Plant Molecular Biology **51:** 577–587, 2003.

Identification of a copper transporter family in *Arabidopsis thaliana*

Vicente Sancenón[1], Sergi Puig[2], Helena Mira[1,3], Dennis J. Thiele[2] and Lola Peñarrubia[1,*]
[1]*Departament de Bioquímica i Biologia Molecular, Universitat de València, Dr. Moliner 50, Burjassot, València, 46100 Spain (*author for correspondence; e-mail penarrub@uv.es);* [2]*Department of Biological Chemistry, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0606, USA;* [3]*Present address: Karolinska Institutet, Sheelevag 1, A1P2, Retzius Building, 17177 Stockholm, Sweden*

Received 25 June 2002; accepted in revised form 18 July 2002

Key words: Arabidopsis, copper transport, heterologous complementation, RT-PCR, ^{64}Cu uptake

Abstract

Despite copper ions being crucial in proteins participating in plant processes such as electron transport, free-radical elimination and hormone perception and signaling, very little is known about copper inward transport across plant membranes. In this work, a five-member family (COPT1–5) of putative *Arabidopsis* copper transporters is described. We ascertain the ability of these proteins to functionally complement and transport copper in the corresponding *Saccharomyces cerevisiae* high-affinity copper transport mutant. The specific expression pattern of the *Arabidopsis COPT1–5* mRNA in different tissues was analyzed by RT-PCR. Although all members are ubiquitously expressed, differences in their relative abundance in roots, leaves, stem and flowers have been observed. Moreover, steady-state *COPT1* and *COPT2* mRNA levels, the members that are most efficacious in complementing the *S. cerevisiae* high-affinity copper transport mutant, are down-regulated under copper excess, consistent with a role for these proteins in copper transport in *Arabidopsis* cells.

Introduction

Copper is an essential micronutrient for living organisms as a cofactor for proteins mostly implicated in redox processes (Koch *et al*., 1997). On the other hand, copper-mediated production of reactive oxygen species and displacement of other metals from their natural binding sites, make copper a potentially toxic substance (Halliwell and Gutteridge, 1990). Thus, aerobic organisms have developed complex homeostatic networks to control the acquisition and use of copper. *Saccharomyces cerevisiae* has proved to be a powerful tool for elucidating the copper homeostasis mechanisms in higher eukaryotes. In this sense, functional complementation experiments have shown that these mechanisms are conserved along pluricellular eukaryotic evolution (Peña *et al*., 1999). In agreement, members of a widespread family of eukaryotic copper transporters (Ctr) have been identified in mammals and plants by sequence homology or by functional complementation in yeasts. All members belonging to the Ctr family contain three predicted transmembrane segments and most possess an amino-terminal Met and His-rich putative metal binding domain (for reviews see Peña *et al*., 1999; Labbé and Thiele, 1999; Harris, 2000; Puig and Thiele, 2002). *S. cerevisiae* has three members of this family (Ctr1–3); two of them (Ctr1 and Ctr3) are redundant for high-affinity copper uptake (Knight *et al*., 1996), and the function of the other (Ctr2) remains unclear (Kamfenkel *et al*., 1995; Portnoy *et al*., 2001).

Copper homeostasis is receiving a growing interest in plant research since it is implicated in responses to the oxidative damage produced by environmental stresses, such as the ambient pollutant ozone and during natural senescence (Himelblau *et al*., 1998; Miller *et al*., 1999; Mira *et al*., 2001). Plants have developed two main systems to chelate excess metals: the low molecular weight metallothioneins (Zhou and Goldsbrough, 1995) and the enzymatically synthesized phytochelatins (Rauser, 1995). Under low copper availability, intracellular copper distribution is performed

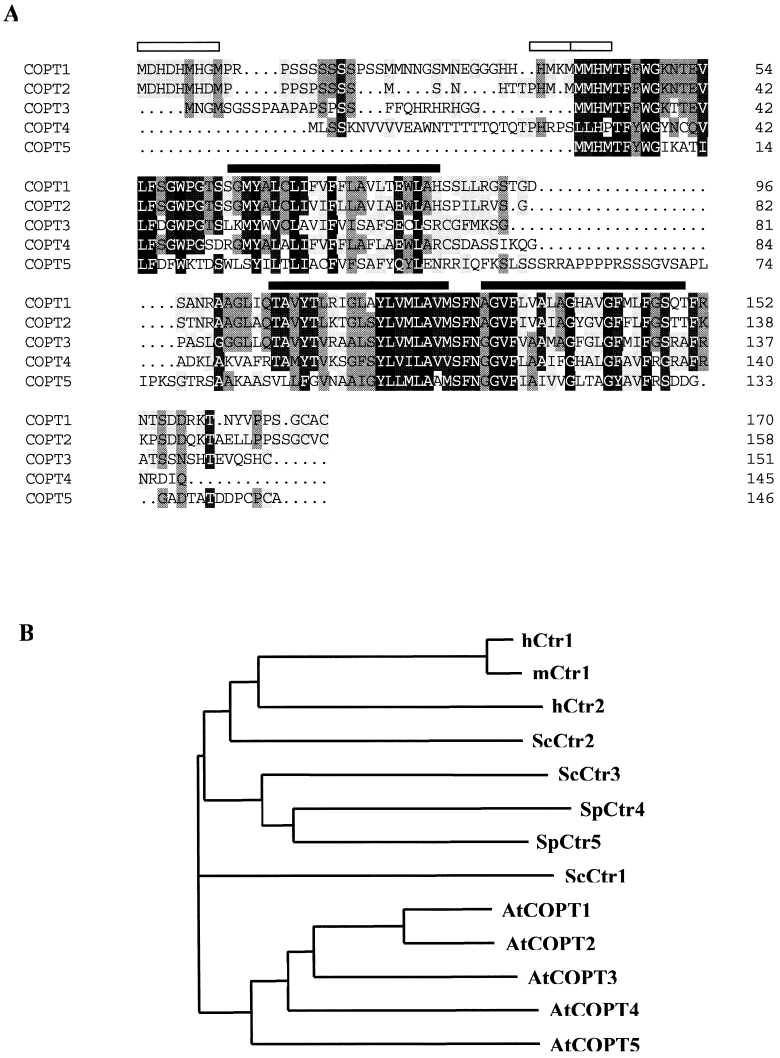

Figure 1. Primary sequence analysis of the COPT family. A. Alignment of the predicted amino acid sequences for the COPT1–5 *Arabidopsis* putative copper transporters. Dynamic programming is used for the optimal multiple alignment (Feng and Doolittle, 1987; Thompson *et al.*, 1994). A bold line indicates predicted transmembrane domains and boxes show Met- and His-rich regions. Conserved amino acids are shaded in a scale of grays. B. Phylogenetic tree of the Ctr family proteins. Abbreviations correspond to the following organisms: *Homo sapiens* (h); *Mus musculus* (m); *Saccharomyces cerevisiae* (Sc); *Schizosaccharomyces pombe* (Sp) and *Arabidopsis thaliana* (At).

by metallochaperones, a set of soluble copper-binding proteins that further direct the metal to its final destination (for a review see Huffman and O'Halloran, 2001; Puig and Thiele, 2002). Thus far the copper chaperone (CCH) is the only metallochaperone described in plants (Himelblau *et al.*, 1998; Mira *et al.*, 2001a, b). Compared to the task performed by homologues in other organisms, CCH should deliver copper to the P-type Cu-transporting ATPase denominated RAN1 (Hirayama *et al.*, 1999) at a post-Golgi compartment. This copper homeostasis-related gene was isolated in a screen for plants with an unusual response to the ethylene antagonist *trans*-cyclooctene, underscoring the critical role of the metal in the ethylene signaling pathway. This role is explained by the fact that the ethylene receptor requires Cu for high-affinity hormone binding (Rodriguez *et al.*, 1999). However, very little is known about copper transporters that could also play an important role in metal tolerance (Salt *et al.*, 1998). A putative *Arabidopsis thaliana* copper transporter (COPT1) has been previously identified by the ability of its cDNA to functionally complement an *S. cerevisiae* mutant defective in high-affinity copper uptake (Kamfenkel *et al.*, 1995). *COPT1* encodes a polypep-

tide of 169 amino acids with 49% similarity to its yeast counterpart Ctr1. The 44 amino-terminal residues of COPT1, enriched in Met and His residues, display similarity to bacterial copper-binding motifs and are predicted to face the extracytoplasmic region (Fox and Guerinot, 1998). However, the expression and function of COPT1 or other potential copper transporters in plants remain mostly unknown.

In this work, we have identified and analyzed five members of the Ctr copper transporter family from *A. thaliana* and characterized their ability to functionally complement the growth defects and copper transport function in the corresponding yeast *ctr1ctr3* mutant. Moreover, we analyzed their specific pattern of expression in different plant tissues, and demonstrated that expression of specific *Arabidopsis* copper transporters is influenced by copper, consistent with a role in copper transport.

Materials and methods

Plant growth conditions and treatments

Seeds of *Arabidopsis thaliana*, ecotype Columbia (Col), were grown in pots and covered with a clear plastic dome. The covered pots were placed in a cold room at 4 °C for 2 days to synchronize germination, and then moved to a growth cabinet at 23 °C with a 16 h photoperiod (65 μmol/m^2 of cool-white fluorescent light). Plants were harvested after times indicated in Figure legends. For copper and bathocuproinedisulfonic acid (BCS, Sigma) treatments, rosette leaves from 4- to 5-week old *Arabidopsis* were excised and then immediately submerged in 1 mM copper sulfate or 1 mM BCS solutions for 18 h.

Screening of the Arabidopsis *cDNA λ-YES expression library*

For the screening of cDNAs encoding *Arabidopsis* copper transporters, the yeast *ctr1*Δ*ctr3*Δ double mutant strain MPY17 (*MATα, ctr1::ura3::kan^R, ctr3::TRP1, lys2-801, his3*) (Peña *et al*., 2000) was transformed with the galactose-inducible cDNA λ-YES expression library (Elledge *et al*., 1991) using the lithium acetate procedure (Gietz *et al*., 1995). The selection process requires non-fermentable carbon sources based on the copper-dependent respiratory defects of this strain (Dancis *et al*., 1994a; Peña *et al*., 2000). Therefore, YPEgal plates (1% yeast extract, 2% bactopeptone, 3% ethanol, 0.02% galactose) were used for the screening, containing a galactose concentration determined sufficient to induce a 10-fold increase in the plasmid basal expression, while still preventing the growth of the untransformed mutant strain. Transformation efficiency was 3×10^5 cells per μg DNA. Transformants were plated in SC-ura (glucose synthetic media lacking uracil) medium, resuspended in sterilized water and transferred to YPEgal medium. Growth-positive colonies were purified through up to 5 successive streakings alternating SC-ura and YPEgal media and the original mutant phenotype was verified in SC-his and SC-trp plates. Clones that passed throughout all the selective rounds were subjected to plasmid rescue by the glass bead procedure and the DNA obtained was introduced into *Escherichia coli* by electroporation (Dower *et al*., 1988). Plasmid DNA prepared from two independent transformants was analyzed by restriction enzyme cleavage and gel electrophoresis and used to retransform the *ctr1*Δ*ctr3*Δ strain. Clones that restored *ctr1*Δ*ctr3*Δ growth in YPEgal were subcloned in pBlueScript II (Stratagene) and sequenced.

Plasmid constructs

COPT1 cDNA was recovered from the λ-YES plasmid and subcloned into the *Eco*RI site of the yeast expression vector p426GPD (Mumberg *et al*., 1995). Primers were designed to PCR the predicted open reading frames of *COPT2* (forward, 5′-CGGGATCCCTATTAACATTAGTATCATGG; reverse, 5′-GGAAT TCAATTGTTCAACAAACGCAGCC), *COPT3* (forward, 5′-CGGGATCCATGAACGGCATGAGTGGATC; reverse, 5′-GGAATTCTCAACAATGTGATTGAACCTCG), *COPT4* (forward, 5′-CGGGATCCCAAATTAAAGGAGATGTTGTCG; reverse, 5′-GGAATTCATTCCATTCTCATAGGGTTTGG) and *COPT5* (forward, 5′-CGGGATCCATGATGCACATGACCTTCTAC; reverse, 5′-GGAATTCTCAAGCACATGGACATGGATC) and subcloned into the *Bam*HI/*Eco*RI sites of p426GPD. *S. cerevisiae CTR1* was amplified with primers CTR1atgS (forward, 5′-GGACCCGGGATGGAAGGTATGAATATGGGT) and CTR1tPN (reverse, 5′-CCCAAGCTTCTGCAGTTAGTTATGAGTGAATTT TTCGGC) and cloned into the *Sma*I/*Hin*dIII sites of p426GPD.

Functional complementation experiments in yeast

MPY17 cells transformed with p426GPD plasmid alone or containing one of the *COPT1–5* or *CTR1* open reading frames were grown in SC-ura to OD_{600nm} =

1.0. Four 10-fold serial dilutions were plated on SC-ura, YPEG (2% ethanol, 3% glycerol-rich medium) and YPEG containing 100 μm $CuSO_4$. Plates were incubated for 3 or 9 days at 30 °C and photographed.

Copper uptake measurements in yeast cells

ctr1Δctr3Δ yeast cells (MPY17 strain) harboring plasmids indicated in the figure legends were grown in SC-ura to OD_{600nm} = 0.5–1.0. $^{64}CuCl_2$ (specific activity 15–30 mCi per μg of copper) was added to 1 ml of culture to a final concentration of 5 μM, and cells were incubated at room temperature or on ice for 10 min. Time course experiments showed that rate of copper uptake was linear during at least 20 min. For metal competition experiments, 25-fold molar excess of non-radioactive $CuCl_2$, $AgNO_3$, $FeCl_2$, $ZnCl_2$, $CdSO_4$ and $MnCl_2$ was independently added to the cell culture prior to ^{64}Cu measurements. Samples were quenched by adding ice-cold EDTA (10 mM final concentration), filtered and washed twice with quenching buffer (10 mM EDTA in 0.1 M Tris-succinate, pH 6.0). Cell-associated ^{64}Cu was quantified in a γ-counter (Packard Cobra II) by using a standard curve. Values obtained on ice were subtracted from room temperature values, and normalized to cell number. Experiments were independently repeated at least twice by triplicate.

RNA and DNA preparation and analysis

Total RNA was isolated from *Arabidopsis* tissues as described by Prescott and Martin (1987). RNA was quantified by UV spectrophotometry and its integrity was visually assessed on ethidium bromide-stained agarose gels. Genomic DNA was prepared from rosette leaves by grinding the tissue in liquid nitrogen and transferring the dust to lysis buffer (Tris-HCl 10 mM, NaCl 0.4 M, EDTA 5 mM, SDS 0.5% w/v, proteinase K 100 μg/ml, pH 7.8). The extract was incubated for 1 h at 37 °C and nucleic acids were further extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. DNA was resuspended in water and purified by differential precipitation with 2.5 M LiCl and RNase digestion. DNA concentration was estimated by measuring A_{260}. For Southern blotting, 30 μg of genomic DNA was digested with the indicated enzyme and fractionated on a 0.8% agarose gel. DNA was blotted onto a Hybond-N membrane (Amersham Pharmacia Biotech), and hybridized at 65 °C with a radiolabeled probe as described by Ausubel *et al.* (1995). Autoradiography was obtained exposing X-Omat films (Eastman Kodak, New York) to the membrane.

Semiquantitative RT-PCR

Total RNA (1–5 μg) isolated from the indicated tissues was first converted to cDNA by reverse transcription with SuperScript II reverse transcriptase (Gibco-BRL) and the anchored oligo$(dT)_{15}$(Roche) and primer 18S (reverse, 5′-CTGGATC CAATTACCAGACTCAA). For PCR reactions, 1.5–10 μl of the diluted (1:5) cDNA products was used as template. Primers were designed to specifically anneal with *COPT1* (forward, 5′-CAATGGATCCATGAACGAAGG; reverse, 5′-CCTGAGGGAGGAACATAGTTAG), *COPT2* (forward, 5′-ACGTGTCAGTGGCTCAACC; reverse, 5′-GACGGCGGAAGAAGCTCGGCGG), *COPT3* (forward, 5′-GATCTTCTCCGGCGGCTCCGG; reverse, 5′-GTGACTGTTACTGCTAGTGGC), *COPT4* (forward, 5′-GGAGATGTTGTCGAGCAAAAACG; reverse, 5′-CCCTATTCCTAAACGCCCGCCC) and *COPT5* (forward, 5′-CTTCTACTGGGGAATCAAAGC; reverse, 5′-GGAATTCTCAAGCACATGGACATGGATC) cDNAs. The reaction was run at 94 °C (30 s), 55 °C (*COPT1–2*) or 58 °C (*COPT3–5*) (30 s), 72 °C (30 s) for 25 (*COP1–2*) or 28 (*COPT3–5*) cycles, which was within the linear reaction window. 18S rRNA was also measured in the same reaction as internal control with 18S primers (forward, 5′-TGGGATACCTGCCAGTAGTCAT; reverse, see above) together with the competimers 18S (having the same sequence as their counterparts, but a 3′-terminal dideoxy nucleotide). To balance for the great excess of 18S rRNA with respect to the message of each member of the COPT family, a series of PCR reactions were performed varying the ratio competimers/(primers + competimers) from 0 to 1. The ratio that yielded the best relation of COPT to 18S intensities, without saturating the detection method employed (see below), was selected for further analysis. Reproducibility of the results was confirmed by running two PCR reactions in parallel containing a 1:2 ratio amounts of cDNA template and subsequently quantifying and normalizing the results in both duplicates.

PCR products were run on a 1.8% agarose gel and stained with ethidium bromide. Relative values of *COPT* messenger levels in each sample were estimated by measuring the signal intensity of the PCR products (see below) and normalized with respect to 18S. An arbitrary value of 100% was assigned to

the highest level of each experiment. Band intensities were analyzed and quantified with the applications imageStore 5000 and GelBlot (Ultra Violet Products).

Computer-assisted sequence analysis

Computer database searches were performed with BLAST software (http://mips.biochem.mpg.de/proj/thal/). Multiple alignment and identity trees were obtained with the informatics application DNAMAN (Lynon Biosoft) and predictions of transmembrane-spanning domains with TMHMM (http://genome.cbs.dtu.dk).

Results

Screening of the Arabidopsis thaliana *cDNAs λ-YES library in the ctr1Δctr3Δ* S. cerevisiae *strain*

COPT1 was previously identified as a putative *Arabidopsis* copper transporter by functional complementation of the *ctr1-3* yeast mutant (Kampfenkel *et al*., 1995). Southern cross-hybridization bands with the *COPT1* probe (results not shown) and sequence analysis of genome databases pointed to a small family of *COPT1*-related transporters in *A. thaliana*. With the aim of finding COPT1 functional homologues able to perform high-affinity copper transport in yeast, we screened an *Arabidopsis* cDNA library for functional complementation of the *S. cerevisiae* double mutant strain *ctr1Δctr3Δ*. This strain was transformed with the *Arabidopsis* cDNA λ-YES expression library and the copper-dependent respiratory ability of the transformants was assessed in YPEgal plates (see Materials and methods). After analyzing ca. 1.3×10^6 transformants, a total of 5 independent positive clones that were able to restore yeast growth in the selection medium were obtained (results not shown). All these clones encoded the COPT1 transporter (Kampfenkel *et al*., 1995), except for an extra codon that codes for Arg-11 and a nucleotide change dictating an amino acid substitution (Pro to Ser) at position 13, both found in the five sequenced clones. These changes are included in Figure 1A and are also in agreement with the *COPT1* sequence at the *Arabidopsis* complete genome sequence (*Arabidopsis* Genome Initiative, 2000). The slight sequence differences described in this study for COPT1 and that previously reported (Kampfenkel *et al.*, 1995) may correspond to ecotype polymorphisms. Whereas those authors used an *Arabidopsis* Landsberg *erecta*-derived expression library, the λ-YES library used in this study was constructed from Columbia plants (Elledge *et al*., 1991).

Table 1. Properties of the COPT family predicted polypeptides.

Name	Chr.	Length	M_r	Code	Accession
COPT1	V	170	18399	At5g59030	AF466373
COPT2	III	158	17058	At3g46900	AF466370
COPT3	V	151	16170	At5g59040	AF466371
COPT4[1]	II	145	16084	At2g37920	AF466372
COPT5	V	146	15784	At5g20650	AF466374

Chromosome number location (Chr.), length expressed as number of residues, predicted molecular mass, entry code of the MIPS *Arabidopsis thaliana* database and accession numbers of the sequenced cDNAs in GenBank. [1]Data refer to the predicted first exon of At2g37920.

Table 2. Percentages of identity between COPT proteins.

	COPT1	COPT2	COPT3	COPT4	COPT5
COPT1	100				
COPT2	75.0	100			
COPT3	53.5	51.7	100		
COPT4	44.8	44.3	40.3	100	
COPT5	33.1	37.0	39.5	31.6	100

Identities have been calculated following the Feng and Doolittle algorithm.

Database search and cloning of Arabidopsis *COPT1 homologues*

We have identified 4 putative ORFs (COPT2–5) with homology to the *COPT1*-encoded protein in the whole genome of *Arabidopsis thaliana* (*Arabidopsis* Genome Initiative, 2000). To further investigate the function of the COPT proteins we designed specific oligos and subcloned the coding region by RT-PCR into the p426GPD expression vector as described in Materials and methods. The constructs obtained were used for sequencing and subsequent functional complementation analysis (see below). Their chromosome localization, predicted lengths, molecular weights, code number at the *Arabidopsis thaliana* MIPS database and GenBank accession numbers are shown in Table 1. The percentages of identity between these COPT family members are shown on Table 2.

We did not observe any difference with the sequences at the *Arabidopsis* MIPS DataBase, except for a G-to-A transition in the position 331 of the COPT4 coding sequence resulting in an Ala-to-Thr substitu-

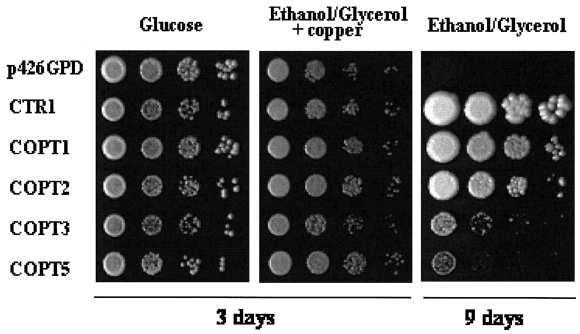

Figure 2. Functional complementation of *ctr1*Δ*ctr3*Δ *S. cerevisiae* mutant by *COPT1, COPT2, COPT3* and *COPT5*. ctr1Δctr3Δ mutant was transformed with vector p426GPD (expression vector, negative control), the same vector expressing yeast *CTR1* (yeast positive control) or the predicted ORF for *COPT1* (plant positive control), *COPT2*, *COPT3 or COPT5.* Cells were grown at 30 °C on glucose (SC-ura) or on ethanol/glycerol plus copper (YPEG + 100 μm $CuSO_4$) plates for 3 days, and on ethanol/glycerol (YPEG) for 9 days.

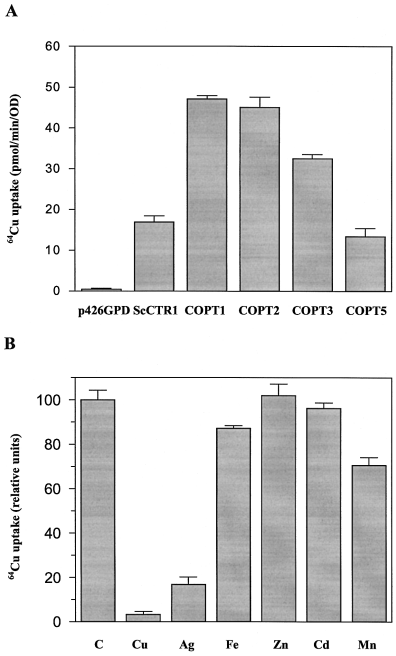

Figure 3. A. *COPT1*, *COPT2*, *COPT3* and *COPT5* restore high-affinity copper transport of the *ctr1*Δ*ctr3*Δ yeast mutant. High-affinity ^{64}Cu uptake rate was measured for the strains detailed in Figure 2 as described in Materials and methods. B. COPT1 transporter metal specificity. Competition of COPT1 ^{64}Cu uptake in *ctr1*Δ *ctr3*Δ yeast cells by 25-fold molar excess of non-radioactive copper, silver, iron, zinc, cadmium, and manganese is shown. Copper accumulation was compared to untreated control cells represented as 100% (C bar). Values were normalized to cell number. Error bars indicate standard deviation for triplicate measurements.

tion in the protein. In addition, our ORF prediction for COPT4 suggests the presence of a unique exon encompassing the first of the two predicted exons of At2g37920 and the 5′ of the intron ending at the first encountered stop codon (data not shown). These modifications with respect to the MIPS *Arabidopsis* database annotation are supported by the following data: first, attempts to detect any two-exon derived transcript by RT-PCR were unsuccessful whereas PCR with genomic DNA as template rendered a product of the expected size; second, a mRNA was detected by RT-PCR that includes a region of the predicted intron of the At2g37920 clone (results not shown); third, conserved polyadenylation signals, both far-upstream element (FUE) and near-upstream element (NUE) (Wu *et al*., 1995; Rothnie, 1996) are present in the predicted intron (results not shown). A multiple alignment of the five sequences and a homology tree are shown in Figure 1. COPT2 presents a high degree of identity with COPT1, whereas the other three members of the family are more distantly related (Table 2). All five proteins predicted from these gene sequences share a similar overall hydrophobicity pattern compatible with a model encompassing three transmembrane-spanning domains (TMD) (Figure 1A).

Functional complementation and copper uptake in the S. cerevisiae ctr1Δctr3Δ *mutant*

To investigate the potential role of the *Arabidopsis* COPT1–5 proteins in copper transport, functional complementation analysis was performed in the *ctr1*Δ*ctr3*Δ *S. cerevisiae* mutant (Figure 2). The putative *Arabidopsis* copper transporters were expressed in yeast under the control of the constitutive glyceraldehide-3-phosphate dehydrogenase (GPD) gene promoter (Mumberg *et al*., 1995). Transformants were able to grow on SC-ura demonstrating the presence of the expression vector (Figure 2, Glucose). The lack of growth in ethanol/glycerol media for the mutant containing p426GPD (negative control) was restored when transformed with the same plasmid expressing yeast *CTR1* (positive control). *COPT1*, as previously described (Kampfenkel *et al*., 1995), and *COPT2* robustly complemented the YPEG growth defect of this yeast strain, while *COPT3* and *COPT5* more modestly restored mutant cell growth (Figure 2, Ethanol/Glycerol). Moreover, addition of 100 μM copper functionally complemented the respiratory deficiency of this strain (Figure 2, Ethanol/Glycerol + copper). Since no transformants were obtained with

the p426GPD-COPT4 plasmid on several attempts, this suggests that expression of COPT4 could be toxic for yeast cells (data not shown). Thus, it is currently unclear whether COPT4 protein will complement the *S. cerevisiae ctr1*Δ*ctr3*Δ growth and copper uptake defects.

To further evaluate the ability of the COPT family members to transport copper across the plasma membrane into the *ctr1*Δ*ctr3*Δ mutant cells, high-affinity ^{64}Cu uptake assays were carried out in yeast cells constitutively expressing COPT1, 2, 3 and 5 (see Materials and methods). In agreement with the growth complementation experiments (Figure 2), *S. cerevisiae ctr1*Δ*ctr3*Δ cells expressing COPT1 and COPT2 showed the highest rate of copper uptake. However, yeast Ctr1 uptake levels were consistently lower than those found for COPT1 and COPT2, despite the high complementation rate conferred by Ctr1 (Figure 2). Furthermore, although COPT3 and COPT5 expression stimulated ^{64}Cu uptake at levels comparable or greater than yeast Ctr1, this robust copper transport activity was not reflected in their rate of growth in YPEG. If the level of copper uptake stimulated by COPT1 expression is considered 100%, COPT3 and COPT5 uptake show a reduction of 30% and 70% respectively (Figure 3). For these assays, vector and cells expressing yeast Ctr1 were used respectively as negative and positive controls. Taken together, these data demonstrate that expression of COPT1, 2, 3 and 5 is able to complement both growth and copper uptake defects associated with yeast mutants defective in plasma membrane high-affinity copper transport.

Metal competition experiments performed with yeast and human Ctr1 (Dancis *et al.*, 1994b; Lee *et al.*, 2002) strongly suggest that the Ctr1 family of high-affinity copper transporters specifically transports reduced Cu(I). We examined the metal specificity of the COPT1 transporter. A 25-fold molar excess of non-radioactive copper, silver, iron, zinc, cadmium, and manganese were independently added to *ctr1*Δ*ctr3*Δ cells expressing COPT1. ^{64}Cu uptake rate was determined and compared to control untreated cells (Figure 3B). Addition of 25-fold molar excess of non-radioactive copper reduced the rate of transport to 3%. Addition of silver, isoelectric to Cu(I), reduced copper uptake to 17%. Among the other metals, only manganese slightly competed for copper transport (70% uptake). These results strongly suggest that the *Arabidopsis* COPT1 transporter is specific for Cu(I).

RT-PCR analysis of COPT1-5-specific expression

To begin to understand the potential roles of COPT family members in *Arabidopsis* plants, we assessed mRNA expression levels in different organs by RT-PCR. The mRNA levels of the five *COPT* genes were independently measured by semiquantitative RT-PCR and normalized to the 18S ribosomal RNA intensity in the same sample (see Materials and methods). A graphic representation of representative results obtained for roots, leaves, stems and flowers from adult plants is shown in Figure 4. With respect to their tissue-specific expression pattern, three different profiles can be distinguished. The patterns of *COPT1* and *COPT2* mRNA levels share some similarities, being mainly expressed in leaves, although *COPT1* is also highly expressed in flowers. *COPT3* and *COPT5* accumulate preferentially in stems, although *COPT5* is also abundant in leaves. *COPT4* is much more abundant in roots compared to other organs.

Response to copper treatment

In both baker's yeast and fission yeast the abundance of the Ctr class of high-affinity copper transporters is regulated at the level of gene transcription by copper availability (Dancis *et al.*, 1994a; Labbé *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1997; Labbé *et al.*, 1999). Furthermore, levels of the *Arabidopsis* CCH copper chaperone mRNA are specifically reduced in plants grown under copper rich as compared to control conditions (Himelblau *et al.*, 1998; Mira *et al.*, 2001). To determine whether COPT1–5 are regulated by copper status, rosette leaves from mature *Arabidopsis* plants were removed and incubated for 18 h in 1 mM $CuSO_4$, 1 mM bathocuproinedisulfonic acid (BCS, a copper chelator) or H_2O as a control. Total RNA from these leaves was used in semiquantitative RT-PCR experiments as described above. Interestingly, in response to copper treatments, both *COPT1* and *COPT2* mRNA levels were strongly reduced, with COPT2 levels being almost undetectable after copper exposure (Figure 5). In contrast, the levels of COPT3, 4 and 5 were unaffected by exposure of leaves to copper. None of the COPT mRNA levels were altered under these conditions in response to copper chelation by BCS.

Discussion

Copper homeostasis belongs to the primitive eukaryotic cellular biochemical pathways substantially con-

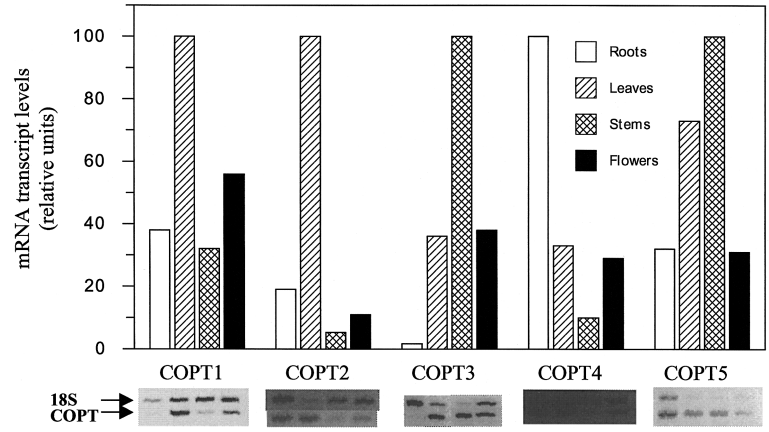

Figure 4. Tissue-specific relative expression levels of COPT1–5 measured by RT-PCR. Total RNAs were prepared from the indicated tissues and subjected to RT-PCR as described in Materials and methods. Experiments were repeated at least three times. Gel bands obtained in a representative experiment corresponding to both COPT1–5 and 18S messages are shown at the bottom. Relative specific expression at different *Arabidopsis* tissues is represented at the histograms considering 100% the highest level measured.

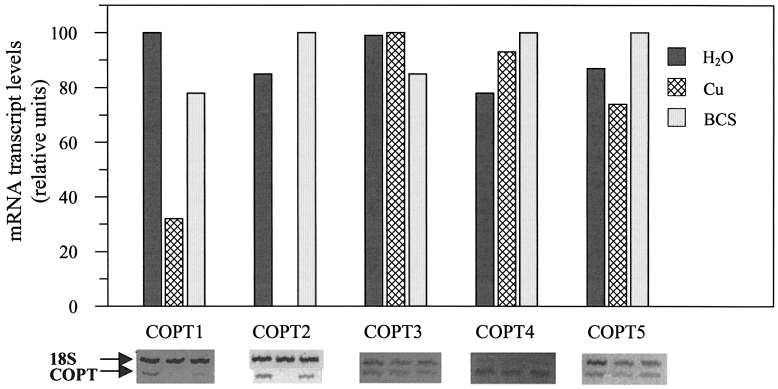

Figure 5. Relative expression levels (%) of COPT1–5 under different copper status measured by RT-PCR. Total RNAs were prepared from the mature excised leaves after 18 h of 1 mM copper sulfate or BCS treatments and subjected to RT-PCR as described in Materials and methods. Experiments were repeated at least three times. Gel bands obtained in a representative experiment corresponding to both COPT1–5 and 18S messages are shown at the bottom. Relative specific expression at different *Arabidopsis* tissues is represented at the histograms considering 100% the highest level measured.

served among distantly diverged organisms such as yeast, animals and plants. Thus, homologues of the yeast Ctr copper transporters family have been found in *Mus musculus* (Lee *et al.*, 2000), *Homo sapiens* (Zhou and Gitschier, 1997; Lee *et al.*, 2000) and *Arabidopsis thaliana* (Kamfenkel *et al.*, 1995) (Figure 1B).

In spite of the fact that we did not obtain *COPT1* homologue clones able to functionally complement *ctr1*Δ*ctr3*Δ yeast mutants when transformed with the *Arabidopsis* cDNAs λ-YES library, *COPT2*, *COPT3* and *COPT5* are able to restore these yeast mutants growth (Figure 2). A possible explanation for these apparently contradictory results could be that these cDNAs are poorly represented in the library.

Conserved residues among these family members are largely restricted to the three transmembrane domains (TMDs) and the region of 22 residues preceding the first TMD, suggesting structural or functional roles for these amino acids. In contrast, the amino-terminal domains and the loop between TMD1 and TMD2 differ in polarity and length, whereas the carboxyl-terminal part of these proteins is mostly unrelated. Homology regions are also maintained when compared to other Ctr members (Labbé and Thiele, 1999; Puig and Thiele, 2002). Both, abundant Cys residues within the TMD in Ctr3 (Peña *et al.*, 2000) and a Met-rich region at the amino-terminal domain in yeast Ctr1 (Dancis *et al.*, 1994a), have been proposed as candidates for copper binding. None of the *Arabidopsis*

family members present abundant Cys residues within the TMDs, although some of them have a CysXCys motif at their carboxy-terminal part. With regard to the amino-terminal region, COPT1 and COPT2 possess two Met- and His-rich boxes, whereas COPT3 and COPT5 have only one of these boxes and none of them are present in COPT4 (Figure 1A).

Taken together, our results support the existence of three COPT categories according to the number of N-terminal Met- and His-rich boxes. The first one, including COPT1 and COPT2 displays the more canonical high-affinity copper transporter features. In parallel with the presence of the two boxes, these transporters are characterized by the complete restoration of yeast mutant growth (Figure 2), which correlates with their high rate of copper transport when expressed in yeast cells (Figure 3A), probably functioning as plasma membrane high-affinity copper transporters. Metal competition experiments suggest that *Arabidopsis* COPT1, similarly to other Ctr1 family members, is a high-affinity transporter with specificity for Cu(I) (Figure 3B).

Despite complementing at a similar rate (Figure 2), COPT1 and COPT2 showed copper uptake rates higher than control Ctr1 (Figure 3A). These apparently contradictory results could be explained by an additional and still uncovered role of copper transporters in copper intracellular delivery, other than simply copper import, and/or a lower plant protein turnover in the yeast system. Alternatively, differences in pH between the uptake experiments (liquid) and the growth assays (YPEG plates) may explain the differences in efficiencies observed. Furthermore, COPT1 and COPT2 display a similar pattern of expression (Figure 4) and are copper-regulated in leaves (Figure 5). Although *COPT1* mRNA was previously reported to be undetectable in roots by northern blot analysis (Kamfenkel *et al*., 1995), RT-PCR data indicate that *COPT1* and *COPT2* are indeed expressed in these nutrient uptake organs. However, a more detailed analysis of tissue and cellular-specific expression, as well as creation of plants in which specific COPT gene expression is silenced, is required to further assess the role of these copper transporters in roots, as well as in other organs.

The second category includes the COPT3 and COPT5 transporters having only one Met- and His-rich box, which show an intermediate level of both complementation and copper transport rate. Although their expression pattern would suggest a predominant role in stem (Figure 4), it would be merely speculative to propose a function for them in plants given the present data and the current state of knowledge about copper transporters in other organisms. Moreover, it is also plausible that the observed lower levels of complementation could be due to incorrect targeting of the proteins that are expressed from a strong promoter in yeast. In this regard, putative target sequences to the chloroplasts and the secretory pathway have been predicted for COPT3 and COPT5, respectively (MIPS *Arabidopsis thaliana* DataBase). Finally, COPT4 represents a third category showing unique features such as potential toxicity when expressed in yeast cells, and high level expression in roots. The potential toxicity of COPT4 in yeast cells could be caused either by a direct or an indirect effect of its expression on essential yeast processes. We have recently shown that an extracellular methionine residue (conserved yeast Ctr1 Met-127) and an M*XXX*M motif within the second transmembrane domain are essential elements for Ctr1-mediated high-affinity copper transport in yeast and human cells (Puig *et al.*, 2002). COPT4 is the only COPT family member that lacks these essential elements suggesting a non-direct role in copper transport. In addition to COPT4, other Ctr family members, mostly differing at their amino-terminal domain, whose role in copper homeostasis remains unsolved, have been described in different organisms, such as Ctr2 from *S. cerevisiae* (Kamfenkel *et al*., 1995) and hCtr2 from *Homo sapiens* (Zhou and Gitschier, 1997). The fact that these putative transporters cannot always substitute for their apparent yeast counterparts may indicate a change in their affinity, a role in the transport of other metals or a subcellular localization inconsistent with a role in copper uptake across the plasma membrane, or a requirement of a co-factor that is absent in yeast. Alternatively, these proteins could have acquired a new function different from copper transport upon losing their metal binding motif. In this sense, *ein2* is a member of the Nramp family of cation transporters that does not display metal-transporting capacity when expressed in heterologous systems, but participates in the ethylene transduction pathway in plants (Alonso *et al*., 1999). Given that copper homeostasis in *Arabidopsis* is becoming a subject of growing interest in plant biology (Himelblau and Amasino, 2000), we expect that the characterization of these putative copper transporters will help to elucidate how copper is incorporated into plant cells and is transported to subcellular proteins and compartments.

Acknowledgements

This work was supported by grants (PB95-0029-C02-02) from DGICYT Spain to L.P., GM41840 from the National Institutes of Health to D.J.T. and a Collaborative Research Grant from the NATO International Scientific Exchange Programmes. V.S., H.M., L.P. and S.P. were recipients of fellowships from the Ministerio de Educación y Cultura and Conselleria d'Educació i Ciència Generalitat Valenciana. We gratefully acknowledge the Mallinckrodt Institute of Radiology at Washington University School of Medicine, St. Louis, MO, for ^{64}Cu with support by the NIH Resource Grant 1R24CA 86307.

References

Alonso, J.M., Hirayama, T., Roamn, G., Nourizadeh, S. and Ecker, J.R. 1999. EIN2, a bifunctional transducer of ethylene and stress response in *Arabidopsis.* Science 284: 2148–2152.

Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. 1995. Current Protocols in Molecular Biology. John Wiley, New York.

Dancis, A., Haile, D., Yuan, D.S. and Klausner, R.D. 1994a. The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiological role in copper uptake. J. Biol. Chem. 269: 25660–25667.

Dancis, A., Yuan, D.S., Haile, D., Askwith, C., Eide, D. Moehle, C., Kaplan, J. and Klausner, R.D. 1994b. Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. Cell 76: 393–402.

Dower, W.T., Miller, J.F. and Ragsdale, C.W. 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucl. Acids Res. 16: 6127–6145.

Elledge, S.J., Mulligan, J.T., Ramer, S.W., Spottswood, M. and Davis, R.W. 1991. λ-YES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc. Natl. Acad. Sci. USA 88: 1731–1735.

Feng, D.F. and Doolittle, R.F. 1987. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. 25: 351–360.

Fox, T.C. and Guerinot, M.L. 1998. Molecular biology of cation transport in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 669–696.

Gietz, R.D., Schiestl, R.H. and Willems, A.R., Woods, R.A. 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11: 355–360.

Halliwell, B. and Gutteridge J.M. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. Meth. Enzymol. 186: 1–85.

Harris, E.D. 2000. Cellular copper transport and metabolism. Annu. Rev. Nutr. 20: 291–310.

Himelblau, E. and Amasino, R.M. 2000. Delivering copper within plant cells. Curr. Opin. Plant Biol. 3: 205–210.

Himelblau, E., Mira, H., Lin, S.-J., Culotta, V.C., Peñarrubia, L. and Amasino, R.M. 1998. Identification of a functional homolog of the yeast copper-binding gene ATX from *Arabidopsis thaliana.* Plant Physiol. 117: 1227–1234.

Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A. and Ecker, J.R. 1999. RESPONSIVE-TO-ANTAGO- NIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in *Arabidopsis*. Cell 97: 383–393.

Huffman D.L., O'Halloran, T.V. 2001. Function, structure, and mechanism of intracellular copper trafficking proteins. Annu. Rev. Biochem. 70: 677–701.

Kampfenkel, K., Kushinr, S., Babychuk, E., Inzé, D. and Van Montagu, M. 1995. Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. J. Biol. Chem. 270: 28479–28486.

Knight, S.A.B., Labbé, S., Kwon, L., Kosman, D.J. and Thiele, D.J. 1996. A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev. 10: 1917–1929.

Koch, K.A., Peña, M.M.O. and Thiele, D.J. 1997. Copper-binding motifs in catalysis, transport, detoxification and signaling. Chem. Biol. 4: 549–560.

Labbé, S. and Thiele, D.J. 1999. Pipes and wiring: the regulation of copper uptake and distribution in yeast. Trends Microbiol. 7: 500–505.

Labbé, S., Zhu, Z. and Thiele, D.J. 1997. Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. J. Biol. Chem. 272: 15951–15958.

Labbé, S., Peña, M.M.O., Fernandes, A.R. and Thiele, D.J. 1999. A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. J. Biol. Chem. 274: 36252–36260.

Lee, J., Prohaska, J.R., Dagenais, S.L., Glover, T.W. and Thiele, D.J. 2000. Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. Gene 254: 87–96.

Lee, J., Pena, M.M.O., Nose, Y. and Thiele, D.J. 2002. Biochemical characterization of the human copper transporter Ctr1. J. Biol. Chem. 272: 4380–4387.

Miller, J.D., Arteca, R.N. and Pell, E.J. 1999. Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. Plant Physiol. 120: 1015–1024.

Mira, H., Martínez-García, F. and Peñarrubia ,L. 2001a. Evidence for the plant-specific intercellular transport of the *Arabidopsis* copper chaperone CCH. Plant J. 25: 521–528.

Mira, H., Vilar, M., Perez-Paya, E., Penarrubia, L. 2001b. Functional and conformational properties of the exclusive C-domain from the *Arabidopsis* copper chaperone (CCH). Biochem. J. 15: 545–549.

Mumberg, D., Müller, R. and Funk, M. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 156: 119–122.

Peña, M..M., Lee, J., Thiele and D.J. 1999. A delicate balance: homeostatic control of copper uptake and distribution. J. Nutr. 129: 1251–1260.

Peña, M.M., Puig, S. and Thiele, D.J. 2000. Characterization of the *Saccharomyces cerevisiae* high-affinity copper transporter Ctr3. J. Biol. Chem. 275: 33244–33251.

Portnoy, M.E., Schmidt, P.J., Rogers, R.S. and Cullotta ,V.C. 2001. Metal transporters that contribute copper to metallochaperones in *Saccharomyces cerevisiae*. Mol. Genet. Genomics 265: 873–882.

Prescott, A. and Martin, C. 1987. A rapid method for the quantitative assessment of levels of specific mRNAs in plants. Plant Mol. Biol. Rep. 4: 219–224.

Puig, S. and Thiele D.J. 2002. Molecular mechanisms of copper uptake and distribution. Curr. Opin. Chem. Biol. 6: 171–180.

Puig, S., Lee, J., Lau, M. and Thiele D.J. 2002. Biochemical and genetic analyses of yeast and human high-affinity copper transporters suggest a conserved mechanism for copper uptake. J. Biol. Chem. 277: 26021–26030.

Rauser, W.E. 1995. Phytochelatins and related peptides. Plant Physiol. 109: 1141–1149.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E. and Bleecker, A.B. 1999. A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. Science 283: 996–998.

Rothnie, H.M. 1996. Plant mRNA 3′-end formation. Plant Mol. Biol. 32: 43–61.

Salt, D.E., Smith, R.D. and Raskin, I. 1998. Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 643–668.

Thompson, J. D., Higgins, D. J. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Woeste, K.E. and Kieber, J.J. 2000. A strong loss-of function mutation in *RAN1* results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell 12: 443–455.

Wu, L, Ueda, T. and Messing, J. 1995. The formation of mRNA 3′-ends in plants. Plant J. 8: 323–329.

Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D.J., Klausner, R.D., Dancis, A. 1997. Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of *FRE1* and *CTR1*. J. Biol. Chem. 272: 17711–17718.

Zhou, J. and Goldsbrough, P.B. 1995. Structure, organization and expresión of the metallothionein gene family in *Arabidopsis*. Mol. Gen. Genet. 248: 318–328.

Zhou, B. and Gitschier, J. 1997. hCTR1: a human gene for copper uptake identified by complementation in yeast. Proc. Natl. Acad. Sci. USA 94: 7481–7486.