Metal tolerance protein MTP6 affects mitochondrial iron and manganese homeostasis in cucumber

Magdalena Migocka1,*, Ewa Maciaszczyk-Dziubinska2, Karolina Malas1, Ewelina Posyniak3 and Arnold Garbiec3

1 University of Wroclaw, Institute of Experimental Biology, Department of Plant Molecular Physiology, Kanonia 6/8, 50–328 Wroclaw, Poland
2 University of Wroclaw, Institute of Experimental Biology, Department of Genetics and Cell Physiology, Kanonia 6/8, 50–328 Wroclaw, Poland
3 University of Wroclaw, Institute of Experimental Biology, Department of Animal Developmental Biology, Sienkiewicza 21, 50–335 Wroclaw, Poland

* Correspondence: magdalena.migocka@uwr.edu.pl

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Abstract

Members of the cation diffusion facilitator (CDF) family have been identified in all kingdoms of life. They have been divided into three subgroups, namely Zn-CDF, Fe/Zn-CDF, and Mn-CDF, based on their putative specificity to transported metal ions. The plant metal tolerance protein 6 (MTP6) proteins fall into the Fe/Zn-CDF subgroup; however, their function in iron/zinc transport has not yet been confirmed. Here, we characterized the MTP6 protein from cucumber, Cucumis sativus. When expressed in yeast and in protoplasts isolated from Arabidopsis cells, CsMTP6 localized in mitochondria and contributed to the efflux of Fe and Mn from these organelles. Immunolocalization of CsMTP6 in cucumber membranes confirmed this association with mitochondria. Root expression and protein levels of CsMTP6 were significantly up-regulated in conditions of Fe deficiency and excess, but were not affected by Mn availability. These results indicate that MTP6 proteins contribute to the distribution of Fe and Mn between the cytosol and mitochondria of plant cells, and are regulated by Fe to maintain mitochondrial and cytosolic iron homeostasis under varying conditions of Fe availability.

Keywords: CDF (cation diffusion facilitator) family, Cucumis sativus, iron, manganese, mitochondria, MTP (metal tolerance protein) family.

Introduction

Iron plays an essential role in many housekeeping cellular functions, including respiration, photosynthesis, Fe–S cluster assembly, and heme synthesis, but it can also be highly reactive and toxic as a catalyst of the Fenton reaction when present in excess (Winterbourn, 1995; Briat et al., 2010). The maintenance of iron homeostasis is particularly relevant for plants, which can find themselves exposed to different Fe regimes and which require continuous Fe delivery for chlorophyll synthesis and photosynthetic reactions. The rate of Fe uptake and storage in plants is also important for human nutrition, since it may significantly affect the yield and nutritional value of crops. Hence, a more comprehensive understanding of the molecular mechanisms that regulate plant iron homeostasis would be beneficial for the breeding or engineering of crops that are enriched in Fe and more tolerant of Fe-deficient soils.

Over the last decade, the molecular mechanisms underlying Fe acquisition and uptake by plants have been identified. They include the key genes involved in two different strategies...
of iron uptake: the reduction strategy used by dicotyledonous and non-graminaceous monocotyledonous species (strategy I), and the chelation strategy used by graminaceous species (strategy II). Iron-uptake strategy I involves genes encoding plasma membrane Fe^{3+}-chelate reductase FRO2 and the Fe^{2+} transporter IRT1 (iron-regulated transporter 1), which are involved in the reduction of soil Fe^{3+} to Fe^{2+} followed by the transport of ferrous ions into the root cells (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2002). The genes involved in iron-uptake strategy II encode proteins involved in the synthesis and secretion of hexadentate Fe^{3+}-chelating compounds (phytosiderophores, mugineic acid) and the specific plasma membrane Fe^{3+}-phytosiderophore importer YSI (yellow stripe 1) (Curie et al., 2001). Members of the YSL (yellow stripe-like) family are also involved in the long-distance transport between different plant tissues and organs (Curie et al., 2009).

In contrast to iron uptake, the partitioning of Fe between organelles in plant cells and the regulation of this process are less well understood. The regulation of intracellular compartmentation of Fe is likely to determine the level of storage and tolerance. A few proteins involved in Fe transport across the vacuolar membrane have already been identified, and these include VIT1 (vacuolar iron transporter) and the MTP8 (metal tolerance protein 8) transporter, which are involved in the loading of iron into the vacuole, and the natural resistance-associated macrophage proteins NRAMP3 and NRAMP4, which release Fe from the vacuoles in response to deficiency (Thomine et al., 2003; Lanquar et al., 2005; Kim et al., 2006; Chu et al., 2017; Eroglu et al., 2017). It has been shown in Arabidopsis that all these four proteins play important roles during embryo development and seed germination. VIT1 is expressed in the endodermal cells surrounding the embryonic vasculature, whereas MTP8 localizes in the hypocotyl cortex cells and sub-epidermal cells of the embryonic cotyledons (Kim et al., 2006; Chu et al., 2017; Eroglu et al., 2017). Both proteins are able to transport Mn in addition to Fe; however, VIT1 is primarily responsible for the storage of Fe in seeds, whilst MTP8 determines the pattern of seed Mn distribution (Kim et al., 2006; Chu et al., 2017; Eroglu et al., 2017). Nevertheless, phenotypic analyses of Arabidopsis vit1, mtp8, and vit1 mtp8 loss-of-function mutants has shown that VIT1 can substitute for MTP8 when the latter is not functional, and vice versa (Chu et al., 2017; Eroglu et al., 2017). In addition, MTP8 is important for Mn stress tolerance and contributes in the detoxification of Mn under Fe-deficiency conditions (Eroglu et al., 2016; Chu et al., 2017). Similar to VIT1 and MTP8, the NRAMP3 and NRAMP4 proteins from Arabidopsis are also able to transport Fe and Mn, and both have been shown to be required for the release of Fe and Mn from the vacuoles of germinating seeds and mature leaves, respectively (Lanquar et al., 2005, 2010). In seeds, NRAMP3 and NRAMP4 are expressed in the endodermal cells of the embryo, similarly to VIT1 (Lanquar et al., 2005; Kim et al., 2006). These three proteins are thought to form a functional module responsible for the vacuolar accumulation of Fe during embryo development and the efficient remobilization of Fe during seed germination (Mary et al., 2015). The activity of this module is critical for the proper growth and development of seedlings under Fe deficiency (Lanquar et al., 2005; Kim et al., 2006). As VIT1, NRAMP3, and NRAMP4 transport Mn, this model may also function in the efficient vacuolar storage and remobilization of Mn in the endodermal cells of Arabidopsis embryos.

Apart from the vacuole, the central roles in the cellular Fe economy of plants are played by plastids and mitochondria, which house the crucial processes of Fe metabolism, including heme biosynthesis, Fe-S cluster assembly, and Fe storage (Jain and Connolly, 2013). As integral parts of electron transfer chains, the Fe-containing compounds are essential for chloroplast photosynthesis and mitochondrial respiration, the major cellular pathways for energy conversion. However, despite its importance, Fe can also be highly reactive and toxic when it accumulates to high levels within the mitochondria and plastids of plant cells. Iron overload has long been associated with oxidative stress, which results from the Fe-catalysed formation of hydroxyl radicals that can damage DNA and proteins (Halliwell et al., 1992). Thus, the Fe content in the mitochondria and plastids must be carefully regulated to ensure an adequate supply while avoiding its over-accumulation to toxic levels.

The molecular mechanisms underlying the transport of Fe in and out of plastids and mitochondria have not yet been fully elucidated. Proteins involved in Fe loading into plant mitochondria and plastids have been recently discovered in rice (Oryza sativa) and Arabidopsis. The rice mitochondrial iron transporter (MIT) belongs to the family of mitochondrial solute carriers (MSCs) involved in the transport of a wide range of substrates across the inner mitochondrial membrane (Bashir et al., 2011). Homologous mitochondrial iron importers have been identified in yeast (MRS3, MRS4) (Li and Kaplan, 2004), mouse (Paradkar et al., 2009), Drosophila (Metzendorf and Lind, 2010), and zebrafish (mioferrin) (Shaw et al., 2006). The Arabidopsis chloroplast Fe-importing permease PIC1 is localized in the inner membrane and has cyanobacterial origin (Duy et al., 2007).

In contrast to Fe importers, the proteins involved in the efflux of Fe from the mitochondria and plastids of plant cells have not yet been determined. Two mitochondrial proteins, Mnt1 and Mnt2, have been shown to be involved in the efflux of Fe from mitochondria in yeast (Li et al., 2014). These proteins belong to the cation diffusion facilitator (CDF) family of divalent-metal transporters found in all kingdoms of life. A global phylogenetic analysis of CDF proteins from various organisms divided members of this family into three subgroups, namely Zn-CDF, Fe/Zn-CDF, and Mn-CDF, based on their putative specificity to the metal ions transported (Montanini et al., 2007). Based on this analysis, the yeast Mnt1 and Mnt2 proteins fall into the Fe/Zn-CDF subgroup. The homologous protein has been found in plants and named MTP6, but its function in iron/zinc transport has not yet been confirmed.

In this study, we investigated the function of the MTP6 protein in cucumber. Using yeast and protoplasts isolated from Arabidopsis cells, we found that CsMTP6 localizes to mitochondria and contributes to the mitochondrial efflux of Fe and Mn. CsMTP6 is ubiquitously expressed in cucumber organs, but the level of transcript and CsMTP6 protein is significantly increased in conditions of Fe deficiency or excess. These results
indicate that plant MTP6 proteins contribute to the redistribution of Fe and Mn between the cytosol and mitochondria of plant cells, and undergo Fe-mediated transcriptional regulation to maintain mitochondrial and cytosolic iron homeostasis under varying conditions of Fe availability.

**Material and Methods**

**Plant material and growth conditions**

Seeds of cucumber (Cucumis sativus cv Krak) were germinated in darkness for 3 d on moist filter paper. The germinated seeds were transferred to hydroponic media containing a control nutrient solution (Migocka et al., 2011) or solutions additionally supplemented with 10 µM MnCl₂ or 1 mM FeSO₄·EDTA, or solutions completely lacking in Mn or Fe. The Fe-free media were additionally supplemented with 100 µM of the Fe chelator bathophenanthroline disulfonate (BPS) to remove any contamination. Plants were grown for 2 weeks in a controlled environment with a day/night regime of 16/8 h (180 µmol m⁻² s⁻¹) and temperature of 24/22 °C. The media were continuously aerated and changed twice during the time of cultivation.

**RNA extraction and real-time PCR**

Total RNA was isolated from cucumber organs using TRI Reagent (Sigma), according to manufacturer's instructions. RNA samples (2 µg) were reverse–transcribed using a High-Capacity cDNA synthesis kit (Applied Biosystems) and subjected to real-time PCR with primers specific for CsMTP6 (forward primer 5′-TGGGATACAGATTCCACCGT-3′ and reverse primer 5′-TTTACTGTTGCGCTGAGCTGTGGAATT-3′) and for the reference gene encoding clathrin adapter complex subunit CACS (forward primer 5′-AAAACCTCGAGATGGAATCATCATAACATTTCTCCTA-3′ and reverse primer 5′-TGGCTTTCTTTCTGGAATGC-3′). Amplification reactions were performed on a Lightcycler 480 (Roche) in a final reaction volume of 20 µl containing 2 µl of cDNA, 10 µl of 2X Real Time 2XPCR Mix SYBR (A&A Biotechnology), and 2 µl of each primer (10 µM) under the following conditions: 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 15 s. For the analysis of real-time PCR data, the relative gene expression was calculated using critical point (Cp) values, which were converted into relative expression values according to the ΔΔCₚ method.

**Cloning of CsMTP6**

The cDNA corresponding to CsMTP6 was cloned by PCR amplification. Total RNA was isolated from the roots of 2-week-old cucumber seedlings that had been grown in standard liquid medium. cDNA was synthesized from the DNase-treated RNA using the SuperScript III (Invitrogen) and oligo-dT primers. For heterologous expression in yeast (Saccharomyces cerevisiae), the coding sequence of the CsMTP6 gene (1500 bp without the stop codon) was amplified from cDNA as the SpeI-SalI fragment using the forward primer 5′-AAAACCTCGAGATGGAATCATCATAACATTTCTCCTA-3′ and the reverse primer 5′-TTTACTGTTGCGCTGAGCTGTGGAATT-3′ (restriction sites in italics) and subcloned into the SpeI and SalI sites of the pUG35 vector (Niedenthal et al., 1996) harboring the URA selective marker, a constitutive MET25 promoter, and the green fluorescent protein (GFP) reading frame located at the 3′-end of the multiple cloning site, to yield C-terminal GFP fusions. For the protein localization assay in protoplasts, the CsMTP6 cDNA was amplified with the 5′-AAAACCTCGAGATGGAATCATCATAACATTTCTCCTA-3′ forward primer and the 5′-TTTACTGTTGCGCTGAGCTGTGGAATT-3′ reverse primer to yield the XhoI-SpeI fragment, which was subcloned into pA7-GFP vector (Voelker et al., 2006) to yield the C-terminal GFP fusion under the control of CaMV 35S promoter. The fidelity of all the constructs was established by sequencing.

** Yeast strains, media, and fluorescence imaging**

The yeast strains used in this study are listed in Table S1 at the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.306s0b; Migocka et al., 2019). The strains were maintained in YPD liquid medium [2% (w/v) glucose, 2% (w/v) bactopeptone, and 1% (w/v) yeast extract]. All yeast transformations were performed as described previously (Migocka et al., 2014), and the transformants were selected on complete synthetic media lacking uracil (SC–Ura), leucine (SC–Leu), or uracil and leucine (SC–Ura–Leu), containing 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose, the required amino acids, and 2% (w/v) agar. The mitochondrial localization of CsMTP6 in yeast mitochondria was determined using a fluorescence microscope (Axio Imager M1, Carl Zeiss) equipped with a 100× oil-immersion objective. The cells were incubated with 100 nM MitoTracker Red (Molecular Probes) for 15 min prior to fixation to label the mitochondria.

**Immunolocalization of CsMTP6 in cucumber cells**

Total microsomes were prepared from cucumber roots according to the procedure previously described by Kabala and Kłobus (2001). Tonoplast-enriched fractions were separated from the microsomal pellet in a discontinuous sucrose density gradient (the interphase between 20/28% sucrose) as described previously (Kabala and Kłobus, 2001). The right-side-out plasma membrane vesicles were separated from the microsomes in an aqueous dextran–polyethylene glycol two-phase system, following previously described methods (Larsson et al., 1997; Migocka et al., 2011). Crude mitochondria- and plastid-enriched fractions were prepared from cucumber roots essentially as described by Viganì et al. (2013). Purified mitochondrial fractions were collected from the 28/40% interface of a Percoll gradient following centrifugation of crude mitochondrial pellets on a 40, 28, and 13.5% (v/v) Percoll step-gradient (Viganì et al., 2013). The distribution of marker membrane proteins in each membrane fraction was examined by western blot analysis with specific antibodies (Agrisera, Vännäs, Sweden) raised
against the plasma membrane (PM) H^+-ATPase (AS07 260), the tonoplast V-ATPase (epsilon subunit) (AS07 213), the mitochondrial COXII cytochrome oxidase (AS04 053A), and the plastidial outer-envelope membrane translocon complex Tox75 (AS06 150). Immunolocalization of CsMTP6 was carried out using a specific antibody raised against a synthetic peptide antigen corresponding to the predicted C-terminal sequence of CsMTP6 (positions 324–337, C+CEGVKGCHRLRGRRA; C, additional cysteine residue for linkage with keyhole limpet hemocyanin KLH). The peptide conjugated to KLH was injected into two rabbits (three immunizations per rabbit). The CsMTP6-specific IgG was purified using Sepharose resin and detected and quantified by an ELISA test. The peptide antigen linked with the antigen peptide, and the purified antibody was detected and quantified by an ELISA test. The peptide antigen and the antibody were synthesized by GeneScript (Piscataway, NJ, USA). Western blot analysis was performed essentially as described previously by Migocka et al. (2011) with a 1:1000 dilution of primary antibodies for CsMTP6, a 1:5000 dilution of the secondary anti-rabbit IgG antibody (dilution of primary antibodies for marker membrane proteins, and a 1:20 000 dilution of the secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (AS09 602, Agrisera). The blots were developed using an enhanced chemiluminescence system (ChemiDoc TMMP Imaging System, Bio-Rad).

Metal and H2O2 sensitivity spot assays

For metal and H2O2 tolerance assays, serial dilutions (OD 600 0.1, 0.01, and 0.001) of cells grown overnight were spotted onto agar plates containing selective media supplemented with different concentrations of heavy metals (FeSO4-EDTA, CoCl2, CuSO4, MnCl2, CdCl2), H2O2, or 100 μM iron chelator BPS (low-Fe media). Plates were incubated at 30 °C for 3–4 d prior to imaging.

Cytosolic gentisate 1,2-dioxygenase assays

The cytosolic gentisate 1,2-dioxygenase (c-GDO)-FLAG fragment was amplified from the pRS426 vector using the forward primer 5’- ATTAAACAG GCCATTAACGC CAAAAA TGCAAGAAG CAAAAC TGAACACGC-3’ and the reverse primer 5’- AACTGATT GGCCGAGGCGGCC TCACCTGTCCATC GTCTTGTGATGTC-3’ introducing two SfiI sites (in italics), respectively, and subcloned into the SfiI-SfiI sites of the yeast expression vector pBT3C carrying a Leu selectable marker (Dualsystems Biotech). The pBT3C-GDO plasmid was then transformed into yeast cells harbouring plasmids pUG35 or pUG35-CsMTP6, and the transformants carrying two vectors were selected on SC–Ura–Leu solid media. Cells were grown overnight to mid-log phase in liquid SC–Ura–Leu medium and then for 8 h in the same fresh medium supplemented with 100 μM FeSO4–EDTA. Following this treatment, cells were homogenized using glass beads in 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride (Sigma–Aldrich). The presence of c-GDO in the extract was confirmed by western blot analysis using antibodies against FLAG (1:10 000, Sigma Aldrich). c-GDO activity was measured spectrophotometrically at 340 nm as described previously (Li et al. 2014), in an assay mixture containing 20 mM Tris–HCl (pH 8.0) and 0.1 mM 2,3-dihydroxy-benzoic acid (gentisic acid) (Sigma–Aldrich). Enzyme activity was calculated using an extinction coefficient of 10.2 cm−1mm−1 and was expressed as nmol of substrate converted per minute per mg of protein.

Mn-SOD assays

For determination of Mn-SOD activity, mitochondria prepared from yeast cells or protoplasts were disrupted by freeze–thaw cycles and suspended in 20 mM HEPES, 1 mM EDTA, 250 mM sucrose, and 0.1% Triton X–100 to release enzymes (Lanza and Nair, 2009). Mn-SOD activity was measured by activity assays essentially as described previously by Weydert and Cullen (2010), using xanthine–xanthine oxidase to generate O2•− and nitroblue tetrazolium (NBT) reduction as an indicator of O2•− production. This assay allows for the indirect determination of Mn-SOD activity based on competition between SOD and NBT, which is reduced by superoxide into blue formazan and results in a measurable change in absorption. The reaction was measured spectrophotometrically at 560 nm using a UV/VIS Carry 100 spectrophotometer (Agilent Technologies). As CuZn-SOD has also been shown to be present in the intermembrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001), an inhibitor, 5 mM potassium cyanide, was added to the reaction assays to completely inactivate CuZn-SOD activity (Asada et al., 1974). One unit of Mn-SOD activity was defined as the amount of enzyme required for the inhibition of the NBT reduction rate to half of the maximum. The specific activity of Mn-SOD was expressed in units per mg protein.

Transformation of Arabidopsis protoplasts and confocal imaging

Arabidopsis cell suspension protoplasts were isolated and transformed essentially as described previously (Thomine et al., 2003). Following transformation, the protoplasts were incubated in the dark at 23 °C for 2–3 d. Confocal fluorescence images were acquired 24–36 h after transformation using an Olympus Fluoview FV1000 (lens, Olympus UPlanSApo 60×/1.35 oil; filter, BF490-590) with excitation at 473 nm. Mitochondria of the protoplasts were counterstained with 25 nM MitoTracker Red CMXRos (Molecular Probes) for 15–60 min.

Mitochondria preparation from Arabidopsis protoplasts

Mitochondria were prepared from Arabidopsis cell suspension protoplasts essentially as described previously by Nishimura et al. (1982), with slight modifications. To measure the accumulation of Fe or Mn in the mitochondria, the transformed protoplasts (2 ml) were incubated with 100 μM FeSO4–EDTA or 100 μM MnSO4 for 12 h in darkness with a slow flotation prior to preparation. The metal-treated protoplasts were gently washed twice with a 10 mM MOPS–KOH buffer (pH 7.2), containing 0.34 M glucose, 0.34 M mannitol, and 1 mM EDTA, and finally suspended in 30 ml of homogenization buffer, consisting
of 10 mM MOPS-KOH buffer (pH 7.2), 0.3 M mannitol, 1 mM EDTA, 0.1% defatted BSA, and 0.6% PVPP. Homogenization was achieved by a mechanical disruption of protoplasts in a glass homogenizer (3-cm diameter) with 10 gentle strokes, and the resulting homogenate was centrifuged at 1000 g for 10 min to pellet the plastids and aggregated organelles. The supernatant was centrifuged at 10 000 g for 10 min and the pellet obtained was gently suspended in 1 ml of a buffer containing 10 mM MOPS-KOH buffer (pH 7.2), 0.3 M mannitol, and 1 mM EDTA. The suspension was layered on top of a discontinuous Percoll density gradient composed of 60/45/28/5% (v/v) Percoll in 20 mM MOPS-KOH buffer (pH 7.2), 0.25 M sucrose, and 1 mM EDTA, and centrifuged for 30 min at 30 000 g. Following centrifugation, the plastidal (28/5% interphase) and mitochondrial (45/28% interphase) fractions were collected and subjected to western blot analysis with antibodies raised against ToC75, COXII, and GFP to confirm the purity of the mitochondrial fraction and the presence of CsMTP6 in mitochondrial membranes.

**Mitochondria preparation from yeast cells**

To determine the metal content in yeast mitochondria, cells transformed with the pUG35 plasmid carrying CsMTP6 or with the empty vector were grown for 48 h in a SC/Glu-Ura medium and then for 12 h in the same media supplemented with 100 µM of the selected metals (FeSO₄-EDTA, CdCl₂, NiCl₂, MnSO₄, CoCl₂, CuCl₂, ZnSO₄). Highly purified mitochondria were isolated from yeast cells as described previously (Gregg et al., 2009) using a four-step sucrose gradient. The intact mitochondria were collected from the 60/32% sucrose interface and subjected to western blot analysis with antibodies raised against CsMTP6, COXII, and GFP to confirm the purity of the mitochondrial fraction and the presence of CsMTP6 in mitochondrial membranes.

**Determination of mitochondrial heavy metal content**

Mitochondria prepared from protoplasts or yeast cells were digested with concentrated HNO₃ at 160 °C for 2 h. The heavy metal content (Fe, Mn, Cu, Zn, Ni, Co, Cd) was measured by flame atomic absorption spectrometry (AAS).

**Protein determination**

Protein contents of all the membrane fractions were determined using Bradford assays (Bradford, 1976).

**Bioinformatics and accession number**

The predicted full coding sequence of CsMTP6 was retrieved from GenBank as described previously (Migocka et al., 2014). The putative CsMTP6 transmembrane domains were predicted using HMMTOP (Tusnády and Simon, 2001) and visualized in TMKPres2.D (Spyropoulos et al., 2004). The mitochondrial targeting peptide was identified using MitoProt II, v.1.101 ([https://bio.tools/MITOPROT-II](https://bio.tools/MITOPROT-II), last accessed on October 19, 2018). The nucleotide and the putative amino acid sequences of CsMTP6 are available in GenBank under the accession number KX118275.

**Statistical analysis**

Student's t-tests, Tukey's test, and ANOVA (MS Excel) were used for statistical analyses.

**Results and discussion**

**Sequence and expression analysis of CsMTP6**

The plant MTP family has been divided into seven groups (1, 5, 6, 7, 8, 9, and 12) based on annotated MTP sequences from Arabidopsis (Gustin et al., 2011). These groups form the three primary clades of the CDF family that differ in their putative substrate specificity: Zn-CDF, Fe/Zn-CDF, and Mn-CDF. Group 6 containing the MTP6 proteins has been classified in the Fe/Zn-CDF group, including bacterial FieF-like and WnfieF-like proteins and the fungal MMT-like proteins, which are involved in Fe transport (Li and Kaplan, 1997; Grünberg et al., 2001; Grass et al., 2005; Li et al., 2014). The genes encoding plant MTP6 proteins have been identified in all the plant genomes sequenced so far except for the those of the red alga *Cyanidioschyzon merolae*, and the green alga *Ostreococcus tauri, O. lucimarinus*, and *Chlamydomonas reinhardtii*, suggesting that the members of group 6 in algae have been lost (Gustin et al., 2011). A gene encoding a protein bearing a close similarity to MTP6-like protein has also been identified in the genomes of the cucumber cultivars Chinese long (acc. no. ACHR01000689) and Borszczagowski (acc. no. ACYN01005409) (Migocka et al., 2014). FGENESH (Find GENES HMM), a Hidden Markov Models (HMM)-based *ab initio* gene-structure prediction program (Salamov and Solovyev, 2000) was used to identify the transcription site, exon/intron boundaries, and the polyadenylation signal sequence of the CsMTP6 gene in these two cultivars and it determined that the cucumber *CsMTP6* gene contains 13 exons and 12 introns and encodes a putative protein of 528 amino acids (Migocka et al., 2014). Based on the sequence retrieved from GenBank, we designed two specific primers to analyse *CsMTP6* expression in the different organs of the cucumber cultivar Krak. *CsMTP6* was differentially expressed in all the vegetative organs, with the lowest expression level in the hypocotyls (Fig. S1, Dataset S1 at Dryad). cDNA prepared from the bulk sample of roots was used to amplify the full cDNAs of *CsMTP6* for the subsequent analysis of *CsMTP6* function and localization in yeast and Arabidopsis protoplasts. Comparative analysis of the cDNA generated *in silico* and the cDNA amplified by PCR revealed the lack of 84 nucleotides encoding a 24-amino acid fragment NLAIIILSESQCFQGSRNLAWPSLFSCKQ in the amplified *CsMTP6* product (Fig. S2A, B at Dryad). There are three possible reasons for this discrepancy; first, the prediction of the *CsMTP6* cDNA by FGENESH might be inaccurate; second, the difference in *CsMTP6* cDNA and protein sequences between the cucumbers Chinese long, Borszczagowski, and Krak may result from a mutational event and reflect some genetic variability between different cultivars; or third, alternative splicing of *CsMTP6* mRNA might occur, giving rise to more than one transcript. Splicing variants of *AtMTP3*, *AtMTP5*, *AtMTP6*, and *AtMTP9* have already been reported and submitted to the Arabidopsis Information Resource (TAIR) database ([https://...](https://...)}
In addition, the existence of multiple transcripts of the MTP1 gene has been reported in the heavy metal hyperaccumulator Thlaspi goingense (TgMTP1t1 and TgMTP1t2) (Persans et al., 2001). In order to confirm the structure of the CsMTP6 transcript and identify its possible alternative splicing variants, we analysed the reassembled genome of the cultivar Chinese long (available at http://cmb.bnu.edu.cn/Cucumis_sativus_v20/, last accessed on October 19, 2018), which was reannotated with RNA-Seq reads from poly(A) RNAs obtained from 10 cucumber tissues (Li et al., 2011). We searched the database for the CsMTP6 gene and found only one transcript annotated as Csa7G395210.1, consisting of 12 exons. The alignment of this transcript and the PCR-amplified CsMTP6 cDNA of the Krak cultivar showed the same length (1503 bp) and 100% identity of both protein-coding sequences (Fig. S2C at Dryad). These results indicated that only one CsMTP6 transcript was present in the cucumber tissues examined and suggested that the differences in the transcripts amplified by PCR and predicted ab initio (Fig. S2A at Dryad) resulted from the inaccuracy of the FGENESH prediction.

In silico analysis of the amino acid sequence deduced from the PCR-amplified CsMTP6 cDNA revealed the presence of four putative transmembrane helices (TMHs) and the mitochondrial targeting sequence (MTS) in the N-terminal domain of CsMTP6 (Fig. 1). The signature sequence characteristic for CDF proteins covered the amino acids 104–147 located within the first TM1 helix and the loop connecting helices TM1 and TM2 of CsMTP6 (Fig. 1). The two Fe/Zn group-specific motifs were identified in the putative TM1 helix (HSVSD) and in the loop connecting helices TM3 and TM4 (HHRAD) (Fig. 1). Based on the X-ray structure of the E. coli YiiP (EcFieF) (Lu and Fu, 2007), the conservative amino acids from both motifs (HD-HD in CsMTP6) are thought to form metal-binding sites. Interestingly, both motifs are located in the transmembrane helices II and V of YiiP (Lu and Fu, 2007). The predicted topology of CsMTP6 (4 TMHs) was quite different from the topology of typical CDF transporter (6 TMHs) (Montanini et al., 2007) and this difference could be a reason for the presence of the HHRAD motif out of the CsMTP6 TMH. However, this predicted topology needs to be experimentally verified to confirm the presence of both motifs in or out of the transmembrane helices. In addition, we did not identify a His-rich region typical for the members of CDF family in CsMTP6 (Montanini et al., 2007). Instead, CsMTP6 had a histidine-serine-rich loop located between TMHs II and III that is typical for sequences belonging to Zrg17-like and MMT-like clusters (Montanini et al., 2007) (Fig. 1).
CsMTP6 localizes to the mitochondria

To confirm the mitochondrial localization of CsMTP6, we tagged the green fluorescent protein (GFP) to the C-terminus of CsMTP6 and investigated the subcellular localization of this fusion protein in protoplasts isolated from Arabidopsis suspension cells. Protoplasts transformed with the CsMTP6-GFP fusion were stained with MitoTracker-Red to simultaneously visualize the mitochondria. As shown in Fig. 2A, the protoplasts containing CsMTP6-GFP displayed dotted green fluorescence signals, which clearly overlapped the regions stained with MitoTracker, confirming that CsMTP6 localizes to the mitochondria. To further confirm this localization in cucumber cells, we performed immunolocalization of CsMTP6 in different subcellular fractions prepared from roots using a specific anti-CsMTP6 antibody. Western blot analysis of the fractions enriched in plasma membranes, tonoplasts, plastids, or mitochondria revealed a specific band migrating at a molecular mass of 35 kDa only in the mitochondrial fractions, consistent with the results obtained from protoplast imaging (Fig. 2B). In comparison, no immunoreactive bands were detected for the other membrane fractions of the cucumber cells. The observed molecular mass was lower than that predicted from the deduced amino acid sequence (~50 kDa), suggesting that the target peptide is removed following the targeting of CsMTP6 to the mitochondrial membrane. To date, members of the plant MTP family have been found to be localized to the tonoplast (Delhaize et al., 2003; Kobae et al., 2004; Arrivault et al., 2006; Migocka et al., 2014, 2015a), the Golgi/prevacuolar system (Delhaize et al., 2007; Peiter et al., 2007; Pedas et al., 2014; Fujiwara et al., 2015), or the plasma membrane (Migocka et al., 2015b; Ueno et al., 2015). The only members of the CDF family that have been found localized to the mitochondria include the two CsMTP6 homologs from yeast, ScMMT1 and ScMMT2 (Li and Kaplan, 1997; Li et al., 2014), suggesting that the yeast and plant members of the Fe/Zn-CDF group can fulfill similar physiological functions. It has been shown that the yeast ScMMT1 and ScMMT2 proteins contribute to the export of Fe from the mitochondria (Li et al., 2014). In order to determine the metal specificity of CsMTP6, we examined the effects of CsMTP6 expression in yeast deficient in different heavy metal transporters.

CsMTP6 expression affects Fe homeostasis in yeast with deletion of mitochondrial iron transporters

Since all the members of Fe/Zn-CDF characterized to date contribute to Fe transport, we first investigated whether CsMTP6 is involved in the transport of Fe when expressed in yeast cells. Over the last few years, a range of organelle Fe transporters have been identified in yeast cells that contribute to cellular homeostasis. These include the MRS3 and MRS4 proteins involved in the mitochondrial Fe import (Mühlenhoff et al., 2003), the vacuolar Fe transporter CCC1 (Li et al., 2001), and the MMT1 and MMT2 proteins involved in mitochondrial Fe export (Li et al., 2014).

The MRS3/4 proteins are members of the mitochondrial solute carrier family that catalyse the transport of Fe into the mitochondria along a concentration gradient (Froschauer et al., 2009; Mühlenhoff et al., 2003), whereas CCC1 is a member of the vacuolar iron transporters (VIT) family involved in the transport of Fe$^{2+}$ and Mn$^{2+}$ into the vacuole, probably by a
H\(^+\) antiport carrier-type mechanism (Li et al., 2001; Kim et al., 2006). In yeast, the CCC1-mediated transport of Fe into the vacuole is the main mechanism of Fe detoxification and tolerance (Li et al., 2001). Deletion of genes encoding MRS3/4 and CCC1 results in two different iron-dependent phenotypes, exhibiting increased sensitivity to low Fe (Δmrs3Δmrs4) due to the impaired import of Fe into the mitochondria, or increased sensitivity to high Fe (Δacc1), resulting from the impaired export of Fe from the cytosol into the vacuole (Li et al., 2001, 2014; Mühlenhoff et al., 2003). The expression of CsMTP6 in both yeast mutants did not complement their Fe-dependent phenotypes (Fig. 3A). Furthermore, the CsMTP6-GFP fusions were observed to co-localize with the mitochondrial networks of both Δacc1 and Δmrs3Δmrs4 cells (Fig. 3B). Mitochondrial localization and the lack of complementation of the Δacc1 and Δmrs3Δmrs4 mutations by CsMTP6 indicated that the cucumber protein was not involved in the sequestration of Fe into the vacuole under high Fe conditions or in the import of Fe into the mitochondria under low Fe conditions.

Since plant MTP6-like proteins belong to the same phylogenetic cluster as the yeast mitochondrial MMT1 and MMT2 proteins, we expected that the expression of CsMTP6 would complement the Δmmt1Δmmt2 mutation or result in the same phenotypes observed when MMT1 and MMT2 are overexpressed. It has been shown previously that a deletion of MMT1 and MMT2 in yeast has no effect on Fe toxicity but results in a decrease in cytosolic Fe and an increased sensitivity to Fe deficiency (Li et al., 2014). The decreased growth of the yeast strain Δmmt1Δmmt2 in Fe-free media is thought to result from the inability of this mutant to use mitochondrial Fe (i.e. to export Fe from mitochondria) under starvation conditions (Li and Kaplan, 1997). As shown in Fig. 3A, the expression of CsMTP6 in Δmmt1Δmmt2 cells reversed the effect of the mutation and rendered the double-mutant more resistant to Fe deficiency. Similar to the Δacc1 and Δmrs3Δmrs4 strains, the CsMTP6-GFP fusion co-localized with the mitochondrial network of the yeast Δmmt1Δmmt2 mutant (Fig. 3B), confirming that CsMTP6 could restore the mitochondrial Fe export in Δmmt1Δmmt2 cells.

To further support the hypothesis that CsMTP6 contributes to the transport of Fe out of the mitochondria, we examined the effects of CsMTP6 expression on the mitochondrial and cytosolic Fe content in wild-type yeast and in the cells deleted for MMT1 and MMT2. Cytosolic Fe was determined based on the activity of cytosolic gentisate 1,2-dioxygenase (c-GDO) following the transformation of cells expressing CsMTP6 with a c-GDO-expressing plasmid. c-GDO is absent in yeast; however, when expressed in yeast, it localizes only in the cytosol and shows Fe\(^{2+}\)-dependent activity using gentisate as a substrate, and therefore the activity of this

Fig. 3. Complementation assay and localization of CsMTP6 in yeast cells defective in vacuolar and mitochondrial iron transporters. (A) Representative images (from four replicates) of serial dilutions of the wild-type (WT) and yeast mutants sensitive to high iron (Δacc1) or low iron (Δmrs3Δmrs4 and Δmmt1Δmmt2) transformed with the empty vector or with CsMTP6. Yeast were grown in SC/Glu-Ura media supplemented with 100 µM BPS (low Fe), 3 mM FeSO\(_4\)-EDTA, or in the control SC/Glu-Ura media. (B) CsMTP6-GFP localization in Δacc1, Δmrs3Δmrs4 and Δmmt1/Δmmt2 cells. Yeast cultured overnight in control liquid SC/Glu-Ura media were diluted to OD\(_{600}\) 0.1 and then grown in liquid SC/Glu-Ura media supplemented with 3 mM FeSO\(_4\)-EDTA (Δacc1) or 100 µM BPS (low Fe, Δmrs3Δmrs4 and Δmmt1Δmmt2) for 8–10 h. 1, Transmission image of the cells expressing CsMTP6-GFP; 2, GFP fluorescence; 3, MitoTracker red fluorescence; 4, merged image. Scale bars are 5 µm. (This figure is available in colour at JXB online.)
enzyme can be used as a measure of cytosolic Fe (Li et al., 2014). The presence of CsMTP6 and c-GDO in yeast was confirmed by western blot analysis (Fig. 4A). Mitochondria isolated from yeast transformants showed major immunoreactive bands migrating just below 70 kDa (Fig. S3 at Dryad), corresponding to the CsMTP6-GFP fusion proteins (~35 kDa for CsMTP6 + 27 kDa for GFP). As shown in Fig. 4B and Dataset S2 at Dryad, the deletion of MMT1 and MMT2 in yeast resulted in a decrease in cytosolic Fe, observed as a decrease in c-GDO activity. In contrast, the mitochondrial Fe content of Δmmt1Δmmt2 cells was increased (Fig. 4C, Dataset S3 at Dryad), confirming the function of MMT proteins in mitochondrial Fe export. Expression of CsMTP6 in wild-type yeast cells markedly increased c-GDO activity and significantly decreased mitochondrial Fe content (Fig. 4B, C, Datasets S2, S3 at Dryad), indicating that CsMTP6 supported endogenous MMT1 and MMT2 proteins in the transport of Fe from the mitochondria to the cytosol. Consistent with this hypothesis, the expression of CsMTP6 in the Δmmt1Δmmt2 mutant reversed the effect of the mutation and restored the mitochondrial and cytosolic Fe levels to those observed in wild-type cells (Fig. 4B, C, Datasets S2 and S3 at Dryad), confirming that the cucumber protein complements the lack of MMT1 and MMT2 and contributes to the transport of Fe out of the mitochondria.

CsMTP6 expression affects the sensitivity of yeast to copper, cobalt, and H2O2

To further confirm that CsMTP6 can be a functional homolog of the yeast proteins MMT1 and MMT2, we investigated the effect of CsMTP6 expression on yeast sensitivity to copper, cobalt, and H2O2. Previous studies have shown that overexpression of MMT1 and MMT2 in wild-type yeast decreases cobalt sensitivity and increases copper and H2O2 sensitivity (Li et al., 2014). In contrast, cells deleted for MMT1 and MMT2 are significantly more sensitive to Cu, and moderately more resistant to Cu (Li et al., 2014). The expression of CsMTP6 in wild-type yeast resulted in a cobalt-resistant phenotype (Fig. 5A) similar to that observed in yeast overexpressing MMT1 and MMT2 (Li et al., 2014). In addition, the expression of CsMTP6 in Δmmt1Δmmt2 cells suppressed their Co-sensitive phenotype (Fig. 5A). The response of the yeast mutant to cobalt stress could have resulted from the CsMTP6-induced modification of the cytosol Fe level. It has been well documented in yeast that cobalt stress immediately and dramatically induces the expression of genes involved in Fe uptake and homeostasis (termed the iron regulon) (Stadler and Schweyen, 2002). This response is mediated by the Aft1 transcriptional factor, which accumulates in the nucleus under Fe starvation and Co excess and activates a set of genes involved in Fe uptake and homeostasis (termed the iron regulon) (Stadler and Schweyen, 2002). Consequently, yeast cells deleted for AFT1 are more sensitive to Co, and this Co-sensitive phenotype is not complemented by the overexpression of MMT1 and MMT2, indicating that AFT1 and not MMT1 and MMT2 is required for Co resistance (Li et al., 2014). The expression of GmMTP6 in Δaft1 cells also did not complement the Co-sensitive phenotype of this mutant strain (Fig. 5A), suggesting that CsMTP6 may not be directly involved in Co transport in yeast cells. Consistent with this hypothesis, the mitochondrial Co content in yeast expressing CsMTP6 was not affected by the activity of the cucumber protein (Fig. 5B, Dataset S3 at Dryad). However, since we did not perform direct measurements of heavy metal transport, additional studies are required to verify the ability of CsMTP6 to transport Co.

Similar to MMT1 and MMT2 overexpression, the expression of CsMTP6 in yeast resulted in Cu-sensitive and H2O2-sensitive phenotypes of the wild-type, a Cu-sensitive phenotype of the Δmmt1Δmmt2 mutant, and a H2O2-sensitive phenotype of the Δfet5Δsmf3 mutant, resulting from a deletion of vacuolar the Fe exporters FET5/SMF3 (Fig. 5C, D). Similar to Co, the mitochondrial Cu content in yeast was not affected by the activity of the cucumber protein (Fig. 5B, Dataset S3 at Dryad). As has already been shown in yeast, increased cytosolic Fe resulting from the overexpression of MMT1 and MMT2 significantly raises the level of cellular oxidants and leads to increased oxidant damage in cells (Li et al., 2014). These results suggest...
that the Cu- and H₂O₂-sensitive phenotypes of yeast expressing CsMTP6 were caused by the changes in cellular redox homeostasis resulting from the CsMTP6-mediated increase in cytosolic Fe, and not by the contribution of the protein in Cu transport. Although the ability of CsMTP6 to transport Cu needs to be verified in direct transport assays, the similar phenotypes resulting from MMT1 and MMT2 overexpression or CsMTP6 expression in yeast support the hypothesis that the plant and yeast homologs affect redox homeostasis in yeast in the same way.

CsMTP6 expression affects the Mn-sensitive phenotype and the mitochondrial Mn level of the yeast mutant K667

A few characterized members of the Fe/Zn-CDF subgroup from bacteria, including FieF from *E. coli* (Grass et al., 2005), MgMamB from *Magnetospirillum gryphiswaldense* (Grünberg et al., 2001), and WmFieF from *Capriavidus metallidurans* (Munkelt et al., 2004), have been shown to be involved in the transport of other metals in addition to Fe, such as Zn²⁺, Co²⁺, Cd²⁺, and Ni²⁺. In order to determine the substrate specificity of CsMTP6, we further investigated whether it was able to complement the mutant phenotype displayed by yeast strains sensitive to Zn (Δzrc1), Cd, Mn, and Ni (K667, Δpmc1Δvxc1Δcnb), or Mn deficiency (Δsmf1). The presence of CsMTP6 in the mitochondria of all transformants was confirmed under fluorescence microscopy (Fig. S4 at Dryad). CsMTP6 did not affect the sensitivity of the Δzrc1 and K667 cells to Zn, Ni, and Cd, and the mitochondrial contents of these metals were not affected by CsMTP6 expression (Fig. S5, Dataset S3 at Dryad). These results suggest that CsMTP6 may not be capable of Zn, Ni, or Cd transport in yeast cells. In contrast, the expression of CsMTP6 in the K667 strain fully complemented the Mn-sensitive phenotype and significantly reduced the mitochondrial Mn content and Mn-SOD activity of this yeast mutant (Fig. 6A–C, Dataset S3 at Dryad), suggesting that CsMTP6 may be involved in the transport of both Fe and Mn out of the mitochondria. Interestingly, the levels of metals determined in the mitochondria of yeast were quite high (nmol concentrations), indicating that they were able to tolerate a certain amount of Zn, Mn, Ni, or Cd. Taking into consideration the metabolic processes occurring in mitochondria, it is assumed that organic acids (citrate, isocitrate, malate, aconitate, oxaloacetate) may be involved in the alleviation of heavy metal toxicity within them by forming Zn-citrate, Zn-malate, Ni-citrate, Cu–citrate, Mn–citrate, and Cd–citrate (Mathys, 1977; Glusker, 1980; Thurman and Rankin, 1982; Godbold et al., 1984; Harrington et al., 1996; Parker et al., 2001). However, based on the results of our metal spot-test assays we may conclude that the capabilities of mitochondria to chelate Zn, Mn, Ni, or Cd are very limited.

Although CsMTP6 complemented the high Mn-sensitive phenotype of the K667 strain, it did not restore the growth of Δsmf1 cells in low-Mn media (Fig. 6D). Smf1p is a cell-surface transporter involved in high-affinity Mn uptake and trafficking in yeast cells, and therefore deletion of SMF1 results in a decrease in the intracellular Mn concentration and increased sensitivity.
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Fig. 6. Effect of CsMTP6 expression on the Mn sensitivity, mitochondrial Mn content, and Mn-SOD activity of yeast. (A) Representative images (from four replicates) of serial dilutions of the wild-type (WT) and K667 mutants transformed with the empty vector or CsMTP6 and grown on either SC/Glu–Ura medium supplemented with MnSO4 or control SC/Glu–Ura medium. (B) Mitochondrial content of Mn in yeast expressing CsMTP6. Data are means (±SD) from separate experiments. (C) Mitochondrial Mn-SOD activity in yeast expressing CsMTP6. Data are means (±SD) from separate experiments, expressed as enzyme units (U). Significant differences were determined using Tukey’s test. (D) Representative images (from four replicates) of serial dilutions of the WT and ∆smf1 mutants transformed with the empty vector or CsMTP6 and grown on either SC/Glu–Ura medium supplemented with EGTA (low Mn medium) or control SC/Glu–Ura medium.

to the Mn chelator EGTA (Supek et al., 1996). The inability of CsMTP6 to complement the sensitivity of ∆smf1 to low Mn indicates that mitochondrial efflux is not able to complement the deficiency in Mn uptake of the mutant cells, or to induce transcriptional pathways that could increase the level of cellular Mn through enhanced influx across the plasma membrane or enhanced efflux from the vacuole. These results are consistent with previous data that indicate that the Mn regulatory pathways identified to date do not all occur at the transcriptional level, but instead also involve post-translational modifications of the localization and turnover of the Smf1p and Smf2p Mn transporters (Liu and Culotta, 1999; Portnoy et al., 2000; Reddi et al., 2009). As CsMTP6 localized in the mitochondria under Mn deficiency (Fig. S4 at Dryad), it was not able to catalyse uptake by ∆smf1 cells. Furthermore, the concentration of Mn detected in the mitochondria of yeast cells was significantly lower compared to that of Fe (Figs 4C, 6B), indicating that their capacity to accumulate and tolerate Mn excess was more limited. Indeed, the complementation of the Mn-sensitive phenotype of K667 cells by CsMTP6 suggests that excess Mn negatively affects the mitochondria of yeast cells. The K667 mutant is deficient in the functional Ca2+-dependent protein phosphatase calcineurin, the vacuolar Ca2+/Mn2+/H+ exchanger Vcx1p (Mnr1), and the vacuolar Ca2+-ATPase Pmc1p (Cunningham and Fink, 1996). Calcineurin is required for the activation of Mn2+/H+ transport activity by Vcx1p (Mnr1) and for the activation of the high-affinity Ca2+/Mn2+-transporting ATPase Pmr1p, and may also prevent the entry of excess Mn2+ into the cell (Farcasanu et al., 1995; Lapinskas et al., 1995; Pittman et al., 2004). Despite the presence of the vacuolar Fe2+ and Mn2+ transporter CCC1 (Li et al., 2001) in K667, the triple Δpmc1Δvcx1Δcnb deletion results in severe impairment of the Mn2+ homeostasis and in an increased sensitivity to Mn. As CsMTP6 restores the growth of K667 cells under high Mn conditions, it may be assumed that the Mn-sensitive phenotype of this strain results from the toxic effect of Mn on mitochondria. As shown in Fig. 6B, C, the Mn content and Mn-SOD activity were higher in the mitochondria of K667 when compared to the wild-type yeast. These results indicate that Mn-induced toxicity can increase the production of reactive oxygen species in the mitochondria of the K667 strain. Mn has already been shown to increase mitochondrial H2O2 production over a physiological as well as toxic range of concentrations in human neuroblastoma cells (Fernandes et al., 2017). In addition, it has been shown in rat primary neuron cultures that toxic Mn concentrations negatively affect the mitochondrial membrane potential and the activity of complex II, and lead to DNA fragmentation that results in apoptotic-like neuronal death (Malecki, 2001). The accumulation of Mn in mitochondria and in mitochondria-rich tissues in vivo also directly interferes with oxidative phosphorylation, probably by binding to the F1-ATPase (Gavin et al., 1992). Hence, the mechanism for Mn-induced toxicity is thought to result from Mn-induced oxidative damage, mitochondrial dysfunction, and the resultant apoptotic cell death (Gavin et al., 1992; Malecki, 2001; Fernandes et al., 2017). Therefore, we can assume that the CsMTP6-mediated Mn efflux from mitochondria and the subsequent CCC1-mediated Mn sequestration in the vacuole could protect the mitochondria of K667 cells from Mn toxicity, and thus render the strain more tolerant to excess Mn. In contrast, the CsMTP6-mediated Fe efflux from mitochondria could not protect Δαα1 cells from excess Fe, as yeast lacking CCC1 are unable to accumulate Fe excess in the vacuole.

In contrast to CsMTP6, the members of the Fe/Zn-CDF subgroup characterized so far, including bacterial FieF-like and WmFieF-like proteins and the fungal MMT-like...
transporters, are probably not capable of Mn transport (Li and Kaplan, 1997; Grünberg et al., 2001; Grass et al., 2005), suggesting functional diversification between the Fe/Zn-CDF transporters from different species. Interestingly, a phylogenomic analysis of CDF proteins from different organisms, including 56 newly identified CDF homologs, found 18 additional clades within the three substrate-specific groups Mn-CDF, Zn-CDF, and Fe/Zn-CDF that contain proteins with potentially new metal specificities (Mn, Fe, Co, Zn, Zn/Cd, Ni/Co) (Cubillas et al., 2013). In this analysis, MTP6 as well as the MMT1 and MMT2 proteins were classified in clade II that contains the Fe-specific CDFs; however, this clade originates from the same ancestor as the neighboring clade I, containing the Mn-specific CDFs (Cubillas et al., 2013). Hence, the broader specificity of MTP6 for Mn and Fe may be an ancient feature that has been retained in some members of clades 1 and 2. In addition, the novel Mn/Fe subfamily has recently been identified in clade VI of CDFs from α-proteobacteria (Cubillas et al., 2014), supporting the hypothesis that some members of the CDF family are capable of transporting both Mn and Fe.

CsMTP6 expression affects mitochondrial Fe and Mn levels in Arabidopsis protoplasts

The similar patterns of CsMTP6 localization in plants and yeast suggested that CsMTP6 contributes to the export of Fe and Mn from the mitochondria of plant cells. To verify the functionality of the cucumber protein in plants, we determined the mitochondrial Fe and Mn contents in Arabidopsis protoplasts expressing CsMTP6-GFP that were pre-incubated with either 100 μM FeSO₄·EDTA or 100 μM MnSO₄ prior to preparation of the mitochondria. The presence of CsMTP6 in the isolated mitochondria was confirmed by western blotting (Fig. 7A). As shown in Fig. 7B, C, and Dataset S4 at Dryad, the Fe and Mn contents in mitochondria containing CsMTP6-GFP were approximately 2-fold lower when compared to mitochondria prepared from protoplasts transformed with an empty vector. In addition, CsMTP6 expression in the protoplasts resulted in a 30% decrease in Mn-SOD activity (Fig. 7D). These results confirmed the hypothesis that CsMTP6 is involved in the efflux of Fe and Mn from the mitochondria of plant cells. Although the direct involvement of CsMTP6 in Fe and Mn extrusion needs to be confirmed, it is not surprising that Mn would share the

Fig. 7. Effects of CsMTP6 expression on mitochondrial Fe and Mn contents and Mn-SOD activity in Arabidopsis protoplasts. (A) Western blot analysis of the purity of mitochondrial and plastidial fractions prepared from protoplasts expressing CsMTP6 using primary antibodies raised against GFP (to detect CsMTP6-GFP), and marker proteins for plastids (Toc75) and mitochondria. The mitochondria and plastid fractions were prepared from protoplasts by discontinuous density gradient centrifugation in Percoll. (B) Fe and (C) Mn content in the mitochondrial fraction prepared from Arabidopsis protoplasts expressing CsMTP6 or transformed with the empty vector. Data are means (±SD) from three separate experiments. (D) Mitochondrial Mn-SOD activity in protoplasts expressing CsMTP6. Data are means (±SD) from three separate experiments, expressed as enzyme units (U). Significant differences were determined using Student’s t-test.
same transporter with Fe. Both metals are known to share the same plasma membrane uptake transport systems, including the IKT1 (Korshunova et al., 1999), YSL2 (Koike et al., 2004), and NRAMP1 transporters (Cailliatte et al., 2010; Castaings et al., 2016). In addition, Fe and Mn share the same tonoplast transporters involved in the vacuolar sequestration (VIT1 and MTP8) or remobilization (NRAMP3 and NRAMP4) of metal ions (Thomine et al., 2003; Lanquar et al., 2005, 2010; Chu et al., 2017; Eroglu et al., 2017). The broader specificity of metal transporters to both metals probably results from the similarity of the two ions: Mn is structurally and chemically similar to Fe due to comparable atomic radii and electron shells, which allows for similar reactions, transport, and trafficking mechanisms by substitution of Fe for Mn (Smith et al., 2017). Therefore, we may assume that both metals can compete for transport by CsMTP6 and that the excess of one metal in the mitochondria should negatively affect the efflux of the other from the mitochondrial matrix. However, the biological relevance of this phenomenon remains to be determined.

The contribution of CsMTP6 to Fe and Mn export from the mitochondria suggests that plant MTP6 proteins may be involved in the protection of mitochondria from excess Fe and Mn and/or in the remobilization of both ions, particularly Fe, from mitochondria under deficiency. Similar to Fe, Mn is also required for the proper function of mitochondria, as a co-factor of the mitochondrial Mn-SOD, an enzyme involved in the protection of mitochondria from the harmful effects of superoxide radicals (Miller, 2012). However, excess Mn, like excess Fe, is toxic for mitochondria and therefore the levels of both must be tightly controlled, and MTP6 proteins could be involved in such control. In order to test this hypothesis, we further measured the transcript and protein levels of CsMTP6 under different Mn and Fe availability.

**Root expression of CsMTP6 varies with different Fe availability**

The levels of CsMTP6 transcript were significantly elevated both by Fe excess and by Fe deficiency, but there was no effect of different Mn availability (Fig. 8A, Dataset S1 at Dryad). Consistent with the results from the expression assay, the mitochondrial level of CsMTP6 was also increased under elevated Fe concentrations and Fe deficit but remained unchanged by different Mn treatments (Fig. 8B). These results indicate that CsMTP6 activity is regulated by Fe but not by Mn, and that the Fe-mediated increase in mitochondrial CsMTP6 content could be important for the maintenance of mitochondrial and cytosolic Fe homeostasis. Interestingly, the Arabidopsis vacuolar transporters NRAMP3 and NRAMP4 are also regulated by Fe but not Mn availability (Lanquar et al., 2010), indicating that the response of plant cells to the two metals involves different signaling pathways.

The enhanced CsMTP6-mediated efflux of Fe from the mitochondria can protect them from the toxic effects of an excess in conditions of Fe toxicity, or can supply the cellular metabolism with Fe in conditions of deficiency. Thus, our results support the current hypothesis on the possible function of plant mitochondria in Fe storage (Vigani et al., 2013).

It has previously been shown that mitochondria, similarly to plastids, are able to accumulate the Fe-storing protein ferritin, and mitochondrial ferritin has been discovered in Arabidopsis, pea, and cucumber (Zancani et al., 2004; Tarantino et al., 2010; Vigani et al., 2013). The localization of ferritin in the mitochondria of cucumber roots indicated that root mitochondria are capable of storing Fe in a non-toxic form that can be remobilized by the cells in case of deficiency. We believe that plant MTP6 proteins are good candidates for the proteins involved in the remobilization of Fe from the mitochondrial pools in plant cells.

To gain insights into the responses of the other Fe organellar transporters to different Fe availability, we have previously measured the root expression of genes encoding the cucumber homologs of Arabidopsis VIT1, MIT1, MIT2, ferroportin, and PIC1 (chloroplast iron permease) under Fe-deficient or -excess conditions (Migocka et al., 2018). In addition, we have studied the expression of another MTP protein of the Fe/Zn subgroup localized in the mitochondria, CsMTP7, and the expression of mitochondrial ferritin under the same conditions; in contrast to CsMTP6, CsMTP7 contributes to Fe loading into the mitochondria when expressed in yeast and Arabidopsis proplasts (Migocka et al., 2018). Protein coding transcripts and protein levels of CsMTP7 and ferritin were significantly and rapidly increased upon exposure to a non-toxic elevated Fe
concentration (500 µM), but were barely detected under Fe deficiency (Migocka et al., 2018). Hence, we assume that CsMTP7 and ferritin work in concert to increase the accumulation of Fe in plant mitochondria under elevated Fe conditions. These results confirm the hypothesis that, similar to plastids and the vacuole, mitochondria have also developed a high capacity for storing Fe. As CsMTP6 is involved in Mn/Fe efflux from mitochondria, we assume that plant MTP6 and MTP7 proteins might fulfill different physiological functions: taking into account the H+ gradient generated in the inner mitochondrial membrane, we may assume that CsMTP7 catalyses the import of Fe(II) into mitochondria via the symport mechanism, whereas CsMTP6 catalyses the export of Fe(II) and Mn(II) from mitochondria through a Fe(II)/(Mn(II))/H+ antiport (Migocka et al., 2018). Based on the effect of Fe on the expression of both proteins, we can hypothesize that CsMTP7 is up-regulated by elevated Fe to increase the mitochondrial content (for Fe storage), whereas CsMTP6 is up-regulated by elevated Fe to remove excess and reduce Fe toxicity, or by Fe deficiency to release Fe stored in mitochondria in order to meet cellular demands. Similar to CsMTP6, the vacuolar iron transporters NRAMP3 and NRAMP4 have been shown to be up-regulated under conditions of low Fe to release Fe stored in the vacuole to meet cellular demands during seed germination (Lanquar et al., 2005). In addition to CsMTP7, iron mitoferrins mediate Fe transport into mitochondria. Nevertheless, both the cucumber genes encoding MITs are up-regulated upon Fe deficiency and are not affected by excess Fe (Migocka et al., 2018), suggesting that they may be crucial for the mitochondrial import of Fe under limiting conditions. The expression of other genes coding for proteins involved in Fe uptake and accumulation in cucumber roots is also altered only upon Fe deficiency (plasma membrane CsIR1, CsFRO2, CsREG2/CsFNP2, chloroplast CsPIC1/CsTIC21) or is not affected by Fe availability (CsVIT1) (Migocka et al., 2018). These results suggest that mitochondrial Fe homeostasis is rapidly regulated by Fe excess and deficiency at the level of the transcription of MITs, MTP6, and MTP7, whereas the accumulation of Fe in the plastids or vacuoles of cucumber roots is not altered following supplementation of Fe. Based on these results, we can conclude that the mitochondria of cucumber roots may play a very important role in maintaining cellular Fe homeostasis, and that the function of the other organelar Fe transporters in the roots may be predominantly related to the response of cucumber to Fe deficiency.

In conclusion, this study supports the hypothesis that plant MTP6 proteins function in mitochondrial Mn/Fe efflux and can thus play an important role in the maintenance of mitochondrial and cytosolic Fe and Mn homeostasis. The mitochondrial localization and the Fe and Mn specificity of CsMTP6 provide novel insights into the functions of MTP proteins in plant cells.

Data deposition

The following tables and figures are available at Dryad Data Repository: https://doi.org/10.5061/dryad.306s0b, last accessed on October 19, 2018.

Table S1. Yeast strains used in this work.

Fig. S1. Organ expression pattern of CsMTP6 in cucumber.
Fig. S2. ClustalW alignment of the CsMTP6 nucleic acid and CsMTP6 amino acid sequences.
Fig. S3. Western blot analysis of mitochondria isolated from yeast expressing CsMTP6.
Fig. S4. Localization of CsMTP6 in Δzrt1, K667, and Δsmf1 cells.
Fig. S5. Effect of CsMTP6 expression on yeast sensitivity to Zn, Ni, and Cd.
Dataset S1. CsMTP6 expression data.
Dataset S2. Data for GDO activity in yeast.
Dataset S3. Data for metal content from mitochondria in yeast.
Dataset S4. Data for metal content in mitochondria of protoplasts.

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References

Arrivaert S, Senger T, Krämer U. 2006. The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. The Plant Journal 46, 861–879.
Asada K, Takahashi M, Nagate M. 1974. Assay and inhibitors of spinach superoxide dismutase. Agricultural and Biological Chemistry 38, 471–473.
Bashir K, Ishimaru Y, Shimo H, Nagasaka S, Fujimoto M, Takanashi H, Tsutsuji N, An G, Nakanishi H, Nishizawa NK. 2011. The rice mitochondrial iron transporter is essential for plant growth. Nature Communications 2, 322.
Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.
Briat JF, Ravet K, Arnaud N, Duc C, Boucheur J, Touraine B, Cellier F, Gaymard F. 2010. New insights into iron homeostasis and oxidative stress in plants. Annals of Botany 105, 811–822.
Caillette R, Schikora A, Briat JF, Mari S, Curie C. 2010. High-affinity manganese uptake by the metal transporter NRAMP1 is essential for Arabidopsis growth in low manganese conditions. The Plant Cell 22, 904–917.
Castaings L, Caquot A, Loubet S, Curie C. 2016. The high-affinity metal transporters NRAMP1 and IRT1 team up to take up iron under sufficient metal supply. Scientific Reports 6, 37222.
Chu HH, Car S, Socha AL, Hindt MN, Punshon T, Guerinot ML. 2017. The Arabidopsis MTP8 transporter determines the localization of manganese and iron in seeds. Scientific Reports 7, 11024.
Cubillas C, Vinuesa P, Tabche ML, Dávalos A, Vázquez A, Hernández-Lucas I, Romero D, García-de los Santos A. 2014. The cation diffusion facilitator protein EmfA of Rhizobium etli belongs to a novel subfamily of Mn²⁺/Fe²⁺ transporters conserved in α-proteobacteria. Metallomics 6, 1808–1815.
Cubillas C, Vinuesa P, Tabche ML, García-de los Santos A. 2013. Phylogenomic analysis of cation diffusion facilitator proteins uncovers Ni²⁺/Co²⁺ transporters. Metallomics 5, 1634–1643.
Cunningham KW, Fink GR. 1996. Calcineurin inhibits Vcx1-dependent H+/Ca2+ exchange and induces Ca2+ ATPases in Saccharomyces cerevisiae. Molecular and Cellular Biology 16, 2226–2237.

Curie C, Cassin G, Couch D, Diod D, Higuchi K, Le Jean M, Mission J, Schikora A, Czernic P, Mari S. 2009. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. Annals of Botany 103, 1–11.

Curie C, Panavieni Z, Loulegue D, Dellaporta SL, Brait JF, Walker EL. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature 409, 346–349.

Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardson AE. 2007. A role for the AtMTP11 gene of Arabidopsis in manganese transport and tolerance. The Plant Journal 51, 198–210.

Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR. 2003. Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. The Plant Cell 15, 1131–1142.

Duy D, Wanner G, Meda AR, von Wirén N, Soli J, Philippar K. 2007. PIC1, an ancient permease in Arabidopsis chloroplasts, mediates iron transport. The Plant Cell 19, 986–1006.

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, USA 93, 5624–5628.

Erogul S, Giehl RFH, Meier B, Takahashi M, Terada Y, Ignatyev K, Andresen E, Kükper H, Peiter E, von Wirén N. 2017. Metal tolerance protein 8 mediates manganese homeostasis and iron reallocation during seed development and germination. Plant Physiology 174, 1635–1647.

Erogul S, Meier B, von Wirén N, Peiter E. 2016. The vacuolar manganese transporter MTP5 determines tolerance to iron deficiency-induced chlorosis in Arabidopsis. Plant Physiology 170, 1030–1045.

Farcasanu IG, Hirata D, Tsuchiya E, Nishiyama F, Miyakawa T, Farcasanu IC, Hirata D, Tsuchiya E, Nishiyama F, Miyakawa T. 1995. Protein phosphatase 2B of Saccharomyces cerevisiae is required for tolerance to manganese, in blocking the entry of ions into the cells. European Journal of Biochemistry 232, 712–717.

Fernandes J, Hao L, Bijli KM, Chandler JD, Orr M, Hu X, Jones DP, Go YM. 2017. Manganese stimulates mitochondrial H2O2 production in SH-SYSY human neuroblastoma cells over physiologic as well as toxicologic range. Toxicological Sciences 155, 213–223.

Froschauer EM, Schweyen RJ, Wiesenberger G. 2009. The yeast mitochondrial carrier proteins Mrp3p/Mrp4p mediate iron transport across the inner mitochondrial membrane. Biochimica et Biophysica Acta 1788, 1044–1058.

Fujitara T, Kawachi M, Sato Y, Mori H, Kutsuna N, Hasezawa S, Maeshima M. 2015. A high molecular mass zinc transporter MTP12 forms a functional heteromeric complex with MTP5 in the Golgi in Arabidopsis thaliana. The FEBS Journal 282, 1965–1979.

Gavin CE, Gunter KK, Gunter TE. 1992. Mn2+ sequestration by mitochondria and inhibition of oxidative phosphorylation. Toxicology and Applied Pharmacology 115, 1–5.

Glusker JP. 1980. Citrate conformation and chelation: enzymic implications. Accounts of Chemical Research 13, 345–352.

Godbold DL, Horst WJ, Collins JC, Thurman DA, Marschner H. 1984. Accumulation of zinc and organic acids in roots of zinc tolerant and non-tolerant ecotypes of Deschampsia caespitosa. Journal of Plant Physiology 116, 59–69.

Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH, Munkelt D. 2005. FieF (Yip) from Escherichia coli mediates decreased cellular accumulation of iron and relieves iron stress. Archives of Microbiology 183, 9–18.

Gregg C, Kyryakov P, Titorenko VL. 2009. Purification of mitochondria from yeast cells. Journal of Visualized Experiments 30, 1417.

Grünberg K, Wawer C, Tebo BM, Schüeler D. 2001. A large gene cluster encoding several magnetosome proteins is conserved in different species of magnetotactic bacteria. Applied and Environmental Microbiology 67, 4573–4582.

Gustin JL, Zanis MJ, Salt DE. 2011. Structure and evolution of the plant cation diffusion facilitator family of ion transporters. BMC Evolutionary Biology 11, 76.

Hallwell B, Gutteridge JM, Cross CE. 1992. Free radicals, antioxidants, and human disease: where are we now? The Journal of Laboratory and Clinical Medicine 119, 589–620.

Hallimond SF, Roberts DJ, Nickless G. 1996. The effect of cadmium, zinc, and copper on the growth, tolerance index, metal uptake, and production of malic acid in two strains of the grass Festuca rubra. Canadian Journal of Botany 74, 1742–1752.

Jain A, Connolly EL. 2013. Mitochondrial iron transport and homeostasis in plants. Frontiers in Plant Science 4, 348.

Kabala K, Kubous G. 2001. Characterization of the tonoplast proton pumps in Cucumis sativus L. root cells. Acta Physiologica Plantarum 23, 55–63.

Kim SA, Punshon T, Lanzirotti A, Li L, Alonso JM, Ecker JR, Kaplan J, Guerinot ML. 2006. Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VTI1. Science 314, 1295–1298.

Kobayase M, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M. 2004. Zinc transporter of Arabidopsis thaliana AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant & Cell Physiology 45, 1749–1758.

Koike S, Inoue H, Mizuno D, Mori S, Nishizawa NK. 2004. OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. The Plant Journal 39, 415–424.

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB. 1999. The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. Plant Molecular Biology 40, 37–44.

Lanquar V, Lelièvre F, Bolte S, et al. 2005. Mobilization of vacuolar iron by AINRP3 and AINRP4 is essential for seed germination on low iron. The EMBO Journal 24, 4041–4051.

Lanquar V, Ramos MS, Lelièvre F, Barbier-Brygoo H, Krieger-Liszak A, Krämer U, Thomine S. 2010. Export of vacuolar manganese by AINRP3 and AINRP4 is required for optimal photosynthesis and growth under manganese deficiency. Plant Physiology 152, 1986–1999.

Lanza IR, Nair KS. 2009. Functional assessment of isolated mitochondria in vitro. Methods in Enzymology 457, 349–372.

Lapinskas PJ, Cunningham KW, Liu XF, Fink GR, Culotta VC. 1995. Mutations in PMR1 suppress oxidative damage in yeast cells lacking superoxide dismutase. Molecular and Cellular Biology 15, 1382–1388.

Larsson C, Widell S, Kjellbom P. 1987. Preparation of high-purity plasma membranes. Methods in Enzymology 148, 558–568.

Li L, Chen OS, McVay Ward D, Kaplan J. 2001. CCC1 is a transporter that mediates vacuolar iron storage in yeast. The Journal of Biological Chemistry 276, 29515–29519.

Li L, Kaplan J. 1997. Characterization of two homologous yeast genes that encode mitochondrial iron transporters. The Journal of Biological Chemistry 272, 28485–28493.

Li L, Kaplan J. 2004. A mitochondrial-vacuolar signaling pathway in yeast that affects iron and copper metabolism. The Journal of Biological Chemistry 279, 33653–33661.

Li L, Miao R, Jia X, Ward DM, Kaplan J. 2014. Expression of the yeast cation diffusion facilitators Mmt11 and Mmt12 affects mitochondrial and cellular iron homeostasis: evidence for mitochondrial iron export. The Journal of Biological Chemistry 289, 17132–17141.

Li Z, Zhang Z, Yan P, Huang S, Fei Z, Lin K. 2011. RNA-Seq improves annotation of protein-coding genes in the cucumber genome. BMC Genomics 12, 540.

Liu XF, Culotta VC. 1999. Post-translation control of Nramp metal transport in yeast. Role of metal ions and the BSD2 gene. The Journal of Biological Chemistry 274, 4863–4868.

Lu M, Fu D. 2007. Structure of the zinc transporter YiiP. Science 317, 1746–1748.

Malecki EA. 1977. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. Brain Research Bulletin 55, 225–228.

Mary V, Schnell Ramos M, Gillet C, et al. 2015. Bypassing iron storage in endodermal vacuoles rescues the iron mobilization defect in the natural resistance associated-macrophage protein3natural resistance associated-macrophage protein4 double mutant. Plant Physiology 169, 748–759.
Mathys W. 1977. The role of malate, oxalate and mustard oil glucosides in the evolution of zinc resistance in herbaceous plants. Physiologia Plantarum 40, 130–135.

Metzendorf C, Lind MI. 2010. Drosophilia mitoferrin is essential for male fertility: evidence for a role of mitochondrial iron metabolism during spermatogenesis. BMC Developmental Biology 10, 68.

Migocka M, Kosieradzka A, Papierniak A, Maciaszkczyz-Dziubinska E, Posniak E, Garbiec A, Filleur S. 2015a. Two metal-tolerance proteins, MTP1 and MTP4, are involved in Zn homeostasis and Cd sequestration in cucumber cells. Journal of Experimental Botany 66, 1001–1015.

Migocka M, Papierniak A, Kosieradzka A, Posniak E, Maciaszkczyz-Dziubinska E, Biskup R, Garbiec A, Marchewka T. 2015b. Cucumber metal tolerance protein CoMTP9 is a plasma membrane H+-coupled antiporter involved in the Mn2+ and Cd2+ efflux from root cells. The Plant Journal 84, 1045–1058.

Migocka M, Papierniak A, Maciaszkczyz-Dziubinska E, Podzik P, Posniak E, Garbiec A, Filleur S. 2014. Cucumber metal transport protein MTP8 confers increased tolerance to manganese when expressed in yeast and Arabidopsis thaliana. Journal of Experimental Botany 65, 5367–5384.

Migocka M, Malas K, Maciaszkczyz-Dziubinska E, Papierniak A, Posniak E, Garbiec A. 2018. Cucumber metal tolerance protein 7 (CoMTP7) is involved in the accumulation of Fe in mitochondria under Fe excess. The Plant Journal 95, 988–1003.

Migocka M, Maciaszkczyz-Dziubinska E, Malas K, Posniak E, Garbiec A. 2019. Data from: Metal tolerance protein MTP6 affects mitochondrial iron and manganese homeostasis in cucumber. Dryad Digital Repository. DOI: doi.org/10.5061/dryad.306fb0b.

Miller AJ. 2012. Superoxide dismutases: ancient enzymes and new insights. FEBS Letters 586, 585–595.

Montanini B, Blaudez D, Jeandroz S, Sanders D, Chalot M. 2007. Localization of the cation diffusion facilitator proteins DmeF and FieF from Deschampsia caespitosa in the intermembrane space of mitochondria. A physiological role of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. The Journal of Biological Chemistry 276, 38084–38089.

Stadler JA, Schwyen RJ. 2002. The yeast iron regulon is induced upon cobalt stress and crucial for cobalt tolerance. The Journal of Biological Chemistry 277, 39649–39654.

Sturtz LA, Diekert K, Jensen LT, Lili R, Culotta VC. 2001. A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for SOD1 in guarding against mitochondrial oxidative damage. The Journal of Biological Chemistry 277, 38084–38089.

Supek F, Supekova L, Nelson H, Nelson N. 1996. A yeast manganese transporter related to the macrophage protein involved in conferring resistance to mycobacteria. Proceedings of the National Academy of Sciences, USA 93, 5105–5110.

Tarantino D, Casagrande F, Soave C, Murgia I. 2010. Knocking out the mitochondrial AtFer4 ferritin does not alter response of Arabidopsis plants to abiotic stresses. Journal of Plant Physiolog 167, 453–460.

Thomine S, Lelièvre F, Debarbieux E, Schroeder JI, Barbiere-Brygoo S, Mathys W. 1977. The role of malate, oxalate and mustard oil glucosides in the evolution of zinc resistance in herbaceous plants. Physiologia Plantarum 40, 130–135.