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BRIEF COMMUNICATION

Genome-wide Analysis of Zinc Transporter Genes of Maize (*Zea mays*)

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Abstract Zinc (Zn) is an essential micronutrient for plants and animals. Zinc-regulated transporters and iron-regulated transporter-like proteins (ZIP) are important zinc transporters in plants with the characteristic ZIP domain (Pfam:PF02535). Although individual genes belonging to the ZIP family had been discovered in various plants, genome-wide analysis of the paralouges (*ZmZIP*) in maize and their relationships with other related genera has so far not been conducted. We performed a genome-wide analysis and identified 12 members of the *ZIP* gene family in maize. Chromosomal locations, motif organization, and biochemical characterizations of proteins, as well as exon–intron, trans-membrane domains of these *ZmZIP* genes were determined, which indicated the structural diversity of *ZmZIP*. Additionally, apart from the identification of the canonical form of the metal binding signature in ZIP domains of the ZmZIP proteins, we also identified a new conserved plant ZIP signature. Further, tissue-specific expressions of those genes were determined by real-time PCR in the flag leaf as well as in 10-day-old-baby kernel among the high and low kernel zinc-containing maize inbreds. We found that overall transcript abundance was higher in the flag leaf than the kernel in both the inbreds for all the members except two, namely *ZmZIP5* and *ZmZIP11* were expressed more in flag leaf of a high-kernel zinc-containing inbreds than a low-kernel zinc-containing inbreds. Therefore, these results provide a basis for further functional characterization of specific *ZmZIP* genes in the future.

Keywords Abiotic stress · Biofortification · Zinc transporter · *Zea mays* · Zinc use efficiency

Introduction

Zinc (Zn), as an essential micronutrient, is a component of more than 300 enzymes involved in plant biological processes. It plays important roles in gene expression and cellular development. However, a pre-requisite to developing zinc biofortified foods is the understanding of the molecular mechanism of zinc uptake by root, transport through stem, and loading to the specified organs such as seed and fruit.

Maize is an important cereal in the world with high production and productivity. Maize is grown in more than 166 countries for various uses, and in diverse seasons and agro-ecological conditions due to its high yield potential. In India, while 25 % of the maize produce is consumed as food, the remaining 75 % is used as livestock and poultry feed. Thus, micronutrient-deficient maize not only affects human beings but also livestock and poultry. Therefore, biofortified maize will be immensely helpful for the eradication of malnutrition as it is sustainable and affordable for the poorest of the poor. It is well documented that several genes control the Zn homeostasis in strategy II model plant rice (Ramesh et al. 2003; Ishimaru et al. 2006, 2007; Lee et al. 2010a, 2010b; Bashir et al. 2012). However, genes responsive to zinc uptake as well as loading in the kernel of maize have not so far been studied, which is a prerequisite for understanding the molecular mechanism of Zn uptake. Members of the ZRT-IRT-like protein (ZIP) family were the first metal transporters to be identified in plants (Eide et al. 1996). Along with other factors, the expressions of ZIP genes are regulated by the tissue metal status of

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the plant (Grotz et al. 1998). Genes with the ZIP domain have also been identified and characterized from other plant species including soybean, *Medicago truncatula*, *Noccaea caerulescens* and *Thlaspi japonica* (Pence et al. 2000; Assunção et al. 2001; Burleigh et al. 2003; Mizuno et al. 2005; Moreau et al. 2002; Plaza et al. 2007). For instance, *NcZNT1* from *Noccaea caerulescens* was shown to mediate high-affinity Zn uptake and low-affinity Cd uptake in yeast (Pence et al. 2000). Homologous genes *TjZNT1* and *TjZNT2* isolated from the Ni hyper-accumulator *Thlaspi japonica* were shown to enhance the transport of Zn, Cd, and Mn (Mizuno et al. 2005).

The complete genome sequences derived from large-scale sequencing projects are important for comparative and functional genomics research, providing the opportunity to scan various gene families. Like any other sequencing project, the complete maize genome sequence also provides a valuable resource for comparative analysis of the gene families. With decoding of the maize genome sequence, several gene families have already been characterized in maize (Liu et al. 2013a, b; Zhang et al. 2013). Although individual zinc-regulated transporters (ZRT) and iron-regulated transporter-like (IRT) protein (ZIP) genes have been extensively studied in yeast (Wu et al. 2011), as well as in higher plants such as rice (Lee et al. 2010b; Lan et al. 2013), genome-wide analysis of the members of this family has yet to be studied in maize.

Therefore, the objectives of present studies were, (1) identification of non-redundant members of maize ZIP family genes, (2) characterization of their biochemical properties, genomic organization, motif analysis, and phylogenetic relationship, and (3) expression analysis of these genes in different genotypes of maize. The results of this work provide a foundation to better understand functional and evolutionary history of the ZIP gene family in angiosperms. In the present study, we identified 12 putative members of maize ZIP genes (*ZmZIPs*) on the basis of ZIP domain and validated their expression among the two inbred lines which differ in Zn use efficiency. This is the first attempt to describe the ZIP gene family along with their expression in maize.

Materials and Methods

Identification, Characterization and Mapping of *ZmZIP* Genes

The complete genome sequence of maize was downloaded from the public database (www.maizesequence.org) and used in this study. *AtZIP1* was the first zinc transporter with ZIP domain (Pfam:PF02535), characterized from *Arabidopsis* (Grotz et al. 1998). Thus, to identify the *ZmZIP* family genes, the *AtZIP1* gene (*AT3G12750*) was used as query against the maize genome sequence using basic local alignment search tool (blastn). The blast search identified the *GRMZM2G045849* gene of maize with the same ZIP domain (Pfam:PF02535). Subsequently, the Hidden Markov Model (HMM) profile of the ZIP domain from the Pfam database (pfam.janelia.org) was then used to search for maize ZIP genes using the blastp program (*E*-value=0.001). The Pfam database was used to ensure that each predicted *ZmZIP* gene encoded the ZIP domain. All confirmed *ZmZIP* were aligned using Clustal W (Thompson et al. 1994) in MEGA 5.05 software (Tamura et al. 2011) to exclude overlapping *ZmZIP* genes. Various biochemical parameters such as length of the protein sequence, isoelectric point (pI), post-translational modification, signal peptide, transmembrane domain (TM), and grand average of hydropathicity (GRAVY) values (Kyte and Doolittle 1982) of the 12 numbers of *ZmZIP* genes were determined using various proteomics tools of ExPySy server (www.expasy.org). Cellular/subcellular targeting sites, were assessed using WoLF PSORT (www.wolfpsort.seq.cbrc.jp).

Each non-overlapping *ZmZIP* gene sequence was then used as a query against the whole maize genome sequence (maizesequence.org), by using the tblastn program and physically positioned on different maize chromosomes. The names of *ZmZIP* genes were given according to their position from the top to the bottom on the maize chromosomes 1 to 10. Thus physical locations of all *ZmZIP* genes were generated (www.maizesequence.org) against a search of the ZIP domain (PF02535).

Synteny analysis between maize and sorghum was conducted locally using the similar method developed for the Plant Genome Duplication Database (Tang et al. 2008). First, blastp was conducted using all ZmZIP proteins to search for potential anchors ($E<1e^{-5}$, top 5 matches) in the sorghum genome. Afterwards, MCscan was employed to identify homologous regions. Finally, syntenic blocks were evaluated by ColinearScan. Alignments with an E value <1 e^{-10} were considered as significant matches.

Phylogenetic and Syntenic Analyses

We identified and retrieved non-redundant protein sequences of ZIP family members of four monocots such as *Brachypodium distachyon*, *Oryza sativa*, *Setaria italic*, *Sorghum bicolor* from the Phytozyme database (www.phytozome.net) that had a ZIP domain (Pfam:PF02535) (Supplementary Table 1). After that, conserved sequences of those proteins were aligned using the ClustalW program in BioEdit software (www.mbio.ncsu.edu/bioedit/bioedit) with default parameters (Hall 1999). Based on the conserved sequences alignment of the proteins, the rooted phylogenetic tree was constructed using MEGA 5.05 software (Tamura et al. 2011), by both the neighbor-joining method (Saitou and Nei 1987) and the minimum evolution method. The reliability of the phylogenetic tree was estimated using

bootstrap values with 1,000 replicates. Using these methods, evolutionary relationships of ZIP family members were established between maize and the four other monocots mentioned above. In contrast, the phylogenetic relationships of *ZmZIP* genes were established on the basis of the ZIP domain only, keeping the rest of the parameters unchanged.

Determination of Exon-intron and *Cis*-elements in the Promoter

To determine the exon–intron organization, genomic and coding sequences (predicted, cDNA when available) of *ZmZIPs* were aligned. To identify TM (trans-membrane) domains of ZmZIP proteins, we used Conpred II (http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/), a consensus prediction method for obtaining transmembrane topology models. To identify the *cis*-elements, promoter sequences from +1 to −1,000 bp of each of the *ZmZIPs* were extracted from www.maizesequence.org and analyzed for stress-responsive cis-elements in the PLACE database (Higo et al. 1999)

Motif Analysis

To further analyze the structure of the ZIP domains, we identified their protein sequence through SMART (www.smart.embl-heidelberg.de) and aligned them using CLUSTALW software. Conserved motifs were identified from the 12 *ZmZIPs* using the MEME 4.6.1/MAST motif search software (Bailey and Elkan 1994; Bailey and Gribskov, 1998) (www.meme.sdsc.edu/meme/cgi-bin/meme.cgi) with the following parameters: (1) distribution of motif occurrences with any number of repetitions, (2) 6 and 60 amino acids as minimum and maximum width of motifs, (3) only motifs with expected value lower than 1×10^{-20}, and (4) a maximum 10 number of motifs per peptide sequence. The functional annotations of these motifs were analyzed by InterProScan (www.ebi.ac.uk/Tools/pfa/iprscan), SMART and the MOTIF search database (genome.jp/tools/motif). Sequence logos of conserved motifs were also generated with WebLogo (Crooks et al. 2004).

In silico Expression Patterns of the *ZmZIPs*

To identify the expression patterns, sequence tags of all *ZmZIPs* were investigated at the available transcriptional level in public domain. Maize ESTs were obtained through blastn searches against the database (www.maizesequence.org). The *ZmZIP* genes were analyzed by using the tblastn program with the following parameters: (1) maximum identity of 95 % and (2) minimum length of 400 bp with *E* value$>10^{-10}$. In addition to the maize EST database, maize expression data of *ZmZIPs* were also extracted from the Maize Assembled Genomic Island (MAGI) (www.magi.plantgenomics.iastate.edu), the Plant Genomic Database (Plant GDB) (www.plantgdb.org) including EST, cDNA and PUTs (Plant GDB unique transcripts) and from the MPSS database (www.mpss.udel.edu/maize).

Expression Analysis of *ZmZIPs* by qPCR

We analyzed the expression levels of *ZmZIP* transcripts by real-time PCR in VQL-2 and CM-145 which were the contrasting high and low kernel zinc-containing maize inbreds, respectively, the former one being an isogenic line of the latter (Chakraborti et al. 2009; Prasanna et al. 2008). The tissue from flag leaf at the age of 10-day-old kernel and 10-day-old baby kernel were both sampled in liquid nitrogen from the plant of those two inbreds, namely VQL-2 and CM-145. Total RNA was extracted from 100 mg of tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The yield and quality of DNAase (Promega Life Sciences)-treated RNA were determined by Nanodrop 1000 (M/S; Thermo Scientific, USA) and 2 % agarose gel electrophoresis in MOPS [3-(N-morpholino) propanesulfonic acid] buffer, respectively. The cDNA was synthesized using 1 μg of RNA with 200 U l^{-1} reverse transcriptase Superscript TM III (Invitrogen), 10 mM dNTPs and 250 ng oligo (dT). The resulting cDNA samples were diluted 20 times (1:20) in RNase-free water, and 2 μl of the diluted cDNA was used in a total reaction volume of 25 μl for determining the relative expression of *ZmZIPs* using QuantiFast SYBR Green PCR Master Mix (Qiagen, India). The primers used to amplify *ZmZIPs* are listed in Supplementary Table 2. Real-time PCR analysis was performed in a 96-well plate using Roche 454 qPCR system (Roche, USA). The thermal cycling conditions of 95 °C for 5 min followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s were used. The expression of each *ZmZIP* gene in various samples was normalized with actin 1 as reference gene (GRMZM2G126010) as an internal control (Zhao et al. 2011). The experiment was performed with at least three independent biological replicates and two technical replicates for each biological replicate. The specificity of the PCR reactions was confirmed by melting curve analysis of the amplicons. The comparative $2^{-\Delta ct}$ [Δ C_T=C_T, gene of interest - C_T, actin 1] method was used to calculate the relative quantities of each transcript in the samples (Schmittgen and Livak 2008). Statistical analyses were conducted using the SAS software of JMP Genomics (SAS Institute, NC, USA).

Results and Discussion

Zinc is an essential micronutrient for plant metabolism and growth. The deficiency of Zn decreases plant growth and affects cereal production and grain quality (Ishimaru et al. 2011), but excess Zn may cause significant toxicity to

biological systems (Ishimaru et al. 2007). Therefore, plants have established a tightly controlled system to balance the uptake, utilization, and storage of these metal ions. The ZRT and IRT-like protein (ZIP) family has been characterized ubiquitously in organisms, including archaea, bacteria, fungi, plants, and mammals, and has been demonstrated to be involved in metal uptake and transport (Ishimaru et al. 2011). ZIP proteins generally contribute to metal ion homeostasis by transporting cations into the cytoplasm (Nozoye et al. 2013). Functional complementation in yeast indicated that ZIP proteins are able to transport various divalent cations, including Fe^{2+}, Zn^{2+}, Mn^{2+}, and Cd^{2+} (Guerinot, 2000). Although ZIP genes have been characterized from several plants, their information on maize is as yet very scanty. With the completion of the maize genome, several gene families have been characterized (Liu et al. 2013a, b; Zhang et al. 2013). Therefore, in the present study, we have identified, through genome-wide in silico analysis, and characterized the *ZmZIP* family genes of maize.

Identification, Characterization and Mapping of *ZmZIPs*

As key Zn transporter, the *ZmZIPs* family plays an important role in Zn homeostasis in maize which affects plant growth and development. We used *Arabidopsis AtZIP1* (AT3G12750) gene for blastn search against the maize genome (maizesequence.org) for the identification of ZIP genes. We identified a maize gene *GRMZM2G045849* which had 54 % similarity with *AT3G12750* with a characteristic ZIP domain (Pfam:PF02535). This ZIP domain is responsible for the transport of Zn metal ions in plants (Grotz et al. 1998). A total of 12 non-redundant putative ZIP family genes were finally identified by a genome-wide survey of maize that significantly had ZIP domains (Table 1). In contrast, Sharma and Chauhan (2008) identified 13 *ZmZIPs*. Although the basis of the identification of *ZmZIPs* was not mentioned in their report, it clearly differed from ours. This may be due to the fact that, at that time, the maize genome had not been fully annotated which led them to identify one redundant sequence as we noticed in that analysis. Such an observation about redundancy of the gene has also been reported elsewhere in identifying the members of the gene family (Jami et al. 2011). Additionally, we also found two proteins, namely GRMZM2G379348 and GRMZM2G045531, in the Phytozome database that were initially identified by a cross-check with the key word 'zinc transporter' in the search; however, these two proteins were finally excluded as either they did not have a ZIP domain or were found to be a truncated protein sequence. Thus, deduced polypeptides of the corresponding 12 *ZmZIP* genes were analyzed for the number of amino acids (length), molecular weight, and isoelectric point (pI). The amino acids number varied from a minimum of 279 (*ZmZIP6*) to maximum of 573 (*ZmZIP12*). Similar amino acid lengths for ZIP genes have also been reported earlier in rice (Chen et al. 2008). The molecular weight of the ZmZIP proteins varied from a minimum of 29.6 kDa (ZmZIP6) to 59.5 kDa (ZmZIP12). Only two proteins, namely ZmZIP1 and ZmZIP12, were found to be large, with a molecular weight of 51.8 and 59.5 KDa, respectively (Table 1). Lengths of the *ZmZIP* genes were found to be minimum of 1,340 bp (*ZmZIP3*) to maximum of 2,430 bp (*ZmZIP12*). It has also been observed that all the genes begin with an initiation codon and end with a stop codon, indicating that they were functional in nature. In addition, the pI value is also considered to be an important biochemical property for *ZmZIPs* because these genes having different acidic or basic features that might respond differentially to various environmental factors (Allagulova et al. 2003). Theoretical pI values

Table 1 Properties of *ZmZIP* genes and their proteins

Gene name	Sequence ID	Chr	Genomic sequence (bp)	ORF length (bp)	Deduced peptides			
					Length (aa)	MW (kDa)	PI	GRAVY
ZmZIP1	GRMZM2G001803	1	6,610	1,899	483	51.8	6.01	-0.207
ZmZIP2	GRMZM2G118821	1	2,205	1,504	381	40.7	9.28	0.425
ZmZIP3	GRMZM2G115190	1	1,419	1,340	361	37.6	8.65	0.600
ZmZIP4	GRMZM2G045849	2	2,490	1,490	367	38.5	7.87	0.553
ZmZIP5	GRMZM2G111300	4	2,687	1,668	386	38.6	7.22	0.642
ZmZIP6	GRMZM2G050484	4	5,598	1,701	279	29.6	6.65	0.719
ZmZIP7	GRMZM2G064382	6	3,170	1,714	402	41.8	6.02	0.436
ZmZIP8	GRMZM2G015955	6	2,579	1,749	387	40.4	6.97	0.528
ZmZIP9	GRMZM2G047762	6	2,240	1,525	341	34.7	5.30	0.707
ZmZIP10	GRMZM2G093276	7	2,010	1,430	397	40.6	6.33	0.551
ZmZIP11	GRMZM2G034551	8	2,898	1,634	396	41.5	6.15	0.449
ZmZIP12	GRMZM5G813470	10	4,270	2,430	573	59.5	8.44	0.793

of *ZmZIPs* varied from a minimum of 5.30 (*ZmZIP9*) to a maximum of 9.28 (*ZmZIP2*) (Table 1). Further analysis of the amino acid compositions of all ZmZIP proteins indicated that they shared the common features; just one exceptional example was *ZmZIP1* which had comparatively low alanin (9 %) and a negative GRAVY (−0.207) value. All the other *ZmZIPs* were found to have a positive GRAVY value (0.425–0.793) (Table 1) indicating the presence of the very hydrophobic nature of peptides which is a common feature for membrane-spanning proteins (Grotz et al. 1998). It has also been found that, except for three genes, namely, *ZmZIP6*, *ZmZIP7*, and *ZmZIP8*, all the other genes had signal peptides indicating that they might be involved for the movement from one organelle to other within the cytoplasm. Based on PROSITE analysis, most of the ZmZIP proteins were predicted to be located in the plasma membrane, except for *ZmZIP4*, *ZmZIP9*, and *ZmZIP7*, *ZmZIP5* proteins which were predicted to be located in cytoplasm and chloroplast, respectively. Similar results were also obtained with rice *OsZIP* genes, where *OsZIP4*, *OsZIP5*, and *OsZIP8* were located in the plasma membrane while others are located in cytoplasm (Chen et al. 2008; Ishimaru et al. 2006, 2007).

The deduced protein sequences when scanned through PROSITE (www.expasy.org/tools/ scanprosite) showed the presence of sites for various post-translational modifications, and other sequence-specific features (Table 2). There were multiple putative phosphorylation sites in these protein sequences, which might have acted as substrates for several kinases in the form of casein kinase II, protein kinase C, tyrosine kinase, and cAMP- and cGMP kinases (Table 2) for these proteins, and this is also well-documented in the literature for the ZIP genes (Aydemir et al. 2012). Further, it had been found that N-Myristoylation was one of the major post transcriptional modifications, the value of which varied from a maximum of 23 to a minimum 7 with no myristoylation with the ZmZIP11 protein. Myristoylation can influence the conformational stability of individual proteins, as well as their ability to interact with membranes or the hydrophobic domains of other proteins. Thus, it plays a critical role in many cellular pathways, especially in the areas of signal transduction, apoptosis, and extracellular export of proteins and membrane transport (Zaun et al. 2012). Therefore, we concluded that it might play an important role in the transport of zinc in maize. Locations of the members in the chromosome are very important and depend on the gene duplications, linkages, and recombinations. To provide a simplified nomenclature for each identified gene, names were given from *ZmZIP1* to *ZmZIP12* to distinguish each *ZmZIPs* (and corresponding proteins) that were denominated as 'ZmZIP', and the followed by a number to represent the gene number according to their locations in the chromosomes 1 to 10 in descending order. Based on available information, standard *ZmZIP* genes were positioned on maize chromosomes (Fig. 1). Although the 3rd, 5th and 9th chromosomes did not have any *ZmZIPs*, yet the maximum of three genes (*ZmZIP1*, *ZmZIP2* and *ZmZIP3*) were each located on chromosome 1 and three (*ZmZIP7*, *ZmZIP8* and *ZmZIP9*) on chromosome 6. Two genes, namely *ZmZIP5* and *ZmZIP6*, were located on chromosome 4. On the other hand, *ZmZIP10* and *ZmZIP12* were located on chromosomes 8 and 10, respectively.

Table 2 Number of predicted post-translational modification sites of *ZmZIP* genes

Gene name	cAMP	Casein	N-Myr	PKC	N-gly	Ami	Tyk
ZmZIP1	3	9	7	6	2	–	–
ZmZIP2	1	1	13	5	2	1	–
ZmZIP3	–	3	10	3	–	–	–
ZmZIP4	–	6	10	5	2	–	–
ZmZIP5	–	2	15	3	–	–	–
ZmZIP6	–	4	13	4	–	–	–
ZmZIP7	1	2	12	3	2	1	–
ZmZIP8	–	7	8	3	1	1	–
ZmZIP9	–	3	12	3	–	1	–
ZmZIP10	–	1	17	1	1	1	–
ZmZIP11	–	2	–	3	11	–	–
ZmZIP12	2	3	23	5	–	–	–

cAMP cAMP - and cGMP-dependent protein kinase C phosphorylation site; *caesin* casein kinase II; *N-Myr* N-myristoylation site; *PKC* protein kinase C phosphorylation site; *N-gly* N-glycosylation site; *Tyk* tyrosine kinase phosphorylation site; *Amid* amidation site

Phylogenetic and Synteny Analysis of *ZmZIPs*

In order to analyze the evolutionary relationship of the ZIP family, a phylogenic tree was constructed. The rooted tree topologies of *ZmZIPs* generated by the two methods were comparable without modifications at branches, and supported by their high bootstrap values, suggesting that we had constructed a reliable rooted tree topology, in which the 12 *ZmZIPs* were grouped into three distinct classes (Supplementary Fig. 1) that were generated by their evolutionary divergence, mostly corresponding to the subgroups identified by motif analysis. On the other hand, the global phylogenetic rooted tree comprised of 5 different monocot species indicated that all the plants had larger numbers of ZIP genes than maize. For instance, *Setaria italic*, *Brachypodium distachyon*, *Sorghum bicolor*, and *Oryza sativa* had 16, 16, 17, and 17, respectively (Fig. 2). Syntenic analysis indicated that all *ZmZIPs* were found to have orthologous sequences in other monocots analyzed and possessed similar ZIP domain. Comparative multiple alignment of amino acid sequences of these monocot ZIP genes common to each orthologous group shared 63 % identity (data not shown). Considering that

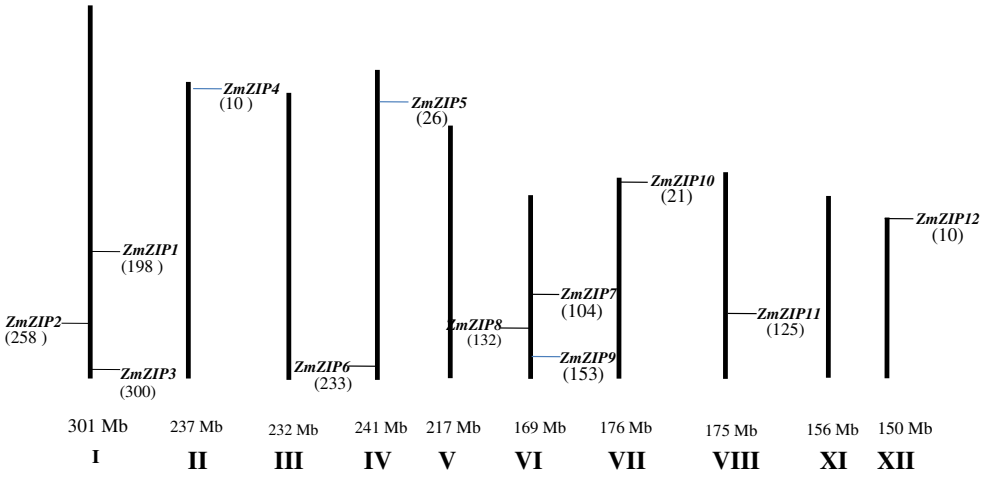

Fig. 1 Locations of the different *ZmZIP* genes in the maize genome. *Roman numerals* indicates different chromosomes of maize and *numbers* in *parentheses* indicate the position of the gene in Mb

orthologs often retained equivalent functions in the course of evolution (Altenhoff and Dessimoz 2009), we examined the orthologous relationships between *ZmZIPs* and *SbZIPs* of sorghum genes using a local synteny-based method. A total of 12 genes from *ZmZIPs* had one or more putative orthologs in sorghum. All of them were classified into the same group as their orthologs in sorghum, further supporting the results of the phylogenetic analysis. Although the reason is not clear, certainly gene duplication might have played an important role in a succession of genomic rearrangements and expansions of this gene family among the different species (Zaun et al. 2012) as indicated by the synteny analysis. It is a

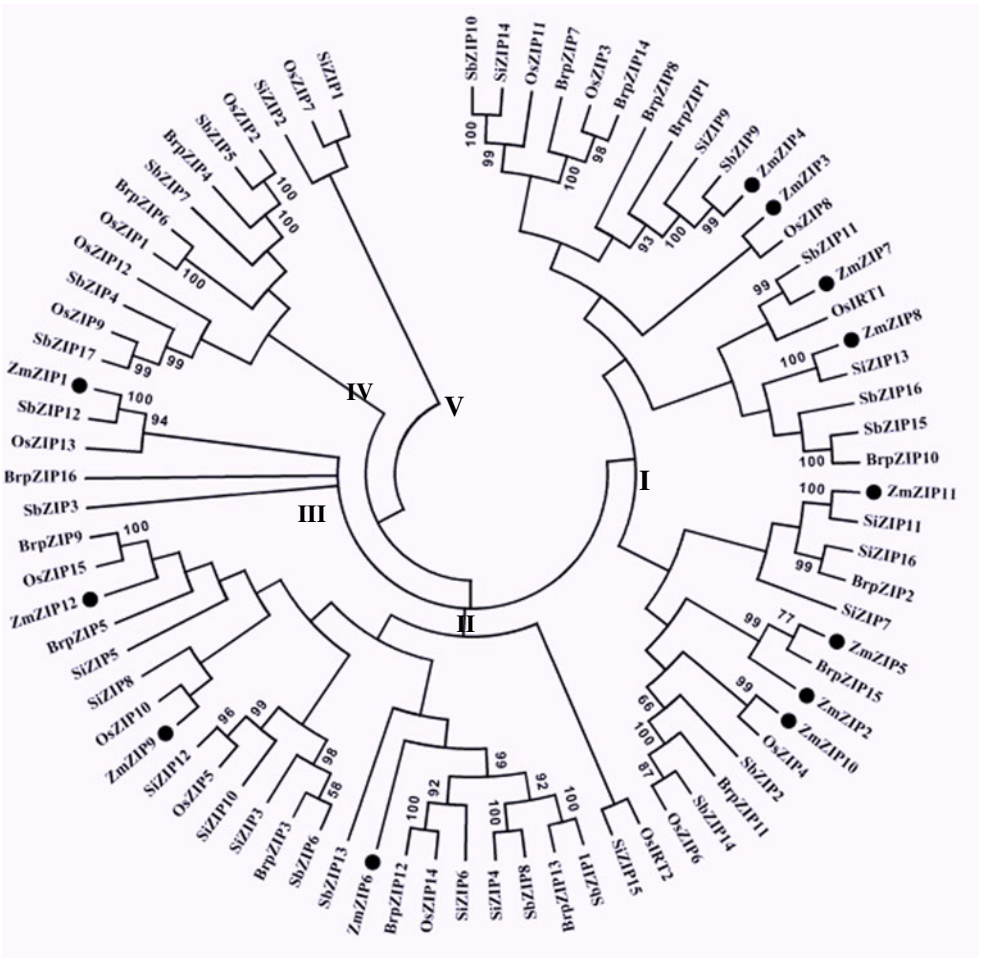

Fig. 2 A rooted, neighbor-joining (NJ)-based tree of the ZIP proteins in selected monocots. The analysis was performed as described in "Materials and Methods"

common phenomenon that has been reported in several studies (Vision et al. 2000; Lynch and Conery 2000; Simillion et al. 2002; Raes et al. 2003). Further, we also compared the chromosomes between maize and sorghum which revealed interesting syntenic relationships among the ZIP genes of both species. Genes of chromosome 1 from maize showed colinearity with sorghum chromosomes 1 and 7, whereas chromosome 6 revealed colinearity with sorghum chromosomes 1, 6, 9, and 10 (Supplementary Fig. 2). Additionally, it is known that gene family expansion occurs through three mechanisms. They are tandem duplication, segmental duplication, and transposition events (Maher et al. 2006). In this study, we found several segmental duplication events occurred among the *ZmZIPs* rather than the other two methods. For an example, *ZmZIP2* and *ZmZIP10* had segmental duplication. Similarly, *ZmZIP8* as well as *ZmZIP5* and *ZmZIP1* and *ZmZIP3* had segmental duplication. It was found that the coding sequences of all the *ZmZIPs* genes were disrupted by introns. Therefore, we concluded that intron loss might accompany the recent evolution of maize *ZmZIP* genes which is also observed in the case of the aldehyde dehydrogenase gene in maize (Zhou et al. 2012). Although gene-order conservation is widely used as the benchmark for orthology prediction (van der Heijden et al. 2007), all 12 *ZmZIPs* genes (100 %) were revealed by the synteny analysis. This findings were also supported by phylogenetic analysis to provide support to interpret putative orthologous or paralogous genes. Although the bootstrapping values for some nodes were not exceptionally high, the reliability of our phylogenetic trees was supported by gene structure and synteny analyses. Similar results have been reported in rice (Nakano et al. 2006), and grape (Zhuang et al. 2009). Genome analysis indicated that whole genome duplication in the ancestral grass genome occurred around 70 million years ago, predating the divergence into panicoid, oryzoid and pooid sub-families (Vision et al. 2000). Analysis of ZIP gene sequences from maize, rice, sorghum and brachypodium in the plaza database (www.bioinformatics.psb.ugent.be/plaza) indicated that these genes might have undergone segmental duplications. The genes in the segmentally duplicated regions were found to be congruent with the orthologous sequences in the phylogeny tree and had high sequence similarity, suggesting that these genes were evolutionarily conserved and may have functional redundancy.

The global phylogenetic tree was divided into five different groups which mostly corroborated the presence of the conserved motif. Although we found 10 conserved motifs as generated by MEME, which is one of the most widely used tools for observation of new sequence patterns in biological sequences and analysis of their significance, yet among them only the 1st, 2nd, 3rd, 4th and 9th motifs were found to have ZIP domains (Supplementary Fig. 3) and the rest did not have any hit in the MOTIF search database (www.genome.jp). This indicated that the 1st, 2nd, 3rd, 4th and 9th motifs were directly involved in Zn metal binding and transport. We found that group I was composed of 35 ZIP genes, all of which contained 8 motifs, i.e. 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 10th motif. Similarly, group II contained 25 ZIP genes, each of which contained 5 motifs (3rd, 5th, 6th, 7th and 8th) and hence formed a different group. Group III consisted of small number of 5 genes with 7 different motifs (1st, 2nd, 4th, 5th, 6th, 7th and 10th). Simultaneously, group IV consisted of 10 ZIP genes with mostly 4 motifs, namely 3rd, 5th, 6th and 7th, except for two genes, *BrpZIP6* and *OsZIP1*, that had one additional 1st motif. Finally, group V was the smallest one with 3 genes (*SiZIP2*, *OsZIP7* and *SiZIP1*) and motif analysis revealed that they were composed of heterogeneous motifs. For example, while *SiZIP2* consisted of 4 motifs (3rd, 5th, 6th and 7th), the other two, *OsZIP7* and *SiZIP1*, had 7 different motifs (1st, 2nd, 3rd, 4th, 5th, 6th and 10th), which might be the reason that both of them were in same clade. Similar phylogenetic classification of the gene family based on the conserved motifs in plants are well-documented in the literature (Jami et al. 2011; Ricachenevsky et al. 2011).

Detection of Exon–intron and *Cis*-elements in the Promoters of *ZmZIPs*

Structural diversity among the members of a gene family depends on the number of exons and introns as well as their length. We also tried to understand the identity among the *ZmZIPs* and found that, in general, the *ZmZIPs* were highly diverse, varyimg from a minimum of 11 % identity between *ZmZIP7* and *ZmZIP1* to a maximum 72 % identity between *ZmZIP2* to *ZmZIP3* genes (Supplementary Table 3). Perhaps the varying length of peptides as indicated for a minimum of 279 to a maximum 573 amino acids (Table 1) might contribute to the structural diversity. These findings also corroborated the earlier finding of Chen et al. (2008) who reported the identify of *OsZIP* genes of rice varied widely, from 17 to 70 %, indicating the highly diverse nature of ZIP genes. The high structural diversity of ZIP genes might be involved in the transport of a variety of cations (Belouchi et al. 1997). Secondly, it has also been observed that the length as well as the number of introns may also contribute to the structural diversity. It has also been revealed that *ZmZIP6* had a maximum 12 introns, whereas *ZmZIP3* had a minimum 2 (Supplementary Fig. 4). Therefore, we concluded that the length as well as the number of introns and corresponding peptide length contributes to the structural diversity of *ZmZIPs* as well documented in the literature (Jain et al. 2006; Nakano et al. 2006; Terol et al. 2006).

The *cis*-elements are important molecular switches involved in the transcriptional regulation of genes during gene expression and may be induced through ABA-dependent and ABA-independent signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki (2005). Previous studies in

Arabidopsis showed the presence of *cis*-elements ZDRE (Zinc Deficiency Responsive Elements) that could respond to zinc deficiency stress (Assunção et al. 2010). Having seen the differential expression of *ZmZIPs*, we analyzed the putative promoter sequence of 1,000 bp from the translational start site to search for stress-responsive *cis*-elements in the PLACE database (Higo et al. 1999). In silico sequence analysis showed that the promoter of each gene contained at least one of the four related putative *cis*-elements, such as zinc deficiency-related elements (ZDRE) (GTCGAC), ABA responsive elements (ABRE) (ACGTG), dehydration responsive elements (DRE/CRT) (G/ACCGCC), and low temperature responsive element (LTRE) (CCGAC) motifs. The promoters of 6 genes, namely *ZmZIP2*, *ZmZIP5*, *ZmZIP7*, *ZmZIP8*, *ZMZIP9* and *ZmZIP11*, contained ZDRE *cis*-elements which are responsible for activation during zinc deficiency stress (Assunção et al. 2010) (Supplementary Table 4).

Analysis of Conserved Motif

Motifs are the most distinctive features of the proteins. A total of 10 motifs containing 6–53 amino acid residues were identified (Supplementary Fig. 1). While most of the *ZmZIPs* were found to have 6–8 TM domains, a typical characteristic feature of Zn transporter protein (Eide 1998), *ZmZIP12* had a maximum of 13, while *ZmZIP1* had 7 TM domains (Supplementary Fig. 5). It is known that Zn transporters can be basically classified into two categories: the cation diffusion facilitator (CDF) transporter family and the ZIP family. The CDF family has common structural characteristics with 6 TM domains containing histidine-rich motifs, which are predicted to be exposed to the cytosol (van der Zaal et al. 1999; Eng et al. 1998). On the other hand, the proteins of the ZIP family are predicted to have 8 TM domains in which the C terminal of peptide is found inside the surface of to plasma membrane, and the N terminal ends of the protein are located on the outside surface of the plasma membrane. However, the important feature shared by most of the ZIP proteins is a long hydrophobic loop located between TM domains III and IV. This region is referred to as the 'variable region' because both its length and sequence showed little conservation among the family members (Guerinot 2000), which has also been found in our analysis (Fig. 3). Additionally, variable region that is shared by several of the ZIP proteins is characterized by the presence of many conserved histidine residues i.e. H-x-H-x-H which is reported to be a putative metal binding sequences

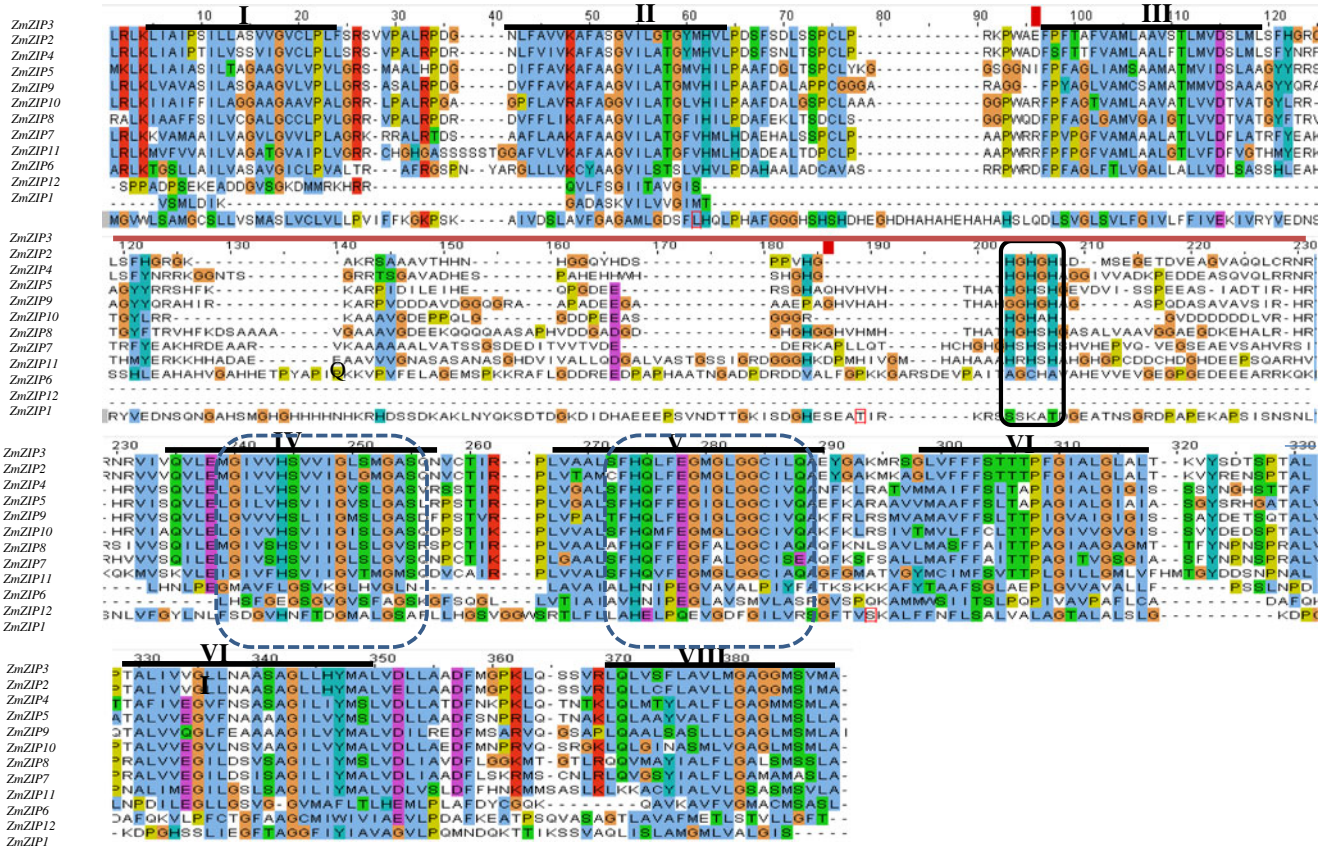

Fig. 3 Multiple sequence alignment of deduced peptides of *ZmZIP* genes obtained by ClustalW. The TM domains indicated as *roman numerals* at the top of the alignments. The *red bar* indicates the 'variable region' between III and IV TMs. The *square* indicates the conserved histidin motif (H-x-H-x-H) in the 'variable' region. The *dotted square* indicates the ZIP motifs

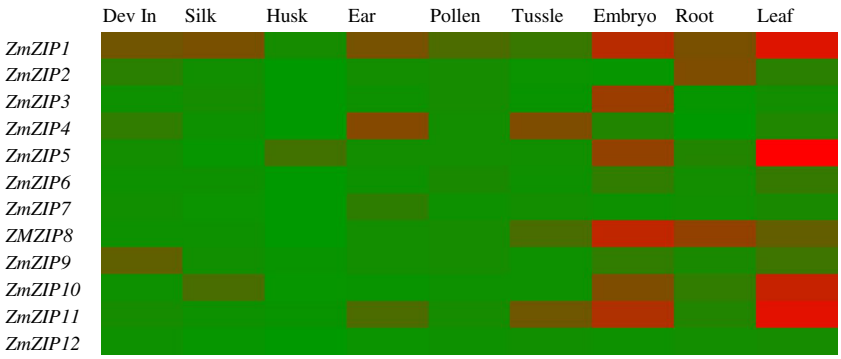

Fig. 4 In silico expression analysis of *ZmZIP* genes across the different tissues. The heat map displaying the transcript abundance was produced from integrating and analyzing of all expressed data (*Dev In* developing inflorescence). *Green* to *deep red* indicate the relative increase of abundance

(Gaither and Eide 2001), similar to the present study (Fig. 3). It is interesting to note that a fairly well-conserved histidyl residue, substituted by polar or semipolar residues, and adjacent to another polar residue, was found at the beginning of the TM domain of V among the *ZmZIPs*. Eide et al. (1996) as well as Zhao and Eide (1996) identified regions in the *IRT1*, *ZRT1*, and *ZRT2* proteins that exhibited the 'HX_3' sequence between putative spanners III and IV. Therefore, it was postulated that HN_3 might be an extra membrane metal ion binding site. Similar sequences have been identified in members of another heavy metal ion transport family, the cation diffusion facilitator (CDF) family (Paulsen and Saier 1997). Further, we also identified two ZIP motifs within the ZIP domain i.e. [LM] GIV [VS] HS VIIG [LVIM] SLG [AV] S and [SA] FH [QN] [VMLF] FEG [MIF] [GA] LGGCI which are present in TM domains IV and V, respectively. While the former one found in the present study was reported earlier as a typical ZIP signature in the TM domain IV in rice (Chen et al. 2008) and various organisms (Eng et al. 1998), the latter has not been documented for any plant system in the literature until now.

In silico Expression Patterns of the *ZmZIP* Genes

After integrating and analyzing all expression data, we found that all the *ZmZIP* genes were divided into 9 groups based on

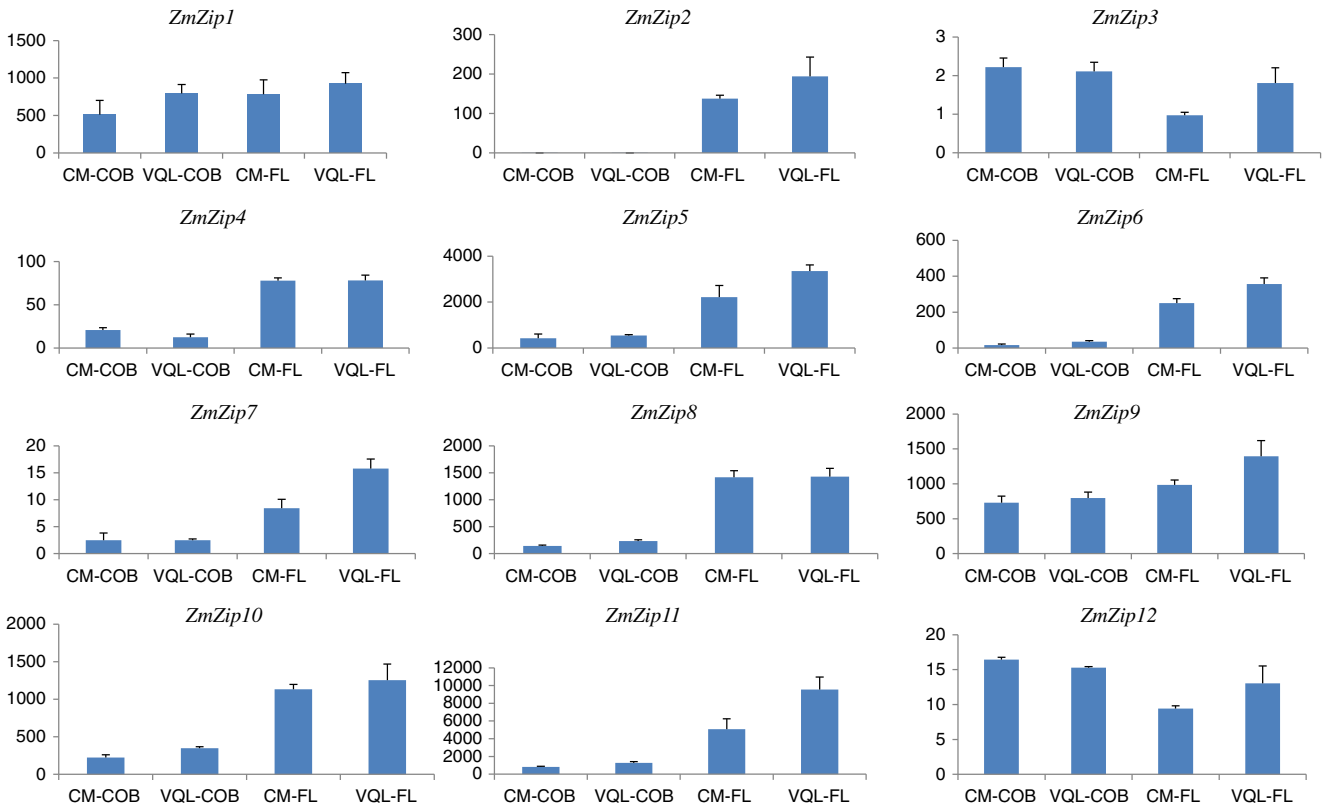

Fig. 5 Relative transcript abundance (on *y-axes*) of *ZmZIP* genes as revealed by qPCR analysis (*COB* 10-day-old kernel, *FL* Flag leaf sampled at 10-day-old kernel, *CM* CM-145, *VQL* VQL-2)

types of tissue that were found as hits in the database search. Differential tissue-specific expression was found among the different *ZmZIP* genes. While maximum expression of *ZmZIP5*, *ZmZIP11*, *ZmZIP1* and *ZmZIP10* was found in leaf, very little expression was found for developing inflorescence, silk, and husk. It had also been found that, while expression of *ZmZIP1* was maximum across a wide range of tissues, the expression of *ZmZIP12* was found to be very low (Fig. 4), which corroborated with low in vivo expression detected by real-time PCR (Fig. 5). Interestingly, *ZmZIP5* and *ZmZIP11* were highly expressed in flag leaf which again was similar with the real-time PCR analysis in the present study (Fig. 5).

Tissue-specific Expression of *ZmZIPs*

Finally, to understand the physiological function of the *ZmZIPs*, the expression pattern was investigated by real-time PCR in some selected tissues. Flag leaves are the major source of phloem-delivered photo-assimilates for developing seeds, and are believed to be one of the sources of remobilized metals for the seeds (Narayanan et al. 2007; Sperotto et al. 2009), which has been experimentally demonstrated in various plants (Uauy et al. 2006; Waters et al. 2009). Similarly, in maize, 10-day-old baby kernels have been found to be metabolically very active (Seebauer et al. 2004), and Zn plays an essential role in embryo and endosperm development (Vallee and Falchuk 1993). Therefore, we selected both tissues i.e. flag leaf and baby kernel, to determine the expression levels of the genes. Our data revealed that, except for *ZmZIP3* and *ZmZIP12*, the overall expression of the other 10 genes was higher in the flag leaf. The *ZmZIP2* transcripts were not detected or were detected below a confidence threshold. The expression levels of *ZmZIP* genes varied considerably, with some genes reaching higher expression levels (*ZmZIP5* and *ZmZIP11*) and others showing very low expression (*ZmZIP7*). Similar behavior of the Zn genes has also been reported in rice, another strategy II plant like maize. In rice, several Zn transporters have been functionally characterized, e.g., *OsIRT1*, *OsIRT2*, *OsZIP1*, *OsZIP3*, *OsZIP4*, and *OsZIP5* (Ishimaru et al. 2006; Lee et al. 2010a; Ramesh et al. 2003). Among them, *OsZIP1*, *OsZIP3*, *OsZIP4*, and *OsZIP5* have been found to be rice Zn transporters induced by Zn deficiency (Ramesh et al. 2003; Lee et al. 2010b; Ishimaru et al. 2006). In situ hybridization analysis has revealed that *OsZIP4* in Zn-deficient rice was expressed in the meristem of Zn-deficient roots and shoots, and also in vascular bundles of the roots and shoots. These results suggest that *OsZIP4* is a Zn transporter that may be responsible for Zn translocation to the plant parts that require Zn. A few members of the ZIP genes of maize have also shown high tissue specificity, i.e. they expressed at very low level (*ZmZIP2*, *ZmZIP5*, *ZmZIP6*, *ZmZIP8* and *ZmZIP11*) in kernel but expressed at a comparatively higher level in the flag leaf. Similar results were also found in rice. The *OsZIP3*, *OsZIP4* and *OsZIP10* gene have shown significantly high levels of expression in flag leaf tissue (Ramesh et al. 2003; Ishimaru et al. 2006), thus suggesting the role of *ZmZIP2*, *ZmZIP5*, *ZmZIP6*, *ZmZIP8* and *ZmZIP11* genes in grain partitioning of Zn ions in maize. Flag leaves are the major source of remobilized metals for developing seeds (Sperotto et al. 2009). We also found that certain genes such as *ZmZIP2*, *ZmZIP3*, *ZmZIP9*, *ZmZIP9* were expressed more in VQL-2. This type of genotypic-dependent expression of Zn transporter is also observed in rice. The *OsZIP4* gene, known as a functional transporter of Zn^{2+} ions in rice (Ishimaru et al. 2006), showed genotype-specific variation in the level of expression in leaf tissue with a higher level of expression in high Zn rice genotypes and a lower level expression in low Zn rice lines. The expression patterns of *ZmZIP2*, *ZmZIP3*, *ZmZIP9*, *ZmZIP9* genes suggested that the genes might play a role in governing the movement of Zn ions in leaf tissue. The expression patterns of *ZmZIP2*, *ZmZIP3*, *ZmZIP9*, *ZmZIP9* genes indicated that acquisition of higher amounts of Zn ions in leaves of high Zn use in an efficient inbreed of maize might enhance its remobilization to developing grains and hence contribute to higher grain Zn values. Similarly, it had been demonstrated that *OsNAC5*, a novel senescence-associated ABA-dependent NAC transcription factor, was highly expressed in flag leaf of rice, and were primarily responsible for mobilizing the Fe and Zn from the flag leaf to the growing kernel (Sperotto et al. 2009). Similarly, in the present study, *ZmZIP5* and *ZmZIP11* were highly expressed in the flag leaf of the VQL-2 inbreed which leads us to conclude that they might play a vital role for the mobilization of Zn from flag leaf to developing kernel for accumulation of high zinc contents in the kernels of VQL-2 (Fig. 5). Therefore, it can be assumed that over-expression of these two *ZmZIP* genes may provide an alternative strategy for the biofortification of crops with Zn.

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