TcOPT3, a Member of Oligopeptide Transporters from the Hyperaccumulator Thlaspi caerulescens, Is a Novel Fe/Zn/Cd/Cu Transporter

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Abstract

Background: Thlaspi caerulescens is a natural selected heavy metal hyperaccumulator that can not only tolerate but also accumulate extremely high levels of heavy metals in the shoots. Thus, to identify the transporters involved in metal long-distance transport is very important for understanding the mechanism of heavy metal accumulation in this hyperaccumulator.

Methodology/Principal Findings: We cloned and characterized a novel gene TcOPT3 of OPT family from T. caerulescens. TcOPT3 was pronouncedly expressed in aerial parts, including stem and leaf. Moreover, in situ hybridization analyses showed that TcOPT3 expressed in the plant vascular systems, especially in the pericycle cells that may be involved in the long-distance transportation. The expression of TcOPT3 was highly induced by iron (Fe) and zinc (Zn) deficiency, especially in the stem and leaf. Sub-cellular localization showed that TcOPT3 was a plasma membrane-localized protein. Furthermore, heterogenous expression of TcOPT3 by mutant yeast (Saccharomyces cerevisiae) complementation experiments demonstrated that TcOPT3 could transport Fe2+ and Zn2+. Moreover, expression of TcOPT3 in yeast increased metal (Fe, Zn, Cu and Cd) accumulation and resulted in an increased sensitivity to cadmium (Cd) and copper (Cu).

Conclusions: Our data demonstrated that TcOPT3 might encode an Fe/Zn/Cd/Cu influx transporter with broad-substrate. This is the first report showing that TcOPT3 may be involved in metal long-distance transportation and contribute to the heavy metal hyperaccumulation.

Introduction

Metal hyperaccumulators can not only tolerate high concentration of heavy metals in the soils but also take them up actively and accumulate and distribute them to appropriate tissues at extreme high levels, thus make them very attractive for the remediation of heavy metal polluted soils [1]. A large amount of different metal hyperaccumulators have been recognized in different regions all over the world [2], among them, Thlaspi caerulescens, a Cd/Zn/Ni hyperaccumulator, has been used as a model plant to study the physiological and molecular mechanisms of heavy metal hyperaccumulation [3-4].

The efficient metal loading and unloading ability in the vascular systems was regarded as the key step of hyperaccumulating process for hyperaccumulators [5]. Nonetheless, knowledge about the mechanisms and proteins involved in transporting heavy metals from soil via the roots and stems into their storage sites is much limited. Transporter families, including ZIP (ZRT/IRT like Protein), Nramp (Natural Resistance and Macrophage Protein), MTP (Metal Tolerance Protein), HMA (Heavy Metal transporting P-type ATPase), ABC-type (ATP-binding cassette), COPT family (high-affinity Cu transporters) as well as YSL (Yellow stripl-Like transporters) families have been proved to involve in transit metal movements, they either act at the plasma membrane (PM) to transport metals into cytoplasm or redistribute metals from intracellular compartments into the cytoplasm [6,7,8,9]. Besides the above transporters families, novel transporters related to metal homeostasis are continuously under discovery, oligopeptide transporter (OPT) family is among those being focused recently [10,11,12]. OPT family is one of three families responsible for peptide transport; the other two are the ATP-binding cassette (ABC-type) transporters superfamily and the

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peptide transporter (PTR) superfamily [10]. Peptides, existing abundantly in xylem and phloem sap, are important for plant growth, development and signaling. Transport of peptides is taken as a more efficient means of nitrogen distribution than transport of individual amino acids, implying the important role of peptide transporters in long-distance transport of nutrients [10]. Besides the peptides, inorganic nutrients and transit metals have been reported as the substrates of peptide transporters in the recent studies. For instance, the ABC transporters AtMRP3 [13], ArATM3 [14] and AtPDR8 [15] are proposed to involve in heavy metal (Cd, Pb) resistance and movement.

The first OPT family is identified in the pathogenic yeast Candida albicans [16] and subsequently in Schizosaccharomyces pombe [17] and Saccharomyces cerevisiae [18]. Later, it is also found in bacteria, plants, and archaea [11], but still not in animals. OPT family is grouped into two distinct subfamilies, the yellow stripe (YS) clade and the peptide transport (PT) clade [11,12]. The function of YSI-like (YSL) transporter on transportation of metal-complex has been documented well in planta. For example, YSLs mediate long-distance transport of specific Fe species [19,20]. zmaYSI cloned from Zea mays is the founding member of YSL family, which functions in root Fe-phytosiderophore (Fe-PS) uptake from the soil [21]. HvYSI encoding a YSL transporter is a specific transporter for iron (III) in barley roots [22]. Since non-Poaceae species do not synthesize phytosiderophores, the substrates of YSL transporters in dicotyledonous plant are suggested to be Fe(II)/Fe(III)-nicotianamine (Fe-NA) functioning in the long-distance transporting system [23,24,25]. NA is suggested to be Fe(II)/Fe(III)-nicotianamine (Fe-NA) functioning in the long-distance transporting system.

We characterized its expression in different tissues, and demonstrated that TcOPT3 was encoded a plasma membrane-localized protein. To study the function of TcOPT3 in Fe and Zn transport, we tested whether expression of a TcOPT3 cDNA could restore the growth of yeast double mutants fet3fet4 (strain DEY1453) and zrt1zrt2 (strain ZHY3), which can not grow on the Fe- and Zn- sequence. We isolated the full-length cDNA from Thlaspi caerulescens by RACE PCR, and designated it as TcOPT3 (Genebank accession no. HQ69904). The coding region of TcOPT3 was 2211 bp, and a corresponding 737 amino-acid sequence was predicted.

TcOPT3 exhibited 79% identity with its homologous AtOPT3, and the deduced protein TcOPT3 showed 95% identity with AtOPT3 (Fig. 1). It contained 14 putative transmembrane domains (TM I-IV, XIV, Fig. 1) and two highly conserved motifs (NPG motif and KIPPR motif, Fig. 1) of OPT family, thus TcOPT3 is likely to be localized to membrane and owns the structure of a transporter protein.

From the rooted phylogenetic tree to compare TcOPT3 with OPTs and YSLs in A. thaliana and the related species. We can see that TcOPT3 belongs to the PT clade, moreover, it clusters together in one branch with the OPTs from T.cabralescens, B. juncea, A. hrota and A. thaliana and is most closely related to BjGT1 (Fig. 2).

**Results**

**Identification of TcOPT3 Gene**

Degenerated primers were designed from the most conserved regions according to the AtOPT3, BgGT1 and zmaGT mRNA sequence. We isolated the full-length cDNA from Thlaspi caerulescens by RACE PCR, and designated it as TcOPT3 (Genebank accession no. HQ69904). The coding region of TcOPT3 was 2211 bp, and a corresponding 737 amino-acid sequence was predicted.

**Tissue- and Organ- Specific Analysis of TcOPT3**

To study the function of TcOPT3 in Fe and Zn transport, we tested whether expression of a TcOPT3 cDNA could restore the growth of yeast double mutants fet3fet4 (strain DEY1453) and zrt1zrt2 (strain ZHY3), which can not grow on the Fe- and Zn-
limited medium respectively [33,34,35]. As expected, expression of TcOPT3 complemented the growth of mutants *fet3 fet4* and *zrt1 zrt2* (Fig. 7) when both metal sources in the medium were provided at a low concentration (10 μM Fe²⁺ or 50 μM Zn²⁺), suggesting that TcOPT3 can transport both Fe²⁺ and Zn²⁺.

**TcOPT3 Contributes to Fe/Zn/Cu/Cd Accumulation**

The role of TcOPT3 in the metal accumulation was further investigated by expressing TcOPT3 and empty vector in the wild-type yeast line (strain DY1455) cultured in the solutions containing 50 μM FeCl₃, 50 μM ZnSO₄, 50 μM CuSO₄ or 20 μM CdSO₄, respectively. Compared with those empty vector-expressing strains, the TcOPT3-expressing grew worse (Fig. 8), meanwhile the corresponding contents of these metal ions were significantly higher as well, suggesting that TcOPT3 can transport Fe²⁺ and Zn²⁺ into yeast cells (Fig. 9).

**Discussion**

The Oligopeptide transporters (OPTs) were initially characterized as small-peptide transporters. However, this has been challenged by their new functions in metal trafficking recently. The first report is from Wintz et al who found that heterologous expression of *AtOPT3* in yeast can transport Cu, Mn and Fe [28]. Later, *AtOPT6* and *AtOPT7* were also proved to transport Cd or Cd-glutathione chelate in yeast [31]. Recently, *AtOPT3* was further demonstrated to be involved in Fe homeostasis [30]. It was showed that heterologously expressed *AtOPT3* increased Cd sensitivity of *S. cerevisiae opt2* mutants and contributed to Cd accumulation [36]. All the above reports indicate the potential role of OPTs in transit metal translocation. *Thlaspi caerulescens*, a Cd/Zn/Ni hyperaccumulator, ecotype of Ganges, from southern France, can accumulate 10 000 mg Cd kg⁻¹ at shoot dry weight base [3,37]. In this study, we cloned and elucidated the function of a novel member of OPT family, *TcOPT3* from *T. caerulescens*.

**Role of TcOPT3 in Metal Transport**

AtOPT3 has been proved to play a role in Fe homeostasis in *A. thaliana* [30]. Here, we also found that heterologous expression of TcOPT3 in *Saccharomyces cerevisiae* rescued the growth of Fe-depleted mutant *fet3 fet4* (Fig. 7), suggesting that TcOPT3 can transport Fe too. As *T. caerulescens* is also a Zn hyperaccumulator, we tested the possible role of TcOPT3 in transporting Zn. As expected, TcOPT3 restored the growth of Zn-uptake-defective mutant *zrt1 zrt2*. Furthermore, heterologous expression of TcOPT3 negatively affected the growth of *S. cerevisiae* when treated with elevated Fe, Zn, Cd, or Cu (Fig. 8) as more Fe, Zn, Cd or Cu was accumulated in the transformed yeast (Fig. 9), suggesting a broader substrates specificity for TcOPT3. The broad substrate specificity of cytoplasmic transition metal importers may ensure their universal potential to fulfill different developmental stages, without perpetuating the consequences of limited metal transporter specificity throughout the plant [7], while the specificity of transition metal export from cytoplasm may serve to establish specificity through the differential storage of transition metals in specific tissues of cell types. Therefore, the specificity of the efflux transporters appears to be more pounced than that of the influx transporters. For example, the Pi⁰-type ATPases HMA3 from *T. caerulescens* showed high specificity for Cd, that serves to efflux Cd into vacoule [38]. As *T. caerulescens* can also accumulate Ni, we tested the possibility of transporting nickel (Ni), and found that though the growth was negatively affected by Ni addition (Fig. S1), the content in the yeast did not change much (Fig. S2), suggesting that TcOPT3 does not transport Ni, and there must be other transporters responsible for Ni hyperaccumulation, such as TcYSL3 [19]. Furthermore, we still found that TcOPT3 did not transport Pb into yeast too (Fig. S1, S2).

Roles of membrane transporters that are responsible for hyperaccumulation include uptake, efflux, translocation and sequestration of transit metals. Metal transporters herein identified include ZIP (ZRT/IRT like Protein), Nramp (Natural Resistance and Macrophage Protein), MTP (Metal Resistance Protein), and HMA (Heavy metal/CpX-type ATPases) families in *A. thaliana* and hyperaccumulators such as *T. caerulescens* and *A. halleri* [4,38,39,40,41]. Transporters of the HMA family and the MTP family that are involved in metal efflux from the cytoplasm, either by movement across the plasma membrane (PM) or into organelles, whereas metal uptake transporters include the NRAMP family and the ZIP families that are transporters either act at the PM to move metals into the cytoplasm or remobilize metals from intracellular compartments into the cytoplasm [6].

In this study, heterologous expression of *TcOPT3* in *S. cerevisiae* increased the sensitivity of yeast by accumulating more metals, indicating that TcOPT3 function as an uptake transporter for heavy metals (Fig. 8, 9).

**Potential Role of TcOPT3 in Plant Long-distance Metal Transport and Hyperaccumulation**

In this study, qRT-PCR results showed that TcOPT3 was preferentially expressed in the aerial parts (stem and leaf) than roots both under normal condition or Fe/Zn deficient conditions (Fig. 3, 5). While in Arabidopsis, *AtOPT3* expressed stronger in flower, leaf and root, but relatively lower in the stem [42]. It may be due to the different growth stage tested, but most probably, the different expression patterns between *AtOPT3* and *TcOPT3* may suggest their different roles in trafficking heavy metals within plants. It was reported that reduced expression of AtOPT3 in the Arabidopsis mutant *opt3-2* resulted in the accumulation of very high levels of Fe in tissues except seeds [30]. Recently, it was showed that heterologously expressed AtOPT3 increased Cd sensitivity of *S. cerevisiae opt2* mutants and contributed to Cd accumulation [36]. Consistent with the role of OPT3 in Cd detoxification, the early-stage seedlings of *A. thaliana* *opt3-3* knockdown allele were extremely sensitive to Cd. In contrast, leaves of hydroponically-grown *opt3-3* mature plants were more tolerant to Cd compared to the wild type [36]. We also found that heterologously expressed TcOPT3 increased heavy metal (Cu/Cd) sensitivity of *S. cerevisiae* and contributed to metal accumulation, suggesting a similar role of TcOPT3 in heavy metal trafficking in plant. However, little evidence from overexpression OPTs in metal distribution is reported to date. Given the different expression
patterns between \textit{TcOPT3} and \textit{AtOPT3}, it is interesting to investigate the potential role of \textit{TcOPT3} in hyperaccumulation in \textit{T. caerulescens}. The tissue specific overexpression of \textit{TcOPT3} is highly desirable in order to verify its potential role in creating metal hyperaccumulators for the purpose of phytoremediation of heavy metal polluted environments in future.

**Figure 2.** Phylogenetic tree of OPT gene transporters based on the amino acid sequences. Dendogram showing sequence comparisons of several known members of the PT family from different species. Analysis was performed using the CLUSTAL X method in MEGA (4.0) using Neighbor-Joining method (Tamura K et al., 2007). Accession numbers are as follows: AtOPT1, NP_200404.1 GI: 15241078; AtOPT2, NP_172464.1 GI:15218331; AtOPT3, NP_567493.5 GI:240255930; AtOPT4, NP_201246.1 GI:15237689; AtOPT5, NP_194389.1 GI:15236800; AtOPT6, NP_194503.1 GI:15234254; AtOPT7, NP_192815.1 GI:15236912; AtOPT8, NP_564525.1 GI:18402162; AtOPT9, NP_200163.1 GI:15238761; AIOPT3, XP_002868139.1 GI:1529800510; AtYSL1, NP_567694.2 GI:79484897; AtYSL2, NP_197826.2 GI:79518939; AtYSL3, NP_200167.2 GI:145359208; AtYSL4, NP_198916.2 GI:42568235; AtYSL5, NP_566584.1 GI:18401590; AtYSL6, NP_566806.1 GI:18405020; AtYSL7, NP_1767501 GI:15218799; AtYSL8, NP_564525.1 GI:18402162; TcYSL1, ABB76761.1 GI:82468791; TcYSL2, ABB86762.1 GI:82468793; TcYSL3, ABB76763.1 GI:82468795; CaOPT1, AAB669628.1 GI:2367386; CaOPT3, ABD17824.1 GI:87045965; ScOPT1, NP_012323.1 GI:6322249; ZmGT, ACL82964.1 GI:220901863 BjGT1, CAD91127.1 GI:30722286. doi:10.1371/journal.pone.0038535.g002
As proposed by Milner and Kochian [4], Zn hyperaccumulation of *T. caerulescens* appears to include at least five physiological events: (1) Increased Zn\(^{2+}\) influx across the root-cell PM; (2) Reduced Zn sequestration in the root-cell vacuole; (3) Increased Zn transport into the xylem and via the xylem to the shoot; (4) Increased Zn\(^{2+}\) influx into leaf mesophyll cells; and (5) Zn and Cd stored primarily in leaf epidermal cells. In *in situ* hybridization results showed that, *TcOPT3* was highly expression in vascular cells both in roots and shoots (Fig. 4). This expression pattern is similar to the previous report on the expression of AtOPT3 in *A. thaliana*, which is also highly expressed in the vascular tissues both in light-grown seedlings and adult plants [42]. As efficient translocation of metals from root to shoot is one of the most important hallmarks of hyperaccumulators. Thus, efficient transporters expressed in loading and unloading tissues are fundamental for hyperaccumulation. For example, HMA4 is characterized metal transporter primarily localized in the root stele. It plays a critical role in loading heavy metals into xylem for long-distance transportation from root to shoot not only in *T. caerulescens* but also in another hyperaccumulating plant *A. hallii* [43,44,45]. *TcHMA3*, another member of HMA family was proved to be a tonoplast-localized transporter highly specific for Cd, which is responsible for sequestration of Cd into the leaf vacuoles, and that a higher expression of this gene is required for Cd hypertolerance in the Cd-hyperaccumulating ecotype of *T. caerulescens* [38]. Uptake transporters responsible for hyperaccumulation, *TcIRT1*, *TcIRT2*, *TcZNT1* and *TcZNT5* of ZIP families in *T. caerulescens*, for instance, were expressed only in roots but not in leaves [46]. The yellow-stripe 1-like (YSL) subfamily is included in the OPT superfamily, some of which was proved to be involved in loading and unloading of nicotianamine-metal chelates from the vascular tissues. *TcYSLs* of YSL transporters from *T. caerulescens*, especially for *TcYSL3* and *TcYSL7*, were expressed in xylem parenchyma and phloem [19]. Furthermore, *TcYSL3* was shown to transport Ni-NA chelates. Here we demonstrated that *TcOPT3* is a plasma membrane-localized protein, and can transport metals into yeast cells (Fig. 6, 8). *TcOPT3* was expressed primarily in shoots, especially under nitrogen deficiency, indicating their specificity in metal uptake and transport in shoots (Fig. 3, 5). As it is highly and constructively expressed in vascular system, including xylem, phloem and vein, it is reasonable to assume that it plays important roles in unloading of nutrients and heavy metals in those tissues, thus consists of an important component in the long-distance transportation system. However, it needs further investigation.

In this study, ecotype Ganges instead of ecotype Prayon was studied. Considered that heavy metal hyperaccumulation varies greatly among different ecotypes, it is interesting to further confirm the expression and functional analysis of *TcOPT3* in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. We have analyzed the amino acids sequence and expression of *TcOPT3*-p preliminarily; however, there is no significant difference between *TcOPT3*-g and *TcOPT3*-p expression pattern. Further studies need to be conducted in the future.

In conclusion, we demonstrated that *TcOPT3* in a metal hyperaccumulator, *Thlaspi caerulescens*, was an Fe/Zn/Cu/Cd influx transporter with non-specificity substrate. This is the first report showing that *TcOPT3* gene may be involved in metal long-distance transport systems that contribute to heavy metal hyperaccumulation.

### Materials and Methods

#### Plant Growth

Seeds of *Thlaspi caerulescens* J. & C. Presl, ‘Ganges’ ecotype, were surface sterilized by 75% alcohol and 10% NaClO₄, then stored at 4°C for 3 days. Seeds were germinated on agar with modified MS medium (with addition 270 μM ZnSO₄ in Murashige and Skoog, Sigma, U.S.) for two weeks at 14-h/25°C day and a 10-h/18°C night regime, a light intensity of 100 μmol photons m⁻² s⁻¹. Then the seedlings were transferred to modified Hoagland nutrient solution (2 mM Ca(NO₃)₂, 0.1 mM KH₂PO₄, 0.5 mM MgSO₄, 0.1 mM KCl, 0.7 mM K₂SO₄, 0.1 mM Fe-EDTA, 10 μM H₂BO₃, 0.5 μM MnSO₄, 10 μM ZnSO₄, 0.2 μM CuSO₄ and 0.01 μM (NH₄)₂MoO₄, pH 5.5).
has been deposited in Genbank with the accession number of HQ699884.

Sequence Comparisons
The predicted amino-acid sequence from TcOPT3 cDNA and other selected OPT protein sequences were aligned using the CLUSTAL W program, version 1.8 (Thompson et al., 1994). The putative trans-membrane domains were predicted by the TMHMM (version 2.0; http://www.cbs.dtu.dk/services/TMHMM/). The putative amino acid sequences were aligned with the program Clustal X Version 1.8 [47] and viewed by GeneDoc version 2.6 [48]. Phylogenetic trees were constructed with the neighbor-joining algorithm using the program with MEGA 4 (http://www.megasoftware.net) [49]. Hydrophilicity plots for TcOPT3 was generated based on the Kyte and Doolittle (1982) method using Protan sequence analysis software (DNASTAR) under default parameters.

Quantitative PCR Analysis of TcOPT3 Expression
Total RNA was isolated from roots, stems and leaves of one-month-old T. caulescens and treated by DNase I (RNase free) as described above. RT-PCR with oligo d(T)-anchor primer were performed with PrimeScript RT (Perfect Real Time) reagent kit described above. RT-PCR with oligo d(T)-anchor primer were performed with PrimeScript RT (Perfect Real Time) reagent kit (TaKaRa, China). For real-time RT-PCR, 2 μl of the diluted (1:5) cDNA products were used as templates with 5 μl SYBR Pre mix (Takara, China), 0.5 μl primers (10 μM each) and 2.5 μl water. Calculation of the ΔCp values was performed as described [50]. Primers were listed in Table 1.

In Situ Hybridization
One-month-old seedlings grown on hydroponic culture were obtained. Plant materials were fixed in potassium phosphate buffer (0.1 M, pH 7.4) containing 4% paraformaldehyde overnight at 4°C, then dehydrated in ethanol series, and embedded in paraffin. A gene-specific fragment containing the 416 bp fragment cross 3'UTR of TcOPT3 was amplified by PCR (primers were shown in Table 1) and cloned into pSPT19 vectors. Sense and antisense probes were synthesized using SP6 and T7 primers with DIG RNA Labeling kit according to the manufacturer’s instructions (Roche, USA). In-situ hybridization was performed as described previously [19,51].

GFP Fusion and Subcellular Localization
For yeast transformation and functional complementation, the Saccharomyces cerevisiae (Meyen) E.C. Hansen knock-out strain fet3Δtet4 (DEY1455; MATa/MATα ade2–1 oc can1 his3 leu2 trp1 ura fet3–2::HIS3 fet4–1::LEU2) [33], zrt1zrt2 (ZHY3; MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3) [34,35]; and its parent strain DY1455 (MATaz ade2–1oc can1 his3 leu2 trp1 ura3) were used, which is defective in low- and high-affinity iron or zinc uptake, respectively. The ORF of TcOPT3 was amplified by PCR with primers (see Table 1 for primer sequences), which was then cloned into the pGEM-T Easy (Promega) vector, digested with NotI, and subsequently cloned into yeast binary vector pFL61 [53] to form TcOPT3-pFL61. Constructs were sequenced to ensure the correct orientations of the inserts and correct sequences. fet3Δtet4 transformants were selected on SD medium lacking uracil (SD-ura) and supplemented with 10 μM FeCl3, zrt1zrt2 transformants were selected on SD-ura and supplemented with 50 μM ZnSO4.

Yeast Complementation
For yeast transformation and functional complementation, the Saccharomyces cerevisiae (Meyen) E.C. Hansen knock-out strain fet3Δtet4 (DEY1455; MATa/MATα ade2–1 oc can1 his3 leu2 trp1 ura fet3–2::HIS3 fet4–1::LEU2) [33], zrt1zrt2 (ZHY3; MATa ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3) [34,35]; and its parent strain DY1455 (MATaz ade2–1oc can1 his3 leu2 trp1 ura3) were used, which is defective in low- and high-affinity iron or zinc uptake, respectively. The ORF of TcOPT3 was amplified by PCR with primers (see Table 1 for primer sequences), which was then cloned into the pGEM-T Easy (Promega) vector, digested with NotI, and subsequently cloned into yeast binary vector pFL61 [53] to form TcOPT3-pFL61. Constructs were sequenced to ensure the correct orientations of the inserts and correct sequences. fet3Δtet4 transformants were selected on SD medium lacking uracil (SD-ura) and supplemented with 10 μM FeCl3, zrt1zrt2 transformants were selected on SD-ura and supplemented with 50 μM ZnSO4.

Figure 4. Localization of TcOPT3 expression by in situ hybridization. (A,C,E,G,I) represents hybridization with TcOPT3 antisense probe. (B,D,F,H,J) shows hybridization with the sense probe (negative control). In situ hybridization of the sense and antisense TcOPT3 probes to sections of Thlaspi caulescens root tissues (A) to (D), stem tissues(E,F,J,L) and leaf tissues(G,H). Abbreviation: c, cortex; ep, epidermis; p, pericycle; ph, phloem; rh, root hair; x, xylem; cb, cambium; ve, vein; me, mesophyll; vc, vascular cambium.

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Figure 5. Effect of element deficiency on mRNA expression of the TcOPT3 gene. Real-time RT-PCR expression analysis of the TcOPT3 gene expression in roots (R), leaves (L) and stems (S) with the treatment of Fe or Zn deficient for 1, 2, 4 days. The ΔCp values were calculated as follows: CP of target gene (TcOPT3) – CP of constitutive control gene (ubiquitin-conjugating enzyme), where the CP value is the fractional cycle number of crossing point (CP). The ΔCp values represent the mean of three technical replicates (±SD) of one experiment representative of three independent experiments. Relative transcript levels (RTL) were calculated as follows: RTL = 2 −ΔCp.

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Figure 6. Sub-cellular localization of TcOPT3 protein. Onion epidermal cells transiently co-transformed with TcOPT3::GFP and pm-rk (Plasma membrane marker). (A) Fluorescence image of epidermal cell expressing the p35S::EGFP fusion protein. (B) Fluorescence image of epidermal cell expressing the pm-rk. (C) Merged fluorescence image of epidermal cell expressing the p35S-TcOPT3::EGFP fusion protein and pm-rk marker. doi:10.1371/journal.pone.0038535.g006

Figure 7. Complementation of the fet3fet4 and ZHY3 (zrt1ztr2) yeasts mutant by T. caerulescens cDNAs. Yeast strains defective in iron uptake (fet3fet4) and zinc uptake (zrt1ztr2) were transformed with pFL61 (empty vector) and pFL61-TcOPT3. Serial dilutions of yeast cells were dropped onto a low-zinc medium (LZM) supplemented with 50 μM ZnSO₄ (A) and a low-iron medium (LIM) supplemented with 10 μM FeCl₃ (B) assayed for growth on SD-ura plates. The entire experiment was performed twice. doi:10.1371/journal.pone.0038535.g007
For complement test, *fel3 fel4* and *zt1 zt2* transformants were grown overnight in liquid SD-ura and supplemented with 10 mM FeCl$_3$ or 50 mM ZnSO$_4$, respectively. Yeast cells were recovered by centrifugation, resuspended in SD-ura (without added Fe or Zn) at an OD$_{600}$ of 1, 0.1, 0.01 and 0.001. Then 10 µL of each culture was spotted on SD-ura plates; plates were incubated at 30°C for 2 d and then photographed.

**Figure 8. Growth of the wild-type (DY1455) and TcOPT3-transformed yeast cells under different metal supplies.** Yeast cells were grown to an OD$_{600}$ of 1.0, then supplemented with 50 µM FeCl$_3$, 50 µM ZnSO$_4$, 20 µM CdCl$_2$ or 50 µM CuSO$_4$ respectively. Data are the means ± SE per experiment (n = 3), P<0.05 by Student’s t-test. doi:10.1371/journal.pone.0038535.g008

**Figure 9. Heavy metal accumulation of wild-type (DY1455) and TcOPT3-transformed yeast cells.** Zn, Fe, Ni, Cd and Cu accumulation in yeast transformants. Metal accumulation was conducted in liquid SD media supplemented with 50 µM FeCl$_3$, 50 µM ZnSO$_4$, 20 µM CdCl$_2$, or 50 µM CuSO$_4$ respectively. Data are the means ± SE per experiment (n = 3), P<0.05 by Student’s t-test. doi:10.1371/journal.pone.0038535.g009
Heavy Metal Tolerance in Yeast

Wild-type yeast DY1455 was transformed with TcOPT3-pFL61, or with pFL61, which served as a negative control. DY1455 transformants were grown on liquid SD-ura medium overnight at an OD 600 of 1. After washed by CaCl₂ and centrifugation, resuspended in SD-ura with 50 mM FeCl₃, 50 mM ZnSO₄, 20 mM CdCl₂, or 50 mM CuSO₄ were grown for another 0, 6, 12, 24 hours. After measured the OD values and washed by CaCl₂ to remove adsorbed transit metals in apoplast cells, aliquots of yeast cells were taken for measurement of metal accumulation.

Elemental Analysis

For metal accumulation, yeast cells were concentrated at 5,000 rpm for 5 min and washed by 0.5 mM CaCl₂ for 5 min twice to remove adsorbed transit metals from the yeast cell walls. Then cell sediments were dried at 70°C for 2 days, and digested in HNO₃. Metal concentration in the digested solution was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES; IRIS/AP optical emission spectrometer).

Statistics

Data were statistically analyzed using analysis of variance (ANOVA) in Origin 8, and tested for significant (P # 0.05) treatment differences using Student’s t-test.

Supporting Information

Figure S1 Growth of the wild-type (DY1455) and TcOPT3-transformed yeast cells under different metal supplies. Yeast cells were grown to an OD600 of 1.0, and then supplemented with 100 mM PbNO₃ or 100 mM NiSO₄ respectively. Data are the means ± SE per experiment (n = 3), P < 0.05 by Student’s t-test. (EPS)

Figure S2 Heavy metal accumulation of wild-type (DY1455) and TcOPT3-transformed yeast cells. Pb and Ni accumulation in yeast transformants. Metal accumulation was conducted in liquid SD media supplemented with 100 mM PbNO₃ or 100 mM NiSO₄ respectively. Data are the means ± SE per experiment (n = 3), P < 0.05 by Student’s t-test. (EPS)

Author Contributions

Conceived and designed the experiments: SJZ YTH FM GXL JLY. Performed the experiments: YTH FM WWC JYY. Analyzed the data: YTH FM SJZ. Contributed reagents/materials/analysis tools: ZYX CYX. Wrote the paper: SJZ YTH.

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Table 1. Primers used for PCR amplification of in TcOPT3 cDNAs, 5’ and 3’ RACE, qRealTime-PCR, and plasmid constructions in order of their first mention in “Materials and Methods”.

| Purpose                                      | Name                | Sequence (5’-3’)                                        |
|----------------------------------------------|---------------------|--------------------------------------------------------|
| RACE PCR                                     | TcOPT3-GSP-F        | CAGTAAACAGACAGGAGAGAT                                  |
|                                              | TcOPT3-GSP-R        | CAGCAGGTTGGCTCAAGGTA                                   |
|                                              | TcOPT3-5’R-1-R      | ACCCTGCTGAGGCCGTAGCTATTA                               |
|                                              | TcOPT3-3’R-1-F      | ACATCACTGGCTGGTCTGATCTG                                |
|                                              | TcOPT3-5’R-2-R      | CGACGATTGATGGCAGAAAGG                                  |
|                                              | TcOPT3-3’R-2-F      | CTTCAAAATGCTCAAGTCTTCT                                  |
| Quantified-RealTime PCR                      | qRT-TcOPT3-F        | CCGAGGACAGACAGGTTG                                    |
|                                              | qRT-TcOPT3-R        | GCGTTGCGCTGGCGATGT                                    |
|                                              | TcUBQ-F             | GGAGCCCCCGTTGGAC                                     |
|                                              | TcUBQ-R             | CGGGGAGGCCGGAGT                                      |
| in situ hybridization                        | TcOPT3-Probe-S      | CCGGAAATTGCTATCTTCCACCGCGGGT                           |
|                                              | TcOPT3-Probe-A      | CCAAGCTTATGGGCAACAGCAGGATG                            |
| Plasmid of GFP fusion                        | TcOPT3-EGFP-F       | AAAAGTACTATGGAGCGAGAAGCTAAT                            |
|                                              | TcOPT3-EGFP-R       | TCCCCGCGGAAACACAGCAGCAGGCTTAACACTCT                    |
| Plasmid of yeast transformation              | TcOPT3-pFL61-F      | ATAGAAATGCGCCCGATGACGACAGAGGCTAA                       |
|                                              | TcOPT3-pFL61-R      | ATAGAAATGCGCCCGCAATCTTAGAAACACAGGACGC                 |

F, Forward; R, Reverse, S, Sense; A, Anti-sense.

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