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Cloning and characterisation of *ZmZLP1*, a gene encoding an endoplasmic reticulum-localised zinc transporter in *Zea mays*

Yao-Guang Xu[A], *Bao-Sheng Wang*[A], *Jing-Juan Yu*[A], *Guang-Ming Ao*[A] *and Qian Zhao*[A,B]

[A]State Key Laboratory of Agribiotechnology, China Agricultural University, Beijing 100193, China.
[B]Corresponding author. Email: zhaoqian@cau.edu.cn

Abstract. The *ZmZLP1* (ZmZIP-like protein) gene was isolated from a cDNA library of *Zea mays* L. (maize) pollen. Bioinformatics analysis indicated that ZmZLP1 shares many characteristics of the ZIP (ZRT/IRT-like protein) family of metal ion transporters. Under general nutrient conditions, the expression of *ZmZLP1* was detected in both mature pollen and, less strongly, in male inflorescences, whereas an induction of the *ZmZLP1* transcript was observed in roots after 12 h of zinc deprivation. The visualisation of GFP showed that ZmZLP1 was targeted to the endoplasmic reticulum (ER). To investigate the gene's functions, we fused ZmZLP1 with the signal peptide of the plasma membrane-localised protein AtIRT1 and transformed this fusion protein into the zinc uptake-deficient yeast (*Saccharomyces cerevisiae*) strain ZHY3 and the wild-type strain DEY1457. The *IRT1-ZmZLP1* transformants grew poorly on zinc-limited medium, and this growth defect was rescued by zinc supplementation, suggesting that ZmZLP1 is responsible for transporting zinc from the ER to the cytoplasm. Further research indicated that ZmZLP1 is involved in the unfolded protein response (UPR) pathway and enhances the heat resistance of yeast cells.

Additional keywords: corn, maize, subcellular localisation, unfolded protein response, zinc deficiency induction, ZIP family.

Introduction

Zinc is a trace nutrient that is indispensable for life, serving as a cofactor for many enzymes and as a key structural motif in transcriptional regulatory proteins. In addition, zinc can potentially modulate the recognition of cellular signals, second messenger metabolism and the functions of protein kinases and phosphatases (Kambe *et al*. 2004; Taylor *et al*. 2004; Ishimaru *et al*. 2005). Zinc cannot passively diffuse across cell membranes. Therefore, specific zinc transporters are required to transport zinc into the cytosol, both from the extracellular environment and from storage compartments (Kambe *et al*. 2004; Taylor *et al*. 2004). Plant genomes encode large families of metal transporters that vary in their substrate specificities, expression patterns and cellular localisation to govern metal translocation throughout the plant. These transporter families include the P_{1B}-ATPase family, the cation diffusion facilitator (CDF) family, the natural resistance associated macrophage protein (NRAMP) family and the ZRT/IRT-like protein (ZIP) family. In mammalian cells, ZIP members facilitate zinc influx into the cytosol, and CDF members facilitate its efflux from the cytosol (Kambe *et al*. 2004). A similar mechanism for zinc transport and trafficking in yeast was reviewed by Eide (2006).

Much progress has been made in uncovering the mechanisms of cellular trafficking of zinc. Msc2 and Zrg17 are two members of the CDF family in *Saccharomyces cerevisiae* and have been shown to be involved in zinc transport into the endoplasmic reticulum (ER). Furthermore, these two gene products have been proposed to form a heteromeric zinc transport complex in the ER membrane (Ellis *et al*. 2004, 2005). YKE4 is another zinc transporter localised to the ER in yeast. It encodes a member of the ZIP family and is orthologous to the KE4 genes in human, mouse, zebrafish, *Drosophila melanogaster* and *Arabidopsis thaliana* (L.) Heynh. (Kumanovics *et al*. 2006). Kumanovics *et al*. (2006) have hypothesised that YKE4 is a bidirectional zinc transporter that is responsible for balancing zinc levels between the cytosol and the secretory pathway.

Protein misfolding in the ER activates a set of intracellular signalling pathways, collectively termed the unfolded protein response (UPR). UPR signalling promotes cell survival by reducing misfolded protein levels (Lin *et al*. 2009). In yeast, zinc deficiency in the ER induces the UPR (Ellis *et al*. 2004). The Msc2, Zrg17 and YKE4 mutants all have an effect on UPR induction (Ellis *et al*. 2004, 2005; Kumanovics *et al*. 2006).

ZIP proteins usually consist of 309–476 amino acid residues with eight putative transmembrane domains (TM), and there is a variable region between TM domains III and IV that contains a potential metal-binding domain rich in conserved histidine residues (Guerinot 2000). ZIP proteins have been identified in many plants including *Arabidopsis thaliana* (Eide *et al*. 1996; Grotz *et al*. 1998; Vert *et al*. 2001, 2002), *Arabidopsis halleri* L. (Weber *et al*. 2004), *Thlaspi caerulescens* J. & C. Presl. (Pence *et al*. 2000; Assuncao *et al*. 2001; Plaza *et al*. 2007), *Pisum sativa* L. (Cohen *et al*. 1998), *Glycine max* L. (Moreau *et al*. 2002), *Lycopersicon esculentum* Mill. (Eckhardt *et al*.

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2001; Schikora *et al*. 2006), *Medicago truncatula* Gaertn. (Burleigh *et al*. 2003; López-Millán *et al*. 2004) and *Oryza sativa* L. (Ramesh *et al*. 2003; Ishimaru *et al*. 2005; Yang *et al*. 2009). Through scanning the available genome sequence, Chauhan (2006) predicted 33 genes involved in iron and zinc transport in *Z. mays*. Nine of these belong to the ZIP family, and of the nine, ZmZIP2 and ZmZIP7 are orthologous to OsZIP2 and OsZIP7, respectively. However, no research about the functions of these ZmZIPs has been reported so far.

In this study, we isolated the *ZmZLP1* (ZmZIP-like protein1) gene from a cDNA library of mature *Z. mays* pollen and demonstrated that *ZmZLP1* is an ER membrane protein whose function is likely to be responsible for transporting zinc from the ER to the cytoplasm.

Materials and methods

Yeast strains and plant material

The yeast strain DEY1457 (wild type) and the zinc uptake-deficient mutant strain ZHY3 were obtained from the Zhen-Hai Han laboratory, China Agriculture University. *Zea mays* L. cv. NongDa108 was given by professor Qi-Feng Xu, China Agriculture University. SiPf40, which is related to the regulation of plant branching, was cloned by Dr Feng XY in our laboratory.

Cloning of the ZmZLP1 gene

A cDNA library was constructed with mRNAs from the mature pollen of *Z. mays* using a cDNA Synthesis kit (Stratagene, La Jolla, CA, USA). About 2.2×10^5 plaques were screened. A partial *SiPf40* cDNA containing the open reading frame from millet was used as the probe. Plaques were lifted and blotted onto a nylon membrane (Amersham Biosciences, Buckinghamshire, UK) according to the recommended procedure and hybridised with ^{32}P-labelled DNA probes (random-primed DNA labelling system; Promega, Madison, WI, USA). Southern blot analysis was performed as described by Sambrook and David (2001).

A 5′-RACE experiment was performed using the RLM-RACE Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Two primers, PR1 and PR2, (Table 1) were synthesised. The amplified fragment was cloned into the pMD18-T plasmid vector (Takara, Otsu, Japan) and sequenced.

Total RNA was extracted from the pollen of *Z. mays* using the procedure developed by Logemann *et al*. (1987). First strand cDNA was synthesised according to the manual of the Reverse Transcription System kit (Promega). Two primers (PZf and PZr) with *Sma*I or *Kpn*I sites were designed for amplifying the complete open reading frame of the *ZmZLP1* gene (FJ812089) (Table 1). The PCR reaction was performed with a denaturation step of 5 min at 95°C, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, 30 s at 72°C and a final extension of 10 min at 72°C. The PCR products were purified, cloned into the pMD18-T vector (Takara) and sequenced.

Northern blot analysis

Total RNA samples were extracted from roots, shoots, leaves, male inflorescences, female inflorescences, pollen and seeds of *Z. mays*. Twenty micrograms of total RNA were electrophoresed on a 1.2% agarose gel containing 0.4 M formaldehyde and transferred to a Hybond-N+ membrane (Amersham). Hybridisation was the same as for the DNA gel blot, with an α-^{32}P-dCTP-labelled *ZmZLP1* probe.

ZmZLP1 expression induction by zinc deficiency

Z. mays seeds were sterilised in 1.5% NaClO for 15 min and washed with distilled water before germination at 28°C. The germinated seeds were cultured in Hoagland nutrient solution in climate chambers with a light/dark cycle of 16/8 h. For Zn-deficiency treatment, the 10-day-old seedlings were transferred to the nutrient solution without $ZnSO_4$. Roots were collected at 0, 6, 12, 24, 48 and 96 h after the Zn-deficiency treatment for semiquantitative RT–PCR assays. PZf and PZr primers were used (Table 1). Two microlitres of PCR product were electrophoresed on a 1.0% agarose gel. The constitutively expressed *actin* gene (EU961034) was amplified as the reference gene using the primers Pact1 and Pact2 (Table 1).

Subcellular localisation

In our subcellular localisation experiments, a *GFP* fragment was amplified using primers pG1 and pG2 from the vector pTAC-gfp, which was provided by the laboratory of Guo-Qin Liu (China Agriculture University, Beijing, China), and inserted into the pROK219 vector to yield the plasmid pROK219-GFP. Four sense primers (PZ047, PZ186, PZ273 and PZ428) and one antisense primer (PZr) for *ZmZLP1* were designed and the stop codon TAG (at 929th site) was deleted. These *ZmZLP1* fragments without the stop codon were cloned into pROK219-GFP to construct the vectors pZ047-GFP, pZ186-GFP, pZ273-GFP and pZ428-GFP, respectively.

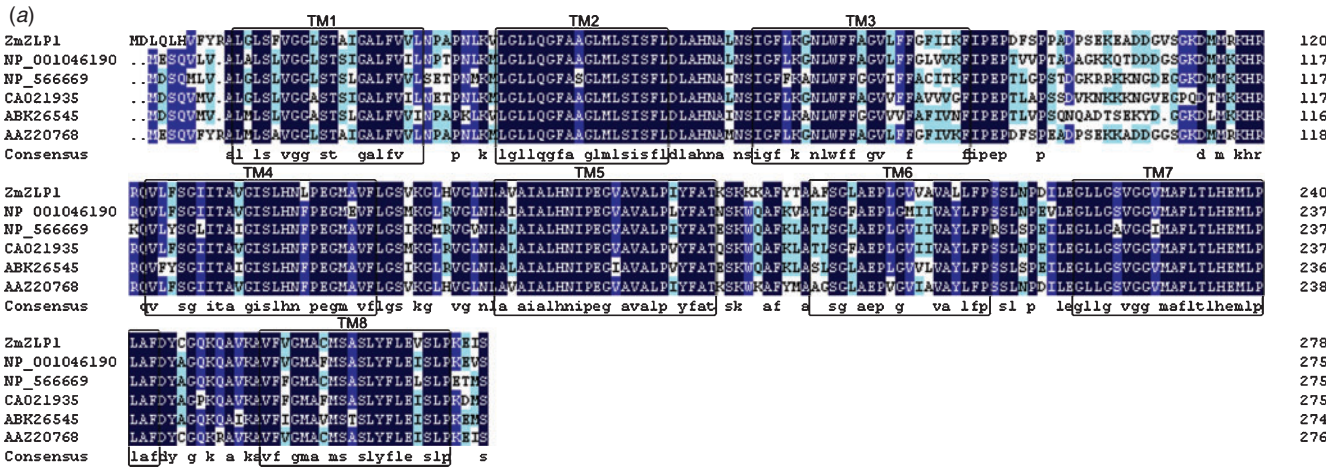
The vectors were purified using the Wizard *Plus* Miniprep DNA Purification System (Promega). Onion inner epidermis was spread on the centre of an MS plate (Hofgen and Willmitzer 1988) and bombarded with 1100 psi of helium at 7 cm distance with a PDS-1000/He (Bio-Rad, Hercules, CA, USA). After bombardment, onion epidermal cells were incubated at 25°C without light for 24 h and then observed under a fluorescent microscope (Olympus, Tokyo, Japan; BX51). The excitation wavelength was 488 nm for fluorescein isothiocyanate excitation, and emission filters were 505/530 nm for GFP fluorescence.

In the experiments for colocalisation of ZmZLP1 and ER-Tracer, a red-orange-fluorescent BODIPY 558/568 conjugate of brefeldin A (Invitrogen, Carlsbad, CA, USA) was used. The *ZmZLP1-GFP* fragment was amplified from pZ047-GFP using primers PZ047 and PG2 (Table 1) and cloned into the pBI121 vector to construct pBI-ZmZLP1-GFP, which was transformed into *Nicotiana tabacum* L. cv. shanxi. The process of tobacco transformation was performed as described by Walkerpeach and Velten (1994). Seven-day-old transgenic seedlings of the T1 generation were used for dyeing with ER tracker. After dyeing at room temperature for over 30 min, the root tips of plants were observed under a fluorescent microscope (Olympus BX51).

Table 1. Oligonucleotides primers used in various gene constructions
ORF, open reading frame

Oligo names	Primer sequences	Features
Primers for *ZmZLP1*		ORF is from 89 to 929
PR1	5′-CACAATAGTCAAATGCAAGAGGC-3′	806 to 827
PR2	5′-CAAAGTGAGGAAAGCCATGACA-3′	773 to 793
PZf	5′-ATCCCCCGGGAAAGCCCAAACCCTAGCA-3′	47 to 63 (*Sma*I underlined)
PZr	5′-GCGGTACCCAAGCTTATCTCCTTTGGTA-3′	907 to 925 (*Kpn*I underlined)
PZ047	5′-ATCCCCCGGGAAAGCCCAAACCCTAGCA-3′	47 to 65 (*Sma*I underlined)
PZ186	5′-ATCCCCCGGGATGCCTGCCCCTAACCTTA-3′	186 to 204 (*Sma*I underlined)
PZ273	5′-ATCCCCCGGGATGAATGCCCTGAATTCTAT-3′	273 to 292 (*Sma*I underlined)
PZ428	5′-ATCCCCCGGGAGATATGATGAGAAAACA-3′	428 to 445 (*Sma*I underlined)
PZb	5′-CGGGATCCGACCTGCAGCTGCATG-3′	93 to 107 (*Bam*HI underlined)
Primers for *actin* of *Zea mays*		
Pact1	5′-GAACCAGAAGGATGCATATGTTG-3′	
Pact2	5′-GGTAATCAGTAAGGTCACGTCCA-3′	
Primers for *GFP*		
PG1	5′-GCGGTACCATGAGTAAAGGAGAAGAACT-3′	*Kpn*I underlined
PG2	5′-GCGAGCTCTTATTTGTATAGTT-3′	SacI underlined
Primers for *ZRT2* of *Saccharomyces cerevisiae*		
PZRT2s	5′-ATCCCCCGGGATGGTTGATCTTATAGCG-3′	*Sma*I underlined
PZRT2a	5′-GCGGTACCTGCCCATTTCCCTAGAAG-3′	*Kpn*I underlined
Primers for the oligonuleotides encoding singal peptide of *IRT1*		
PI1	5′-ATCCCCCGGGATGGCTTCAAATTCAGCACTTCTC ATGAAAACAATCTTCCTCGTGCTCA-3′	*Sma*I underlined
PI2	5′-CGGGATCCTGAAGTTGCTGGAGAGATTGCAAAAG AGACAAAAATGAGCACGAGGAAGAT-3′	*Bam*HI underlined
Primers for *ACT1* of *Saccharomyces cerevisiae*		
PACT1F	5′-TGAGGTCATTGGGTCTTTCG-3′	For real-time PCR
PACT1R	5′-CAACTCAGCGGCGGAGATTA-3′	For real-time PCR
Primers for *HAC1* of *Saccharomyces cerevisiae*		
PHAC1s	5′-CGCAATCGAACTTGGCTATC-3′	For semiquantitative PCR
PHAC1a	5′-TATATCGTCGCAGAGTGGGTC-3′	For semiquantitative PCR
PHAC1F	5′-AAACTGGCTGACCACGAAGA-3′	For real-time PCR
PHAC1R	5′-GTATCAGACGACGAGTGCGA-3′	For real-time PCR
Primers for *CMD1* of *Saccharomyces cerevisiae*		
CMD1s	5′-AGCCTTTGCCCTCTTTGATA-3′	For semiquantitative PCR
CMD1a	5′-AGCAGCGAATTGTTGAATGT-3′	For semiquantitative PCR

Fig. 1. Molecular characterisation of ZmZLP1. (*a*) Alignment analysis of *ZmZLP1* with its homologues AAZ20768, ABK26545, NP_001046190, NP_566669 and CAO21935. Transmembrane domains (TMs) are indicated. (*b*) Transmembrane segments as predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed 19 February 2009). (*c*) Phylogenetic tree of ZmZLP1 with some plant ZIP family members. Protein sequences were aligned using Clustal X and the phylogenetic tree was drawn with the MEGA program. Species designations and corresponding GenBank accession numbers: *Setaria italica* (SiPf40: AAZ20768); *Arabidopsis thaliana* (AtIRT1: AAB01678; AtIRT2: NP_001031670; AtIRT3: NP_564766; AtZIP1: AAC24197; AtZIP2: AAC24198; AtZIP3: AAC24199; AtZIP4: AAB65480; AtZIP5: AAL38432; AtZIP6: AAL38433; AtZIP7: AAL38434; AtZIP8: AAL83293; AtZIP9: AAL38435; AtZIP10: AAL38436; AtZIP11: AAL67953; AtZIP12: AAL38437; AtIAR1: AF216524); *Oryza sativa* (OsIRT1: AB070226; OsIRT2: AB126086; OsZIP1: AY302058; OsZIP2: AY302059; OsZIP3: AY323915; OsZIP4: AB126089; OsZIP5: AB126087; OsZIP6: AB126088; OsZIP7: AB126090; OsZIP7a: AY275180; OsZIP8: AAP88588; OsZIP9: BAF17731; OsZIP10: BAF19808); *Zea mays* (ZmZIP1: AZM4_91600; ZmZIP2: AZM4_112284; ZmZIP3: AZM4_113214; ZmZIP4: AZM4_62845; ZmZIP5: AZM4_12175; ZmZIP6: AZM4_52445; ZmZIP7: AZM4_22482; ZmZIP8: AZM4_102146; ZmZIP9: AZM4_114899); *Lycopersicon esculentum* (LeIRT1: AAF97509; LeIRT2: AAF97510); *Pisum sativum* (PsRIT1: AAC17441); *Glycine max* (GmZIP1: AY029321); *Medicago truncatula* (MtZIP1: AY339054; MtZIP2: AY007281; MtZIP3: AY339055; MtZIP4: AY339056; MtZIP5: AY339057; MtZIP6: AY339058; MtZIP7: AY339059); *Cucumis sativus* (CsIRT1: AY590764); *Saccharomyces cerevisiae* (YKE4: NP_012241; ZRT1: CAA96975; ZRT2: CAA97701; ZRT3: NP_012746). The ZmZIP genomic sequences of *Zea mays* contigs were from http://maize.tigr.org/release5.0/azm5.shtmL and were annotated for open reading frames using the gene prediction algorithms of FGenesH (http://www.softberry.com); the others are in GenBank (http://www.ncbi.nlm.nih.gov/).


(a)
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
ZmZLP1
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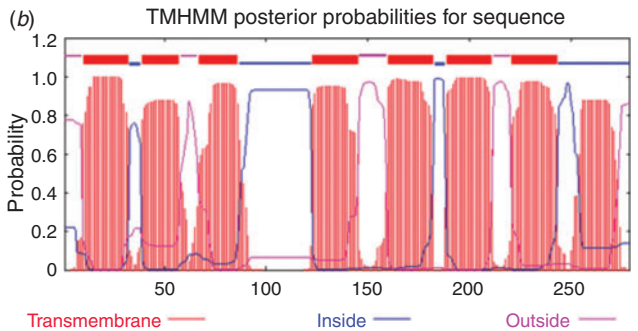
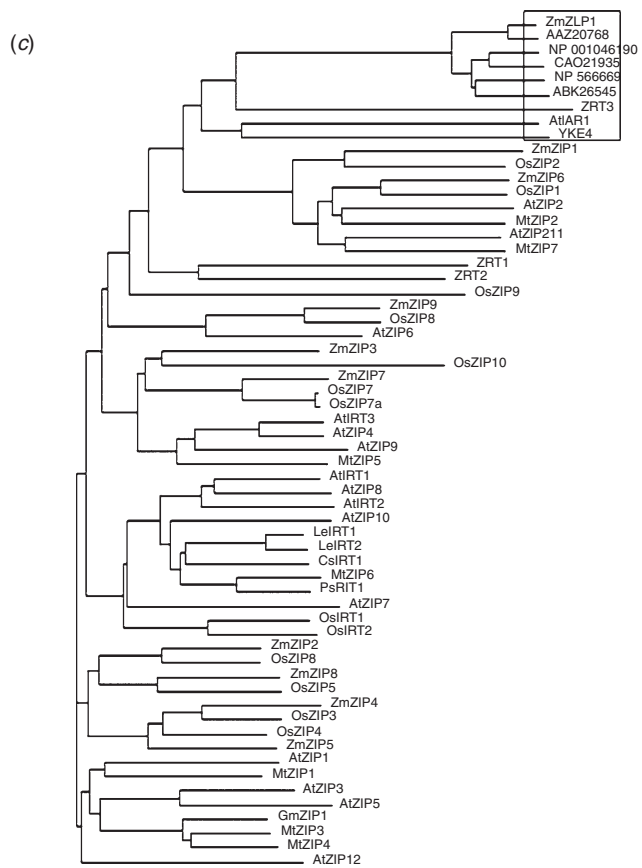
NP_566669
CA021935
ABK26545
AAZ20768
Consensus


(b)
TMHMM posterior probabilities for sequence
Probability
1.2
1.0
0.8
0.6
0.4
0.2
0
50
100
150
200
250
Transmembrane
Inside
Outside


(c)
ZmZLP1
AAZ20768
NP 001046190
CAO21935
NP 566669
ABK26545
ZRT3
AtIAR1
YKE4
ZmZIP1
OsZIP2
ZmZIP6
OsZIP1
AtZIP2
MtZIP2
AtZIP211
MtZIP7
ZRT1
ZRT2
OsZIP9
ZmZIP9
OsZIP8
AtZIP6
ZmZIP3
OsZIP10
ZmZIP7
OsZIP7
OsZIP7a
AtIRT3
AtZIP4
AtZIP9
MtZIP5
AtIRT1
AtZIP8
AtIRT2
AtZIP10
LeIRT1
LeIRT2
CsIRT1
MtZIP6
PsRIT1
AtZIP7
OsIRT1
OsIRT2
ZmZIP2
OsZIP8
ZmZIP8
OsZIP5
ZmZIP4
OsZIP3
OsZIP4
ZmZIP5
AtZIP1
MtZIP1
AtZIP3
AtZIP5
GmZIP1
MtZIP3
MtZIP4
AtZIP12

Yeast transformation

For the functional complementation of the yeast mutant ZHY3 (*zrt1zrt2*), the *ZRT2* gene was cloned from the cDNA of *S. cerevisiae* using the primers PZRT2s and PZRT2a (Table 1). Total RNA from *S. cerevisiae* (DEY1457 stain) was prepared using the fast RNA extraction from yeast cells kit (BioDev, Beijing, China) and first strand cDNA synthesis was performed according to the manual of the reverse transcription system kit (Promega). Then, *ZmZLP1* containing the complete ORF and *ZRT2* fragments was cloned into the pFL61 vector. The resulting constructs were named pFL61-ZmZLP1 and pFL61-ZRT2.

To clone the signal peptide of AtIRT1 (AAB01678), two primers, PI1, containing a *Sma*I site, and PI2, containing a *Bam*HI site (Table 1), were designed and used to perform the PCR reaction according to the following parameters: 95°C 5 min for denaturing DNA; 2 cycles of 94°C 30 s, 55°C 30 s, 72°C 30 s; 72°C 5 min for a final extension. The *ZmZLP1-GFP* fragment with *Bam*HI and *Sac*I was obtained by PCR using the primers PZb and PG2 (Table 1) from pZ047-GFP and was fused to the 92 bp of the *AtIRT1* fragment in the pMD18-T vector (Takara). The PCR product of *IRT1-ZmZLP1-GFP* was amplified using the primers PI1 and PG2 and then cloned into pROK219. We named the resulting plasmid pROK219-IRT1-ZmZLP1-GFP. This vector was transformed into onion epidermal cells by particle bombardment. The processes of particle bombardment and fluorescence observation were performed as described above. In succession, *IRT1-ZmZLP1-GFP* and *IRT1-ZmZLP1* fragments were digested from pROK219-IRT1-ZmZLP1-GFP with *Sma*I and *Sac*I or *Kpn*I and cloned into pFL61 to complete the construction of pFL61-IRT1-ZmZLP1-GFP and pFL61-IRT1-ZmZLP1.

Yeast transformation in this study applied the lithium acetate-based method described by Gietz *et al.* (1992). Transformed cells were selected on s.d. medium containing amino acid supplements without uracil (6.7 g L^{-1} of yeast nitrogen base without amino acids, 20 g L^{-1} of glucose, necessary auxotrophic supplements).

Induction of UPR in ZmZLP1 *transformants*

ZHY3 or DEY1457 transgenic strains were cultured in YPD liquid medium overnight. Cells were collected by centrifugation and resuspended with 3 mL of fresh YPD or s.d. liquid medium to OD600 ~0.4. Yeast cells were cultured to OD600 ~1.0 with shaking at 30°C or 37°C. Total RNA of yeast cells was extracted using the fast RNA extraction from yeast cells kit (BioDev) and first strand cDNA was synthesised for semiquantitative PCR and real-time PCR.

The primers in the semiquantitative PCR for *HAC1* (YFL031W) and *CMD1* (YBR109C) are shown in Table 1. *CMD1* was used as a reference gene. PCR reactions were performed for 5 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 53°C for *CMD1* or at 56°C for *HAC1*, 30 s at 72°C and a final extension of 10 min at 72°C. Two microlitres of PCR product were electrophoresed on a 1.0% agarose gel. Semiquantitative RT–PCR experiments were repeated at least three times independently.

Real-time RT–PCR was performed using the CFX96 real-time system (BioRad) and SYBR Premix EX Taq (Takara). The *ACT1* gene (V01289) was used as a reference gene. The primers for *ACT1* and *HAC1* were designed using the Primer 5.0 program (Table 1) to produce PCR products of ~200 bp in length. PCR reactions contained primers at a concentration of 200 nM each, 20 ng cDNA and 1 × SYBR Premix EX Taq (Takara) in a total volume of 20 μL. QRT–PCR was conducted with an initial denaturation at 95°C for 5 min, followed by 45 cycles of 10 s at 95°C, 5 s at 58°C, and 10 s at 72°C. After amplification, melting curves were created to confirm that a single PCR product was produced in each reaction. At least three repetitions with each sample were conducted. The *HAC1* gene expression fold-change was calculated according to the following formula: $\text{ratio} = 2^{[\Delta CP \text{ target (sample} - \text{control)} - \Delta CP \text{ ref (sample} - \text{control)}]}$ (Pfaffl 2001). In this formula, 'target' refers to the *HAC1* gene; 'ref' refers to the *ACT1* gene; 'sample' refers to the 37°C or zinc-limited treatment, and 'control' refers to non-treatment.

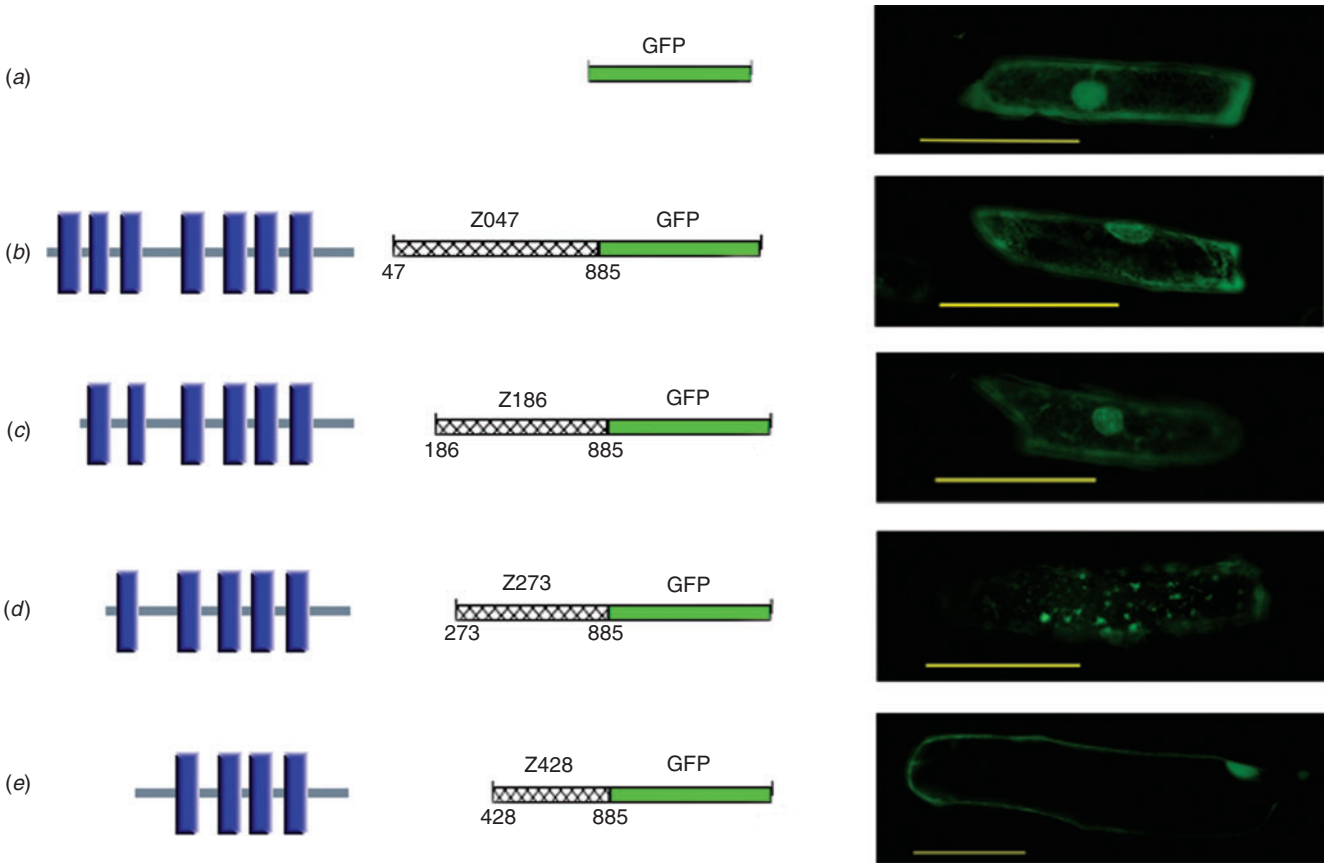
Results

Cloning and bioinformatic analysis of ZmZLP1

The *SiPf40* gene of millet (*Setaria italica*, GenBank accession no: AAZ20768) encodes a 29.4 kDa protein with sequence similarity to the ZIP (ZRT/IRT-like protein) family, which is related to plant branching (Xia *et al.* 2005). In order to identify a gene in *Z. mays* orthologous to *SiPf40*, a cDNA library of mature *Z. mays* pollen was constructed and screened using *SiPf40* cDNA as a probe. A positive clone named *ZmZLP1* (ZmZIP-like protein1) was obtained. To fill the cDNA, the 5′-RACE technique was applied and a ~400-bp fragment was amplified. The full-length cDNA was obtained by RT–PCR. *ZmZLP1* contains an open reading frame 864 bp in length, encoding a predicted polypeptide of 278 amino acids. It is highly similar to *SiPf40* from millet, showing 91% identity at the nucleic acid level and 94% identity at amino acid level.

The BLAST results using the blastp program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, accessed 15 February 2008) indicated that twelve genes from plants were homologous to *ZmZLP1*. They shared 48.5% identity at the amino acid level (data not shown). Furthermore, five gene products (GenBank accession no: AAZ20768, ABK26545, NP_001046190, NP_566669 and CAO21935) showed more similarly to ZmZLP1, possessing 79.59% identity (Fig. 1*a*). These came from *S. italica*, *Picea sitchensis*, *O. sativa*, *A. thaliana* and *Vitis vinifera*, respectively. Few functional studies of these genes have been reported. These results suggest that ZmZLP1 and its homologues were present abroad in dicotyledon and monocotyledon. More than two ZmZLP1 homologues have been found in *O. sativa* and *Arabidopsis*, which indicates that there are presumably other genes similar to *ZmZLP1* in *Z. mays*.

Transmembrane domain prediction using TMHMM 2.0 program and a conserved domain search in NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd, accessed 19 February 2009) indicated that the ZmZLP1 protein has seven predicted transmembrane domains (TM) and a so-called variable cytosolic



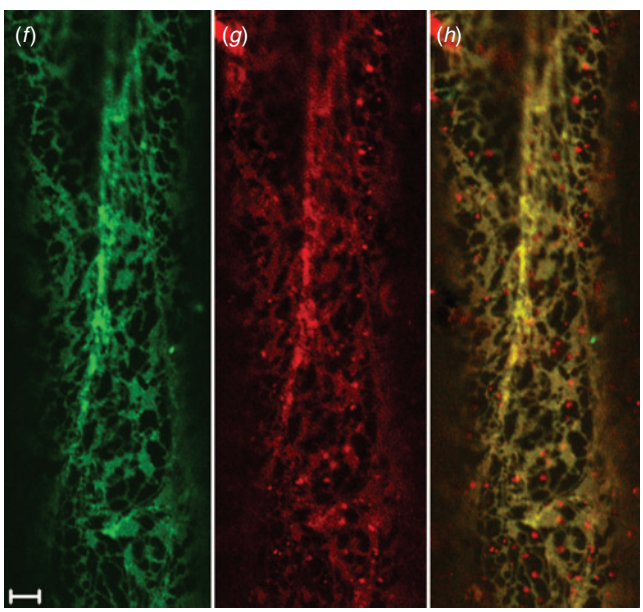


Fig. 2. Subcelluar localisation of ZmZLP1. (*a–e*), Construction and localisation of various ZmZLP1::GFP fusion proteins in onion epidermal cells. (*a*) GFP driven by the CaMV 35S promoter was detected in plasma membrane (PM), endoplasmic reticulum (ER) and nucleus; Full-length (*b*) or lacking the first TM domain (*c*) ZmZLP1::GFP fluoresced in the ER; ZmZLP1 lacking the first two (*d*) or three (*e*) Transmembrane domains (TMs) fused with GFP lose the correct localisation. Photos were taken with a fluorescent microscope (Olympus BX51). Scale bar indicates 50 µm. (*f–h*) Colocalisation of ZmZLP1::GFP with the ER tracer, red-orange-fluorescent BODIPY 558/568 conjugate of brefeldin A (Invitrogen). The fluorescence of ZmZLP1::GFP (*f*) and BODIPY 588/568 (*g*) were merged (*h*). Pictures were taken with a Nikon laser confocal microscope. Scale bar indicates 5µm.

loop between TM3 and TM4 (Fig. 1*a*, *b*), which are typical features of the ZIP (ZRT/IRT-like protein) family. However, unlike many ZIP proteins, the variable region of ZmZLP1 is not rich in histidine residues.

According to published articles and the GenBank database, four ZIP family members have been found in *S. cerevisiae* (Eide 2006; Kumanovics *et al*. 2006), sixteen in *A. thaliana* (Grotz *et al*. 1998; Maser *et al*. 2001; Grotz and Guerinot 2006), thirteen in *O. sativa* (Ishimaru *et al*. 2005), seven in *M. truncatula* (Burleigh *et al*. 2003; López-Millán *et al*. 2004), two in *Lycopersicon esculentum* (Eckhardt *et al*. 2001; Schikora *et al*. 2006) and one each in *P. sativum* (Cohen *et al*. 1998), *G. max* (Moreau *et al*. 2002) and *Cucumis sativus* (Yang *et al*.2009). By scanning the available *Z. mays* genome sequence, Chauhan (2006) identified 33 *Z. mays* genes predicted to be involved in iron and zinc transport. Nine of these genes belonged to the ZIP family. ZmZLP1 had low homology to these nine putative ZmZIPs (data not shown). The 54 proteins named above, along with ZmZLP1 and its homologues, were aligned with the program Clustal × (version 1.8) and a phylogenetic tree was constructed using the program PhyloDraw (version 0.82) (Fig. 1*c*). The results revealed that the ZmZLP1 cluster was closely related to AtIAR1, YKE4 and ZRT3. *AtIAR1* and *YKE4* are homologous genes and they are both subcellularly localised to the ER (Lasswell *et al*. 2000; Kumanovics *et al*. 2006). AtIAR1 is required for sensitivity to several IAA-amino acid conjugates in *Arabidopsis* and is presumed to inhibit the conjugate hydrolases through transporting zinc, copper or other inhibitory metals out of the ER (Lasswell *et al*. 2000). YKE4 is a bidirectional zinc transporter in the ER of yeast and is involved in balancing the zinc levels between the cytosol and the secretory pathway (Kumanovics *et al*. 2006). ZRT3 is a ZIP member localised to the vacuole and is responsible for regulating vacuolar zinc storage in *S. cerevisiae* (MacDiarmid *et al*. 2000). ZmZLP1 shared 11.67, 8.25 and 28.28% amino acid sequence identity with AtIAR1, YKE4 and ZRT3, respectively. The phylogenetic tree demonstrated that ZmZLP1 is closely related to these zinc transporters, which localise to intracellular organelles.

These results of this bioinformatic analysis suggest that ZmZLP1 is a potential member of the *Z. mays* ZIP protein family and is probably involved in zinc transport, specifically intracellularly.

Subcellular localisation of ZmZLP1

To figure out the subcellular localisation of ZmZLP1, four expression vectors containing ZmZLP1 fragments of various lengths fused with GFP were constructed and named pZ047-GFP, pZ186-GFP, pZ273-GFP and pZ428-GFP. In succession, they were transformed into onion epidermal cells by particle bombardment. The fluorescence visualisation showed that GFP fluoresced in the cytoplasm, nucleus and plasma membrane (PM) of cells (Fig. 2*a*). The full-length ZmZLP1, as the same as SiPf40 (Xia *et al*. 2005), was targeted to the ER (Fig. 2*b*) and still kept the proper localisation when its first predicted TM domain, which consisted of the *N*-terminal 61 amino acid residues, was removed (Fig. 2*c*). After truncating the first two or three TMs, ZmZLP1 seemed to lose its localisation (Fig. 2*d*, *e*).

For stable expression, a *ZmZLP1-GFP* vector driven by the CaMV 35S promoter (pBI-ZmZLP1-GFP) was constructed and transformed into tobacco plants. The T1 transgenic plants were used in colocalisation analysis. As Fig. 2*f* shows, ZmZLP1 colocalised to the ER with the ER-Tracker, red-orange-fluorescent BODIPY 558/568 (Invitrogen), which was consistent with observations in onion epidermal cells (Fig. 2*b*).

Expression pattern of ZmZLP1

We examined the expression pattern of *ZmZLP1* by northern blot hybridisation using total RNA from roots, shoots, leaves, young male inflorescences, female inflorescences before pollination, mature pollen and seeds of *Z. mays*. *ZmZLP1* mRNA was detected in the mature pollen and only weakly in male inflorescences (Fig. 3*a*).

At a transcriptional level, the expression of many ZIP genes such as ZRT1, AtIRT1 and OsZIP1 have been induced by zinc or iron starvation (Zhao and Eide 1996; Vert *et al*. 2002; Ramesh *et al*. 2003). Considering its shared structural features with the ZIP transporters, we speculated that *ZmZLP1* might also respond to zinc status. To verify this hypothesis, we grew wild-type *Z. mays* seedlings under zinc-deficient conditions and detected *ZmZLP1* transcripts using semiquantitative RT–PCR. An induction of *ZmZLP1* expression was observed in *Z. mays* roots after 12 h of zinc deprivation (Fig. 3*b*), which suggests that the *ZmZLP1* gene plays a role in zinc homeostasis in *Z. mays*.

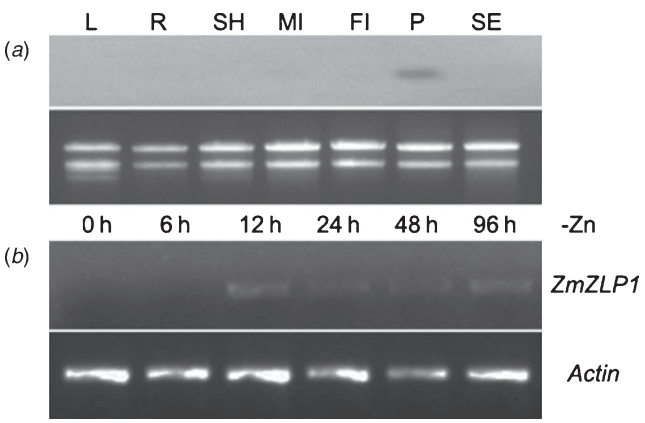


Fig. 3. Expression pattern of *ZmZLP1*. (*a*) RNA gel blot analysis of the total RNA prepared from leaves (1), roots (R), shoots (SH), young male inflorescences (MI), female inflorescences before pollination (FI), pollen (P), seeds (SE). A full-length *ZmZLP1* fragment was used as a probe. *ZmZLP1* transcript was detected in pollen and weakly in young male inflorescences. (*b*) Semiquantitative RT–PCR analysis of *ZmZLP1* in *Z. mays* roots after zinc deficiency. Total RNA was extracted from the roots of *Z. mays* seedlings that were grown in the absence of zinc for 0, 6, 12, 24, 48 or 96 h. PCR reactions were carried out for 5 min at 95°C, followed by 27 cycles of 40 s at 94°C, 40 s at 54°C (*ZmZLP1*) or 52°C (*Action*), 40 s at 72°C, and a final extension of 10 min at 72°C. The experiment was repeated twice, with RT–PCR reactions repeated three times independently.

ZmZLP1 alters zinc homeostasis in transgenic yeast

The fact that ZmZLP1 was localised to the ER and expressed in *Z. mays* roots in response to zinc deficiency led us to propose that ZmZLP1 is likely to be responsible for zinc transport in the ER. In order to test this hypothesis, the vector pFL61-ZmZLP1 was constructed and transformed into both the wild-type yeast strain DEY1457 and the zinc uptake-deficient mutant ZHY3 (*zrt1zrt2*). Yeast cells containing the empty vector (pFL61) were the negative controls and the transgenic *ZHY3* with the *ZRT2* gene, which encodes the zinc transporter of *S. cerevisiae*, was the positive control (Zhao and Eide 1996). The transgenic yeast was cultured on s.d. medium supplemented with zinc or EDTA at 30°C for 36 h. No remarkable difference was observed between the transformants with *ZmZLP1* and the negative controls (Fig. 4). This result indicated that ZmZLP1 could not complement the function of the ZRT1 and ZRT2 genes in yeast.

To determine if the subcellular localisation of ZmZLP1 resulted in the failure to complement, a DNA fragment encoding the signal peptide of IRT1 (AAB01678), a known plasma membrane-localised multispecific metal ion transporter in *Arabidopsis* (Vert *et al*. 2002), was cloned and fused with *ZmZLP1*. The transient expression analysis of the *IRT1-ZmZLP1-GFP* fusion gene driven by the CaMV 35S promoter in onion epidermal cells confirmed that ZmZLP1::GFP had been localised to the plasma membrane (Fig. 5*b*). Then the *IRT-ZmZLP1-GFP* and *IRT1-ZmZLP1* fragments were digested and cloned into the pFL61 vector. The resulting vectors were transformed into the ZHY3 yeast strain and fluorescence visualisation indicated that IRT1::ZmZLP1:: GFP successfully to target the PM (Fig. 5*c–e*). ZHY3 containing *IRT1-ZmZLP1* was cultured on s.d. media supplied with $ZnSO_4$ or EDTA. The yeast with *IRT1-ZmZLP1* was unable to grow on s.d. media supplemented with 0–100 μM EDTA but survived on zinc-supplemented media (Fig. 4). These results suggest that IRT1::ZmZLP1 might be responsible for transporting zinc from the inside to the outside of cells. To do further research, we transformed the pFL61-IRT1-ZmZLP1 vector into the DEY1457 (wild

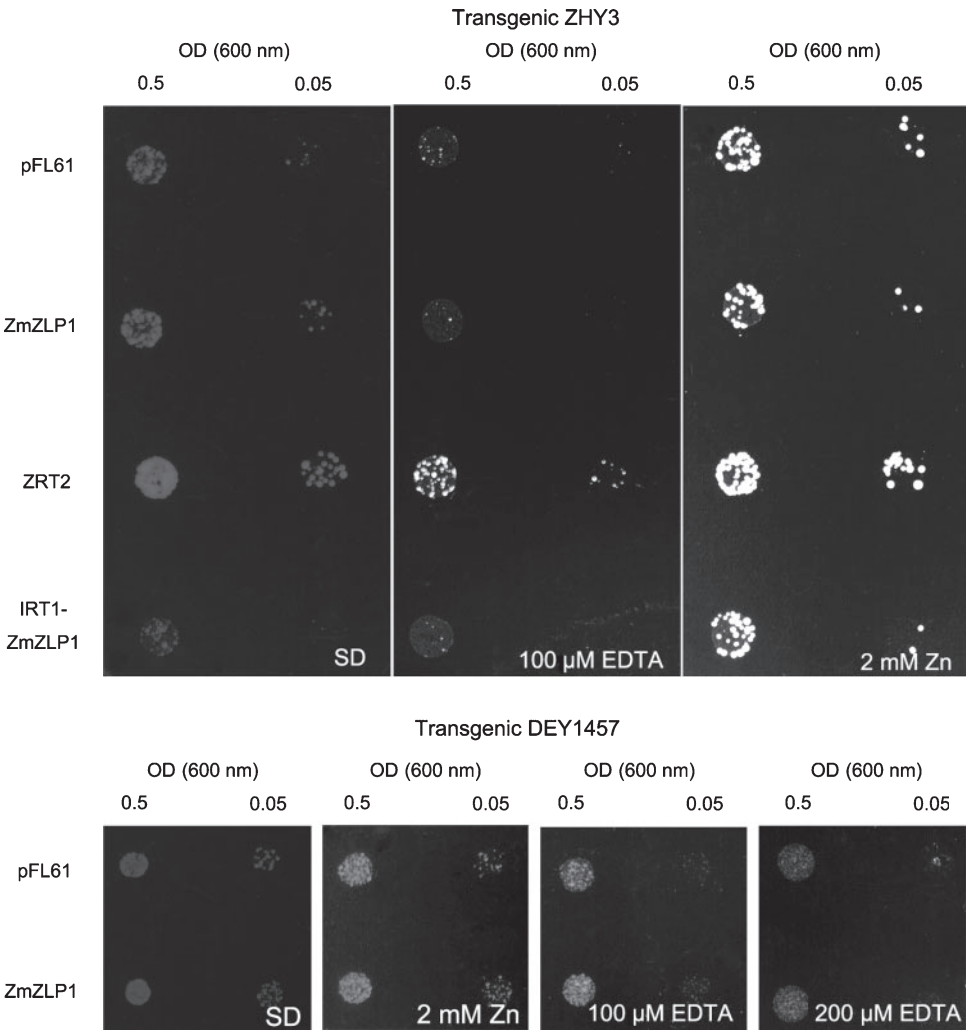


Fig. 4. Growth of transgenic yeast strains with *ZmZLP1* at 30°C. ZHY3 or DEY1457 transgenic cells with *ZmZLP1*,*ZRT2*, *IRT1-ZmZLP1* or *pFL61* were cultured in YPD liquid media over night, serially diluted and spotted onto s.d. media supplemented with 2 mM $ZnCl_2$ or 100 μM EDTA.

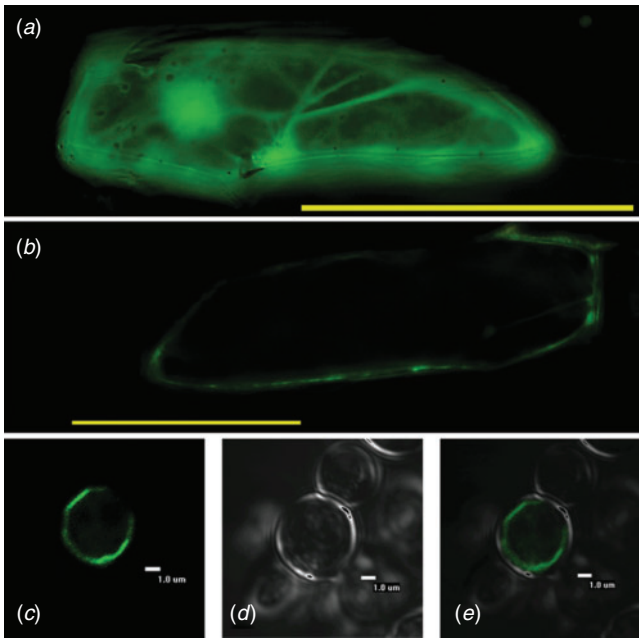


Fig. 5. Subcellular localisation of IRT1::ZmZLP1::GFP. Single *GFP* (*a*) and *IRT1-ZmZLP1-GFP* (*b*) driven by the CaMV 35S promoter were transformed into onion epidermal cells. IRT1::ZmZLP1::GFP fusion protein fluoresced in the plasma membrane of onion cells. (*c*) IRT1::ZmZLP1::GFP fluoresced in the yeast (ZHY3) cells (*d*), the same cells as in (*c*) visualised with transmitted light to show cell boundaries. (*f*), merged image of (*c*) and (*d*). Images were captured with a laser confocal microscope (Olympus BX51). (*a*, *b*), scale bar is 50 µm; (*c*–*e*), scale bar indicates 1 µm.

type) yeast strain and observed the growth of transformants on s.d. media supplemented with 0, 500, 750 µM EDTA. Similar results were observed in that DEY1457 with the pFL61-IRT1-ZmZLP1 vector resembled the control (with pFL61) on s.d. media but grew more weakly on the media supplemented with 500 or 750 µM EDTA compared with the control (Fig. 6). These results suggest that ZmZLP1 is probably a protein that ships zinc ions out of the ER. This is consistent with the opinion that ZIP proteins transport zinc or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm, as proposed by Eide (2006).

Growth of transgenic yeast at higher temperature

Deletion of YKE4, a known zinc transporter localised to the ER, resulted in a growth defect in yeast grown at an elevated temperature (36−38°C) on high zinc media (3.5–5.5 mM zinc) (Kumanovics *et al*. 2006). Therefore, we assumed that *ZmZLP1* transgenic cells had a phenotype contrary to that of the *yke4* mutant. DEY1457 with *ZmZLP1* was spotted onto YPD plate with or without $ZnSO_4$ and grown at 30 or 37°C for 36 h. As we hypothesised, *ZmZLP1* cells grew well at 37°C whether on YPD plates or high zinc media, but the control spots grew poorly (Fig. 7). This suggests that the transformation of *ZmZLP1* enhances resistance to heat stress.

ZmZLP1 *affects the UPR in transformants*

Zinc deficiency in yeast induces the UPR, a system normally activated by unfolded ER proteins. An indirect way to estimate the zinc levels in the ER is to test the transcript levels of the genes in the UPR pathway (Ellis *et al*. 2004). *HAC1* encodes the transcription factor that binds the unfolded protein response element (UPRE), which is found upstream of all genes that respond to the UPR in yeast (Kaufman 1999). We measured the transcript levels of *HAC1* in ZHY3 and DEY1457 yeast strains transformed with *ZmZLP1* or the empty vector pFL61 cultured in YPD (zinc sufficiency) or s.d. liquid media (zinc deficiency) at 30 or 37°C (heat stress). Semiquantitative RT–PCR was applied and the constitutively expressed gene *CMD1*, which encodes a calcium ion binding protein, was used as the reference. The results demonstrated that the *HAC1* levels in *ZmZLP1* transformants were higher than in the controls under zinc deficiency or heat stress (Fig. 8*a*).

To obtain a more precise profile of *HAC1* expression in *ZmZLP1* transformants, quantitative real-time PCR was conducted. Figure 8*b* shows the histograms for the mean values and standard errors of triplicate experiments conducted with different RNA preparations. Our real-time PCR results were consistent with the semiquantitative RT–PCR results (Fig. 8*a*).

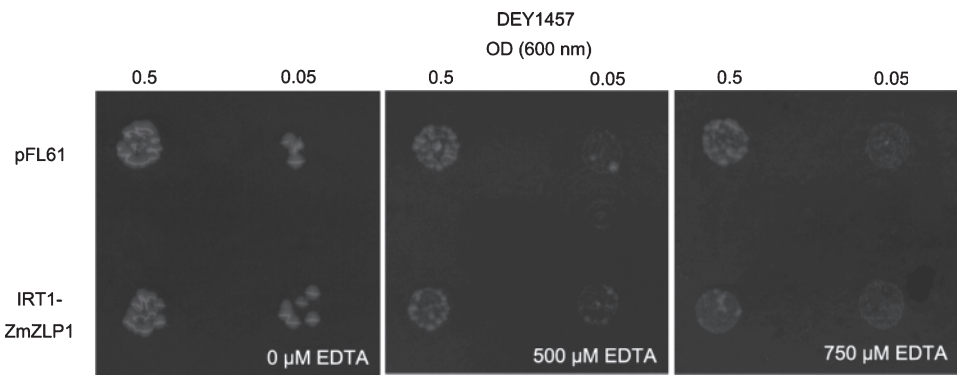


Fig. 6. Suppressed growth of DEY1457 cells transformed with *IRT-ZmZLP1*. DEY1457 transgenic cells were cultured on s.d. medium supplemented with 0, 500 or 750 µM EDTA. Similar to ZHY3 transgenic cells (Fig. 4), IRT1-ZmZLP1 in DEY1457 suppressed the growth of yeast cells in zinc-limited media (containing 500–750 µM EDTA). Photos were taken with an image scanner (Amersham).

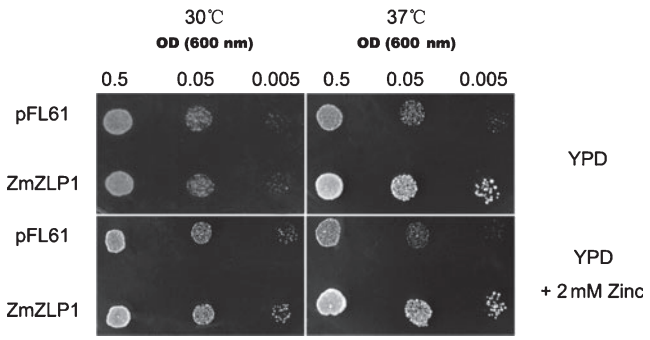


Fig. 7. *ZmZLP1* transformation enhances the resistance of yeast cells to heat stress. DEY1457 transgenic cells containing *ZmZLP1* or the empty vector (pFL61) were grown to mid-log phase in YPD, serially diluted, spotted onto the YPD medium with or without zinc supplementation, and grown at 30°C or 37°C for 36 h. *ZmZLP1* cells grew better than the control at 37°C on YPD media, with or without a zinc supply.

Compared with growth at 30°C in YPD media, *HAC1* expression was upregulated both in *ZmZLP1* transformants and in the control cells when deprived of zinc (s.d. media) or subjected to heat stress (37°C). In addition, higher levels of *HAC1* were observed in *ZmZLP1* transformants than in the control cells under normal growth conditions, which suggests that zinc levels in the ER of *ZmZLP1* transformants was lower than in the transgenic cells with the empty vector (Fig. 8). Thus, ZmZLP1 is probably involved in exporting zinc from the ER (Fig. 8).

Discussion

Unlike most ZIP family members that have a HRD (Guerinot 2000), ZmZLP1 lacks a histidine-rich domain in the variable region. This conserved motif was considered to be a potential metal-binding domain (Guerinot 2000). However, definitive evidence for the function of HRD is still absent. Both positive and negative effects of the participation of the HRD in metal

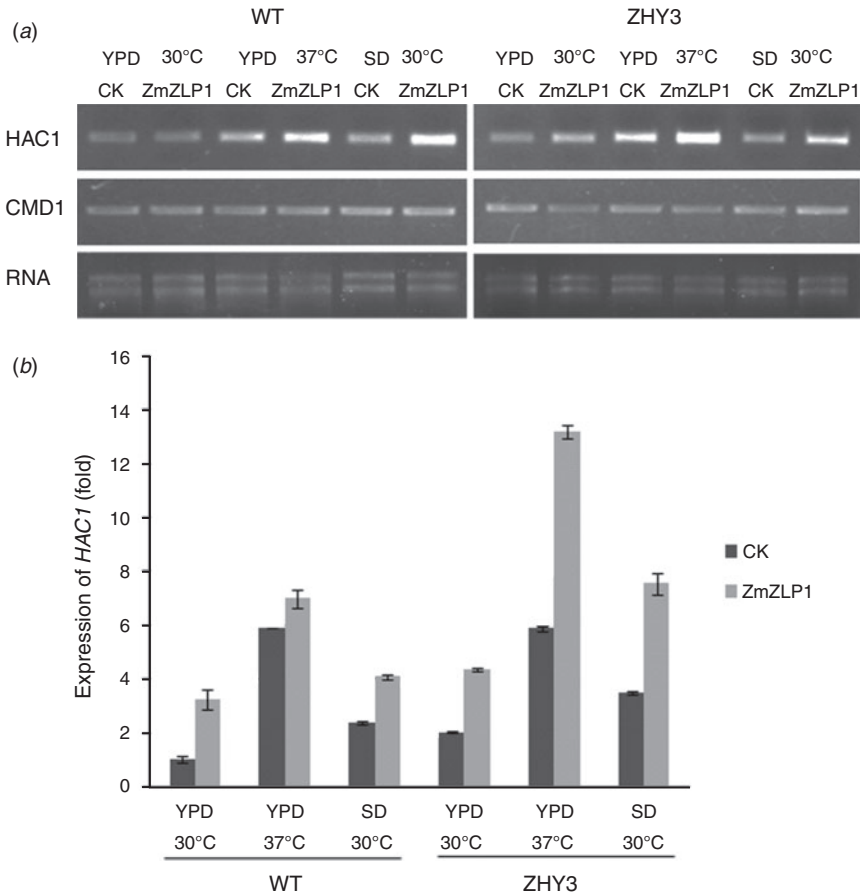


Fig. 8. Expression analysis of *HAC1* in ZHY3 or DEY1457 yeast cells transformed with pFL61-ZmZLP1 and pFL61. (*a*) Yeast cells were cultured in YPD medium and induced through zinc deficiency or heat stress. Total RNA was extracted using the Fast RNA Extraction from Yeast Cells kit (BioDev). PCR reactions were conducted for 5 min at 95°C, followed by 25 cycles of 30 s at 94°C, 30 s at 53°C (*CMD1*) or 56°C (*HAC1*), 30 s at 72°C, and a final extension of 10 min at 72°C. RT–PCR experiments were repeated three times independently. *HAC1* transcript levels increased in *ZmZLP1* transgenic cells compared with the control. (*b*) Quantitative real-time RT–PCR analysis. Transcript levels of *HAC1* in *ZmZLP1* transgenic yeast cells increased under conditions of zinc deficiency or heat stress and were higher than the controls.

binding and transport have been reported (Nishida *et al*. 2008). According to our results, ZmZLP1, which lacks the HRD, is a candidate zinc transporter localised to the ER. This supports the opinion that the HRD is not a crucial factor in binding metal.

As shown in Fig. 2, ZmZLP1 localised to the ER, so did SiPf40 (Xia *et al*. 2005) and NP_566669 (M-Y Wang and M Yuan, unpubl. data). Little research has been reported about the localisation of the other homologues compared with ZmZLP1 at Fig. 1. The signal peptide prediction using the method of hidden Markov models indicates that the signal peptide cleavage sites of these homologues all lie between glycine (G) and alanine (A) of TM1 (data not shown). Some differences lie in the putative signal peptides between ZmZLP1 and its homologues, both length and sequence, which suggest that these differences are not the crucial factors for proper localisation.

DEY1457 transformed with the *IRT1-ZmZLP1* fusion gene grew well on s.d. medium, suggesting that the capability of ZmZLP1 to ship out Zn is far less effective than that for zinc uptake of ZRT in yeast cells (Fig. 6). When the key zinc-uptake transporter proteins of yeast, ZRT1 and ZRT2, were deficient, IRT1::ZmZLP1 was more sensitive to zinc limitation. Different growth states were observed between the ZHY3 transformants with *IRT1-ZmZLP1* and the control on s.d. medium without EDTA (Fig. 4). Moreover, the growth suppression of the *IRT1-ZmZLP1* transformant on s.d. medium could be released by supplying a mere 20 μM $ZnCl_2$ (data not shown), demonstrating that the ability of IRT1::ZmZLP1 to transport zinc out of cells is not powerful. All these results are similar to the research about the *yke4* gene, which is considered to be an inessential yeast gene (Kumanovics *et al*. 2006).

As shown in Fig. 7, yeast cells with pFL61 grew poorly at 37°C, whereas *ZmZLP1* transformants grew well, suggesting that ZmZLP1 enhances yeast resistance to heat stress. Heat stress could cause unfolded protein to accumulate in the ER, triggering the unfolded protein response (Kaufman 1999), so we hypothesised that ZmZLP1 regulates transgenic cells resistance to heat stress through the UPR pathway. Evidence supporting the hypothesis lay in the fact that *HAC1* expression was upregulated by heat stress and the transcript level of *HAC1*, which encodes the transcription factor that binds to the UPR element, was higher in *ZmZLP1* transformants than in the controls under normal nutritional conditions (YPD media) (Fig. 8).

Both ZmZLP1 and YKE4 are ZIP proteins localised to the ER. Δ*yke4* cells have been demonstrated to be sensitive to high zinc levels (Kumanovics *et al*. 2006), whereas *ZmZLP1* transformants seemed not to be. At elevated temperatures, Δ*yke4* cells grew well on YPD but poorly on high zinc media (Kumanovics *et al*. 2006). However, *ZmZLP1* transformants grew well at 37°C on YPD media with or without zinc supplementation (Fig. 7). In addition, YKE4 was deemed to be a bidirectional zinc transporter, although no proof has been found that ZmZLP1 is capable of importing zinc into the ER. The difference between the growth of *ZmZLP1* transformants and the *yke4* mutant is probably because ZmZLP1 and YKE4 execute different functions in the ER zinc mechanism. Moreover, *ZmZLP1* is a gene from *Z. mays* while *YKE4* is from *S. cerevisiae*. Thus, interspecific diversity might be another reason for these differences.

Although ZmZLP1 shares high similarity with SiPf40, differences between them should not be overlooked. Expression data showed that *ZmZLP1* transcript was detected in the mature pollen and weakly in male inflorescences (Fig. 3); however, its homologue *SiPf40* expressed in all tissue and preferentially in millet hypocotyl and bud (Liu *et al*. 2009). In addition, distinct functions of them have been found. SiPf40 upregulated the number of plant branching (Liu *et al*. 2009) while overexpression of *ZmZLP1* gene did not cause extra branches in tobacco (data not shown). This suggests that Pf40 family members might perform diverse functions.

Several lines of evidence support the hypothesis that ZmZLP1 is involved in zinc metabolism. First, the results of bioinformatics analysis suggest that ZmZLP1 is a member of the ZIP family of metal ion transporters. Second, *ZmZLP1* expression in root is induced by zinc deficiency. Third, ZmZLP1 targeted to the plasma membrane through fusion to the signal peptide of AtIRT1 resulted in a growth defect of the transgenic yeast strains in zinc-limited media. Finally, in the UPR pathway, *HAC1* transcripts increased in *ZmZLP1* transformants, suggesting that zinc levels were reduced in the ER of *ZmZLP1* transformants. In summary, *ZmZLP1* encodes a putative ZIP transporter localised to the endoplasmic reticulum and its expression in roots is induced by zinc starvation. ZmZLP1 might be responsible for zinc transport from the lumen of the ER to the cytoplasm.

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References

Assuncao AGL, Costa Martins PDA, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell & Environment* **24**, 217–226. doi:10.1111/j.1365-3040.2001.00666.x

Burleigh SH, Kristensen BK, Bechmann IE (2003) A plasma membrane zinc transporter from *Medicago truncatula* is up-regulated in roots by Zn fertilization, yet down-regulated by arbuscular mycorrhizal colonization. *Plant Molecular Biology* **52**, 1077–1088. doi:10.1023/A:1025479701246

Chauhan RS (2006) Bioinformatics approach toward identification of candidate genes for zinc and iron transporters in maize. *Current Science* **91**, 510–515.

Cohen CK, Fox TC, Garvin DF, Kochian LV (1998) The role of iron-deficiency stress responses in stimulating heavy-metal transport in plants. *Plant Physiology* **116**, 1063–1072. doi:10.1104/pp.116.3.1063

Eckhardt U, Mas Marques A, Buckhout TJ (2001) Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. *Plant Molecular Biology* **45**, 437–448. doi:10.1023/A:1010620012803

Eide DJ (2006) Zinc transporters and the cellular trafficking of zinc. *Biochimica et Biophysica Acta* **1763**, 711–722. doi:10.1016/j.bbamcr.2006.03.005

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 5624–5628. doi:10.1073/pnas.93.11.5624

Ellis CD, Wang F, MacDiarmid CW, Clark S, Lyons T, Eide DJ (2004) Zinc and the Msc2 zinc transporter protein are required for endoplasmic reticulum function. *The Journal of Cell Biology* **166**, 325–335. doi:10.1083/jcb.200401157

Ellis CD, MacDiarmid CW, Eide DJ (2005) Heteromeric protein complexes mediate zinc transport into the secretory pathway of eukaryotic cells. *The Journal of Biological Chemistry* **280**, 28811–28818. doi:10.1074/jbc.M505500200

Gietz D, St. Jean A, Wood RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **20**, 1425. doi:10.1093/nar/20.6.1425

Grotz N, Guerinot ML (2006) Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta* **1763**, 595–608. doi:10.1016/j.bbamcr.2006.05.014

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 7220–7224. doi:10.1073/pnas.95.12.7220

Guerinot ML (2000) The ZIP family of metal transporters. *Biochimica et Biophysica Acta* **1465**, 190–198. doi:10.1016/S0005-2736(00)00138-3

Hofgen R, Willmitzer L (1988) Storage of competent cell for *Agrobacterium* transformation. *Nucleic Acids Research* **16**, 9877. doi:10.1093/nar/16.20.9877

Ishimaru Y, Suzuki M, Kobayashi T, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2005) OsZIP4, a novel zinc-regulated zinc transporter in rice. *Journal of Experimental Botany* **56**, 3207–3214. doi:10.1093/jxb/eri317

Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M (2004) Overview of mammalian zinc transporters. *Cellular and Molecular Life Sciences* **61**, 49–68. doi:10.1007/s00018-003-3148-y

Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes & Development* **13**, 1211–1233. doi:10.1101/gad.13.10.1211

Kumanovics A, Poruk KE, Osborn KA, Ward DM, Kaplan J (2006) YKE4 (YIL023C) Encodes a bidirectional zinc transporter in the endoplasmic reticulum of *Saccharomyces cerevisiae. The Journal of Biological Chemistry* **281**, 22566–22574. doi:10.1074/jbc.M604730200

Lasswell J, Rogg JE, Nelson DC, Rongey C, Bartel B (2000) Cloning and characterization of IAR1, a gene required for auxin conjugate sensitivity in *Arabidopsis*. *The Plant Cell* **12**, 2395–2408.

Lin JH, Li H, Zhang Y, Ron D, Walter P (2009) Divergent effects of PERK and IRE1 signaling on cell viability. *PLoS ONE* **4**, e4170. doi:10.1371/journal.pone.0004170

Liu Y, Feng X, Xu Y, Yu J, Ao G, Peng Z, Zhao Q (2009) Overexpression of millet ZIP-like gene (*SiPf40*) affects lateral bud outgrowth in tobacco and millet. *Plant Physiology and Biochemistry* **47**, 1051–1060. doi:10.1016/j.plaphy.2009.08.007

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* **163**, 16–20. doi:10.1016/0003-2697(87)90086-8

López-Millán AF, Ellis DR, Grusak MA (2004) Identification and characterization of several new members of the ZIP family of metal ion transporters in *Medicago truncatula. Plant Molecular Biology* **54**, 583–596. doi:10.1023/B:PLAN.0000038271.96019.aa

MacDiarmid CW, Gaither LA, Eide D (2000) Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae. The EMBO Journal* **19**, 2845–2855. doi:10.1093/emboj/19.12.2845

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, *et al*. (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiology* **126**, 1646–1667. doi:10.1104/pp.126.4.1646

Moreau S, Thomson RM, Kaiser BN, Trevaskis B, Guerinot ML, Udvardi MK, Puppo A, Day DA (2002) GmZIP1 encodes a symbiosis-specific transporter in soybean. *The Journal of Biological Chemistry* **277**, 4738–4746. doi:10.1074/jbc.M106754200

Nishida S, Mizuno T, Obata H (2008) Involvement of histidine-rich domain of ZIP family transporter TjZNT1 in metal ion specificity. *Plant Physiology and Biochemistry* **46**, 601–606. doi:10.1016/j.plaphy.2008.02.011

Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens. Proceedings of the National Academy of Sciences of the United States of America* **97**, 4956–4960. doi:10.1073/pnas.97.9.4956

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Research* **29**, e45. doi:10.1093/nar/29.9.e45

Plaza S, Tearall KL, Zhao FJ, Buchner P, McGrath SP, Hawkesford MJ (2007) Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens. Journal of Experimental Botany* **58**, 1717–1728. doi:10.1093/jxb/erm025

Ramesh SA, Shin R, Eide DJ, Schachtman DP (2003) Differential metal selectivity and gene expression of two zinc transporters from rice. *Plant Physiology* **133**, 126–134. doi:10.1104/pp.103.026815

Sambrook J, David WR (2001) 'Molecular cloning: a laboratory manual.' 3rd edn. (Cold Spring Harbor Laboratory Press: New York)

Schikora A, Thimm O, Linke B, Buckhout TJ, Muller M, Schmidt W (2006) Expression, localization, and regulation of the iron transporter LeIRT1 in tomato roots. *Plant and Soil* **284**, 101–108. doi:10.1007/s11104-006-0046-8

Taylor KM, Morgan HE, Johnson A, Nicholson RI (2004) Structure-function analysis of HKE4, a member of the new LIV-1 subfamily of zinc transporters. *The Biochemical Journal* **377**, 131–139. doi:10.1042/BJ20031183

Vert G, Briat JF, Curie C (2001) *Aribidopsis IRT2* gene encodes a root-periphery iron transporter. *The Plant Journal* **26**, 181–189. doi:10.1046/j.1365-313x.2001.01018.x

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat JF, Curie C (2002) IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell* **14**, 1223–1233. doi:10.1105/tpc.001388

Walkerpeach CR, Velten J (1994) Agrobacterium-mediated gene transfer to plant cells: co-integrate and binary vector systems. In 'Plant molecular biology manual'. (Eds SB Gelvin, RA Schilperoort) pp. 1–19. (Kluwer: Dordrecht, The Netherlands)

Weber M, Harada E, Vess C, Roepenack-Lahaye EV, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthese, a ZIP transporter and other genes as potential. *The Plant Journal* **37**, 269–281. doi:10.1046/j.1365–313x.2003.01960.x

Xia YF, Zhao Q, Yu JJ, Ao GM (2005) Study of subcellular localization of PF40. *Progress in Biochemistry and Biophysics* **32**, 1020–1025.

Yang X, Huang J, Jiang Y, Zhang HS (2009) Cloning and functional identification of two members of the ZIP (Zrt,Irt-like protein) gene family in rice (*Oryza sativa* L.). *Molecular Biology Reports* **36**, 281–287. doi:10.1007/s11033-007-9177-0

Zhao H, Eide D (1996) The yeast ZRT1 gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 2454–2458. doi:10.1073/pnas.93.6.2454

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