinese Academy of Agricultural Sciences. Tissue samples collected included roots, basal stem, leaf sheath, and leaf blade at the vegetative stage, and roots, basal stem, lower leaf sheath, lower leaf blade, flag leaf sheath, flag leaf blade, node I, node II, inter node II, peduncle, rachis, spikelet, husk, and seed at the reproductive stages. A single plant was regarded as a biological replicate and three biological replicates were used.

The total RNA was extracted by Trizol (Vazyme Biotech Co. Ltd., Nanjing, China). The total RNA was then reverse-transcribed using the HiScript Q RT SuperMix for qPCR kit (Vazyme Biotech Co. Ltd., Nanjing, China). The AceQ Universal SYBR qPCR Master Mix kit (Vazyme Biotech Co. Ltd., Nanjing, China) was used for quantitative analysis [42]. The primers used for qRT-PCR are given in Supplementary Table S2.

### *2.6. Expression of Candidate Genes in Yeast*

The $Zn^{2+}$ translocation ability of each candidate gene protein was examined via a yeast complementation assay. The yeast strain used was the Zn-uptake-deficient double-mutant ZHY3 (*MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*). The open reading frames of all candidate genes were amplified from the full-length cDNA of rice cv. Nipponbare, and the primer sequences used are shown in Supplementary Table S3. Each candidate gene was ligated into a *pYES2* vector with the correct direction.

Using a yeast transformation kit (Coolaber Technology Co. Ltd., Beijing, China), the empty vector *pYES2,* positive control *OsZIP5,* or candidate gene vector was introduced into the ZHY3 yeast cells. The transformed yeast was selected on a SD medium without uracil (SD-U). Positive clones were cultured in the SD-U liquid medium until the early logarithmic phase, concentrated, and washed three times with sterile ultrapure water. The yeast cell suspensions of ZHY3 transformed with empty vector *pYES2,* positive control *OsZIP5,* or candidate genes were serially diluted (1:10), and 6 µL of the cell suspension was spotted onto solid media containing 1, 3, or 100 µmol/L $ZnSO_4$. The growth phenotypes were evaluated by culturing the plates at 30 °C for three days.

The $OD_{600}$ of the overnight yeast cells in the SD-U liquid medium was adjusted to 1.0 with sterile distilled water. Subsequently, 20 µL cell suspensions were added to 20 mL liquid SD-U media containing 3 or 100 µmol/L $ZnSO_4$ in each bottle. The $OD_{600}$ was determined at the indicated time.

### *2.7. Sequence Analysis of LOC_Os02g06010 in Four Parental Lines and 30 Other Rice Varieties*

DNA samples of the four parental lines were extracted from the seedling samples by CTAB. The DNA samples were then used as templates to amplify the full-length genomic sequence and promoter of *LOC_Os02g06010* via the KOD-FX polymerase (Toyobo, Japan) using specific primers (Supplementary Table S4). The PCR products were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China). *LOC_Os02g06010* sequences of the four parental lines were aligned and analyzed using DNAMAN.

The accessions were genotyped using the Illumina HiSeq 2000 (PE150) (50X) by Berry Genomics Corporation; the average sequencing depth of each accession genome was 50× [40]. The sequence of *LOC_Os02g06010* in different rice varieties was analyzed according to the sequencing data.

## 3. Results

### *3.1. Distribution of $Zn^{2+}$ Concentration in Shoots and Grains of MAGIC Population*

We evaluated the $Zn^{2+}$ accumulation in the 215 lines of the MAGIC population and its four parental lines. The zinc concentrations in shoots of the vegetative growth period (3 weeks after being transferred) and brown rice at the mature stage were determined, respectively. The $Zn^{2+}$ concentrations in shoots and brown rice were highest in the parental line B, and the lowest in the parental line D (Figure 1A,B). The MAGIC population exhibited significant phenotypic variation in $Zn^{2+}$ concentrations in shoots and brown rice. The distributions of the two traits were approximately normal (Figure 1A,B).

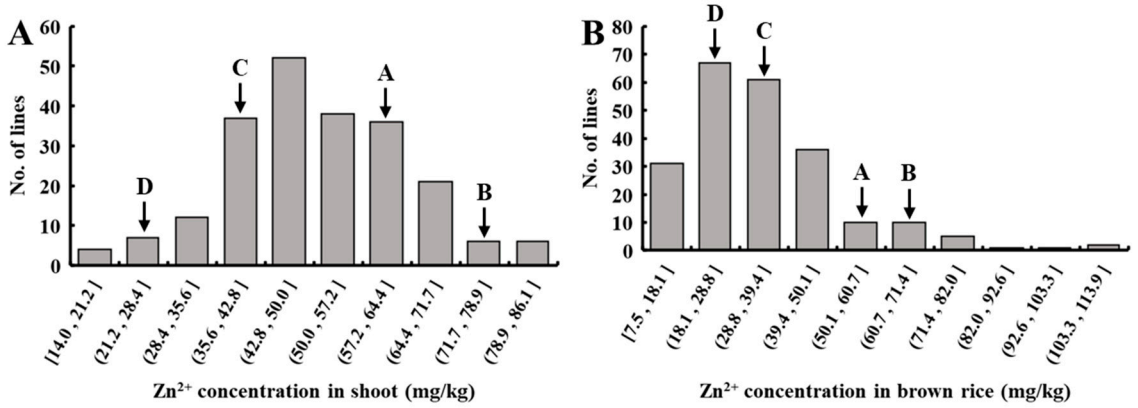


**Figure 1.** Distribution of $Zn^{2+}$ concentration in shoots at the seedling stage and in grains at the mature stage in the DC1 population. A pot experiment was carried out in the greenhouse of the Agricultural Genomics Institute in Shenzhen, Chinese Academy of Agricultural Sciences. The shoot and grain samples were collected at the seedling stage (3 weeks after transplanting) and the mature stage, respectively. Frequency distribution of (**A**) $Zn^{2+}$ concentration in shoots at the seedling stage, and (**B**) $Zn^{2+}$ concentration in brown rice at the mature stage.

The correlation between Zn concentration in the shoots of seedlings and concentration in the mature brown rice was positive and moderate (correlation coefficient was 0.39; Figure 2).

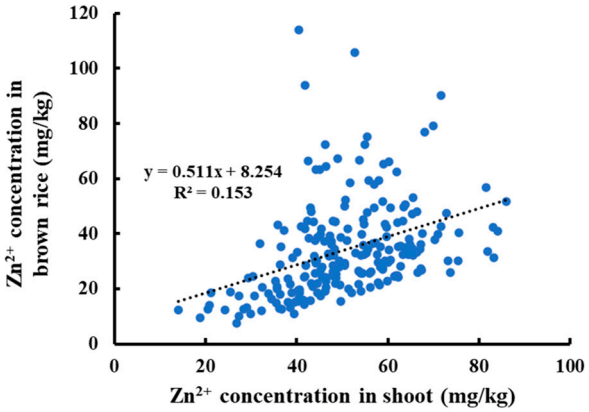


**Figure 2.** The relationship between $Zn^{2+}$ concentration in shoots at the seedling stage and in grains at the mature stage in the DC1 population.

### *3.2. Mapping of QTLs Associated with $Zn^{2+}$ Concentration in Shoots and Grains*

Five QTLs associated with $Zn^{2+}$ concentration in shoots were located on Chromosomes 1, 2, 3, and 8. They explained 4.5% (*qSZn1*), 5.3% (*qSZn2-1*), 3.7% (*qSZn2-2*), 3.9% (*qSZn3*), and 5.7% (*qSZn8*) of the phenotypic variation, respectively (Table 1, Figure 3). Six QTLs were identified as being associated $Zn^{2+}$ concentration in brown rice, which were distributed on Chromosomes 2, 3, 5, 7 and 8, and explained 5.7% (*qGZn2*), 5.8% (*qGZn3*), 5.5% (*qGZn5-1*), 5.5% (*qGZn5-2*), 6.1% (*qGZn7*), and 8.9% (*qGZn8*) of phenotypic variation, respectively (Table 1, Figure 3). *qSZn2-1* was co-located with *qGZn2*, and *qSZn3* was co-located with *qGZn3*. The *qSZn2-2* and *qGZn5-1* regions covered the genes *OsYSL14* and *OsZIP7*, respectively (Table 1).

**Table 1.** QTLs associated with $Zn^{2+}$ concentration in shoots at the seedling stage and grains at the mature stage in the MAGIC population.

| QTLs | Chr. | Position (bp) | *p*-Value | Contribution | Gene Symbol | References |
|---|---|---|---|---|---|---|
| $Zn^{2+}$ concentration in shoot | | | | | | |
| *qSZn1* | 1 | 5911595 | 0.001835 | 4.5% | | |
| *qSZn2-1* | 2 | 3312324 | 0.000793 | 5.3% | | |
| *qSZn2-2* | 2 | 23649858 | 0.004561 | 3.7% | *OsYSL14* | [43] |
| *qSZn3* | 3 | 35337103 | 0.003697 | 3.9% | | |
| *qSZn8* | 8 | 7947702 | 0.000537 | 5.7% | | |
| $Zn^{2+}$ concentration in brown rice | | | | | | |
| *qGZn2* | 2 | 3178843 | 0.002451 | 5.7% | | |
| *qGZn3* | 3 | 35466303 | 0.002126 | 5.8% | | |
| *qGZn5-1* | 5 | 7576955 | 0.003099 | 5.5% | *OsZIP7* | [24–26] |
| *qGZn5-2* | 5 | 15660281 | 0.003082 | 5.5% | | |
| *qGZn7* | 7 | 22050741 | 0.001602 | 6.1% | | |
| *qGZn8* | 8 | 26287654 | 7.33E-05 | 8.9% | | |

Note: Significant SNPs within a physical distance of 1.5 Mb were delineated into a single QTL. Positions of the most significant SNPs are based on rice reference sequence MSU V 7.0. Chr.: Chromosome; Contribution: phenotypic variance explained.

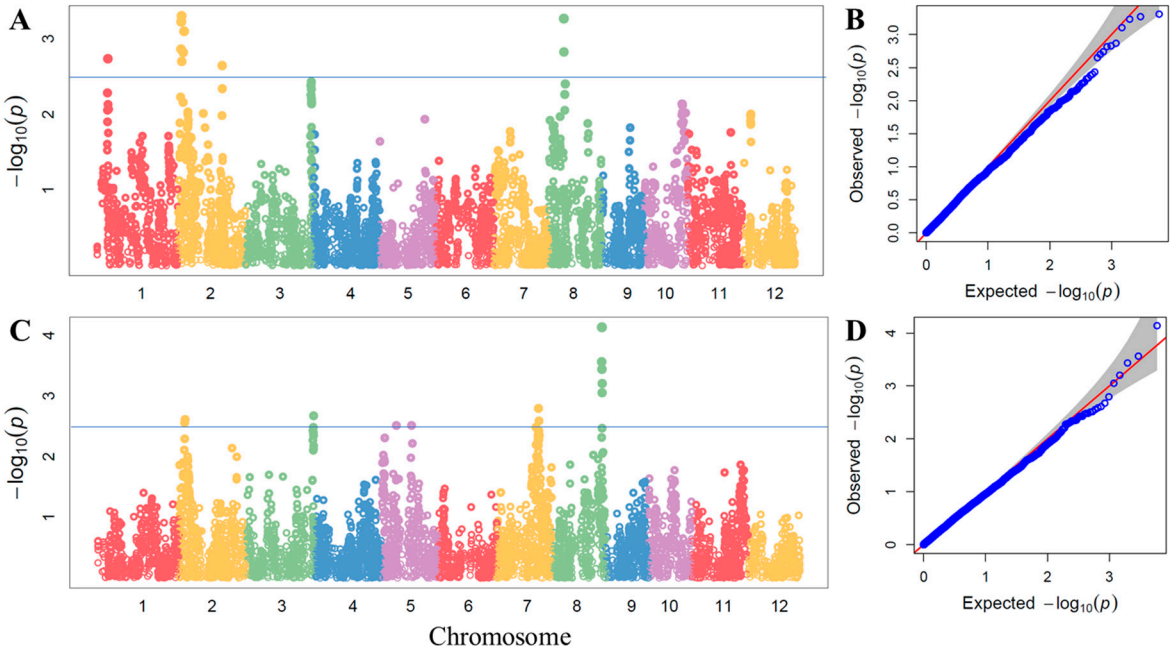


**Figure 3.** GWAS of $Zn^{2+}$ concentration in shoots at the seedling stage and in grains at the mature stage in the MAGIC population. Manhattan and quantile–quantile plots of $Zn^{2+}$ concentration in shoots at the seedling stage (**A**,**B**), and $Zn^{2+}$ concentration in brown rice at the mature stage (**C**,**D**).

### *3.3. Identification of Candidate Genes Related to $Zn^{2+}$ Accumulation in Shoots and Grains*

The QTLs *qSZn1*, *qSZn2-1*/*qGZn2*, *qSZn3*/*qGZn3*, *qGZn7*, and *qGZn8* were relatively more relevant for $Zn^{2+}$ concentration in shoots, concentration in brown rice, or both (Table 1, Figure 3). Based on annotation (http://rice.plantbiology.msu.edu/index.shtml) and literature information, 16 genes were chosen as the candidate genes responsible for $Zn^{2+}$ accumulation (Table 2). Among them, 15 encode proteins with transmembrane structure (Supplementary Figure S1). Six genes, namely *LOC_Os02g06010* (*qSZn2-1*/*qGZn2*), *LOC_Os07g37510* (*qGZn7*), *LOC_Os08g41370*, *LOC_Os08g42020*, *LOC_Os08g42150*, and *LOC_Os08g42170* (*qGZn8*) were found to be induced by $Zn^{2+}$ deficiency (Figure 4).

**Table 2.** Annotations of the selected candidate genes from promising QTLs detected for $Zn^{2+}$ accumulation.

| MSU ID | Annotation |
|---|---|
| *qSZn1* | |
| *LOC_Os01g10600* | aquaporin protein, putative, expressed |
| *LOC_Os01g10990* | integral membrane protein DUF6 containing protein, expressed |
| *LOC_Os01g11250* | potassium channel KAT1, putative, expressed |
| *qSZn2-1/qGZn2* | |
| *LOC_Os02g06010* | integral membrane protein, putative, expressed |
| *LOC_Os02g06540* | transporter family protein, putative, expressed |
| *qSZn3/qGZn3* | |
| *LOC_Os03g62200* | ammonium transporter protein, putative, expressed |
| *LOC_Os03g62270* | MATE efflux family protein, putative, expressed |
| *LOC_Os03g62650* | ion channel DMI1-like, chloroplast precursor, putative, expressed |
| *LOC_Os03g62750* | inner membrane protein, putative, expressed |
| *qGZn7* | |
| *LOC_Os07g37320* | transporter family protein, putative, expressed |
| *LOC_Os07g37454* | urate anion exchanger, putative, expressed |
| *LOC_Os07g37510* | organic cation transporter-related, putative, expressed |
| *qGZn8* | |
| *LOC_Os08g41370* | amino acid permease family protein, putative, expressed |
| *LOC_Os08g42020* | zinc ion binding protein, putative, expressed |
| *LOC_Os08g42150* | zinc transporter 2 precursor, putative, expressed |
| *LOC_Os08g42170* | zinc transporter 2 precursor, putative, expressed |

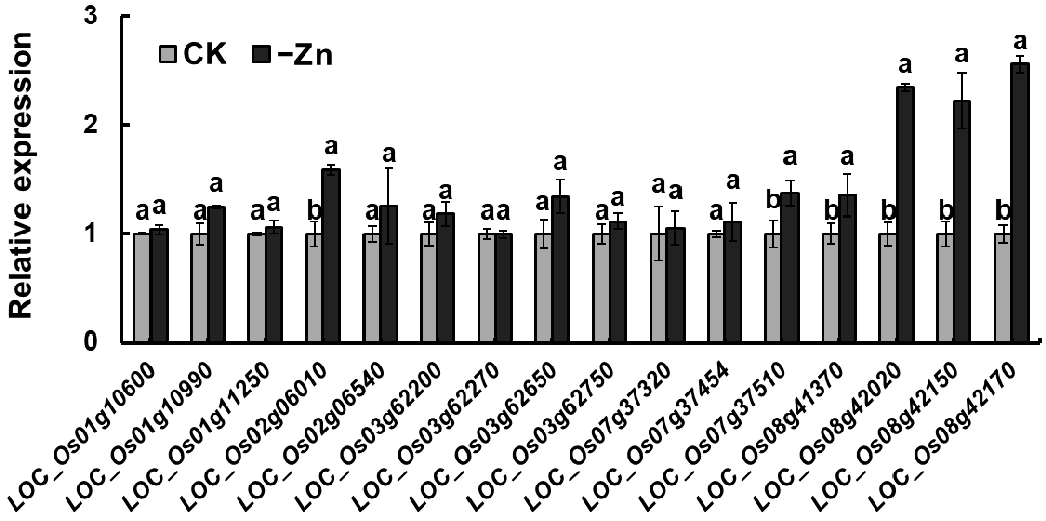


**Figure 4.** Relative expression of 16 candidate genes under different $Zn^{2+}$ concentrations. The seedlings of rice cv. Nipponbare at the three-leaf stage grown in 1/4 strength IRRI solution with 0.77 μM $Zn^{2+}$ (CK) or without $Zn^{2+}$ (−Zn) for three weeks were used. RNA was extracted from rice roots. Values are mean ± SE (n = 3). The different letters above the bars indicate significant difference between the control and treatments at $p < 0.01$.

To determine the $Zn^{2+}$ transport activity of the expressed proteins of six candidate genes, expression levels of the candidate genes in the yeast mutant strain ZHY3 were compared with the empty vector *pYES2* and positive control *OsZIP5*. In terms of growth, there were no significant differences between the ZHY3 transformed by the six candidate genes, *OsZIP5* and *pYES2* under the control medium and the solid medium containing 100 μM $Zn^{2+}$ (Supplementary Figure S2). There was no significant difference between the *LOC_Os07g37510-*, *LOC_Os08g41370-*, *LOC_Os08g42020-*, *LOC_Os08g42150-*, *LOC_Os08g42170-*, and *pYES2*-transformed ZHY3 on the solid medium containing 1 μM or 3 μM $Zn^{2+}$ either (Supplementary Figure S2), suggesting that these proteins may not have $Zn^{2+}$ transport activity. The growth of *LOC_Os02g06010*-transformed ZHY3 was

significantly higher than that of *pYES2*-transformed ZHY3 in the solid medium containing 1 μM or 3 μM $Zn^{2+}$ after 3 days (Supplementary Figure S2; Figure 5A), suggesting that LOC_Os02g06010 may have $Zn^{2+}$ transport activity. Experiment using a liquid medium with different $Zn^{2+}$ concentrations confirmed that the growth of ZHY3 under 3 μM $Zn^{2+}$ supply was increased by *LOC_Os02g06010* (Figure 5A,B).

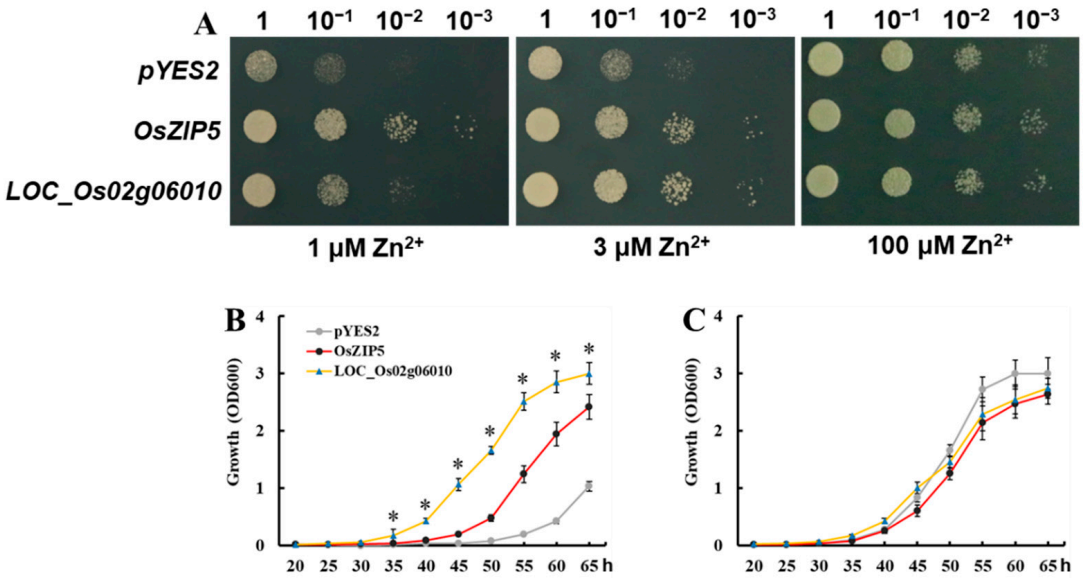


**Figure 5.** Complementation of the yeast mutant strain ZHY3 by *LOC_Os02g06010.* (**A**) Overnight yeast cell suspensions of ZHY3 transformed with empty vector *pYES2*, *OsZIP5*, or *LOC_Os02g06010* were serially diluted (1:10) and spotted onto solid media containing 1, 3 or 100 μmol/L $ZnSO_4$. Pictures were taken after three days growth at 30 °C. Yeast strains were grown in liquid media with (**B**) 3 μmol/L $ZnSO_4$ or (**C**) 100 μmol/L $ZnSO_4$ for 65 h. The absorbance at 600 nm ($OD_{600}$) of cell cultures was measured every 5 h. Values are mean ± SE ($n$ = 3). The asterisks (*) above the bars indicate significant difference between lines at $p < 0.01$.

### *3.4. Expression Pattern and Sequence Analysis of LOC_Os02g06010 in Parental Lines*

The difference of *LOC_Os02g06010* in the four parental lines was amplified by PCR and sequenced. The coding region sequence of *LOC_Os02g06010* showed no difference between the four parental lines (Figure 6A). The sequence in the 2000 bp region of the upstream of the ATG and the 3′-UTR sequence after the TGA of *LOC_Os02g06010* were identical between A, B, and C (Figure 6A). However, D had four single-nucleotide mutations in the first intron, three single-nucleotide mutations in the second intron, and one single-nucleotide mutation in the 3′-UTR (Figure 6A).

The sequence of *LOC_Os02g06010* in different rice varieties was analyzed according to the sequencing data, and SNP: −951 bp and SNP: −830 bp in the intron region of the upstream of the ATG were found in the accessions. The two SNPs of most accessions were the same as those of A, B, and C (ABC-TYPE), and only seven accessions were the same as D (D-TYPE). We selected 30 accessions of ABC-TYPE and seven accessions of D-TYPE to measure the $Zn^{2+}$ concentration in brown rice (Supplementary Table S5). It was found that the $Zn^{2+}$ concentration in brown rice of ABC-TYPE was 13.1% higher than that of D-TYPE (Supplementary Figure S3).

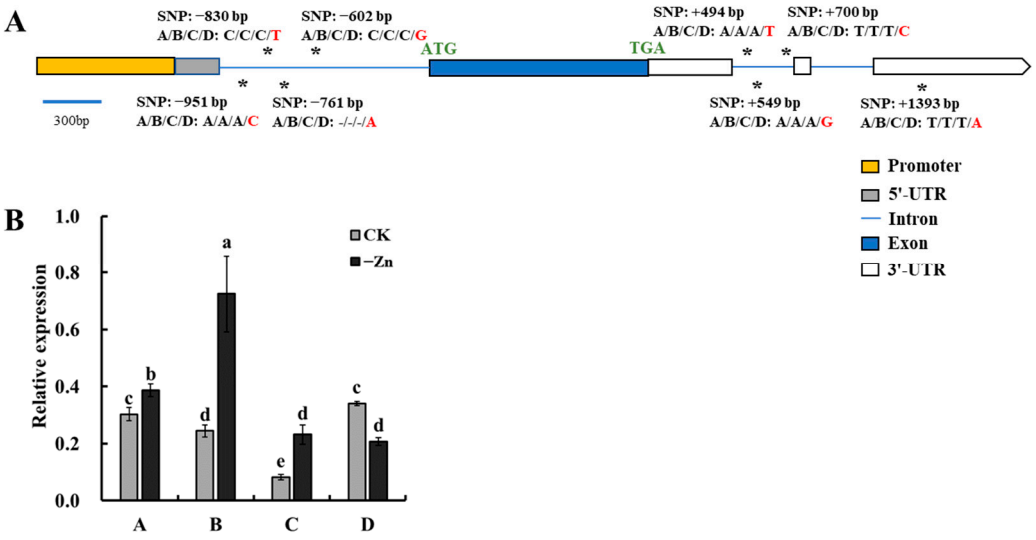


**Figure 6.** Sequence and expression of *LOC_Os02g06010* in the four parental lines of the MAGIC population. (**A**) Gene structure of *LOC_Os02g06010* and polymorphism locations (*: SNP). The SNPs are underlined with asterisks. (**B**) Relative expression of *LOC_Os02g06010* in four parental lines under different $Zn^{2+}$ concentrations. The seedlings of four parental lines at three-leaf stage, grown in the 1/4 strength IRRI solution with 0.77 μM $Zn^{2+}$ (CK) or without $Zn^{2+}$ (-Zn) were used. RNA was extracted from rice roots. Values are mean ± SE (n = 3). The different letters above the bars indicate significant difference between the control and treatments at $p < 0.01$.

The expression pattern of *LOC_Os02g06010* in response to $Zn^{2+}$ deficiency was further analyzed in four parental lines. Compared with the normal $Zn^{2+}$ supply, the expression of *LOC_Os02g06010* was significantly increased by $Zn^{2+}$ deficiency in A, B, and C, while it was significantly reduced in D (Figure 6B).

### *3.5. Expression of LOC_Os02g06010 in Different Organs at Different Growth Stages*

To further analyze the biological functions of *LOC_Os02g06010*, its expression levels in different organs at different stages were investigated. *LOC_Os02g06010* was expressed in all the measured tissues at different growth stages, and was strongly expressed at the flowering stage (Figure 7).

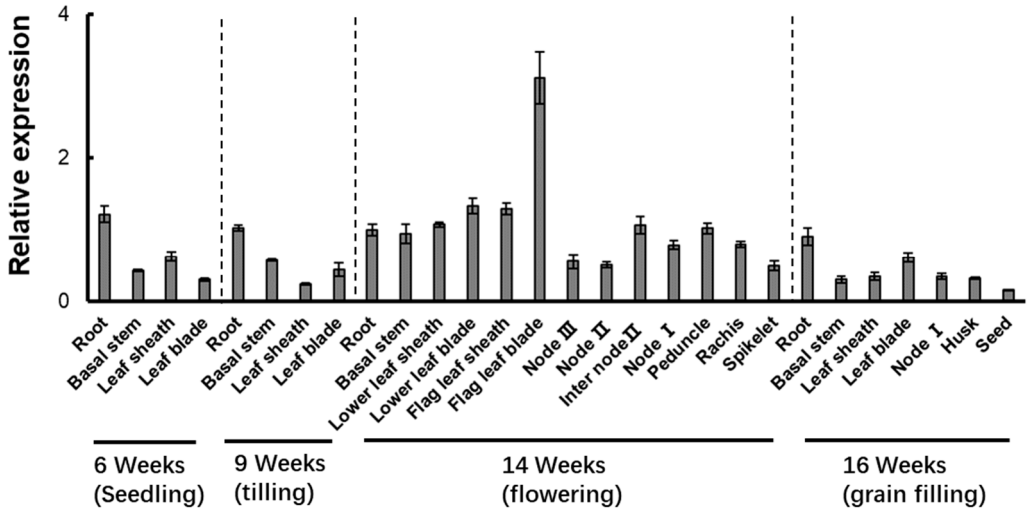


**Figure 7.** Relative expression of *LOC_Os02g06010* in different organs at different growth stages. Samples were collected from cv. Nipponbare grown in a paddy field. Values are mean ± SE (n = 3).

## 4. Discussion

A set of rice MAGIC populations has been developed by IRRI to better integrate QTL discovery and breeding. Their use in QTL mapping for a range of agronomic traits has been previously reported [36–39,44]. We previously identified QTLs associated with the toxicity tolerance of rice to three essential metals (Fe, Zn, and Al) by using three of the MAGIC populations, including DC1, DC2, and eight-way populations, genotyped using a 55 K SNP array [37]. In this study, we found that the four parents of the DC1 population displayed substantial differences in $Zn^{2+}$ concentrations in shoots at the seedling stage and in grains at the mature stage (Figure 1A,B). GWAS was conducted using a mixed linear model for the two traits.

Five QTLs for the concentration of $Zn^{2+}$ in shoots at the seedling stage, and six QTLs for the concentration of $Zn^{2+}$ in brown rice at the mature stage were identified (Table 1). These QTLs did not co-locate with those QTLs for $Zn^{2+}$ concentration in leaves or shoots at the mature stage reported by Norton et al. [35] and Yang et al. [29]. This surprising inconsistency may be attributed to the obvious differences in genetic populations, growth conditions, and growth stages for sample collection. However, QTLs *qSZn2-2* and *qGZn5-1* identified in this study are physically very close to the known zinc-affecting genes (Table 1), suggesting that our study was of good quality. Functional analysis of some *YSL* members has shown that they are not only involved in Fe uptake but also in the transport and homeostasis of other transition metals such as $Zn^{2+}$ [45]. The expression level of *OsYSL14* in flag leaves exhibited significant correlations with Fe and/or Zn concentrations in the seeds [43]. *OsZIP7* expression in *Arabidopsis* resulted in a 25% increase in shoot Zn concentrations compared to nontransformed plants [25]. *OsZIP7* functions in xylem loading in roots and intervascular transfer in nodes to deliver Zn to the grain in rice [24]. Two QTLs for the two traits were co-located; *qSZn2-1* was co-located with *qGZn2* and *qSZn3* was co-located with *qGZn3*. This could explain the moderate and positive correlation observed between the two traits (Figure 2).

Screening of 16 candidate genes for five important QTLs, chosen based on annotation and literature information for response to $Zn^{2+}$ deficiency, identified six responsive genes (Figure 4). Among them, only *LOC_Os02g06010* was found to have $Zn^{2+}$ transport activity, using the transformation of the yeast mutant strain ZHY3. In rice, many genes such as OsZIP1, OsZIP3, OsZIP5, and OsZIP9 have been proven to have $Zn^{2+}$ transport activity in yeast [23,27,46]. *LOC_Os02g06010* codes for an integral membrane protein, with a domain of unknown function domain (DUF) and transmembrane structure (Supplementary Figure S1).

Further functional analysis of *LOC_Os02g06010* in four parental lines showed that the expression of *LOC_Os02g06010* was significantly induced in A, B, and C but inhibited in D under zinc-deficient conditions (Figure 6B). The different expression patterns of *LOC_Os02g06010* in the parental lines may have caused the difference in zinc accumulation among the four parental lines. To find the reasons for the different expression levels of the four parental lines, we analyzed the promoter sequence and genome sequence of *LOC_Os02g06010*. The coding region of *LOC_Os02g06010* showed no difference between the four parental lines (Figure 6A). In the 3′-UTR and the introns, parental lines A, B, and C were identical, while the parental line D showed one and seven single-nucleotide mutations, respectively. (Figure 6A). The UTR region does not encode amino acids, but plays an important role in the regulation of gene expression. In post-transcriptional regulation, the noncoding regions of mRNA (including 5′-UTR and 3′-UTR, especially 3′-UTR) determine the translation speed, stability, subcellular localization, degradation, and other features of mRNA [47–49]. The mutation of the 3′-UTR in the rice *OsCKX2-2* gene can affect CKX enzyme activity to regulate rice development and grain size [50]. Intron retention, as a component of regulated gene expression programs, could increase transcription levels by affecting the rate of transcription, nuclear export, and transcript stability [51,52]. An intron was converted into one that increased mRNA accumulation 24-fold and reporter enzyme activity 40-fold relative to the intronless control by introducing 11 copies of the more active TTNGATYTG motif in *Arabidopsis* [53]. An intron-derived motif strongly increased gene

expression from transcribed sequences via a splicing independent mechanism in *Arabidopsis* thaliana [54]. An intron-containing plasmid increased the level of GUS enzyme activity 10- to 40-fold and 80- to 90-fold compared with the intronless plasmid, pBI221, in transgenic rice protoplasts and transgenic rice tissues, respectively [55]. The intron elevated GUS gene expression mainly at the mRNA accumulation level, but also stimulated enhancement at the translational level in rice [56]. The 5′ UTR intron of the rice *rubi3* gene enhanced GUS reporter gene activity in transgenic lines about 29-fold [57]. Wu et al. [58] found that the addition of the maize *Ubi* intron 1 significantly enhanced the *OsMT2b* promoter activity in rice embryos, suggesting that the natural variations observed in the introns or UTRs may cause differences in expression. SNP: −951 bp and SNP: −830 bp were found in the sequenced varieties, and the $Zn^{2+}$ concentration in brown rice of ABC-TYPE was higher than that of D-TYPE (Supplementary Table S5; Supplementary Figure S3). It is speculated that SNP: −951 bp and SNP: −830 bp in the first intron region of the upstream of the ATG may be the key sites for regulating *LOC_Os02g06010* expression. *LOC_Os02g06010* was expressed in all the measured tissues of different growth stages of rice, and was more strongly expressed at the flowering stage (Figure 7). Taken together, the present results suggest that *LOC_Os02g06010* may play an important role in $Zn^{2+}$ accumulation in *indica* rice. Further characterization of *LOC_Os02g06010* is needed to elucidate its functional significance in $Zn^{2+}$ uptake, translocation, and accumulation in rice.

## 5. Conclusions

In this study, we used a MAGIC population to locate QTLs related to $Zn^{2+}$ concentration in shoots at the seedling stage and in grains at the mature stage. Five QTLs (*qSZn1*, *qSZn2-1*, *qSZn2-2*, *qSZn3*, and *qSZn8*) were detected to be relevant for $Zn^{2+}$ concentration in shoots at the seedling stage, and explained 3.7–5.7% of the phenotypic variation. Six QTLs (*qGZn2*, *qGZn3*, *qGZn5-1*, *qGZn5-2*, *qGZn7*, and *qGZn8)* were detected to be relevant for $Zn^{2+}$ concentration in grains at the mature stage, and explained 5.5–8.9% of the phenotypic variation. Sixteen candidate genes were predicted, and *LOC_Os02g06010* was selected as our candidate gene through qRT-PCR and yeast heterologous functional complementation verification test. The expression of *LOC_Os02g06010* was significantly induced in A, B, and C, but inhibited in D by $Zn^{2+}$ deficiency. The coding region of *LOC_Os02g06010* showed no difference between the four parental lines. In the 3′-UTR and introns, parental lines A, B, and C were identical, while the parental line D showed one and seven single-nucleotide mutations, respectively. SNP: −951 bp and SNP: −830 bp in the first intron may be the key sites for regulating LOC_Os02g06010 expression. *LOC_Os02g06010* was strongly expressed at the flowering stage of rice. These results suggest that *LOC_Os02g06010* may play important roles in $Zn^{2+}$ accumulation in *indica* rice.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2077-0472/11/1/70/s1, Table S1: Description of the four parental lines used for developing the MAGIC DC1 population, Table S2: Primers used for qRT-PCR, Table S3: Primers used to amplify the open reading frames of six candidate genes and *OsZIP5*, Table S4: Primers used to amplify the full length of *LOC_Os02g06010* and its promoter, Table S5: Detailed information of 37 rice accessions, Figure S1: Predicted topological models of proteins encoded by 16 candidate genes, Figure S2: Complementation of the yeast mutant strain ZHY3 by *OsZIP5* and six candidate genes, Figure S3: Haplotype analyses of *LOC_Os02g06010*.

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