

Genome-wide identification of ZIP gene family members in wheat

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Abstract

Background: The plant ZIP (Zn-regulated, iron-regulated transporter-like protein) transporter family is one of the most essential gene families regulating the uptake, transport and accumulation of microelements, which play important roles in plant growth, development and biofortification. Although the ZIP family has been systematically studied in many plant species, the significance of this family in wheat is not well understood at present.

Results: Through a genome-wide search based on the latest wheat reference sequence (IWGSC_V1.1), 58 TaZIP genes were identified. Most of these genes were represented by two to three homoalleles, which were named TaZIP_-A, TaZIP_-B, TaZIP_-D. Protein structure analysis revealed that most TaZIP proteins contain more than six transmembrane (TM) domains and that the distance between TM-3 and TM-4 is variable. Furthermore, the TaZIP proteins clustered into four groups in a phylogenetic tree, and the proteins belonging to the same group shared similar exon-intron structures and conserved motifs. Expression pattern analysis revealed that most TaZIP genes were significantly highly expressed in root, and that nine TaZIP genes were up-regulated at the grain filling stage. When exposed to ZnSO₄ and FeCl₃ solutions, TaZIP genes showed different expression patterns, and 16 TaZIP genes were identified as candidate high-affinity Zn transporter genes and 23 as low-affinity Zn transporter genes. Finally, using yeast complementation analysis three TaZIP genes were demonstrated to have the capacity to transport Zn and Fe.

Conclusion: This study systematically analyzed the genomic organization, gene structures and expression profiles of TaZIPs. The findings not only provide candidates for further functional analysis, but also contribute to a better understanding of the regulatory roles of ZIPs in wheat.

Background

Zinc (Zn) is a microelement essential for plant normal growth and development. It plays an important role in diverse biochemical processes and is also an essential component of biological metabolic enzymes involved in the regulation of enzyme activity [1,2]. Iron (Fe) is another essential microelement; it is an auxiliary group of many enzymes in plants, such as cytochrome oxidase, peroxide and catalase, and plays an important role in respiratory electron transport [3,4,5]. Both Zn and Fe are indispensable for plant photosynthesis [6]. Although Zn and Fe are vital for plant growth and development, excessive levels of Zn and Fe result in significant toxicity to biological systems [7]. Therefore, plants have evolved multiform transport systems to equipose the absorption, utilization and storage of these metal ions [8,9]. These systems include the ZIP (Zn-regulated, iron-regulated transporter-like protein), CDF (Cation-Diffusion Facilitator), and HMA (Heavy Metal ATPase) proteins [10].

Generally, ZIP transporters are composed of 326 to 425 amino acid residues, and most ZIP transporters contain eight transmembrane (TM) domains with a variable number of amino acids between TM regions III and IV [11]. In this variable region, there are many histidine residues, which are associated with the

binding and transport of metal ions [11]. In addition, metal ions and transporters can form octahedral, tetrahedral and plane structures [12]. Recently, many ZIP genes have been identified; these genes encode proteins with the ability to transport various divalent cations, including Fe^{2+} , Zn^{2+} , Mn^{2+} , and Cd^{2+} [11,13]. So far, 16 ZIP genes have been found in rice and Arabidopsis, respectively [14,15,16].

In Arabidopsis, *AtIRT1* was the first functionally identified ZIP gene, and is mainly expressed in the root and enriched nickel (Ni) under iron deficiency [17]. In yeast complementation assays, *AtIRT1* was shown to transport Fe and Zn, and under Fe-deficient conditions, *irt1* mutant plants showed severe etiolation, with an iron content in etiolated leaves only about 30% of that of wild type; this etiolation phenotype could be alleviated when mutant plants were transformed with an iron transporter gene [18,19]. The function of *AtIRT2* is highly similar to that of *AtIRT1*; *AtIRT2* could restore the transport activity of an iron-deficient yeast mutant [20]. The functions of *AtZIP1* and *AtZIP2* have also been reported. *AtZIP1* is mainly expressed in the root and leaf vein, while *AtZIP2* is highly expressed in the root column [21]. The proteins encoded by these two genes are located in the vacuole membrane and plasma membrane, respectively. *AtZIP1* participates in the reactivation of metal ions transported from vacuoles to the root cytoplasm, while *AtZIP2* participates in root absorption of Mn and Zn. Both proteins play an important role in the transport of Mn and Zn from roots to leaves [21]. Extensive studies have also shown that some ZIP genes are also involved in response to Zn-deficiency in Arabidopsis [22,23].

In rice, *OsIRT1* and *OsIRT2* are mainly responsible for Fe transport [24]. Under Fe-deficient conditions, the expression of these genes increase significantly in roots. Overexpression of *OsIRT1* increases the resistance to Fe-deficient stress and the sensitivity to excessive Zn and Cd [25,26,27]. Rice overexpressing *OsIRT1* showed no significant difference from the wild type at the seedling stage, but at the reproductive stage, overexpression plants had shorter and fewer tillers as well as decreased yield, while the contents of Fe and Zn in grains increased [22], suggesting that overexpression of *OsIRT1* improved the accumulation of Fe and Zn in rice grains, but at the same time resulted in yield reduction. The function of *OsIRT2* is similar to that of *OsIRT1*, but it has a lower transshipment capacity [26]. The family members *OsZIP1*, *OsZIP3*, *OsZIP4*, and *OsZIP5* were also shown to transport of Zn in rice [12,28,29,30].

Since ZIP proteins were first demonstrated to be the key proteins involved in plant absorption and transport of Zn and Fe, they have been extensively studied in model plants, such as rice and Arabidopsis. However, little is known about this family in wheat. Wheat is a major staple crop worldwide, feeding approximately 40% of the world's population [31]. Although the average yield and total production of wheat have been greatly increased with the help of improved breeding and cultivation programs, wheat nutritional quality, particularly the contents of the microelements Zn and Fe, has not been enhanced to meet humans' needs. It is reported that about two billion people in South Asia and Sub-Saharan Africa suffer from Zn and Fe deficiency, which has been termed "hidden hunger" [32]. Increasing the Zn and Fe content in wheat grains through genetic engineering breeding is a convenient, cost-effective and sustainable way to solve this problem. Identification and mining of the genes regulating the uptake, transport and enrichment of Zn and Fe is crucial for breeding wheat with high Zn and Fe content. Thus, we have systematically analyzed the genomic organization, gene structures and expression profiles of

ZIP gene family members in wheat at the genome level, with the aim of discovering candidates for further functional study and genetic improvement applications.

Results

Identification and classification of ZIP genes in wheat

The updated wheat reference genome sequence and protein information (IWGSC_V1.1) were downloaded from the Ensemble plant database (http://plants.ensembl.org/Triticum_aestivum/Info/Index). The ZIP HMM file, which is specific to the ZIP protein family, was downloaded from the Pfam database. Based on a whole genome search, a total of 58 ZIP genes were identified (Table 1) (Additional file 1, 2, 3, and 4), of which 44 genes were orthologous to ZIP genes rice; these genes were named following the rice nomenclature (Fig. 1). The remaining genes were named *TaZIP17* to *TaZIP30* according to their locations on the chromosome defer to the principles of 1A to 7D (Fig. 2). We found that the TaZIPs were unevenly distributed on the chromosomes, with no ZIP genes on the fifth chromosome group (Fig. 1). The amino acid lengths of the TaZIP proteins ranged from 185 to 577, and the proteins contained 3 to 13 TM domains. Most TaZIPs contained 7 to 9 TM domains and the distance between TM-3 and TM-4 was variable. The TaZIP proteins were predicted to be localized on the plasma membrane.

The phylogenetic relationships between the 58 wheat ZIPs and 16 rice ZIP proteins were determined (Fig. 2, File 5). In a phylogenetic tree these ZIP proteins were clustered into four groups: ZIPI (37 wheat proteins and 9 rice proteins), ZIPII (3 wheat proteins and 1 rice proteins), ZIPIII (6 wheat proteins and 2 rice proteins) and ZIPIV (12 wheat proteins and 4 rice proteins). The largest group, ZIPI, included *TaZIP17* to *TaZIP30*. Six wheat ZIPI proteins were closely related to *OsIRT1* and *OsIRT2*, suggesting that these six TaZIPs may share similar functions in Fe transport. Three homoeologous copies (A, B and D) of *TaZIP4* clustered together with *OsZIP3* and *OsZIP4* on the same branch in the ZIP1 group. Group ZIPII consisted of *OsZIP6*, *TaZIP6-A*, *TaZIP6-B* and *TaZIP6-D*. Eight and 16 proteins were assigned to groups ZIPIII and ZIPIV, respectively.

TaZIP gene structures and conserved motifs

To obtain a preliminary understanding of the functions of TaZIPs, we further analyzed their gene structures and identified conserved motifs (Fig. 3). The sizes of the wheat ZIP genes ranged from 836 bp to 14,494 bp (Additional file 1). The numbers and lengths of introns were the main factors accounting for the variation in gene size. The number of introns varied from 0 to 11, and the number of exons ranged from 1 to 11. Although *TaZIP27* was the largest gene, *TaZIP16-A,B,D* and *TaZIP13-A,B,D*, which are all members of ZIPIV, had the most exons (up to 11). The shortest gene, *TaZIP28*, had only one exon. The genes with closer phylogenetic relationships clearly shared more similar gene structures.

Using the MEME tool, we identified 11 conserved motifs in the wheat ZIP proteins (Fig. 3). Members of the same groups also had similar conserved motif organizations. All TaZIP proteins except *TaZIP5-A* and *TaZIP27* had motif 3, which contains a histidine residue that is used to bind to metal ions for

transmembrane transport. All TaZIP proteins in group ZIP I had motifs 1 to 7, except three truncated proteins (*TaZIP5-A*, *TaZIP27* and *TaZIP28*). All group II members had the same motifs except *TaZIP6-D*, which contained motif 7. Motif 8 was only found in group IV proteins. All proteins in this group contained both motifs 3 and 8, but *TaZIP11-A, B, D* only had motif 3. (Additional file 4) Most TaZIP proteins also had motif 11, but the location of this motif varied between proteins.

Expression patterns of TaZIP genes in four tissues and at different grain filling stages

Overexpression of some ZIP genes has been shown to increase Zn and Fe content, and thus improve the quality of the grain and fruit. The wheat cultivar Zhongmai175 is an elite variety with a high Zn and Fe content [33]. To analyze the expression patterns of TaZIP genes in the grains of this wheat cultivar, we extracted RNA from wheat grains at different filling stages. We also analyzed the tissue-specific expression profiles of all TaZIP genes, except for 19 genes, for which we unfortunately could not design a suitable primer for fluorescence quantitative PCR (Additional file 6).

Publicly available RNA-seq datasets were also used to investigate the expression of TaZIP genes in different tissues, including the root, stem, leaf and grain. A total of 39 genes were found to be expressed in these tissues. Most of them were highly expressed in root but lowly expressed in grains; for example, *TaIRT1-D* was expressed most highly in the root but expressed at the lowest levels in grain (Fig. 4). Four genes (*TaZIP4-B*, *TaZIP4-A*, *TaZIP6-D*, *TaZIP14-B*) showed highest expression in the stem, and three genes (*TaZIP19*, *TaZIP6-A*, *TaZIP2-A*) showed highest expression in the leaf. The expression levels of all group III and IV genes, except *TaZIP16-A* and *TaZIP16-B*, were relatively high in all four tissues (Fig. 4), and they were particularly higher expressed in root. Differential expression between homoeologous genes was also observed. For example, *TaIRT2-A* and *TaIRT2-B* were highly expressed in grain, but *TaIRT1-D* was lowly expressed in this tissue.

To better understand the expression patterns of TaZIP genes at the grain filling stage, we compared the gene expression levels at different DAF (days after flowering) relative to those at 7 DAF as a control (Additional file 7). Analysis of expression patterns at 14 DAF, 21 DAF and 28 DAF revealed significant differences in the expression levels of TaZIPs during grain filling (Fig. 4). A total of 31 TaZIP genes were down-regulated at the grain filling stage, with the lowest expression levels observed at 28 DAF. Eight TaZIP genes were highly expressed during the grain filling stages, but displayed diverse patterns of up-regulation. Six group I genes were up-regulated, and *TaZTP7-B* was significantly more highly expressed than the others. *TaIRT2-A* and *-D* were found to be highly expressed at the grain filling stage as well. No group ZIP II genes displayed high expression at this stage. *TaZIP14-B* and *TaZIP14-A* were unique among group III genes in that their expression was up-regulated at the grain filling stage. The expression level of *TaZIP14-B* was very high, while *TaZIP14-A* was moderately expressed. In group IV, both *TaZIP13-B* and *TaZIP13-D* were highly expressed but these genes had distinct expression patterns; *TaZIP13-B* expression levels increased rapidly during the filling stage while *TaZIP13-D* displayed moderate expression *TaZIP13-B*.

Expression profiles of TaZIP genes under Zn or Fe stress

The change in TaZIP gene expression in response to treatment with solutions containing different concentrations of ZnSO₄ and FeCl₃ was also analyzed. Almost half of the ZIP genes were up-regulated under low concentrations of ZnSO₄, but the expression trend differed among the four groups and also within the same group as well. As shown in Fig. 5, 12 group I genes were highly expressed under 0.05 µmol/L ZnSO₄, but the expression levels were lower under the 0.5 and 50 µmol/L conditions. The expression of these genes was suppressed by increasing of Zn stress. At the same time, 14 genes were up-regulated under 0.50 µmol/L ZnSO₄. Moreover, the expression levels of nine ZIP genes (*TaZIP21*, *TaZIP5-D*, *TaZIP5-B*, *TaZIP8-B*, *TaIRT1-D*, *TaIRT2-D*, *TaIRT2-A*, *TaZIP10-B*, *TaZIP10-A*) increased in response 50 µmol/L ZnSO₄. All genes in groups ZIPII and ZIPIII were up-regulated in response to low concentrations of ZnSO₄. The gene *TaZIP14-D* was up-regulated by 0.5 µmol/L ZnSO₄ solution, but it was down-regulated by 50 µmol/L. *TaZIP14-A* were peculiar was suppressed under 50 µmol/L ZnSO₄. In group IV, two genes (*TaZIP13-D* *TaZIP13-B*) were very highly expressed under 0.5 µmol/L ZnSO₄. Although the gene *TaZIP13-B* was expressed only moderately under 0.05 µmol/L, it was up-regulated in response to other concentrations of ZnSO₄.

The expression patterns of TaZIPs under different concentrations of FeCl₃ were also analyzed. As shown in Fig. 5, low concentrations of FeCl₃ promoted the expression of 13 ZIP genes, and these genes were further suppressed or moderately up-regulated under 0.5 and 50 µmol/L FeCl₃. Another 16 genes were highly expressed under 0.05 and 0.5 µmol/L FeCl₃ solutions. Six genes were expressed highly under FeCl₃ concentrations of 0.05 and 0.5 µmol/L. The genes *TaZIP9-A*, *TaZIP13-D* and *TaZIP7-A*, *-B*, *-D* were up-regulated under high concentrations of FeCl₃. As the Fe transporter in wheat, *TaIRT1-D* was reported to be a key gene involved in the transport of iron ions, but its expression level under the FeCl₃ treatment was lower than that under the ZnSO₄ treatment. The expression patterns of *TaIRT2-A* and *-B* were similar to that of *TaIRT1-D*.

Functional analysis of three TaZIPs by complementation in yeast cells

To reveal the biological functions of the TaZIPs, three genes (*TaZIP9-A*, *TaIRT2-A*, *TaZIP13-D*) were cloned for yeast complementation analysis under ZnSO₄ and FeCl₃ treatment. Three rice genes, *OsZIP3*, *OsZIP5* and *OsIRT1*, which have been demonstrated to be involved in the transport of Zn and Fe [15, 16, 34], were also cloned to use as positive control.

Three *Saccharomyces cerevisiae* yeast strains were used in this experiment: the wild type strain 1455, the *zrt1zrt2* double mutant (ZHY3) and the *fet3fet4* double mutant (DEY1453), to verify that the three wheat genes were capable of restoring the ability of the mutant yeast to transport Zn and Fe. The full-length cDNAs of the three TaZIP genes were cloned and expressed in the two double mutants. The ZHY3 yeast cells transformed with the TaZIP genes grew on the SD media plus 0.4 mM EDTA, and the transformed 1453 cells grew on SD with 50 mM MES. As shown in the Fig. 6a, ZHY3 cells transformed with the TaZIP and OsZIP genes grew on the SD media containing three different concentrations ZnSO₄, indicating that the TaZIP proteins reversed the growth defect in the ZHY3 yeast mutant as efficiently as the functionally

characterized OsZIP proteins. This reversal was especially evident for TaZIP13-D. Similarly, the transformed 1453 cells grew on SD media containing different concentrations FeCl₃ and 50 mM MES. As expected, the growth defect was reversed with the expression of TaZIP and OsZIP genes (Fig. 6b). Cells transformed with TaZIP13-D showed the strongest growth under Fe-limited conditions. Expression of TaIRT2-D could also allow growth of transformed 1453 cells on SD media containing FeCl₃. The fact that TaZIP9-A, TaZIP13-D, and TaIRT2-D could complement the growth phenotypes of the zinc transporter mutant *zrt1zrt2* and iron transporter mutant *fet3fet4* effectively suggests that they have the ability to transport Zn and Fe.

Discussion

Zn and Fe are microelements that are essential for normal plant growth. When plants cannot absorb enough Zn and Fe, they will show phenotypes such as etiolation, withering, and even death [20]. Low available Zn and Fe content in soil is the main reason for plant Zn deficiency [35]. About 30% of the world's agricultural area is low in Zn, which affects grain yield and the Zn concentration in grains [36]. Plants achieve sustained Zn uptake from the environment using a dual-transporter system, which includes high-affinity and low-affinity Zn transporters called ZIPs [37]. This protein family has been reported in many species, including Arabidopsis, rice, barley, and maize [14,38,39,40,41]. To the best of our knowledge, this important family has not been well studied in wheat.

In this study, we identified 58 ZIP genes in wheat by performing a genome-wide search. The genes were distributed on all chromosomes except the 5 chromosome group, which demonstrates that the localization of the ZIP family is uneven in wheat. This uneven distribution might be due to specific retention and dispersion of TaZIPs during polyploidization. The lengths of the wheat ZIP genes were different and there were a variable number of amino acids between TD-III and TD-IV [11]. All TaZIP proteins were predicted to be localized in the plasma membrane, which is consistent with ZmZIPs, *AtIRT3* and *AtZIP4* and *HvZIP7* [14,16,39,42]. There are also ZIP proteins located on the vacuolar membrane, such as *AtZIP1* and *OsZIP6* [12,26]. The plasma membrane is an important site for the transport of metal ions including Zn²⁺ and Fe²⁺, which are quickly assimilated from fluctuating environments [43]. Thus, the predicted plasma membrane localization of all TaZIP proteins suggests that wheat could rapidly absorb Zn and Fe from the environment.

Most of the TaZIP genes were mainly expressed in roots, and others were mainly expressed in the leaf or stem. This result suggests that most TaZIP genes likely absorb and transport Zn and Fe in the root. The root takes up these elements, which are then translocated to the shoots. Several studies have revealed that the Zn and Fe taken up by the root are primarily delivered to different tissues through a phloem-tropic mode [44]. When the mineral elements arrive at the leaf and stem, the ZIP proteins that are mainly expressed in these tissues transport them into cells, where it is required to maintain normal plant growth and development.

The content of Zn and Fe in the grain is one of the most important indexes used to evaluate the quality of wheat [45]. Zn and Fe accumulation in grain generally occurs during the grain filling stage [46]. Several studies have demonstrated the relationship between the overexpression of ZIP genes and the resistance to mineral deficiency [28,47]. The relationship between the content of Zn and Fe in cereals and the overexpression of ZIP genes has also been reported [16,22]. In this study, the expression of ZIP genes during the grain filling stage was investigated. Nine genes were up-regulated at this stage, implying that these genes may take part in the accumulation of Zn and Fe in grain. It was found that overexpression of *OsIRT1* in rice enhanced the content of Zn and Fe in seed, but in this study we found that the orthologous gene *TaIRT1* was down-regulated in wheat grains. We suspect that because *TaIRT1* and *TaIRT2* produce similar proteins that transport Zn and Fe, *TaIRT2-A* and *TaIRT2-D* suppress the expression of the *TaIRT1* genes.

The expression patterns of ZIP genes under Zn and Fe deficiency have been examined in several studies, and most ZIP genes have been shown to be up-regulated under these conditions [14,16,48]. For example, *AtZIP1-5*, *AtZIP9-12* and *AtIRT3* have been shown to be up-regulated under Zn-deficiency conditions, and *OsIRT1* and *OsIRT2* are up-regulated under Fe-deficiency conditions. The ZIP transporter is a dual-transporter system, which consists of both high-affinity and low-affinity Zn transporters [36,49]. The high affinity system is saturated at about 0.1 $\mu\text{mol/L}$, and the uptake of Zn by the low-affinity system increases linearly between the concentrations of 0.5 to 50 $\mu\text{mol/L}$ [28,47]. A previous study found that the Zn uptake system in wheat is also a dual-uptake system [49]. As far as we know, no study has reported the expression patterns of ZIP genes under different concentrations of Zn^{2+} and Fe^{3+} . Our study revealed that about half of TaZIP genes were most highly expressed in the 0.05 $\mu\text{mol/L}$ ZnSO_4 solution and the rest of the genes were mostly highly expressed under 0.5 $\mu\text{mol/L}$ ZnSO_4 . TaZIP genes also displayed similar expression patterns in FeCl_3 solutions. We regard the 16 TaZIP genes that were most highly expressed in the 0.05 $\mu\text{mol/L}$ ZnSO_4 solution as encoding high-affinity Zn transporters, and the remaining TaZIP genes as encoding low-affinity Zn transporters. Actually, some of high-affinity Zn transporters in wheat were also up-regulated in another two concentration solutions .

In yeast, the high-affinity transporter gene (*Zrt1*) is responsible for the uptake of Zn in Zn-limiting media. When Zn is abundant in external media, *Zrt1* is repressed and the low-affinity transporter (*Zrt2*) mediates Zn uptake [37]. ZHY3 is yeast *zrt1zrt2* double mutant that cannot grow normally in SD media containing ZnSO_4 . *fet3fet4* DEY1453 is another mutant that cannot grow normally in the SD media containing FeCl_3 . Inserting a functional gene into these mutants will reverse the growth defect. Yeast complementation assays have been used in many studies to demonstrate that ZIP proteins are able to reverse the growth defects in *zrt1zrt2* and *fet3fet4* double mutants [14,16]. In this study, we assayed the ability of three genes, *TaZIP9-A*, *TaIRT2-A* and *TaZIP13-D*, to rescue the growth defects in the *zrt1zrt2* and *fet3fet4* double mutants. These genes were chosen because they belong to different groups and were highly expressed during the grain filling stage and under different ZnSO_4 and FeCl_3 treatments. All three genes rescued the growth defects of the yeast mutants, revealing that the proteins encoded by these genes transport Zn and Fe effectively.

Conclusions

This is the first study to report the genomic organization, gene structures, phylogenetic relationships and expression profiles of ZIP gene family members in wheat. A total of 58 TaZIP genes were identified. Gene structure and protein motif analysis indicated that TaZIP genes with closer phylogenetic relationships share similar exon-intron structures and conserved motifs. The expression patterns of TaZIP genes differed in different tissues and at different stages of grain filling. TaZIP genes also had different expression patterns in response to Zn and Fe treatment, and Zn- or Fe- responsive TaZIPs were identified. Three TaZIP genes were also shown to have the ability to reverse the growth defects of yeast Zn and Fe transport mutants. This study not only provides candidates for further functional analysis, but also contributes to a better understanding of the regulatory roles of ZIPs in wheat.

Methods

Plant materials

The wheat line ZhongMai175 was used in this study which is an elite variety that contains high Zn levels in grain [33]. A total of four rows were planted with this wheat line at the experimental station of Northwest A&F University, Yangling, China (34°20'N, 108°24'E). At the flowering stage, we chose one plant in each row as materials. Grains from each plant were collected at 7 DAF and frozen in liquid nitrogen. Samples were then collected every week until the grain matured. A total of four samples were collected in this study: 7DAF, 14DAF, 21DAF and 28DAF.

In the laboratory, the ZhongMai175 was cultured in a glass garden with filter paper, which was placed in a climate chamber (RXZ-500D-LED, Ning Bo) with a light/dark cycle of 16/8 h and 24 °C for ten days. The whole wheat were Collected and treated with different concentrations (0.05, 0.5, 5, and 50 $\mu\text{mol/L}$) of ZnSO_4 and FeSO_4 solution for 1 h.

Identification and bioinformatic analysis of TaZIP genes

The wheat ZIP gene and protein sequences were downloaded from Ensemble Plants (<http://plants.ensembl.org/index.html>) (PF02535) was downloaded from the Pfam v31.0 database (<http://pfam.xfam.org/>). This profile was used to search against plant protein sequences using a threshold of $E < 1e-5$ [50]. Blast and manual corrections were then performed to remove alternative events and redundant sequences. Then these proteins sequences were compared to those in the corresponding protein database from WPD (<http://www.wheatprotein.cn/index.html>). The rice OsZIP genes were downloaded from the NCBI database (<https://www.ncbi.nlm.nih.gov/>) as described by Chen and Tiong [15,16]. The neighbor-joining phylogenetic tree was constructed with MEGA 7 and EvolView (<http://www.evolgenius.info/evolview/>) based on the protein sequences of wheat and rice ZIPs with 1000 bootstrap replicates.

Gene structure and conserved motif analyses

Gene structure analysis was conducted using GSDS (<http://gsds.cbi.pku.edu.cn/>). Conserved protein motifs were predicted using the MEME Suite web server (<http://meme-suite.org/>), with the maximum number specified as 11 and the optimum width of motif sets specified as 5 to 200 amino acids.

RNA isolation and real-time PCR

Total RNA was isolated from grains and leaves with the RNAPrep Pure Plant Kit (TIANGEN, China) and from seedlings with TRIZOL (Takara, China). cDNA synthesis was performed in a 20- μ L reaction mixture containing 1 μ g of total RNA and mixture from the TIANscript RT Kit (TIANGEN, China). The real-time PCR mixture contained 1 μ L cDNA, 1 μ L of forward and reverse primers (Additional file 6), and 17 μ L SYBR Green (TIANGEN). The reaction was performed in an ABI7300 Real Time Thermal Cycler and repeated three times. The $2^{-\Delta\Delta C_t}$ method was used for fluorescence quantitative data analysis.

Cloning of three TaZIP genes and OsZIP genes

The coding and open reading frame sequences were obtained from the Wheat Sequence Database (<https://wheat-urgi.versailles.inra.fr/Tools>). The primers for cloning the three TaZIP genes (Additional file 7) were designed using Oligo 7 (Molecular.Biology.Insights, USA). In this step, RNA extracted from ZhongMai175 seedlings was used for cDNA synthesis. The PCR reaction mixture volume was 50 μ L and contained 5 μ L cDNA template, 2.5 μ L of forward and reverse primers (Additional file 7), 25 μ L of 2 \times Master Mix (NEB, USA) and 15 μ L of nuclease free water. The reaction was performed on a DNA amplification machine (Thermo Fisher Scientific, USA). The cycling conditions were as follows: initial denaturation at 98 $^{\circ}$ C for 30 s, followed by 35 cycles of denaturation at 98 $^{\circ}$ C for 10 s, annealing at 60 $^{\circ}$ C for 20 s, extension at 72 $^{\circ}$ C for 30 s, and final extension at 72 $^{\circ}$ C for 2 min. After amplification, 7 μ L of Purple 2-Log Ladder (NEB) was added to the PCR products, which were separated on a 1.5% agarose gel for 30 min at 120 V. After separation, PCR products were purified with the Universal DNA Purification Kit (TIANGEN), ligated into the cloning vector pLB (TIANGEN) and sequenced.

Yeast complementation assay

Specific primers were designed for PCR amplification and expression vector construction. The PCR procedure was the same as described above except that annealing was performed at 70 $^{\circ}$ C for 20 s. PCR products were inserted into the *Bam*H1 site of the yeast expression vector pDR195 (PLASMID, China). The constructs were sequenced and subsequently transformed into yeast competent cells prepared according to Gietz and Schiestl [52]. The following three yeast strains were used in this experiment: DY1455 (*MATa ade6 can1 his3 leu2 trp1 ura3*), *fet3fet4* DEY1453 (*MATa/MATa ade2/+ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2*), and *zrt1zrt2* ZHY3 (*MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3*) (provided by Rumei Chen, Agricultural Biotechnology Institute, Chinese Academy of Agricultural Sciences (CAAS)). *pDR195-TaZIP* constructs were transformed into DEY1453 and ZHY3 using the lithium acetate conversion method described by Gietz and Schiestl [52]. To ensure the correctness of the experiment, three rice genes, *OsIRT1*, *OsZIP5* and *OsZIP7*, were transformed into yeast competent cells as positive controls: . The wild-type strain DY1455

harboring pDR195 was used as another positive control. The empty vector pDR195 was used as a negative control. Transformed cells were spread on selective SD-URA solid medium without corresponding amino acids. For verifying the functions of the genes, we diluted the yeast liquid cultures to OD₆₀₀ values of 1, 0.1, 0.01, and 0.001, and dropped 10 µL of the culture onto different media. The yeast strain *zrt1zrt2* ZHY3 was grown on SD/–ura medium (pH 4.4) supplemented with 0.4 mM EDTA and 250 µM or 300 µM ZnSO₄. The yeast strain *fet3fet4* was grown on SD/–ura medium (pH 5.5-5.8) containing 50 mM 2-(N-morpholino) ethanesulfonic acid supplemented with 0, 50 or 100 µM FeCl₃.

Abbreviations

ZIP: Zn-regulated, iron-regulated transporter-like protein

CDF: Cation-Diffusion Facilitator

HMA (Heavy Metal ATPase)

TM: Transmembrane

ORF: Opening reading frame

Declarations

Ethics approval and consent to participate

The plant materials used here were grown in the greenhouse of NWAUFU for research use only. Wheat is not listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora Appendices I, II and III (valid from 4 April 2017, <https://cites.org/eng/app/appendices.php>). Collection of plant materials complied with the institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WJZ conceived and designed the project; SL contributed to RT-PCR, bioinformatics analysis gene cloned real-time RT-PCR and yeast complementation and writing of the manuscript. ZHL analysed the RT-PCR data and made the Gene expression heat map. HJL and LLG collected the plant materials and extracted the RNA. XJN and SCC contributed to revisions of the manuscript. All authors have read and approved the manuscript.

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Additional Files

Additional file 1: The detailed information of TaZIP genes. (docx 196kb)

Additional file 2: The detailed information of OsZIP proteins. (docx 15kb)

Additional file 3: The detailed information of Primer for fluorescent quantitation. (xlsx 30kb)

Additional file 4: The detailed information of Primer for gene cloning, vector construction. (xlsx 11kb)

Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Figures

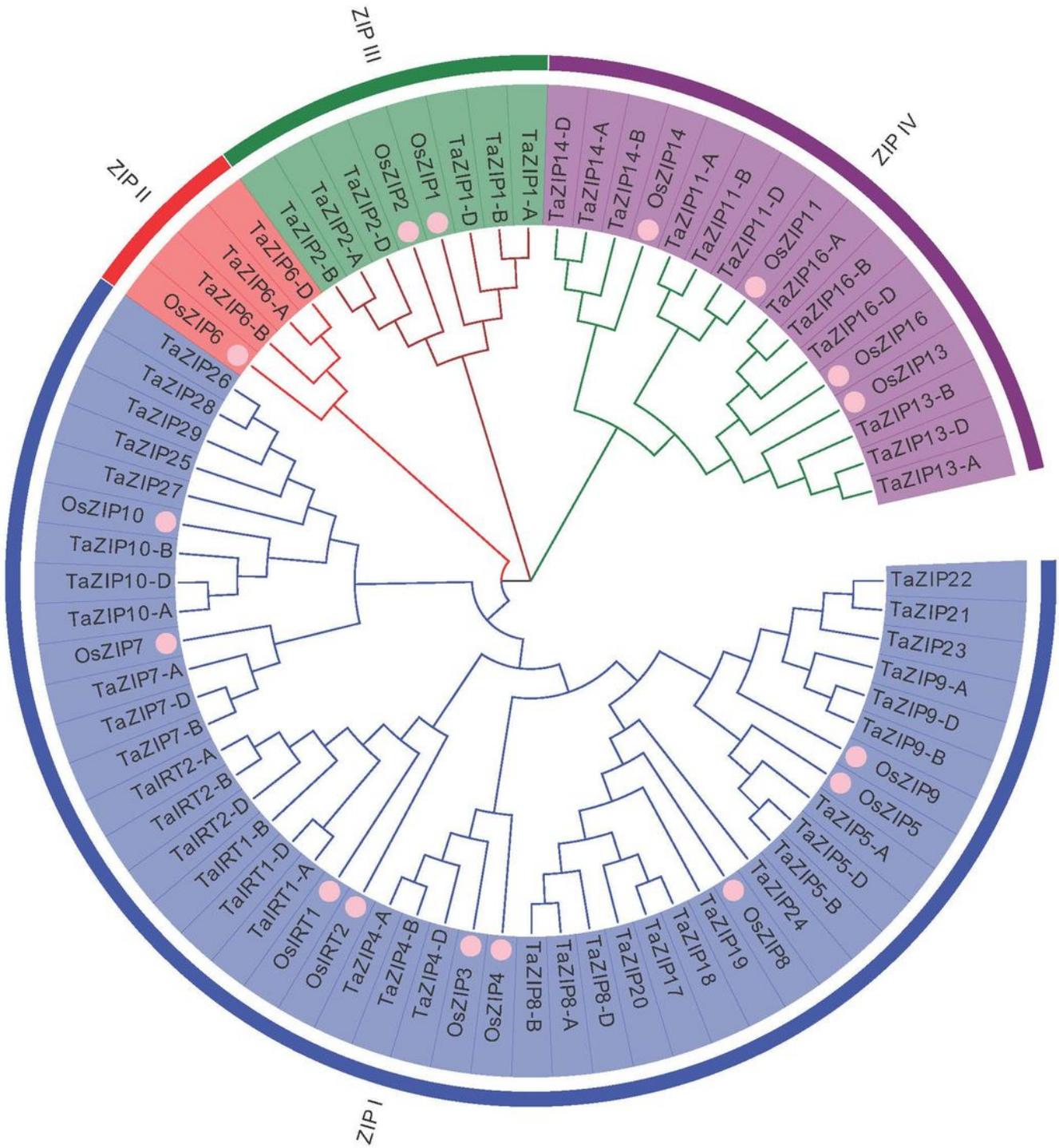


Figure 1

Phylogenetic tree of ZIP proteins constructed using the neighbor-joining method. The four different groups are indicated by different colors. OsZIP proteins are indicated by pink circles. (PDF 34kb)

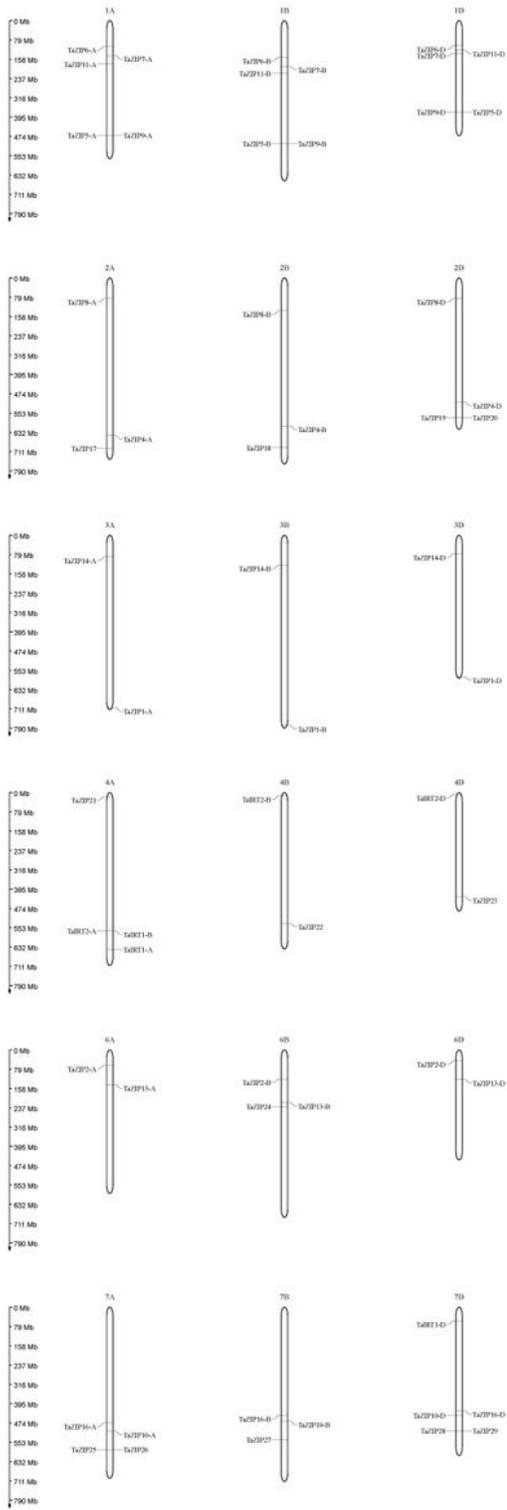


Figure 2

Chromosome locations of wheat TaZIP genes. (PDF 219kb)

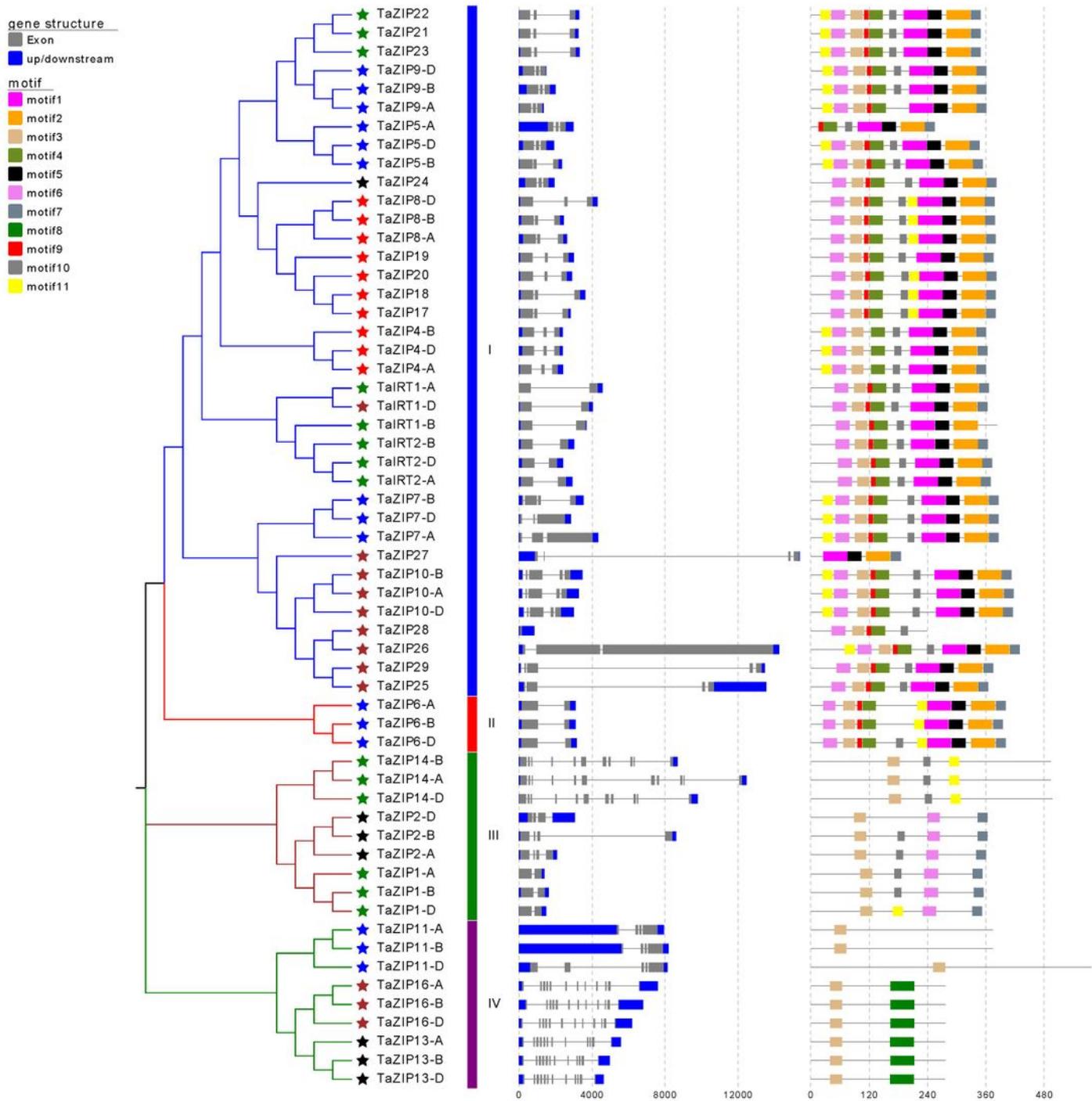


Figure 3

TaZIP gene structures and motifs in TaZIP proteins. Gene structures are shown to the right of the phylogenetic tree. Blue boxes represent TaZIP UTRs, gray boxes represent exons, and gray lines represent introns. Protein motifs are shown to the right of the gene structures. The boxes in different colors represent different motifs, and the gray lines represent non-conserved sequences. (PDF 28kb)

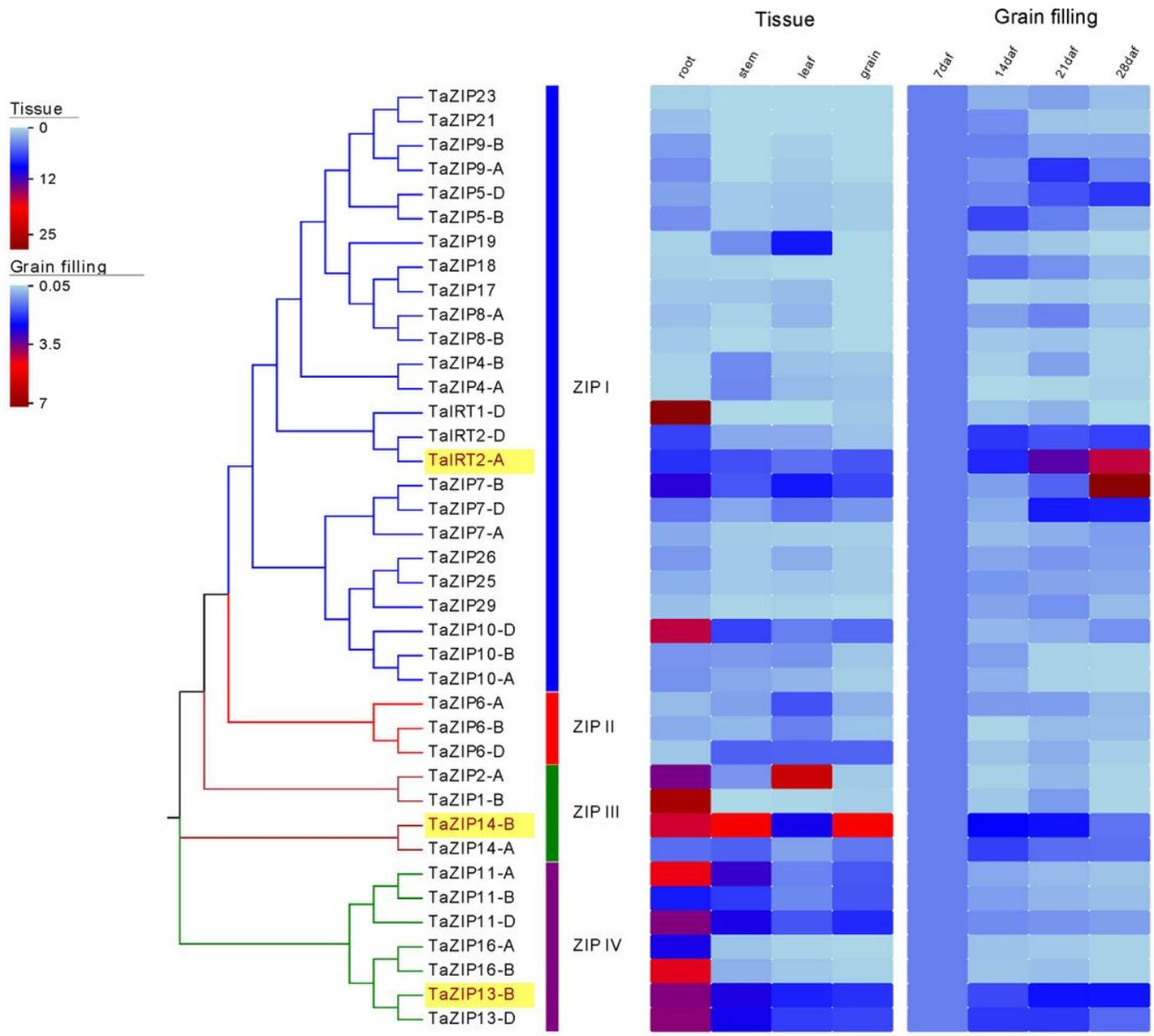


Figure 4

Heat map of the expression profiles of wheat TaZIP genes in different tissues and at different grain filling stages. RNA-seq data were obtained from roots, leaves, stems, and grains of the Chinese Spring cultivar. The expression levels at different grain filling stages were obtained by real-time fluorescence quantification PCR. (PDF 208kb)

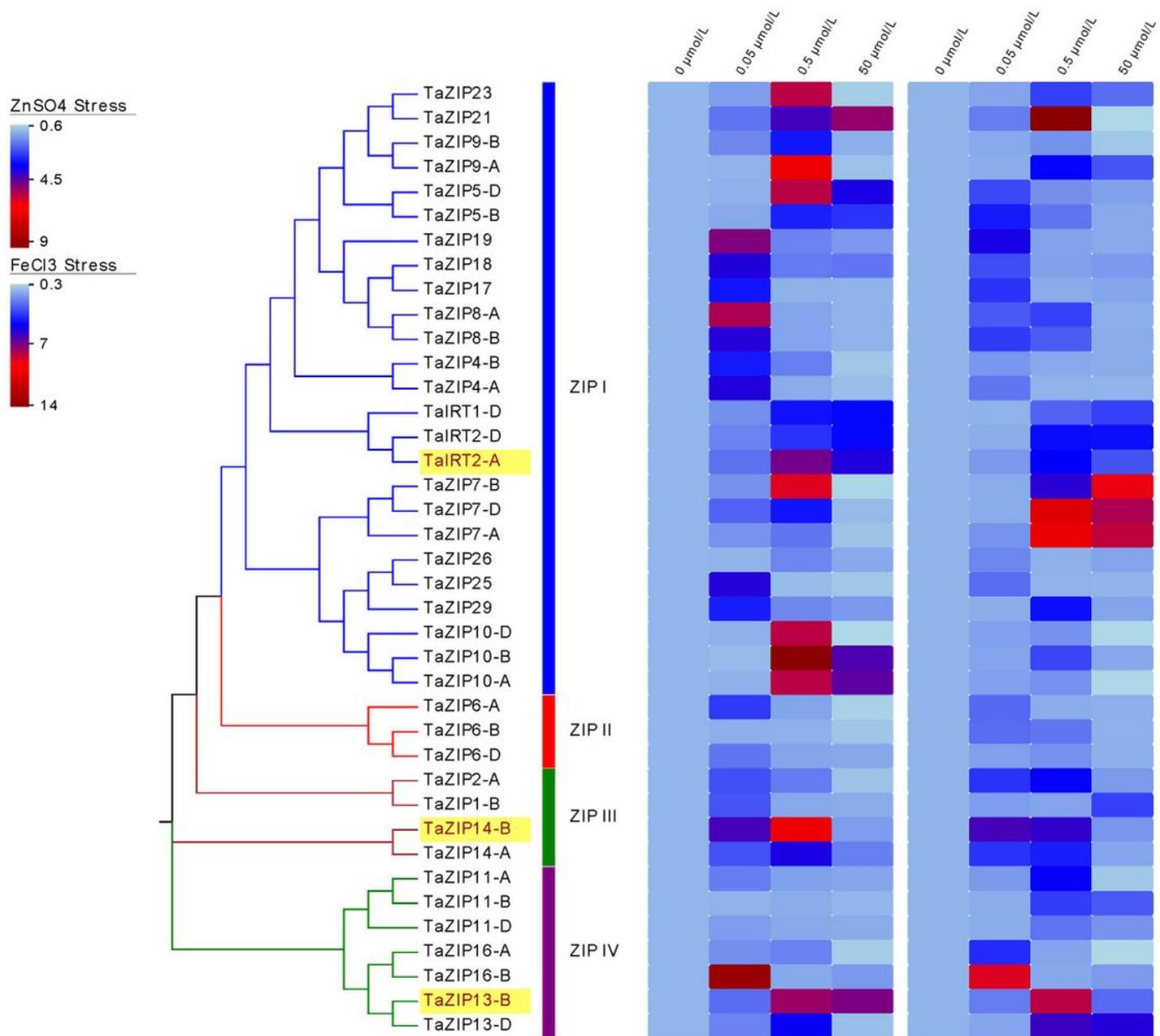
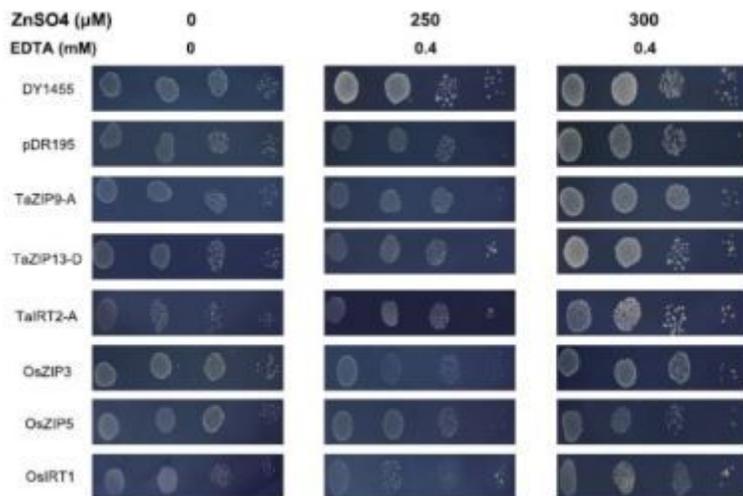


Figure 5

Heat map of the expression profiles of wheat TaZIP genes treated with solutions containing different concentrations of Zn²⁺ or Fe³⁺. (PDF 23kb)

a.



b.

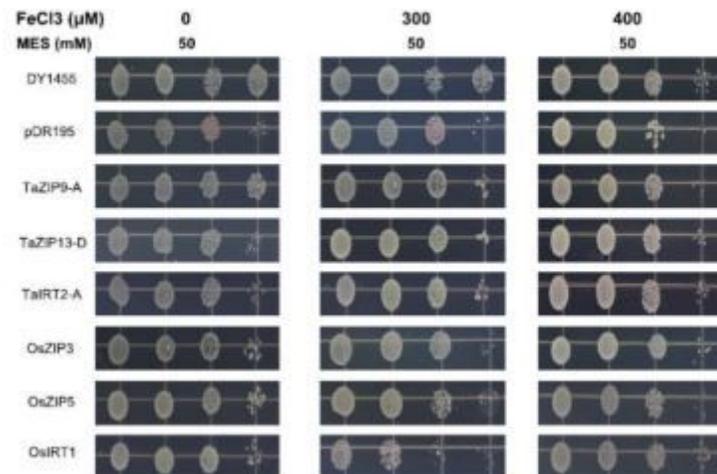


Figure 6

Functional complementation of yeast Zn and Fe transport mutants by TaZIPs under different pH conditions. The Zn transport mutant *zrt1zrt2* (a) (PH 4.4) and the Fe transport mutant *fet3fet4* (b) (PH 5.5-5.8) were transformed with the expression vector pDR195 carrying TaIRT2-D, TaZIP9-A, or TaZIP13-D or a functionally characterized ZIP gene, OsZIP5, OsZIP8 or OslRT1. The wild-type strain DY1455 transformed with pDR195 was used as a positive control, and the yeast *zrt1zrt2* or *fet3fet4* mutant transformed with the empty vector pDR195 was used as a negative control. (PDF a: 7624kb; PDF b: 7740kb)

Supplementary Files

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