COPT6 Is a Plasma Membrane Transporter That Functions in Copper Homeostasis in Arabidopsis and Is a Novel Target of SQUAMOSA Promoter-binding Protein-like 7*

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Background: Copper uptake is tightly regulated to prevent deficiency while avoiding toxicity.

Results: AtCOPT6 localizes to the plasma membrane, is regulated by copper availability, interacts with itself and AtCOPT1, and regulates response to copper limitation and excess.

Conclusion: AtCOPT6 is a novel SPL7 target that functions in copper homeostasis in Arabidopsis.

Significance: Identification and characterization of copper transporters are crucial for understanding of copper homeostasis.

Among the mechanisms controlling copper homeostasis in plants is the regulation of its uptake and tissue partitioning. Here we characterized a newly identified member of the conserved CTR/COPT family of copper transporters in Arabidopsis thaliana, COPT6. We showed that COPT6 resides at the plasma membrane and mediates copper accumulation when expressed in the Saccharomyces cerevisiae copper uptake mutant. Although the primary sequence of COPT6 contains the family conserved domains, including methionine-rich motifs in the extracellular N-terminal domain and a second transmembrane helix (TM2), it is different from the founding family member, Ctr1p. This conclusion was based on the finding that although the positionally conserved Met106 residue in the TM2 of COPT6 is functionally essential, the conserved Met27 in the N-terminal domain is not. Structure-function studies revealed that the N-terminal domain is dispensable for COPT6 function in copper-replete conditions but is important under copper-limiting conditions. In addition, COPT6 interacts with itself and with its homolog, COPT1, unlike Ctr1p, which interacts only with itself. Analyses of the expression pattern showed that although COPT6 is expressed in different cell types of different plant organs, the bulk of its expression is located in the vasculature. We also show that COPT6 expression is regulated by copper availability that, in part, is controlled by a master regulator of copper homeostasis, SPL7. Finally, studies using the A. thaliana copt6-1 mutant and plants overexpressing COPT6 revealed its essential role during copper limitation and excess.

Copper is a redox-active transition element that serves as an essential micronutrient for all living organisms (1–3). It acts as a cofactor for enzymes involved in electron transfer reactions and, thus, participates in important biological processes such as respiration, photosynthesis, and scavenging of oxidative stress. In addition, copper is involved in the perception of ethylene, nitrogen metabolism, molybdenum cofactor synthesis, cell wall remodeling, and response to pathogens in plants (1, 4, 5). However, free copper ions are toxic to cells in excess due to their ability to promote the formation of free radicals through the Fenton reaction and increased malfunction of important proteins, either through thiol capping or displacement of metal co-factors of metalloenzymes (1, 6–8). Therefore, organisms have evolved sophisticated mechanisms for maintaining copper homeostasis to prevent deficiency while avoiding toxicity.

The response to copper deficiency in plants includes reallocation of intracellular copper and induction of the expression of copper uptake systems. In Arabidopsis thaliana, this regulation is largely attributable to the activity of the transcription factor SPL7 (SQUAMOSA promoter-binding protein-like 7) and its downstream targets, microRNAs (9–13). When copper is limited, SPL7 up-regulates expression of miRNA398, which in turn down-regulates expression of two main isoforms of the major copper enzymes, the cytosol Cu/Zn-superoxide dismutase (SOD), CSD1, and the chloroplast stroma SOD, CSD2 (9–13). As CSD1 and CSD2 transcripts and the activity of the encoded proteins decrease, their superoxide-scavenging functions are replaced by an increase in gene expression and total enzyme activity of a plastid-localized Fe-SOD, FSD1 (11, 14). The miRNA-mediated copper economy model has been proposed, where energy-related electron transport functions receive priority in copper delivery over the major copper enzymes (1, 11, 15, 16).

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4 The abbreviations used are: SOD, superoxide dismutase; BCS, bathocuproine disulfonate; MYTH, split ubiquitin membrane yeast two-hybrid system; BIFC, bimolecular fluorescence complementation; ICP-MS, inductively coupled plasma mass spectroscopy; Mets motif, methionine-rich N-terminal motif; qRT-PCR, quantitative RT-PCR; EGF, enhanced green fluorescent protein; TM, transmembrane; ‡‡ MS, half-strength Murashige and Skoog; UBP, ubiquitin-specific protease.
Simultaneously, plants regulate copper homeostasis by controlling its uptake into cells. Copper uptake in plants, the green alga *Chlamydomonas reinhardtii*, yeast, *Drosophila*, and humans is maintained mainly through the tight regulation of the expression and stability of copper transporters of the CTR/COPT family (17–25). The founding members, Ctr1p, Ctr2p, and Ctr3p, were identified in *Saccharomyces cerevisiae* (17–19, 25). Ctr1p and Ctr3p localize to the plasma membrane, are functionally redundant, and mediate high affinity copper uptake from the external medium during copper deficiency (17, 18, 25). Expression of the *CTR1* gene is subject to copper metalloregulation; it is transcriptionally induced under copper-deficient conditions via a transcription factor, Mac1, and is posttranscriptionally degraded under copper-replete conditions (26–28). Ctr2p is located on the vacuolar membrane and mobilizes copper from the vacuole during copper deficiency (19). CTR/COPT family members have conserved structural features that include three putative transmembrane (TM) helices, with the N and C termini located toward the extracellular space and cytosol, respectively; methionine-rich N-terminal motifs (Mets motifs); and M and G motifs located toward the extracellular space and cytosol, respectively; methionine-rich N-terminal motifs with the N and C termini located toward the extracellular space (29). CTRs are homologous, physically interact with other CTRs, and localize to the plasma membrane. They regulate copper homeostasis in response to toxic copper concentrations (31, 33, 34). CTR/COPT proteins homotrimmerize to form a pore within the membrane to transport copper across the lipid bilayer but can also form heterocomplexes with other CTR/COPT family members and/or other proteins (5, 29, 30, 35, 36).

In plants, the CTR/COPT family is best characterized in *A. thaliana* and *Oryza sativa* and is represented by six and seven members, respectively (32, 36). *A. thaliana* COPT1 and COPT2 fully suppress the copper deficiency-associated respiratory defect of the *S. cerevisiae ctra1Δctr3Δ* mutant, whereas COPT3 and COPT5 partially complement this phenotype (22–24). Subsequent studies showed that COPT1 localizes to the plasma membrane, mediates copper influx, and, similar to CTR proteins from other organisms, is highly specific for copper (30, 23). Studies in *A. thaliana* showed that COPT1 plays a predominant role in copper acquisition from the soil via root tips (20, 22). In contrast, COPT5 localizes to the tonoplast and prevacuolar compartment and functions by remodeling copper from these organelles during copper deficiency (37, 38). Expression of COPT1 and COPT2 mRNA is up-regulated in copper-deficient conditions (22), and these responses are controlled by the transcription factor, SPL7 (12). COPT6 is a newly identified member of the CTR/COPT family of *A. thaliana* transporters (32), and its function in copper homeostasis has not yet been elucidated.

We show that COPT6 is a plasma membrane copper uptake transporter that is distinct from its *S. cerevisiae* counterpart, Ctr1p, and plays an important role in *A. thaliana* during copper deficiency and excess.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions**—All plant lines used in the study were in the *A. thaliana* Columbia (Col-0) background. Seeds of the *copt6-1* (SALK 083438) T-DNA insertion allele were obtained from the *Arabidopsis* biological Resource Center (39). Seeds of the *spl7-1* mutant were obtained from Dr. Shikanai (Kyoto University), and this mutant has been described (12). Before growing different *A. thaliana* lines on solid medium, seeds were surface-sterilized with 75% (v/v) ethanol before soaking in a solution containing 1.8% bleach (made up by diluting household Clorox) and 0.1% Tween 20. Sterilized seeds were rinsed with sterile water and sowed on half-strength Murashige and Skoog (½ MS) medium (pH 5.7) with 0.1% (w/v) sucrose and 0.7% (w/v) agar (Sigma A1296) supplemented with or without 45 μM CuCl2 or the indicated concentrations of the specific copper chelator, bathocuproine disulfonate (BCS). After stratification at 4°C for 2 days in darkness, seeds were germinated, and seedlings were grown vertically for 10 days (at 22°C; 12-h light/12-h dark photoperiod at photosynthetic photon flux density of 120 μmol m−2 s−1) before subsequent analyses.

**RNA Extraction and cDNA Synthesis**—Root and shoot tissues from 10-day-old seedlings grown under the indicated conditions were separated and flash-frozen in liquid nitrogen before homogenization in liquid nitrogen using a mortar and pestle. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. Genomic DNA in total RNA samples was digested with DNAse I (Roche Applied Science) prior to first strand cDNA synthesis using the QPCR cDNA synthesis kit (Bio-Rad).

**Plasmid Construction**—The vectors YES3 (40) and SAT6-N1-EGFP (41) were modified into Gateway destination vectors with the Gateway® vector conversion system (Invitrogen) and designated accordingly as YES3-Gate and SAT6-N1-EGFP-Gate.

To generate the YES3-EGFP vector, the EGFP DNA sequence was amplified from the SAT6-N1-EGFP vector using primer pairs that generated SmaI restriction enzyme recognition sites at 5’ and 3’ ends of the PCR product (supplemental Table S1). The PCR product was then subcloned into SmaI/SmaI site-diagnosed YES3 vector. The resulting YES3-EGFP was converted into YES3-EGFP-Gate with the Gateway® vector conversion system (Invitrogen).

The COPT6 cDNA with or without the stop codon was amplified by RT-PCR from RNA isolated from *A. thaliana* leaves. The primers (supplemental Table S1) added attB sites on resulting PCR products, which were then introduced into corresponding vectors by recombination cloning (Invitrogen). Site-directed mutagenesis of COPT6 was performed directly on a YES3-Gate-COPT6 vector with QuikChange® II site-directed mutagenesis kit (Stratagene). The mutagenic oligonucleotides (supplemental Table 1) were designed to substitute conserved methionines to alanine codons in the Mets motif of the predicted extracellular domain or TM2 of COPT6 or to delete 81 bp corresponding to 27 amino acids of the extracellular N-terminal domain (supplemental Fig. 1). Mutagenesis was...
confirmed by sequencing the cDNA regions encompassing each mutation.

The cDNA encoding the full-length Ctr1p, and Ctr1p with
M127L and M260A point mutations were amplified by PCR
from the 416TEF vector (courtesy of Dr. Dennis Thiele, Duke
University) using primer pairs (supplemental Table 1). All
inserts were introduced into the DONR222 entry vector before
recombination with the YES3-Gate destination vector men-
tioned above.

Functional Complementation of the S. cerevisiae Copper
Uptake-deficient ctr1Δctr2Δctr3Δ Mutant Strain—S. cerevisiae
SEY6210 (MATa ura3-52 leu2-3,112 his3Δ200 trp1Δ901 lys2-801 suc2Δ9) wild-type and ctr1Δctr2Δctr3Δ triple mutant
(MATa ura3-52 his3Δ200 trp1Δ901 ctrl::ura3::Knr ctrl2::HIS3
ctrl3::TRP1) strains that were used for functional complemen-
tation assays were the generous gift of Dr. Dennis Thiele (Duke
University). Yeast cells were transformed with the YES3-Gate-
COPT6 vector or YES3-Gate lacking the cDNA insert using the
Frozen-EZ Yeast Transformation II kit (Zymo Research).

Transformants were selected for uracil prototrophy on YNB
medium containing 6.7% (w/v) yeast nitrogen base without
amino acids (Difco), 0.77% (w/v) CSM-URA, 0.5% (w/v) NaCl,
10-fold diluted, and spotted onto YPEG medium containing 1%
(w/v) yeast extract, 2% (w/v) bacto-peptone, 3% (v/v) glycerol,
10% yeast extract (w/v), and 2% glucose (w/v) agar.

Respiration competence was evaluated by testing the ability of
transformants to grow on the non-fermentable carbon
sources, glycerol and ethanol (17, 31). Transformants were
grown in liquid YNB-URA to an A600nm = 1.0–1.1, serially
10-fold diluted, and spotted onto YPEG medium containing 1%
(w/v) yeast extract, 2% (w/v) bacto-peptone, 3% (v/v) glycerol,
2% (v/v) ethanol, and 2% (w/v) agar and the indicated concen-
trations of CuSO4. Plates were incubated for 3 days at 30 °C.

Quantitative Real-time (qRT)-PCR and Data Analysis—
Prior to qRT-PCR analysis, primer and cDNA concentrations
were optimized to reach the target and normalizing gene ampli-
fication efficiency of 100 ± 10%. One microliter of 15-fold
diluted cDNA was used as a template for qRT-PCR in a total
volume of 10 µl containing a 500 nM concentration of each PCR
primer, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM each
dNTP, and 1.25 units of iTaq DNA polymerase in 1Q SYBR
Green Supermix (Bio-Rad). PCR was carried out using the
CFX96 real-time PCR system (Bio-Rad). The thermal cycling
parameters were as follows: denaturation at 95 °C for 3 min,
followed by 39 cycles of 95 °C for 10 s and 55 °C for 30 s. Ampli-
con dissociation curves (i.e., melting curves) were recorded after
cycle 39 by heating from 60 °C to 95 °C with 0.5 °C increments
and an average ramp speed of 3.3 °C s⁻¹. Real-time PCR exper-
iments were conducted using three independent biological
samples, each consisting of three technical replicates (42),
unless indicated otherwise. Data were normalized to the
expression of ACTIN 2. The ΔΔCT was calculated using the
CFX Manager Software, version 1.5 (Bio-
Rad). Statistical analysis was performed using the Relative
Expression Software Tool (REST; Qiagen) (43).

Subcellular Localization and Fluorescent Microscopy—For
studies of subcellular localization in S. cerevisiae, COPT6
was fused at the C terminus with the EGFP in the YES3-EGFP-Gate
vector and expressed under the control of the constitutive
phosphoglycerate kinase (PGK) gene promoter. The resulting
COPT6-EGFP construct and the empty YES3-EGFP-Gate vec-
tor were transformed into S. cerevisiae ctrl1Δctr2Δctr3Δ triple
mutants using the Frozen-EZ Yeast Transformation II kit
(Zymo Research). Plasma membranes were stained with FM
4-64 dye, an endocytic marker that is also used to visualize the
plasma membrane after short term staining at 0 °C (44–46).
Three milliliters of overnight culture were pelleted by centrif-
ugation, and concentrated cells were resuspended in 1 ml of
ice-cold liquid YPD medium containing 20% peptone (w/v),
10% yeast extract (w/v), and 2% glucose (w/v), pH 6.0. The
sample was cooled on ice for 5–10 min before adding 3 µl of DMSO
and FM 4-64 at a final concentration of 7.5 µM. The sample was
incubated at 4 °C before dispensing on precooled slides topped
with 1% agarose for visualization. Epifluorescence images were
collected using an Axio Imager M2 microscope (Zeiss).

For studies of the subcellular localization of COPT6 in Ara-
bidopsis protoplasts, the full-length COPT6 cDNA without the
stop codon was fused at the C terminus with the modified green
fluorescent protein (EGFP) using the SAT6-N1-EGFP-Gate
vector and expressed under the control of the cauliflower
mosaic virus 35S promoter. The resulting 35Spro-COPT6-EGFP
construct or SAT6-N1-Gate, lacking the cDNA insert, was
transfected into A. thaliana protoplasts isolated from leaf mes-
ophyll tissue using previously established procedures (47–49).
Plasma membranes were stained with 50 µM FM 4-64 as
described (45). EGFP- and FM 4-64-mediated fluorescence and
chlorophyll autofluorescence were visualized using FITC (for
EGFP) or rhodamine (FM 4-64 and chlorophyll) filter sets of
the Axio Imager M2 microscope equipped with the motorized
Z-drive (Zeiss). z stack (1.3-µm-thick) images were collected with
the high resolution AxioCam MR Camera and then three-
dimensionally deconvoluted using an inverse filter algorithm
of the Zeiss AxioVision 4.8 software. Images were processed using
the Adobe Photoshop software package, version 12.0.

Bimolecular Fluorescence Complementation (BiFC) Assays
and Confocal Microscopy—The vectors used in the BiFC assay,
UC-SPYNE and UC-SPYCE, contained the N- or C-terminal
fragments of yellow fluorescent protein, YFPN and YFPC, respec-
tively, allowing expression of fused proteins under the control
of the 3SS promoter (50). To generate COPT6-YFPN and
COPT6-YFPC fusion constructs, the C terminus of the COPT6
coding sequence was fused with YFPN or YFPC in the
UC-SPYNE or UC-SPYCE vectors, respectively. The coding
regions of COPT1 and COPT5 were amplified without stop
codons and used to generate COPT1-YFPN and COPT5-YFPC.
The resulting constructs and UC-SPYNE and UC-SPYCE vec-
tors lacking cDNA inserts were co-transfected in the indicated
combinations into A. thaliana leaf protoplasts as described
(47–49). Protoplasts were analyzed using a Zeiss 710 confocal
microscope. The YFP fluorophore was excited with a 514-nm
laser, and the emission was recorded in the range of 524–595
nm and 650–715 nm for YFP and chlorophyll, respectively. To
allow comparison of the relative brightness between different
BiFC experiments, the zoom, pinhole, detector gain, amplifier
offset, frame size, scan speed, scan average, and laser power
were kept consistent between samples. Images were processed
using the Zen 2009 LE software of the Zeiss 710 confocal micro-
scope and the Adobe Photoshop software package, version 12.0.
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**RESULTS**

The COPT6 Polypeptide Contains the Key Features of the CTR/COPT Family—The *A. thaliana* genome possesses six genes encoding putative CTR/COPT transporters that are designated COPT1 to 6. Of the six family members, COPT6 has been identified in the *A. thaliana* genome only recently (32), thus making COPT6 the least studied member of the *A. thaliana* CTR/COPT family. Analysis of the amino acid sequence identity and similarity of COPT6 to other CTR/COPT family members of *A. thaliana* disclosed that COPT2 and COPT1 are the closest COPT6 homologs, sharing 75%/79% sequence identity/similarity and 71%/75% identity/similarity, respectively (supplemental Fig. 1). In addition, COPT6 clusters in one clad with COPT1 and COPT2 when their amino acid sequences are subjected to phylogenetic analysis (Fig. 1A), suggesting that COPT6 may have similar functions to COPT1 and COPT2 in copper homeostasis.

Computer algorithm-assisted analysis of the COPT6 polypeptide membrane topology and conserved motif organization
revealed an extracellular N-terminal domain; a membrane domain encompassing three TM helices, where TM2 and TM3 are separated by 3 amino acids; and an intracellular C-terminal domain (Fig. 1B). The COPT6 polypeptide includes the MXXXM$_2$GXXG signature of CTR/COPT proteins; its N terminus and TM2 contain Mets and MXXXM motifs, respectively, whereas TM3 harbors the GXXXG motif (Fig. 1C). These features are conserved in the majority of the CTR/COPT proteins and are also present in COPT1 and COPT2. However, unlike COPT1 and COPT2, COPT6 does not possess the cysteine-rich CXC motif at the C terminus (Fig. 1C).

**Heterologously Expressed COPT6 Complements the Growth Defect of the Triple ctr1Δctr2Δctr3Δ S. cerevisiae Mutant on Non-fermentable Growth Medium**—Despite the presence of key motifs conserved in the CTR/COPT family, COPT6 lacks the CXC motif at the C terminus (Fig. 1C), which was shown to play an important role in copper homeostasis in Ctr1p (31, 33, 34). This motif is also present in A. thaliana homologues COPT1 and COPT2, prompting us to test whether the lack of CXC motif in COPT6 still results in a protein capable of transporting copper. In this regard, S. cerevisiae strains harboring mutations in copper uptake genes have been used as a versatile and well defined model system for functional analysis of putative copper transporters from various species. Yeast lacking copper uptake systems cannot grow on non-fermentable carbon sources, such as glycerol and ethanol (YPEG medium), due to low levels of intracellular copper. This deficiency results in the inability of cytochrome c oxidase to obtain its copper cofactor, which, in turn, causes a defect in the mitochondrial respiratory chain unless copper is exogenously supplied to the medium (17, 61).

The function of COPT6 in copper transport was tested through complementation studies in the S. cerevisiae ctr1Δctr2Δctr3Δ triple mutant strain, which lacks high affinity plasma membrane transporters Ctr1p and Ctr3p, and a vacular membrane transporter, Ctr2p (19). Growth of ctr1Δctr2Δctr3Δ mutant and wild-type strains expressing an empty YES3-Gate vector and the ctr1Δctr2Δctr3Δ mutant transformed with YES3-Gate harboring the COPT6 cDNA insert was compared on medium with glucose (YNB-URA) or ethanol/glycerol (YPEG) as a carbon source. All yeast alleles were able to grow on YNB-URA (Fig. 2A). As shown previously (19), ctr1Δctr2Δctr3Δ cells did not grow on YPEG medium unless the medium was supplemented with exogenous copper (Fig. 2A). In contrast, ctr1Δctr2Δctr3Δ cells expressing COPT6 grew on YPEG regardless of copper supplementation, and their growth was comparable with that of wild-type cells transformed with the empty vector (Fig. 2A).

To determine the subcellular localization of COPT6 in the heterologous system, we fused it at the C terminus to EGFP of the YES3-EGFP-Gate vector and transformed this construct or the empty vector expressing EGFP only into the...
 grown on YNB-URA. In contrast, the wild-type cells accumulated 20.7 ppm copper when grown at 10 and 20 μM CuSO₄, respectively, than mutant cells expressing the empty vector and 3- and 4-fold more copper than the empty vector-expressing wild type (Fig. 3). These data indicate unambiguously that A. thaliana COPT6 is a copper uptake transporter.

A Positionally Conserved Methionine Residue in TM2 (Met⁴⁷⁰), but Not a Positionally Conserved N-terminal Methionine Residue (Met³⁷⁷), Is Essential for COPT6 Function—

Elegant genetic and biochemical studies have shown that positionally conserved methionine residues within the hydrophilic N-terminal extracellular domain of S. cerevisiae Ctr1p, Met²²⁷, and in TM2, Met²⁶⁰, are important for extracellular copper binding and uptake, respectively (31). To test whether the corresponding residues in COPT6 (Met⁷⁷ in the N terminus and Met¹⁰⁶ in TM2; supplemental Fig. 1) are also essential for function, we converted Met²²⁷ and Met¹⁰⁶ in the full-length COPT6 wild-type protein to alanine, M27A and M106A, respectively, and transformed these mutant alleles into the ctr1Δctr2Δctr3Δ mutant. Concurrently, the ctr1Δctr2Δctr3Δ mutant was transformed with full-length Ctr1p or the previously described point mutants with Met¹²⁷ and Met²⁶⁰ substituted to leucine or alanine (M127L or M260A, respectively) as controls (31). We then compared the ability of wild-type COPT6, wild-type Ctr1p, and their mutant alleles to suppress the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG medium.

We found that all yeast alleles grew well on YPEG medium supplemented with 100 μM CuSO₄ (Fig. 4). As expected, expression of the full-length wild-type COPT6 and Ctr1p complemented the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG medium; however, expression of M106A-substituted COPT6 did not (Fig. 4). Consistent with previous findings (31), the Ctr1p mutant allele with a point mutation in the corresponding Met¹⁰² residue (M260A) also did not complement the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG (Fig. 4). We also tested whether the adjacent conserved Met¹⁰² residue in TM2 of COPT6 is essential for COPT6 function. However, substituting Met¹⁰² to alanine did not appear to alter COPT6 function because the ctr1Δctr2Δctr3Δ mutant expressing the M102A allele grew on YPEG medium as well as mutant cells expressing wild-type COPT6 or Ctr1p (Fig. 4). These results show that the function of one of two methionine residues within the TM2 is conserved throughout the CTR/COPT proteins in yeast and plants.

Consistent with previous findings of the essential role of a positionally conserved Met¹²⁷ residue in the N terminus of Ctr1p (31), expression of the M127L mutant allele of Ctr1p did not complement the respiratory deficiency of the ctr1Δctr2Δctr3Δ mutant on the YPEG medium (Fig. 4). Surprisingly, substitution of the corresponding Met²⁷ (M27A) residue in COPT6 complemented the ctr1Δctr2Δctr3Δ mutant growth defect on YPEG medium, suggesting that this residue in the N terminus of COPT6 is not required for COPT6 function. In addition, mutation of the semi-conserved, adjacent Met⁴² (M22A) in the N terminus (supplemental Fig. 1) also comple-

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\text{ctr1Δctr2Δctr3Δ mutant. The COPT6-EGFP construct was functional because, unlike the empty vector, it rescued growth of the ctr1Δctr2Δctr3Δ mutant on YPEG medium (Fig. 2A). The COPT6-EGFP-mediated fluorescence was observed at the cell periphery (Fig. 2B), where it overlapped with FM 4-64 dye, which stained the plasma membrane after short term labeling conducted in low temperatures (44–46). These data suggested that COPT6-EGFP localizes to the plasma membrane in the heterologous system. A fraction of COPT6-EGFP degraded, resulting in EGFP accumulation inside the vacuole and in endoplasmic reticulum (Fig. 2B), as frequently observed for overexpressed plasma membrane proteins (62, 63). Nevertheless, the plasma membrane-localized COPT6-EGFP was sufficient to complement the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG medium (Fig. 2A). The ability of heterologously expressed COPT6 to rescue the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG medium, along with its plasma membrane localization, suggest that COPT6 is involved in copper uptake.}

**Heterologously Expressed COPT6 Facilitates Copper Accumulation in ctr1Δctr2Δctr3Δ S. cerevisiae Cells—**To ascertain that COPT6 rescues the growth defect of the ctr1Δctr2Δctr3Δ mutant on YPEG medium by transporting copper, we analyzed the copper content of the ctr1Δctr2Δctr3Δ mutant expressing the YES3-Gate vector with or without the COPT6 cDNA and wild-type cells expressing the YES3-Gate vector. Yeast cells were grown on YNB-URA medium supplemented with the indicated concentrations of CuSO₄, and copper content was analyzed by ICP-MS. We found that empty vector-expressing wild-type cells accumulated 20.7 ± 0.5 ppm of copper when grown on YNB-URA. In contrast, the ctr1Δctr2Δctr3Δ mutant accumulated 6-fold less copper than the empty vector-expressing wild type (Fig. 3). These data are consistent with previous findings showing that CTR proteins are important for copper transport (17, 25, 31). Expression of COPT6 in the ctr1Δctr2Δctr3Δ mutant increased its ability to accumulate copper to the level of the wild-type strain (Fig. 3). Supplementing YNB-URA with the indicated concentrations of copper increased copper accumulation in all yeast alleles (Fig. 3). Nevertheless, ctr1Δctr2Δctr3Δ cells expressing COPT6 accumulated 2- and 3-fold more copper when grown at 10 and 20 μM CuSO₄, respectively, than mutant cells expressing the empty vector and 3- and 4-fold more copper than the empty vector-expressing wild type (Fig. 3). These data indicate unambiguously that A. thaliana COPT6 is a copper uptake transporter.
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**FIGURE 4. The conserved Met residue within the TM2 is essential for COPT6 function.** S. cerevisiae wild-type (Wt) and ctrlΔctr2Δctr3Δ mutant cells (ctr1,2,3Δ) were transformed with the empty vector, ctrlΔctr2Δctr3Δ mutant cells were also transformed with the vector containing the full-length COPT6, S. cerevisiae CTR1 DNA inserts (COPT6 and CTR1, respectively), COPT6 lacking the extracellular N-terminal domain (NTDΔ), Met-to-Ala-substituted COPT6 alleles (M22A, M27A, M102A, and M106A), or Met-to-Leu and Met-to-Ala-substituted CTR1 alleles (M127L and M260A). Yeast alleles were grown, diluted as indicated in the legend to Fig. 3, and spotted on YPEG medium with or without 100 μM CuSO_4 (100 Cu) or 10 μM BCS (10 BCS), as indicated on the right.

**A. thaliana COPT6 Is a Copper Transporter**

The Methionine-rich N Terminus Is Required for COPT6 Function under Conditions of Copper Limitation—Previous studies suggested that Met within the methionine-rich regions (Mets motifs) of the N terminus of Ctrlp is involved in extracellular copper binding prior to its transport into the cell (31). Our data indicate that the corresponding positionally conserved Met or adjacent semiconserved Met are dispensable for COPT6 function. To determine the function of the N terminus of COPT6, we decided to generate a COPT6 mutant allele without its extracellular N-terminal domain (NTDΔ). We then tested the respiratory competence of the ctrl1Δctr2Δctr3Δ mutant expressing NTDΔ-COPT6 compared with the mutant expressing full-length COPT6 or Ctrlp proteins. Surprisingly, cells expressing NTDΔ-COPT6 were still able to grow on YPEG medium (Fig. 4), suggesting that the N-terminal region of COPT6 is not absolutely essential for the function of the transporter. However, we noticed that the cell density of NTDΔ-COPT6-expressing cells on YPEG medium was lower than that of cells expressing the full-length COPT6 or Ctrlp (Fig. 4). We then tested if limiting bioavailable copper through the addition of a specific copper chelator, BCS, to the YPEG medium would alter the growth of cells expressing NTDΔ-COPT6. We found that the growth of cells expressing NTDΔ-COPT6 was severely impacted when extracellular bioavailable copper was limited by the addition of BCS (Fig. 4, YPEG + 10 BCS). These results suggest that the extracellular N-terminal domain of COPT6 plays an important role in copper homeostasis under copper-limiting conditions. Future studies will reveal the specific amino acid residue(s) involved in copper binding.

COPT6 Localizes to the Plasma Membrane in A. thaliana Protoplasts—Members of the CTR/COPT family in different species associate with different endomembranes, including the plasma membrane or endosomal vesicles/vacuoles, and contribute to copper homeostasis either by copper uptake from the external medium or copper release from internal compartments during deficiency (19, 20, 64, 65). Our studies of the subcellular localization of COPT6 in S. cerevisiae showed that in this heterologous system, it localizes to the plasma membrane. Subsequently, we sought to determine its localization in A. thaliana. The coding sequence of COPT6 was fused at the C terminus to EGFP in the SAT6-EGFP-N1-Gate vector and transiently expressed under the control of the constitutive cauliflower mosaic virus 35S promoter in A. thaliana protoplasts. As a control, protoplasts were transfected with the empty SAT6-EGFP-N1-Gate vector.

EGFP-mediated fluorescence was present at the periphery of COPT6-EGFP-transfected protoplasts and did not overlap with chlorophyll autofluorescence (Fig. 5A). To ascertain the plasma membrane localization of COPT6-EGFP, transfected protoplasts were co-stained with FM 4-64. After the short term labeling at 4°C, FM 4-64 stained the plasma membrane (Fig. 5B, FM 4-64+Ch0), and FM 4-64-mediated fluorescence overlapped with COPT6-EGFP-mediated fluorescence but not with chlorophyll-mediated fluorescence (Fig. 5B). In protoplasts transfected with the empty vector, EGFP was present as a soluble protein in the cytosol, and its fluorescence did not overlap with chlorophyll autofluorescence (Fig. 5C). These results are consistent with the plasma membrane localization of COPT6.

We also co-transfected protoplasts with the SAT6-EGFP-N1-Gate vector containing a COPT6-EGFP fusion and BIN20 vector containing a plasma membrane marker, PIP2A, fused to mCherry (66). Fluorescence signals originated from COPT6-EGFP and PIP2A-mCherry co-localized at the periphery of transfected protoplasts (supplemental Fig. 2), suggesting that COPT6 similar to PIP2A localizes to the plasma membrane.

**Analyses of the copt6-1 Mutant and Transgenic Plants Ectopically Expressing COPT6**—Our studies in S. cerevisiae showed that COPT6 is involved in copper uptake (Figs. 2A and 3). To study the role of COPT6 in planta, we used a T-DNA insertion allele, copt6-1 (SALK 083438) and two transgenic lines, 3SS_pro-COPT6-1 and 3SS_pro-COPT6-2. Wild-type plants and a transgenic line expressing the EarlyGate201 vector without the COPT6 cDNA insert (3SS_pro) were used as controls for the copt6-1 mutant- or COPT6-overexpressing lines, respectively.

Sequencing of the copt6-1 genomic DNA fragment revealed a T-DNA insertion 110 bp upstream of the COPT6 start codon. qRT-PCR analysis disclosed a 10- and 8-fold decrease of COPT6 mRNA in roots and shoots, respectively, in the copt6-1 allele (Fig. 6A). In contrast, transcript abundance of COPT6 was 1,000- and 30-fold higher in roots and shoots of 3SS_pro-HA-COPT6-1 transgenic plants compared with plants expressing the empty vector (Fig. 6B). Expression of COPT6 in the 3SS_pro-
HA-COPT6-2 transgenic line was 7,000 and 170 times higher in roots and shoots, respectively (Fig. 6B).

SDS-PAGE and immunoblot analyses of total membrane proteins isolated from leaves of transgenic plants revealed the anti-HA antibody reactive protein with an apparent molecular mass of 37 kDa in extracts from 35Spro-HA-COPT6-1 and 35Spro-HA-COPT6-2 transgenic lines but not from the empty vector-expressing plants (Fig. 6C). Consistent with the qRT-PCR results (Fig. 6B), the level of anti-HA immunoreactive polypeptide was higher in the 35Spro-HA-COPT6-2 line than in the 35Spro-HA-COPT6-1 line (Fig. 6C). It is noteworthy that the molecular mass of COPT6-HA is considerably larger than its predicted size of 16.9 kDa. It is possible that this aberrant migration is a consequence of posttranslational modifications (i.e., O-glycosylation) that were shown for the COPT6 homolog from S. cerevisiae, Ctr1p (31).

**FIGURE 5.** Subcellular localization of COPT6 in A. thaliana protoplasts. A. thaliana leaf protoplasts were transfected with the vector expressing the COPT6-EGFP fusion (A and B) or vector expressing EGFP without the COPT6 cDNA insert (C). To visualize the plasma membrane, COPT6-EGFP-transfected protoplasts were co-stained with FM 4-64 (B). EGFP-mediated fluorescence, derived from COPT6-EGFP (COPT6) or EGFP (EGFP) and FM 4-64-mediated fluorescence and chlorophyll autofluorescence (FM 4-64 + Chl and Chl, respectively) were visualized using FITC or rhodamine filter sets. Superimposed images of COPT6-EGFP- and FM 4-64-mediated fluorescence and chlorophyll autofluorescence (Overlay) were created to demonstrate that green fluorescence derived from COPT6-EGFP co-localizes with FM 4-64. Scale bar, 10 µm.

**FIGURE 6.** Characterization of the copt6-1 mutant allele and transgenic lines ectopically expressing 35Spro-HA-COPT6. A. qRT-PCR analysis of 10-day-old copt6-1 seedlings (copt6-1) indicates a 10- and 8-fold decrease of the abundance of the COPT6 transcript in roots and shoots, respectively, compared with the wild-type (Wt) seedlings. B, abundance of the COPT6 transcript in roots and shoots of two transgenic lines, 35Spro-HA-COPT6-1 (COPT6-1) and 35Spro-HA-COPT6-2 (COPT6-2). Results are presented relative to the expression of COPT6 in plants transformed with the empty vector, designated as 1. Error bars, S.E. (n = 9). *, statistically significant differences (p ≤ 0.05) of the mean values. C, immunoblot analysis of the COPT6-HA polypeptide in transgenic plants expressing EarleyGate201 vector lacking the cDNA insert (35Spro) and two lines (COPT6-1 and COPT6-2) expressing 35Spro-HA-COPT6. The apparent molecular weight of COPT6-HA is indicated on the right.
The superoxide-scavenging functions of CSD1 and CSD2 during copper deficiency are replaced by an increase in gene expression of FSD1 (11, 16). These morphological and molecular phenotypes of copper excess or deficiency were observed in A. thaliana seedlings grown in the presence of $45 \mu M$ CuCl$_2$ or $750 \mu M$ BCS (supplemental Fig. 3, A and B).

Root length of 10-day-old seedlings was used as a measure of tolerance or sensitivity to these growth conditions. The average lengths of primary roots of the copt6-1 mutant and the 35S$_{pro}$-COPT6 transgenic line cultured at 1/2 MS were the same as of their corresponding controls, the wild type- and the empty vector-expressing plants, respectively (Fig. 7A). We also did not find statistically significant differences in copper accumulation in roots (not shown) or shoots (Fig. 7B) of these plant lines. In contrast, roots of 35S$_{pro}$-COPT6-2 transgenic line, which manifested the highest level of the COPT6 transcript and polypeptide (Fig. 6, B and C), were 1.2-fold longer than empty vector-expressing plants (Fig. 7A). Although internal copper content of roots of this plant line did not differ from the other plant lines (not shown), its shoots accumulated 1.5-fold more copper than the empty vector-expressing plants (Fig. 7B). These data suggest that significantly increased expression of COPT6 in these plants was beneficial for plants even at copper-sufficient conditions.

We then tested the response of different plant lines to copper limitation or excess. As would be expected for copper-limiting conditions, primary roots were shorter in all plant lines grown on 1/2 MS supplemented with BCS than on 1/2 MS without supplements (Fig. 7, A and C). However, there was no difference between the root length of the copt6-1 mutant and wild-type plants in copper-limiting conditions (Fig. 7C). This result was not very surprising because of the possible functional redundancy of COPT6 with other COPT family members. In contrast, roots of 35S$_{pro}$-COPT6-1 and 35S$_{pro}$-COPT6-2 transgenic plants grown in copper-limiting conditions were 1.3- and 1.4-fold longer, respectively, compared with the empty vector-expressing plants (Fig. 7C), suggesting that overexpression of COPT6 was beneficial for plant growth also when copper was scarce.

Supplementation of 1/2 MS medium with a toxic concentration of CuCl$_2$ inhibited root growth of all plant lines in comparison with 1/2 MS without supplementation (Fig. 7, A and D).
COPT6-overexpressing transgenics and the copt6-1 mutant showed opposite responses to toxic copper; roots of plants overexpressing COPT6 were significantly shorter, whereas roots of the copt6-1 mutant were longer when compared with corresponding controls (Fig. 7D). It is important to note that the sensitivity of COPT6-overexpressing plants to copper excess correlated with the level of COPT6 overexpression; the 35Spre-COPT6-2 line was more markedly sensitive and overexpressed COPT6 to a higher degree compared with 35Spre-COPT6-1.

The improved growth of COPT6 transgenics in copper-limiting conditions and their increased sensitivity to copper excess and the decreased sensitivity of the copt6-1 mutant to copper excess not only show that COPT6 functions in copper homeostasis in planta but also further support the notion that tight control of the expression of copper transporters is needed for preventing copper deficiency while avoiding toxicity.

Expression of COPT6 Is Regulated by Copper Status—Regulation of the mRNA expression of CTR/COPT family members by copper status is among the major mechanisms of the control of copper homeostasis in the cell. Therefore, we sought to determine whether the abundance of COPT6 mRNA in Arabidopsis would be altered in response to changes in copper supply as well. Wild-type A. thaliana (Col-0) seedlings were grown on ½ MS medium alone (control) or with 45 μM CuCl₂ (copper excess) or 500 μM BCS (copper deficiency).

Analyses of COPT6 transcript abundance in A. thaliana cultured on control ½ MS medium showed that COPT6 is mainly expressed in leaves (Fig. 8A). The transcript abundance of COPT6 increased in both roots and shoots of BCS-treated A. thaliana wild-type plants (Fig. 8B). In contrast, copper excess affected the transcript abundance of COPT6 only in shoots and not in roots (Fig. 8B). Based on these results, we concluded that COPT6 expression is differentially regulated in roots and leaves of A. thaliana in response to alterations in copper availability.

Expression of COPT6 Depends, in Part, on SPL7—A recent microarray study revealed that up-regulation of the expression of many copper deficiency-responsive genes in Arabidopsis, including COPT1 and COPT2, is attributable to the activity of the transcription factor, SPL7 (12). SPL7 activates the transcription of its targets via binding to the GTAC motif (also known as copper-responsive elements) in the transcription regulation regions of copper-responsive genes (12, 69). Because COPT6 is not represented on the Agilent Arabidopsis 3 Oligo Microarray used previously (12), we sought to determine whether the transcriptional response of COPT6 to low copper availability (Fig. 8B) is controlled by SPL7 as well.

In this regard, we identified two GTAC motifs in a region of <200 bp upstream of the COPT6 start codon using the PLACE prediction software, suggesting that COPT6 might be an SPL7 target. We then used the spl7-1 mutant allele of A. thaliana (12) and tested whether decreasing copper availability by supplementing the growth medium with BCS would up-regulate COPT6 expression in this mutant background as it did in the wild type (Fig. 8B). It was found that the transcript abundance of COPT6 was still significantly increased in roots of the BCS-treated spl7-1 mutant (Fig. 8C). In contrast, expression of COPT6 mRNA was not up-regulated in shoots of the BCS-treated spl7-1 mutant (Fig. 8C). Based on these findings, we concluded that SPL7 is essential for the transcriptional
response of COPT6 to copper limitation in shoots, whereas other transcription regulators might be involved in its response in roots of A. thaliana.

The Expression Pattern of COPT6 in A. thaliana—To investigate the sites of COPT6 action in A. thaliana, the COPT6 promoter sequence (COPT6pro) was fused to the uidA reporter gene encoding β-glucuronidase (GUS), and this COPT6pro-GUS construct was transformed into wild-type A. thaliana. From three transgenic lines exhibiting the same pattern of GUS activity, we selected one representative line for subsequent studies. Consistent with results of qRT-PCR analysis (Fig. 8A), the majority of GUS activity was observed in leaves of young seedlings (Fig. 9A). Although the GUS staining was observed throughout different cell types of lateral roots, it was absent in the primary root and at the root tips of lateral roots (Fig. 9B). We also observed strong GUS staining in the vasculature in leaves, in leaf trichomes and trichome basal cells (Fig. 9, C and D). The histochemical analysis of mature plants revealed that COPT6 is also expressed in sepals and petals of inflorescence (Fig. 9E). Analysis of the COPT6pro activity in reproductive organs disclosed COPT6pro activity in stigma and ovary, pollen grains, and filaments of stamens (Fig. 9, E and F). GUS activity in young siliques was less abundant and was primarily located in a gynophore, vasculature, a central replum, funiculus, style, and stigma surfaces (Fig. 9, G and H). Because the bulk of COPT6pro activity is concentrated in the shoots and reproductive organs, it is tempting to suggest that COPT6 may play a primary role in copper delivery and/or redistribution in above ground tissues.

COPT6 Interacts with Itself and with COPT1 at the Plasma Membrane—CTR/COPT proteins exist as homo- and/or heterocomplexes on the cellular membranes (5, 29, 30, 35, 36). Here we tested whether COPT6 would interact with itself by using split ubiquitin-based MYTH and BiFC approaches (51, 70, 71).

In the MYTH approach, modified ubiquitin is split into C- and N-terminal halves (Cub and NubG) and is fused to membrane-bound bait or prey proteins, respectively. Interactions of bait and prey proteins cause Cub and NubG to reconstitute ubiquitin. The presence of reconstituted ubiquitin is recognized by ubiquitin-specific proteases (UBPs) that release an artificial transcription factor, PLV (protein A-LexA-VP16), which is fused to the C terminus of ubiquitin (CubPVL). Interactions are monitored by the PLV-induced expression of lexA-driven reporter genes, ADE2, HIS3, and lacZ (51, 52).

Interactions using the MYTH approach can only be detected when CubPVL and Nub fusions are located in the cytosol (51). Based on computer algorithm analysis of the predicted membrane topology, the C terminus of COPT6 is in the cytosol, whereas the N terminus is located extracellularly (Fig. 1B). Therefore, in order to detect COPT6-COPT6 interactions, NubG and CubPVL were fused to the C terminus of the full-length COPT6, generating COPT6-NubG and COPT6-Cub-
PVL, and co-expressed in *S. cerevisiae*. To detect false positives due to self-activation, COPT6-CubPVL was co-expressed with pNubG lacking the COPT6 cDNA insert. Our data show that regardless of whether interactions were monitored as colony formation on selective medium (SC) or by β-galactosidase activity, interactions occurred only in cells that co-expressed COPT6-CubPVL and COPT6-NubG constructs (Fig. 10A). These interactions were not caused by self-activation because they did not occur in cells that co-expressed COPT6-CubPLV and pNubG (Fig. 10A).

The BiFC method is based on the observation that N- and C-terminal halves of enhanced yellow fluorescent protein,
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YFP<sup>N</sup> and YFP<sup>C</sup>, respectively, reconstitute a functional fluorophore when brought into proximity by two interacting proteins fused to the YFP fragments (70, 71). For BiFC assays, the full-length COPT6 was fused at the C terminus with the N- or C-terminal halves of YFP to generate COPT6-YFP<sup>N</sup> or COPT6-YFP<sup>C</sup>, and both constructs were co-transfected into *A. thaliana* protoplasts. For negative controls, protoplasts were co-transfected with YFP<sup>N</sup> and YFP<sup>C</sup> vectors without the COPT6 insert (Vector-YFP<sup>N</sup>/Vector-YFP<sup>C</sup>) or with COPT5-YFP<sup>N</sup> and COPT6-YFP<sup>C</sup> (COPT5-YFP<sup>N</sup>/COPT6-YFP<sup>C</sup>). COPT5 has been shown to localize to the vacuolar membrane (37, 38) and should not interact with the plasma membrane-localized COPT6 protein. Interactions were visualized by following the fluorescence pattern of the reconstituted YFP fluorophore. Strong yellow fluorescence was observed at the plasma membrane only in COPT6-YFP<sup>N</sup> and COPT6-YFP<sup>C</sup> co-transfected protoplasts and not in protoplasts co-transfected with Vector-YFP<sup>N</sup>/Vector-YFP<sup>C</sup> or COPT6-YFP<sup>C</sup>/Vector-YFP<sup>N</sup> (Fig. 10B). Chlorophyll autofluorescence was also detected using the YFP filter set, but it did not overlap with yellow fluorescence associated with the plasma membrane in COPT6-YFP<sup>N</sup>/COPT6-YFP<sup>C</sup>-co-expressing protoplasts. These results suggest that COPT6 interacts with itself at the plasma membrane in BiFC assays.

We also tested if COPT6 will form heterologous interactions with COPT1, which is located at the plasma membrane and has a well established role in copper homeostasis (22, 23). Protoplasts co-transfected with COPT1-YFP<sup>N</sup> and COPT6-YFP<sup>C</sup> (COPT1-YFP<sup>N</sup>/COPT6-YFP<sup>C</sup>) showed a faint yellow fluorescence pattern at the plasma membrane, which did not overlap with chlorophyll autofluorescence (Fig. 10B). This suggests that COPT6 interacts with COPT1. Collectively, these results show that COPT6 at a minimum interacts with itself and COPT1 at the plasma membrane.

**DISCUSSION**

The essential, yet potentially toxic, nature of copper exemplifies the careful balance required to prevent deficiency while avoiding toxicity in most organisms. Among the central mechanisms in controlling copper homeostasis is the regulation of copper uptake. In this work, we characterized COPT6, a newly identified member of the CTR/COPT family in *A. thaliana*. The primary sequence of COPT6 contains the family-conserved methionine-rich motifs of which residues corresponding to Met<sup>37</sup> and Met<sup>106</sup> in *S. cerevisiae* Ctr1p for copper binding and transport functions, respectively. In addition, the C terminus of Ctr1p and two closest COPT6 homologs, COPT1 and COPT2, contain the CXC motif (Fig. 1C). This motif in Ctr1p is also involved in copper binding and transfer to cyttoplasmic copper chaperones and in protein degradation during copper excess (31, 33, 34). In contrast, the primary sequence of COPT6 lacks the CXC motif (Fig. 1C). Nevertheless, COPT6 suppresses the copper-deficient phenotype of the *ctr1Δctr2Δctr3Δ* mutant and confers copper accumulation (Figs. 2 and 3), suggesting that the CXC motif is not important for its transport activity. Whether it plays a regulatory role under copper excess as was proposed for *S. cerevisiae* Ctr1p (31, 33, 34) is yet to be determined.

Consistent with the role of the positionally conserved methionine residue in TM2 of the CTR/COPT proteins (31), the positionally conserved Met<sup>106</sup> in TM2 is required for COPT6 function (Fig. 4). However, it was surprising to find that the conserved Met<sup>27</sup> in the N-terminal extracellular domain as well as the extracellular domain itself were dispensable for COPT6 function when copper was still available in the medium. We found, however, that the extracellular domain is required for COPT6 function when availability of external copper was depleted by the addition of BCS (Fig. 4), suggesting that this domain is important for COPT6 function during high affinity copper uptake under copper limitation. Because the positionally conserved Met<sup>27</sup> and adjacent semiconserved Met<sup>22</sup> were dispensable for COPT6 function in the presence of BCS in the medium (Fig. 4), we suggest that other residues within the extracellular domains may be involved in copper coordination. In this regard, the N terminus of COPT6 contains five methionine and three histidine residues in addition to Met<sup>22</sup> and Met<sup>27</sup> (supplemental Fig. 1), which can also coordinate copper ions prior their transport via COPT6.

The role of COPT6 in copper homeostasis in planta was tested by using an *A. thaliana* knockdown allele, *copt6-1*, and two transgenic lines ectopically expressing COPT6, 35S<sub>puro</sub>-HA-COPT6-1 and 35S<sub>puro</sub>-HA-COPT6-2. Knocking down or overexpressing COPT6 had opposite effects on plant growth during copper limitation or excess; *copt6-1* plants were more sensitive to copper limitation while more tolerant to copper toxicity (Fig. 7, C and D). Furthermore, as expected, 35S<sub>puro</sub>-HA-COPT6-1 and 35S<sub>puro</sub>-HA-COPT6-2 plants were more tolerant of copper limitation but more sensitive to copper excess (Fig. 7, C and D). Given that our studies in yeast show that COPT6 is an uptake transporter, it is likely that the observed responses of the mutant and transgenic plants to copper availability result from impaired copper uptake and/or tissue partitioning in the mutant and increased copper transport and/or tissue partitioning in transgenic lines.

The transcript abundance of COPT6 is significantly higher in leaves than in roots (Fig. 8A), suggesting its primary role in maintaining copper homeostasis of above ground tissues. We also found that the transcript abundance of COPT6 decreases in leaves but not in roots when copper concentration in external medium reaches its toxic limits (Fig. 8B), suggesting that COPT6 expression must be tightly controlled in leaves to protect the photosynthetic apparatus from copper overload. We hypothesize that COPT6 expression in roots is not altered by high copper because it is already relatively low under control conditions, and thus COPT6 contribution to copper uptake would be minimal under copper-replete or -excess conditions. In contrast, because of the low expression in roots under control conditions, COPT6 expression is up-regulated under copper-limiting conditions to provide an adequate copper supply to plants (Fig. 8, A and B).

The histochemical analysis of the spatial distribution of the COPT6 promoter activity in transgenic plants expressing the COPT6<sub>puro</sub>-GUS construct shows that although COPT6 is expressed in different cell types, its expression is concentrated in the vasculature (Fig. 9), where the majority of plant copper is stored to ensure rapid remobilization in response to increased...
copper demand (37). Therefore, it is tempting to speculate that COPT6 is involved in copper partitioning between different plant organs for its delivery to copper-requiring functions. Because COPT6 is also expressed in different cell types of lateral roots except for root tips (Fig. 9), its function in copper uptake from external solution cannot be excluded. In addition, the finding that COPT6 transcript abundance increases in roots and leaves of *A. thaliana* under copper-deficient conditions (Fig. 8B) further support the suggestion that COPT6-mediated copper influx might be important when copper availability is limited.

Similar to *S. cerevisiae* Ctr1p, COPT6 interacts with itself on the plasma membrane (Fig. 10). However, unlike Ctr1p, which does not form heterocomplexes with its closest homolog, a plasma membrane transporter, Ctr3p (31), COPT6 interacts with COPT1 (Fig. 10B). However, because COPT6 functions in copper uptake without COPT1 in *S. cerevisiae* (Figs. 2 and 3), the biological significance of COPT6-COPT1 interactions is yet to be determined. It is possible that COPT1-COPT6 interactions are important for copper uptake in specific *A. thaliana* cell types (e.g. in trichomes and/or pollen that co-express COPT6 and COPT1) (Fig. 9) (22).

The transcriptional response of some of the CTR/COPT family members is under tight transcriptional control exerted by Mac1 or SPL7 transcriptional factors in *S. cerevisiae* and *A. thaliana*, respectively (12, 27, 28). Microarray analysis revealed that *A. thaliana* COPT1 and COPT2 are among the SPL7 targets (12). Because COPT6 was not represented on the Agilent *Arabidopsis* 3 Oligo Microarray used previously (12) but is subjected to transcriptional regulation by copper status (Fig. 8B), we tested whether COPT6 is among the SPL7 targets as well. Our studies using the *spl7*-1 mutant of *A. thaliana* revealed that the transcript abundance of COPT6 increases independently of SPL7 in roots of *A. thaliana* (Fig. 8C), suggesting involvement of other transcription factors in this organ. In contrast, the COPT6 transcriptional response to copper limitations in the shoot absolutely depends on SPL7 (Fig. 8C). Although the transcriptional regulation region of COPT6 contains two copper-responsive elements essential for SPL7 binding, whether COPT6 is a direct SPL7 target is not known. It is noteworthy that SPL7 is expressed mainly in roots, where it suggested to sense copper availability (12). Nevertheless, many SPL7 targets (12), including COPT6, are induced by copper deficiency in shoots, suggesting a complex mechanism of SPL7-dependent regulation of the transcriptional copper deficiency response.

COPT6 has been annotated as a vacuolar membrane protein based on studies of the *A. thaliana* vacuole proteome (72). However, our data showing that 1) COPT6-EGFP, heterologously expressed in *S. cerevisiae* and transiently expressed in *A. thaliana* protoplasts is associated with the plasma membrane, 2) COPT6 interacts with the plasma membrane-localized COPT1 but not with vacuolar membrane-localized COPT5 in BiFC assays, 3) heterologously expressed COPT6 complements the *S. cerevisiae* copper uptake mutant, and 4) transgenic plants that overexpress COPT6 are more sensitive to copper excess are consistent with the suggestion that COPT6 localizes to the plasma membrane and is involved in copper uptake rather than in vacuolar sequestration. To reconcile our experimental data with the results of Carter et al. (72), we suggest that the detection of COPT6 in the vacuolar proteome may reflect a vacuole-mediated degradation pathway of COPT6, as was shown for its *S. cerevisiae* counterpart Ctr1p (73). To conclude, COPT6 is a plasma membrane copper uptake transporter and is a novel SPL7 target that is essential for maintaining plant growth during extreme copper conditions, acting, possibly, by controlling copper uptake and partitioning to copper requiring functions.

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