

# Two *Chlamydomonas* CTR Copper Transporters with a Novel Cys-Met Motif Are Localized to the Plasma Membrane and Function in Copper Assimilation <sup>W</sup>

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**Inducible high-affinity copper uptake is key to copper homeostasis in *Chlamydomonas reinhardtii*. We generated cDNAs and updated gene models for four genes, *CTR1*, *CTR2*, *CTR3*, and *COPT1*, encoding CTR-type copper transporters in *Chlamydomonas*. The expression of *CTR1*, *CTR2*, and *CTR3* increases in copper deficient cells and in response to hypoxia or Ni<sup>2+</sup> supplementation; this response depends on the transcriptional activator CRR1. A copper response element was identified by mutational analysis of the 5' upstream region of *CTR1*. Functional analyses identify *CTR1* and *CTR2* as the assimilatory transporters of *Chlamydomonas* based on localization to the plasma membrane and ability to rescue a *Saccharomyces cerevisiae* mutant defective in high-affinity copper transport. The *Chlamydomonas* CTRs contain a novel Cys-Met motif (CxxMxxMxxC-x<sub>5/6</sub>-C), which occurs also in homologous proteins in other green algae, amoebae, and pathogenic fungi. *CTR3* appears to have arisen by duplication of *CTR2*, but *CTR3* lacks the characteristic transmembrane domains found in the transporters, suggesting that it may be a soluble protein. Thus, *Chlamydomonas* CTR genes encode a distinct subset of the classical CTR family of Cu(I) transporters and represent new targets of CRR1-dependent signaling.**

## INTRODUCTION

Copper is an essential micronutrient for most organisms, especially in respiring eukaryotes, due to its being a cofactor in electron transfer proteins and in enzymes that catalyze redox reactions or oxygen chemistry, such as cytochrome oxidase, ferroxidases, and Cu/Zn superoxide dismutases. The availability of copper in the environment or in the nutrient source may vary widely, however, and copper deficiency is a challenge that many organisms encounter, a challenge they have met through the evolution of sophisticated adaptive mechanisms. At the same time, free copper ions have the potential to participate in Fenton chemistry resulting in the production of reactive oxygen species. Copper assimilation must therefore be tightly regulated so as to meet but not exceed cellular requirements (reviewed in Eide, 1998; Puig and Thiele, 2002; Winge, 2002; Merchant et al., 2006; Pilon et al., 2006).

Genetic and biochemical studies in a number of model organisms have established the existence in eukaryotes of two types of transmembrane copper transporters, the copper transporting P-type ATPases that function in copper distribution within the cell (Lutsenko and Petris, 2003) and the CTR family copper transporters (Puig and Thiele, 2002; De Feo et al., 2007) that

function in copper acquisition from the environment. The CTR family copper transporters do not use energy from ATP hydrolysis (Lee et al., 2002); Cu is transported down a concentration gradient because intracellular copper is immediately sequestered.

Genes encoding CTR-type copper transporters were first identified in *Saccharomyces cerevisiae*. Ctr1p and Ctr3p are localized to the plasma membrane (Dancis et al., 1994; Peña et al., 2000) and are functionally redundant; disruption of both *CTR1* and *CTR3* is required to abolish high-affinity copper uptake, although in most laboratory strains *CTR3* is already disrupted by the *Ty* transposon (Knight et al., 1996). Measurements of Cu<sup>64</sup> uptake by *S. cerevisiae* indicate that the uptake system in that organism has an affinity of ~4 μM for copper (Lin and Kosman, 1990; Dancis et al., 1994). The cell-surface Fe(III)/Cu(II) reductase *FRE1* is required for maximal Cu uptake, suggesting that the translocated species is Cu(I) (Hassett and Kosman, 1995; Knight et al., 1996). Ctr1p and Ctr3p hand off copper to cytosolic chaperones (Xiao and Wedd, 2002; Xiao et al., 2004) for incorporation into target proteins, such as the mitochondrial cytochrome c oxidase subunit Cox2p and the mitochondrial and cytosolic Cu/Zn superoxide dismutases (reviewed in Cobine et al., 2006), and for transfer to distributive transporters, such as Ccc2, which transfers copper into the lumen of the secretory pathway where it is incorporated into newly synthesized proteins, such as the plasma membrane ferroxidase Fet3p (Yuan et al., 1997). Upregulation of assimilatory components is the first-line response to copper deficiency (reviewed in Labbé and Thiele, 1999); expression of *CTR1*, *CTR3*, and *FRE1* is activated by the Cu-sensing transcription factor Mac1p that binds to copper response elements (CuREs) in the promoter regions of these genes (reviewed in Rutherford and

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Bird, 2004). A third CTR-type transporter, Ctr2p, mobilizes stored copper from the vacuole under conditions of copper deficiency (Rees et al., 2004).

CTR family copper transporters are also present in animal and plant cells. A human homolog of Ctr1p, hCTR1 (Zhou and Gitschier, 1997) is localized to the plasma membrane and mediates saturable Cu(I) uptake with a  $K_m$  of  $\sim 2$  to  $4 \mu\text{M}$  (Eisses and Kaplan, 2002; Lee et al., 2002). Knockout of the homologous murine *CTR1* is lethal (Lee et al., 2001). *Drosophila melanogaster* expresses three *Ctr1* genes. Ctr1-A and -B localize to the plasma membrane and are important in copper acquisition; *Ctr1-B* is transcriptionally activated in response to copper deprivation (Zhou et al., 2003; Turski and Thiele, 2007). A six-member gene family (*COPT1-6*) has been reported in *Arabidopsis thaliana* (Sancenón et al., 2003). Of these, *COPT1* and *COPT2* are upregulated in copper deficiency, and *COPT1* knockdown plants contain decreased levels of copper (Sancenón et al., 2004; Puig et al., 2007), but roles for the individual transporters have yet to be defined. In plants, Cu must also be distributed to the chloroplast; in *Arabidopsis*, the P-type ATPase transporters *PAA1* and *PAA2* translocate Cu across the envelope and thylakoid membranes, respectively (Shikanai et al., 2003; Abdel-Ghany et al., 2005).

The green alga *Chlamydomonas reinhardtii*, a unicellular eukaryote, has proved a particularly valuable model for studies of adaptation to copper deficiency (Hanikenne et al., 2009). The major copper proteins in copper replete *Chlamydomonas* are plastocyanin, a thylakoid lumen-localized copper protein that transfers electrons from the cytochrome *b<sub>6</sub>f* complex to photosystem I, the mitochondrial cytochrome *c* oxidase subunit COX2b, and the plasma membrane ferroxidase FOX1, which is important under conditions of iron deficiency (Herbik et al., 2002; La Fontaine et al., 2002; Chen et al., 2008). Like other green algae, *Chlamydomonas* appears to have reduced its baseline copper requirement by dispensing with the non-essential CuZnSODs that are almost ubiquitous in plant and animal cells, relying instead on one FeSOD and five MnSODs (Asada et al., 1977; Allen et al., 2007a).

Acclimation of *Chlamydomonas* to copper deficiency involves a number of changes in plastid-localized metabolic pathways. The best studied is the replacement of plastocyanin by cytochrome *c<sub>6</sub>*. This occurs via transcriptional activation of the *CYC6* gene and degradation of plastocyanin (Merchant et al., 1991; Li and Merchant, 1995), which ensures maintenance of photosynthetic electron flow in the absence of an otherwise essential copper protein. Genes encoding enzymes in the tetrapyrrole biosynthetic pathway are also regulated coordinately with the *CYC6* gene. This includes *CPX1* for coproporphyrinogen oxidase and *CRD1* and *CTH1* representing isozymes of Mg-protoporphyrin IX monomethylester cyclase (Quinn et al., 1999; Moseley et al., 2000; Allen et al., 2008). Each of the genes is controlled by CRR1, an SBP domain transcription factor, which binds to CuREs associated with the 5' upstream regions and activates transcription. The CuREs contain a GTAC core sequence that is critical for CuRE function (Quinn et al., 1999, 2000, 2002; Eriksson et al., 2004; Kropat et al., 2005).

The existence of a regulated high-affinity copper uptake system has also been demonstrated in *Chlamydomonas*. Cells

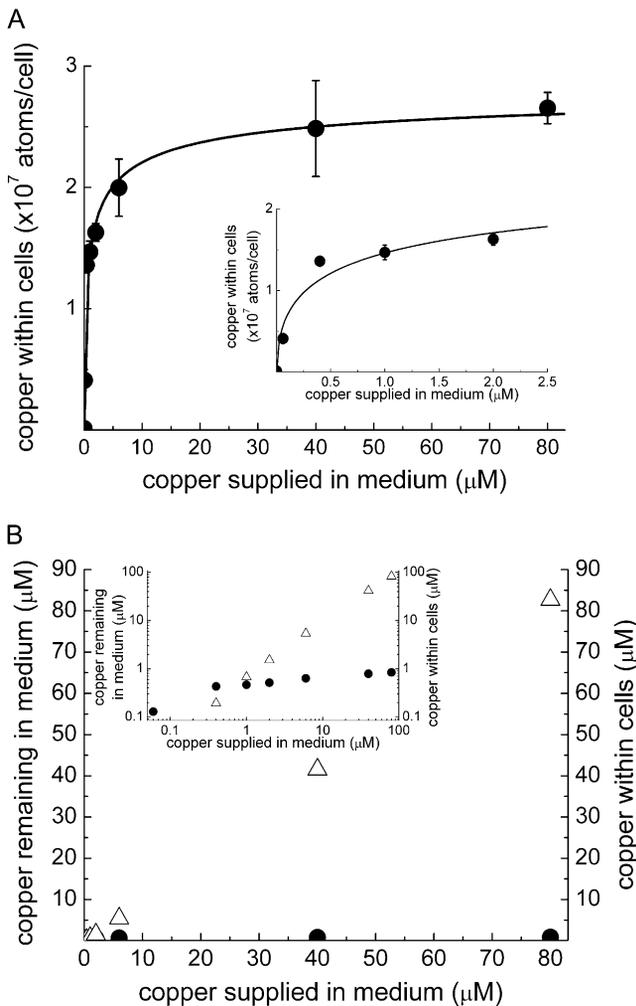
grown under conditions of copper deficiency are more sensitive to the toxic effects of Ag(I) than are cells grown with sufficient copper, suggesting that Ag(I) enters the cell via an inducible transporter (Howe and Merchant, 1992). Copper-deficient *Chlamydomonas* responds to 2 to 5 nM concentration of copper ions in the medium, again indicative of a high-affinity uptake system (Merchant et al., 1991). This system likely consists of a cell surface Cu(II) reductase that is more active in copper-deficient than in copper replete cells and a transport component. Measurements of Cu<sup>64</sup> uptake by whole cells indicate the presence of a copper uptake system that exhibits saturable kinetics. The kinetics indicate a 20-fold higher  $V_{max}$  for Cu uptake in copper-deficient cells compared with copper-replete ones, but a  $K_m$  of  $\sim 0.2 \mu\text{M}$  in both conditions, suggesting the presence of a single copper transporter whose expression is regulated by Cu availability (Hill et al., 1996).

The recent completion of the *C. reinhardtii* genome (Merchant et al., 2007) and cataloging of its metal transporters (Hanikenne et al., 2009) allowed us to employ a candidate gene approach to the identification of components of the copper assimilation pathway. In this article, we demonstrate that *Chlamydomonas* expresses three CTR family copper transporters, encoded by the *COPT1*, *CTR1*, and *CTR2* genes and a fourth gene, *CTR3*, encoding a protein that is not a conventional copper transporter but is related to CTR1 and CTR2 and may contribute to copper uptake.

## RESULTS

### Regulated High-Affinity Copper Uptake in *Chlamydomonas*

*Chlamydomonas* has a remarkable capacity to extract even trace amounts of copper from growth media. In previous work, we noted that just 1 to 10 nM copper provided in the growth medium could support assembly of holoplastocyanin (Merchant et al., 1991) and that *Chlamydomonas* grown in medium containing limiting amounts of copper was able to deplete that medium of copper down  $\leq 1.6$  nM (Hill et al., 1996). To determine whether Cu uptake was under homeostatic control, we grew *Chlamydomonas* in TAP medium containing various amounts (0 to  $80 \mu\text{M}$ ) of added copper and measured both the amount of copper left in the media and the amount of copper taken up by the cells. Figure 1 shows that *Chlamydomonas* cells are able to deplete copper-deficient media down to under 0.2 nM copper (corresponding to the detection limit), but once cells have taken up enough copper to support maximal plastocyanin production ( $\sim 69$  nM Cu when cultures contain  $4.6 \times 10^6$  cells/mL; Hill and Merchant, 1995), no further depletion of copper from the medium is observed. This is as expected, since copper uptake in excess of requirements would be deleterious for the cell. Taken together, these observations indicate that *Chlamydomonas* has a high-affinity copper uptake system that is induced under conditions of copper deficiency and repressed under conditions of copper sufficiency. The amounts of iron and manganese in cells of *Chlamydomonas* changed little as the copper concentration was varied, suggesting that the uptake system is copper specific (Supplemental Figure 2).



**Figure 1.** Regulated Copper Assimilation in *Chlamydomonas*.

(A) and (B) Cells were inoculated into medium containing the indicated amounts of copper (added to the medium at the time of inoculation) and collected at a density of  $3 \times 10^6$  cells/mL. Each point is the average of three independent experiments. Errors shown are those for the average of experimental triplicates. Similar data were obtained for a second set of experimental triplicates.

(A) Copper content of cells as a function of initial copper concentration. The Cu content of cells is expressed on a per cell basis. The inset shows an expanded view of the points at low Cu concentrations.

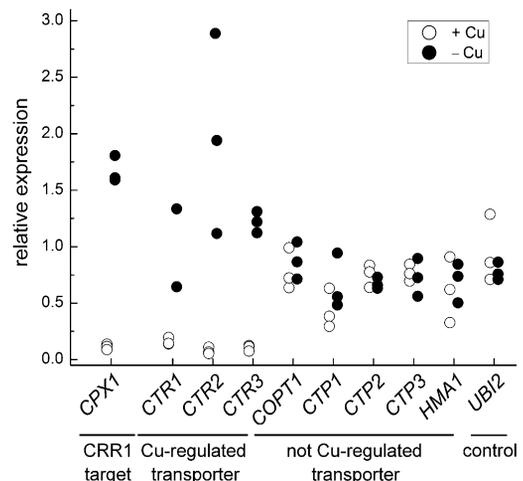
(B) Copper content of cells and spent medium as a function of initial copper concentration. The amount of Cu remaining in the medium is indicated by the open triangles and the amount taken up by the cells by the filled circles. To give an indication of the fraction of Cu taken up by the cells, the Cu content of cells is expressed in terms of concentration corresponding to the volume of the culture. The inset shows an alternative (log/log) plot of the data. Error bars are smaller than the symbols used to represent the data.

### Three Candidate CTR Family Copper Transporters in *Chlamydomonas* Are Regulated by Copper and by CRR1

To identify potential copper transporters in *Chlamydomonas*, we queried the draft genome (initially version 1.0 and subsequently

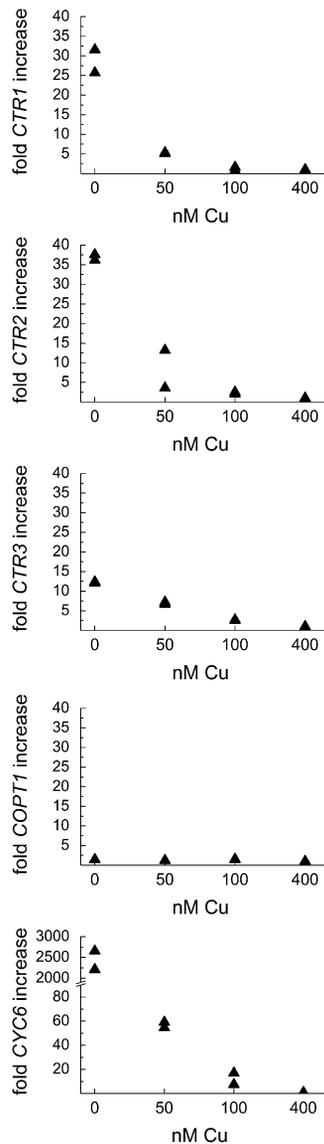
versions 2.0 and 3.0) using BLAST to identify gene models corresponding to homologs of copper transporters of other organisms, including fungi, plants, and animals (Merchant et al., 2006; Hanikenne et al., 2009). We identified four candidate CTR family copper transporters, corresponding to protein IDs 196101, 196096, 196115, and 196102 in the version 3.0 draft genome. These were named *CTR1*, *CTR2*, *CTR3*, and *COPT1*, respectively, the latter on the basis of its close similarity to *COPT1* of *Arabidopsis thaliana*. We also identified four candidate copper-transporting P-type ATPases, corresponding to protein IDs 206047, 205938, 195962, and 195998. These were named *CTP1*, *CTP2*, *CTP3*, and *HMA1*, respectively, the latter on the basis of its similarity to the chloroplast copper transporter *HMA1* of *Arabidopsis*.

To assess the relevance of these candidate proteins in *Chlamydomonas* copper assimilation, we analyzed their expression in response to variation in copper nutrition (Figures 2 and 3). *CTR1*, *CTR2*, and *CTR3* are substantially upregulated under conditions of copper deficiency, with levels of their mRNAs increasing up to 20-, 30-, and 15-fold, respectively, in three separate experiments (Figure 2). By contrast, the abundance of *COPT1*, *CTP1*, *CTP2*, *CTP3*, and *HMA1* mRNAs did not change significantly under the conditions tested. The pattern of copper-responsive expression of *CTR1*, *CTR2*, and *CTR3*, but not of *COPT1*, was confirmed by RNA gel blot hybridization (Figure 4A) and recapitulated the pattern noted for the *CYC6*, *CPX1*, and



**Figure 2.** Accumulation of mRNAs Encoding Candidate Copper Transporters in Response to Cu Nutrition.

RNA from copper-deficient (filled circles) and copper-replete (open circles) cells was analyzed by real-time PCR for the abundance of transcripts encoding various copper transporters. Primers used are listed in Supplemental Table 1 online. *Chlamydomonas* strain 2137 was grown in TAP medium with or without added copper to  $\sim 3 \times 10^6$  cells/mL. The fold difference in abundance of each mRNA is shown after normalization to *CBLP* and is relative to a reference mixture of the six RNA samples (three +Cu and three -Cu) from three independent experiments. Each data point is the average of technical triplicates and represents one experiment.



**Figure 3.** Copper Concentration Dependence of *CTR1*, *CTR2*, *CTR3*, and *COPT1* mRNA Accumulation.

*Chlamydomonas* strain 2137 was grown in TAP medium containing the indicated amounts of supplemental copper. Cells were collected at  $\sim 1 \times 10^7$  cells/mL for total RNA preparation. RNA was analyzed by real-time PCR as described in Methods and the relative abundance of mRNAs calculated relative to the sample at 400 nM added copper. Two independent experiments were performed, and each data point represents the average of technical triplicates.

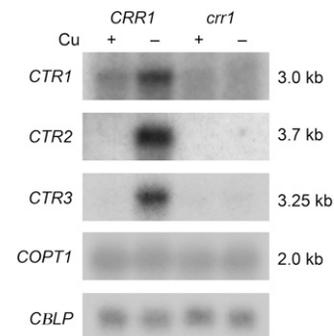
*CRD1* genes, which are established targets of the CRR1- and CuRE-dependent nutritional copper signaling pathway (Figure 3) (Quinn and Merchant, 1995; Quinn et al., 1999, 2000; Moseley et al., 2000; Eriksson et al., 2004; Kropat et al., 2005). Therefore, we tested whether the *CRR1* locus was required for their upregulation in Cu-deficient cultures. Indeed, *crr1-1* cells growing under conditions of copper deficiency failed to increase

transcription of *CTR1*, *CTR2*, or *CTR3* beyond the basal levels observed in +Cu wild-type cells (Figure 4A). We conclude that *CTR1*, *CTR2*, and *CTR3* represent three further targets of the CRR1-mediated copper deficiency response pathway in *Chlamydomonas*.

#### Improved Gene Models for *CTR1*, *CTR2*, *CTR3*, and *COPT1*

The sizes of the *CTR1*, *CTR2*, *CTR3*, and *COPT1* transcripts (3.0, 3.7, 3.25, and 2 kb, respectively; Figure 4) indicated that the existing gene models for *CTR1*, *CTR2*, *CTR3*, and *COPT1* were unlikely to be correct. Therefore, we sought to extend and correct the original models by sequencing PCR products amplified from cDNA and by 5'-rapid amplification of cDNA ends (RACE) (see Supplemental Methods online). The final models, shown schematically in Supplemental Figure 1A, have been incorporated into the version 3.1 draft *Chlamydomonas* genome at the Joint Genome Initiative browser and the mRNA sequences deposited in GenBank (accession numbers DQ021453, DQ646486, EU915720, and DQ646487, respectively).

Analysis of the improved gene models suggests that the *CTR3* gene has been formed by partial duplication of *CTR2*; the two genes lie only 31.7 kb apart. Exons 1 to 4 of *CTR3* map closely onto exons 1 to 4 of *CTR2* (79% identity, 85% similarity, and 62% identity; 75% similarity over two segments of 182 and 95 amino acids, respectively, separated by Pro/Ser-rich segments of differing lengths). By contrast, there is little sequence similarity between exons 5 to 8 of *CTR3* and exons 5 to 8 of *CTR2*. Interestingly, exons 6 and 7 of *CTR3* exhibit significant sequence similarity to exons 3 and 4 of both *CTR3* (63% identity and 72% similarity over 104 amino acids) and *CTR2* (60% identity and 73% similarity over 96 amino acids), suggesting that exons 6 and



**Figure 4.** Potential CTR Family Copper Transporters in *Chlamydomonas*.

Transcript sizes and CRR1-dependent increase of *CTR1*, *CTR2*, and *CTR3* transcripts in copper-deficient cells. Wild-type (CC125) and *crr1-1* mutant strains were grown in TAP medium containing either 0 (–Cu) or 2  $\mu$ M (+Cu) copper. Five micrograms of total RNA was loaded in each lane and analyzed by blot hybridization. The expression of *CBLP* was monitored as a loading control. Transcript sizes are shown at right and were estimated from a standard curve of RNA markers (Gibco BRL 0.24 to 9.5 kb).

7 of *CTR3* may have arisen from exons 3 and 4 of either *CTR2* or *CTR3* by a second duplication event.

We also determined that a 264-nucleotide region of *CTR2*, previously assigned intron status, is actually an exon; its unusual protein sequence, rich in Ala, Pro, and Ser, may have evaded exon prediction programs. The *COPT1* gene is G+C rich, even by *Chlamydomonas* standards (average 64%; Merchant et al., 2007). The last 250 nucleotides of the coding sequence has a G+C content of 77%, and a 50-nucleotide segment of this region has 92% G+C.

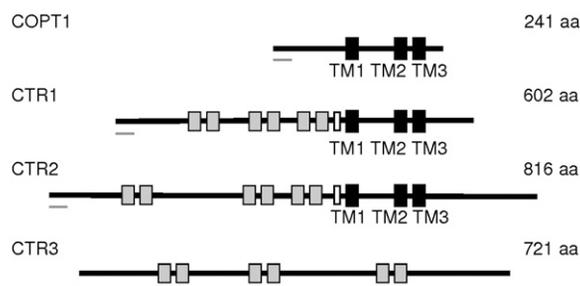
Full-length cDNAs for *CTR2*, *CTR3*, and *COPT1* were assembled using standard molecular biology strategies (see Supplemental Methods online). The *CTR1*, *CTR2*, *CTR3*, and *COPT1* gene products were then analyzed by in vitro transcription-translation of full-length cloned cDNAs. Migration of the polypeptides produced corresponded closely with those expected for the predicted gene products (*CTR1*, 67 kD; *CTR2*, 90 kD; *CTR3*, 80 kD; *COPT1*, 27 kD; see Supplemental Figure 1B online).

### Features of the *CTR1*, *CTR2*, *CTR3*, and *COPT1* Proteins

The *CTR* family copper transporter proteins differ widely in size and degree of sequence similarity but have a well-defined domain structure. A central domain is defined by three membrane-spanning  $\alpha$ -helices, the first (TM1) and second (TM2) of which are separated by a cytoplasmic loop, while only a few residues separate TM2 and TM3. TM2 contains the motif MxxxM (where x is any amino acid), and TM3 the motif GxxxG, together forming the characteristic motif MxxxM-x<sub>13</sub>-GxxxG motif (De Feo et al., 2007). Hydrophilic N-terminal and C-terminal domains of various sizes are oriented to the extracellular environment and the cytoplasm, respectively. The N-terminal domain is generally rich in metal binding amino acids like His, Met, and (more rarely) Cys. The C-terminal domain tends to contain Cys and/or His in motifs such as CxC, shown to bind copper ions and transfer them to cytosolic copper chaperones (Xiao and Wedd, 2002; Xiao et al., 2004). All these features are present in *CTR1*, *CTR2*, and *COPT1* of *Chlamydomonas*. A schematic view of the four proteins is shown in Figure 5. All four are predicted to have cleavable N-terminal signal sequences.

*COPT1* is similar to vascular plant and vertebrate *CTRs* in that it has fairly small N- and C-terminal domains (~70 and 15 amino acids, respectively). The N-terminal domain contains His and Met in two motifs, HxxHxxHxxH and HxHxxxMxMxM, while the motif CH is located close to the C terminus. Public draft genome data (www.jgi.doe.gov) for *Volvox carteri*, a green alga closely related to *Chlamydomonas*, indicate the presence of a *COPT1* homolog in this organism.

*CTR1*, on the other hand, has a fairly large C-terminal extramembrane domain (~80 amino acids) that has the likely metal binding motif CxCxH close to its C terminus, and it also has a large N-terminal extramembrane domain (~380 amino acids), which contains six highly distinctive Cys- and Met-containing motifs, the most common form of which appears to be CxxMxxMxxC-x<sub>5/6</sub>-C (Figure 6). We have termed these Cys-Met motifs and have identified a number of other proteins



**Figure 5.** Structural Organization of the Predicted *CTR1*, *CTR2*, *CTR3*, and *COPT1* Proteins.

Black bars represent the protein chains, black boxes represent predicted membrane-spanning  $\alpha$ -helices (TM1, TM2, and TM3), and gray lines underneath the protein chains designate predicted N-terminal signal sequences. Light-gray boxes indicate the positions of novel Cys-Met motifs in *CTR1*, *CTR2*, and *CTR3*. White boxes indicate the position of a conserved MxMxxH motif in *CTR1* and *CTR2*. Sizes of the predicted precursor proteins are given at right.

(candidate copper transporters) in which they occur, especially in the green algae and in amoebae. *CTR2* also has a large C-terminal domain (~190 amino acids) containing both the motif CC and a His-rich motif, H-x<sub>3</sub>-H-x<sub>4</sub>-H-x<sub>6</sub>-HHxxHxxH, and a large N-terminal extramembrane domain (~490 amino acids) that, like that of *CTR1*, contains six Cys-Met motifs. The *Chlamydomonas* *CTR1*, *CTR2*, and similar proteins also exhibit another potential metal binding motif, MxMxxH, located ~15 amino acids from the N-terminal end of TM1 (Figure 6). The position of this motif relative to TM1 is similar to that of the Met-127 residue of *S. cerevisiae* Ctr1p, which has been shown to be critical for the function of that protein (Puig et al., 2002).

As mentioned previously, the *Chlamydomonas* *CTR3* gene lies close to *CTR2* (~31.5 kb away) and appears to have arisen from it by partial duplication. Like *CTR1* and *CTR2*, the *CTR3* protein has six Cys-Met motifs, but in other respects, it differs significantly. Hydrophobicity analyses suggest that the protein does not contain any membrane-spanning  $\alpha$ -helices and may be a soluble protein. Furthermore, although it has the MxxxM-x<sub>13</sub>-GxxxG motif characteristic of *CTR*-type copper transporters, this overlaps the fifth Cys-Met motif, the sequence in the region being CNMMPYMPGC-x<sub>8</sub>-GxxxG. It also does not contain the conserved MxMxxH motif described above. We conclude that *CTR3* is probably not a typical copper transporter, but the observed upregulation of *CTR3* under conditions of copper deficiency suggests that *CTR3* has some role in *Chlamydomonas* copper homeostasis. We note the presence of a *CTR3*-like protein (PID 95441) in *V. carteri*; in this case, the gene encoding it lies adjacent to *V. carteri* *CTR2*.

### The *Chlamydomonas* *CTR1* and *CTR2* Proteins Are High-Affinity Copper Transporters

The potential of the *C. reinhardtii* *COPT1* and *CTR* genes to encode high-affinity copper transporters was tested by



### *Chlamydomonas* CTR1 and CTR2 Are Localized to the Plasma Membrane and Are More Abundant in Copper-Deficient Cells

To address the question of CTR1 and CTR2 localization, membranes were prepared from cells grown with or without added copper (2  $\mu$ M) and separated in a two-phase aqueous polymer system into plasma membranes and a fraction containing all other membrane species. The efficiency of this separation was checked by immunoblotting with antibodies to proteins of known localization: CRD1/CTH1, thylakoid membrane and chloroplast envelope (Allen et al., 2008); cytochrome *f*, thylakoid membrane; COX2b, mitochondrial inner membrane; TOM20, mitochondrial outer membrane (Werhahn et al., 2001); FOX1 ferroxidase, plasma membrane (Herbik et al., 2002); and the *Chlamydomonas* plasma membrane H<sup>+</sup> ATPase (Norling et al., 1996). This analysis showed that the plasma membrane fraction was largely free of contamination with other membranes (estimated at  $\leq 1\%$  for thylakoid membrane by immunoblotting for cytochrome *f*; data not shown) (Figure 8A). The accumulation of COX2b and ferroxidase, both copper proteins, is strongly dependent on copper availability (La Fontaine et al., 2002).

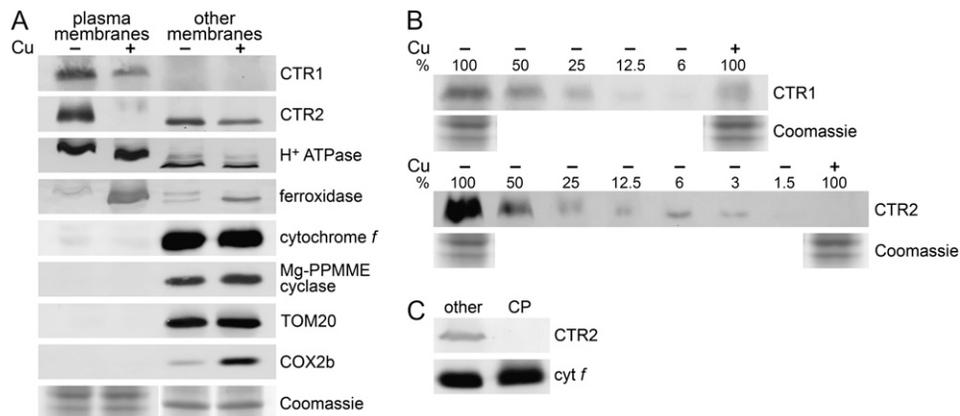
When we probed these membrane fractions with antibodies against CTR1, we observed a prominent band in the  $-Cu$  plasma membranes. This migrated at  $\sim 64$  kD, which corresponds closely with the size expected for the CTR1 polypeptide after cleavage of the predicted signal sequence (Figure 8A). Competition experiments confirmed that this band was CTR1 (data not shown). CTR1 was located exclusively in the plasma membrane fractions. The abundance of CTR1 is approximately threefold

higher under conditions of copper deficiency (Figure 8B), in general agreement with the results obtained for the Cu-dependent changes in *CTR1* mRNA abundance.

Antibodies to CTR2 also recognized a prominent band in  $-Cu$  plasma membranes. Its molecular mass was estimated at 85 kD, which corresponds well with that expected for the CTR2 polypeptide after cleavage of the predicted signal sequence (Figure 8A). Competition experiments confirmed that this band was CTR2. Accumulation of CTR2 in the plasma membrane fraction was strongly dependent on copper availability, the amount in copper-deficient cells being at least 30 times higher than in copper-sufficient cells (Figure 8B). This corresponds closely with the change in RNA abundance for the *CTR2* mRNA (Figures 2 and 4). Some CTR2 was also detected in the "other membranes" fraction from both  $-Cu$  and (to a lesser extent)  $+Cu$  cells. CTR2 was not detected in chloroplast membranes (Figure 8C). It is possible that CTR2 localizes similarly to the *Dictyostelium discoideum* p80 and human Ctr2 proteins, which are found in both plasma membrane and endocytic compartments (Ravanel et al., 2001; Bertinato et al., 2008).

### *CTR1*, *CTR2*, and *CTR3* Are Transcriptionally Regulated by Copper

Since the RNAs for *CTR1*, *CTR2*, and *CTR3* are regulated coordinately with those for *CYC6* and *CPX1* that are known targets of CRR1, which is a master regulator of nutritional Cu homeostasis (Eriksson et al., 2004), we tested the *CTR* genes for their transcriptional regulation by copper and for the presence of CuREs. Reporter constructs (Figures 9 and 10) were introduced

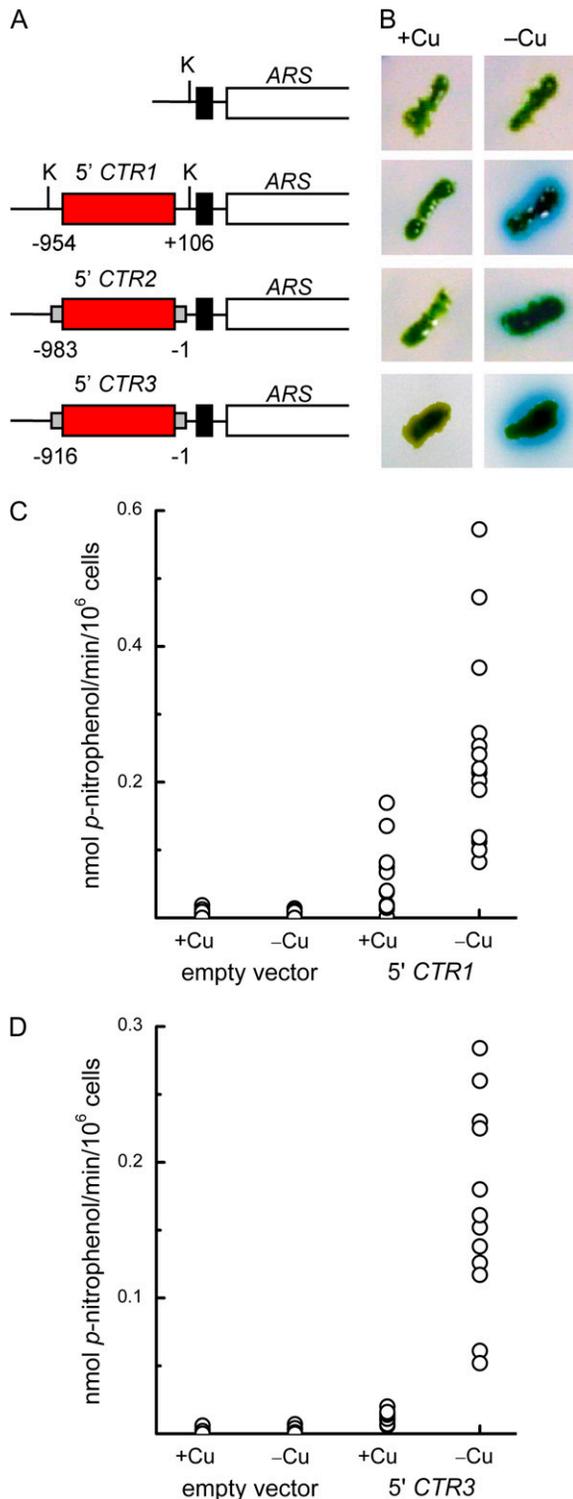


**Figure 8.** Localization of *Chlamydomonas* CTR1 and CTR2 and Abundance under Conditions of Copper Deficiency and Repletion.

**(A)** Localization. *Chlamydomonas* strain CC425 was grown in TAP medium containing either 0 ( $-Cu$ ) or 2  $\mu$ M ( $+Cu$ ) added copper. Membranes were isolated and separated into a plasma membrane fraction and a fraction containing all other membranes. Protein (2 to 20  $\mu$ g) from each fraction was separated by denaturing gel electrophoresis and transferred to nitrocellulose for immunoblot analysis with anti-CTR1, anti-CTR2, antiplasma membrane H<sup>+</sup> ATPase, anti-FOX1 ferroxidase (plasma membrane), anticytochrome *f* (thylakoid membrane), anti-CRD1 Mg-protoporphyrin monomethyl ester cyclase (thylakoid membrane and chloroplast envelope), anti-TOM20.3, and anti-COX2b (mitochondrial outer and inner membranes, respectively). Protein loadings were normalized by Coomassie blue staining (representative bands shown). Molecular masses of markers are indicated in kilodaltons.

**(B)** Abundance of CTR1 and CTR2 in copper deficiency and repletion. Plasma membranes (100% = 20  $\mu$ g protein) were analyzed as described above. Percentages on the left are the fraction of the  $-Cu$  sample that was applied for quantitation.

**(C)** Further localization of CTR2. The  $-Cu$  "other membranes" fraction shown in **(A)** and chloroplast membranes isolated from *Chlamydomonas* strain CC425 grown in  $-Cu$  TAP medium were analyzed as described above. Protein loadings were normalized by immunoblotting for cytochrome *f*.



**Figure 9.** Copper-Responsive Arylsulfatase Expression from *CTR1-ARS1a*, *CTR2-ARS1a*, and *CTR3-ARS1a* Reporter Gene Constructs.

**(A)** Schematic of reporter gene constructs. White segments represent the *ARS1a* reporter gene, black the minimal *TUB2* promoter, red the 5' flanking DNAs, and gray the *attB1* and *attB2* sites of the Gateway-

into the Arg auxotroph *Chlamydomonas* strain CC425 and Arg prototrophs tested for arylsulfatase expression on TAP plates with or without added copper using the chromogenic substrate X-sulfate (de Hostos et al., 1988).

Constructs pJD100-5'CTR1, pJD100GW-5'CTR2, and pJD100GW-5'CTR3 all gave rise to cotransformants that exhibited copper-responsive arylsulfatase expression. A single expressing colony from each transformation is shown in Figure 9B. By contrast, no colony arising from the pJD100 control transformation showed significant arylsulfatase activity.

We screened cotransformants by PCR to check cotransformation frequencies and for the presence of full-length reporter construct DNAs. Approximately 50% of cotransformants carried full-length reporter DNA, and ~40% of these were arylsulfatase expressers (see Supplemental Table 2 online). All cotransformants exhibiting copper-responsive arylsulfatase expression carried full-length reporter construct DNA.

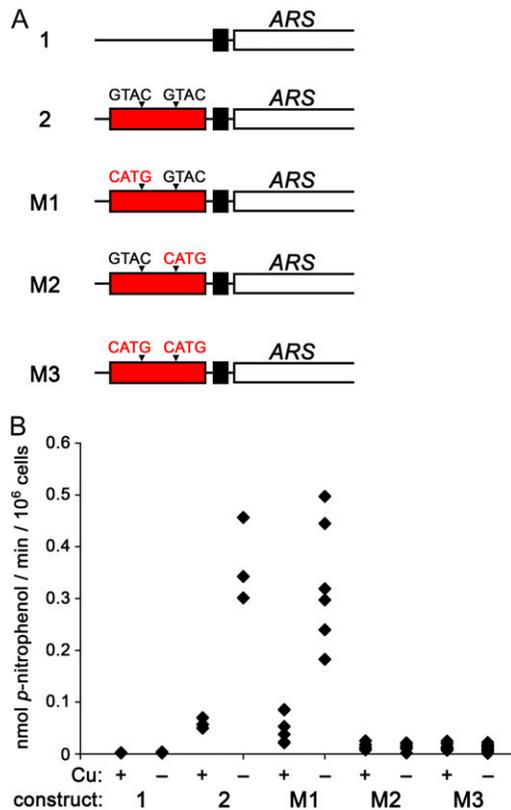
Quantitation of arylsulfatase expression by enzyme assay showed clearly that the 5' flanking sequences from *CTR1* and *CTR3* confer copper-responsive expression on the reporter gene (Figures 9C and 9D). Furthermore, the average increases in arylsulfatase expression in copper-deficient over copper-replete cells were ~7- and 15-fold, respectively, which shows that the magnitude of regulation observed in these experiments recapitulates that observed for the endogenous genes in the real-time PCR experiments described above. Unfortunately, all transformants carrying the pJD100GW-5'CTR2 construct showed very low arylsulfatase activity, precluding quantitation of reporter gene expression.

The *CYC6*, *CPX1*, and *CRD1* genes contain CuREs, with the core sequence GTAC, that serve as binding sites for CRR1 (Quinn and Merchant, 1995; Quinn et al., 1999, 2000; Allen et al., 2008). Analysis of the DNA region upstream of the *CTR1* 5' end revealed the presence of two GTAC motifs, a proximal one at -58/-82 and a distal one at -306/-330 relative to the two transcription starts determined by 5'-RACE. To confirm that at least one of these GTACs might function as CuREs, we tested constructs in which the GTAC core of each potential CuRE was mutated, either singly (constructs M1 and M2 in Figure 10) or together (construct M3). The GTAC sequence was altered to CATG, a change that abolishes copper-responsive gene expression (Quinn et al., 2000).

adapted pJD100GW. K, restriction sites for *KpnI*. The numbers beneath each 5' flanking DNA segment indicate positions relative to the transcription start sites determined by 5'-RACE.

**(B)** Arylsulfatase activity on plates. Single representative transformants growing on agar-solidified TAP medium with (+Cu) or without (-Cu) added copper (2  $\mu$ M) were stained for arylsulfatase activity by spraying with a solution of the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulfate.

**(C)** and **(D)** Quantitation of arylsulfatase expression by enzyme assay. Transformants carrying the *CTR1* or *CTR3* 5' flanking DNA fused to the *ARS1a* reporter gene were grown to mid to late log phase ( $5 \times 10^6$  to  $2 \times 10^7$  cells/mL) in TAP medium with (+Cu) or without (-Cu) added copper (2  $\mu$ M) and culture supernatants assayed for arylsulfatase activity using *p*-nitrophenol sulfate. Activities are expressed as nanomoles of *p*-nitrophenol liberated per minute per  $10^6$  cells.



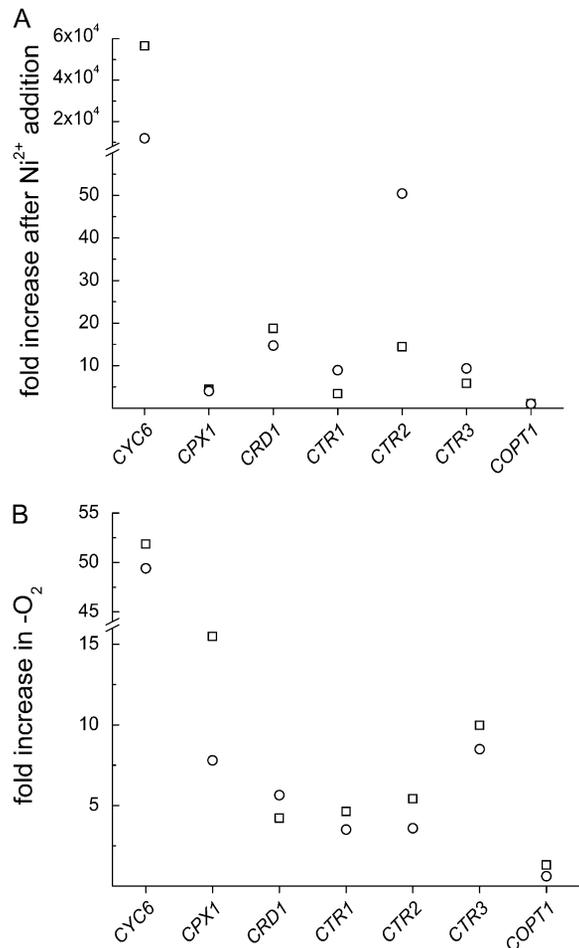
**Figure 10.** Copper-Responsive Arylsulfatase Expression from a *CTR1-ARS1a* Reporter Gene Construct Requires a CuRE.

**(A)** Schematic of reporter gene constructs. White segments represent the *ARS1a* reporter gene, black the minimal *TUB2* promoter, and red the 5' flanking *CTR1* DNA, showing the wild-type and mutated GTAC sequences.

**(B)** Quantitation of arylsulfatase expression by enzyme assay. Transformants carrying wild-type and mutated *CTR1* 5' flanking DNA fused to the *ARS1a* reporter gene were grown to mid to late log phase ( $5 \times 10^6$  to  $2 \times 10^7$  cells/mL) in TAP medium with (+Cu) or without (-Cu) added copper (2  $\mu$ M) and culture supernatants assayed for arylsulfatase activity using *p*-nitrophenol sulfate. Activities are expressed as nanomoles of *p*-nitrophenol liberated per minute per  $10^6$  cells.

In line with the results reported above, ~23% of the colonies arising from cotransformation of the construct pJD100-5'*CTR1* exhibited copper-responsive arylsulfatase expression, while no colony arising from the pJD100 control transformation showed significant arylsulfatase activity. Approximately 20% of the colonies arising from cotransformation of the construct pJD100-5'*CTR1*-M1, in which the distal GTAC had been mutated (Figure 10, construct M1), also exhibited copper-responsive arylsulfatase expression. By contrast, no colonies resulting from cotransformation of *Chlamydomonas* with either construct pJD100-5'*CTR1*-M2, in which the proximal GTAC was mutated (Figure 10, construct M2), or construct pJD100-5'*CTR1*-M3, in which both GTACs were mutated (Figure 10, construct M3), showed significant arylsulfatase activity, even though the cotransformation frequencies for all constructs were similar (see

Supplemental Table 3 online). Quantitation of arylsulfatase expression by enzyme assay confirmed the results described above, that is, that mutation of the distal GTAC (construct M1) has no effect on copper-responsive expression of the arylsulfatase reporter, but mutation of the proximal GTAC (construct M2) abolishes the copper response, as does mutation of both GTACs (construct M3) (Figure 10B). On the basis of these results, we



**Figure 11.** Effects of Hypoxia and Nickel Supplementation on *CTR1*, *CTR2*, *CTR3*, and *COPT1* mRNA Abundance.

**(A)** Regulation by nickel. Strains CC125 (boxes) and 2137 (circles), both wild-type laboratory strains, were grown in standard TAP medium. Nickel chloride was added to a final concentration of 50  $\mu$ M when the culture reached a density of  $4 \times 10^6$  per mL. RNA was isolated 5 h later and analyzed by real-time PCR using *CBLP* for normalization. Each point shows the relative mRNA abundance in nickel treated versus untreated cells.

**(B)** Regulation by oxygen. Strains CC125 (boxes) and 2137 (circles) were grown in standard TAP medium. At  $4 \times 10^6$  cells per mL, cultures were divided into two. One culture was bubbled with a reduced concentration of O<sub>2</sub> (2% air + 96% N<sub>2</sub> + 2% CO<sub>2</sub>) for 24 h, and the other was maintained in 98% air + 2% CO<sub>2</sub>. RNA was analyzed by real-time PCR using *CBLP* for normalization. Each point shows the relative mRNA abundance in reduced versus normal oxygen.

conclude that the proximal GTAC sequence represents the core of a CuRE. A similar result has been reported for *CYC6*, the 5' upstream sequence of which contains two GTAC sequences, one at -51 and one at -106 relative to the transcription start site. Using a similar approach to that described above, it was shown that mutagenesis of the GTAC sequence at -51 eliminated copper-responsive expression of the arylsulfatase reporter gene, while mutagenesis of the one at -106 did not (Quinn et al., 2000).

### **CTR1, CTR2, and CTR3 Are Induced by Hypoxia and by Nickel Ions**

All genes known thus far to be targets of the CRR1- and CuRE-dependent nutritional copper signaling pathway (i.e., *CYC6*, *CPX1*, and *CRD1*) are also induced by hypoxia and addition of nickel salts to the growth medium (Moseley et al., 2000; Quinn et al., 2000, 2002, 2003). When we tested the abundance of mRNAs for *CTR1*, *CTR2*, and *CTR3* in *Chlamydomonas* grown under conditions of hypoxia and 5 h after Ni<sup>2+</sup> addition to normoxic cultures, we found that they were coordinately regulated with *CYC6*, *CPX1*, and *CRD1*, reinforcing the point that *CTR1*, *CTR2*, and *CTR3* are targets of CRR1. By contrast, the level of the *COPT1* mRNA was unchanged in either condition (Figure 11).

## **DISCUSSION**

### **Copper Assimilation in *Chlamydomonas***

The acquisition of mineral nutrients is essential for life, and microorganisms generally have a suite of assimilatory pathways that enable selective uptake of a potentially limiting resource in competition with other species. The genome of *Chlamydomonas* revealed a repertoire of transporters related to those in plants, animals, and bacteria (Merchant et al., 2007). When we surveyed and classified putative metal transporters, we noted two families of candidate copper transporters: the P-type ATPases that function to pump copper against a concentration gradient (Lutsenko and Petris, 2003), often into compartments where copper proteins are synthesized, and members of the CTR family (Puig and Thiele, 2002; De Feo et al., 2007). Our focused study revealed four candidate CTR molecules (Figure 4) in contrast with two molecules noted in a prior whole-genome survey by simple automated BLAST search (Hanikenne et al., 2005). This is most likely attributed to the quality of the previous models. Each of the four models in this work is fully supported by cDNA analysis (see Results and Supplemental Methods online). The presence in the *Volvox* genome of orthologs of each CTR gene indicates that expansion of the gene family and diversification of function occurred prior to the divergence of species in the Volvocales.

One protein, COPT1, is most closely related to similar proteins found in the *Arabidopsis* genome (Sancenón et al., 2003), while three others, CTR1, CTR2, and CTR3, form a distinct subset of the CTR family. In this work, we establish CTR1 and CTR2 as plasma membrane-localized high-affinity copper transporters. Their localization (Figure 8), pattern of expression, and magnitude of regulation in copper-deficient *Chlamydomonas* cells (Figures 2 and 3) suggest that they are responsible for the

inducible copper uptake activity measured for *Chlamydomonas* (Hill et al., 1996). The increase in RNA and protein abundance noted in this work corresponds well with the 20-fold increase in the  $V_{max}$  for copper uptake.

Since the CTR molecules use Cu(I) as a substrate, it is likely that the assimilatory pathway must be associated with a cupric reductase as well. This is well-established for *S. cerevisiae* (Hassett and Kosman, 1995; Martins et al., 1998) where a subset of the FRE reductases function in copper assimilation. In *Arabidopsis*, at least one FRO gene is induced by copper deficiency, suggesting that it might function in copper assimilation or distribution via one of the *Arabidopsis* COPT proteins (Mukherjee et al., 2006). Four candidate reductases have been annotated in the *Chlamydomonas* genome (Allen et al., 2007b), but it is not yet known whether one or more of these participates in copper uptake. Activity assays indicate only twofold increase in cell surface cupric reductase activity in copper-deficient *Chlamydomonas* cells (Hill et al., 1996). The requirement for a reductase may thus not be as strong in some of the habitats occupied by *Chlamydomonas* species.

Copper assimilation in *Chlamydomonas* is highly selective. Even in the presence of four to five orders of magnitude higher concentrations of iron, zinc, and manganese in the medium, copper-deficient *Chlamydomonas* cells do not overaccumulate other transition metals (see Supplemental Figure 2 online). This suggests that the CTRs, with their specificity for Cu(I) rather than Cu(II), are the major and perhaps only route for copper assimilation.

### **Discovery of a Novel Metal Binding Cys-Met Motif**

Our analyses of the three CTRs of *Chlamydomonas* revealed a novel motif that we have named the Cys-Met motif to distinguish it from the Mets motifs that occur in previously characterized members of the CTR family, such as CTR1 of *S. cerevisiae*. The N-terminal extramembrane domains of the *Chlamydomonas* CTR1 and CTR2 each contain six of these distinctive Cys and Met-containing motifs, the most common form of which appears to be CxxMxxMxxC-x<sub>5/6</sub>-C. The CTR1 N-terminal domain, expressed in *Escherichia coli* as a thioredoxin fusion protein, binds to immobilized Cu(II), Co(II), and Zn(II) ions, indicating the metal binding potential of the domain (see Supplemental Figure 3 online). Cys-Met motifs are also present in the N-terminal extramembrane domains of CTR-type copper transporters from a number of other organisms. Genome data for *Volvox carteri* indicate that this organism possesses two CTR family transporters (PIDs 59175 and 121329) that are likely orthologs of *Chlamydomonas* CTR1 and CTR2, although their N-terminal domains are slightly shorter and contain only four and five Cys-Met motifs, respectively. Similarly, public draft genome data for the green alga *Chlorella* NC64A (www.jgi.doe.gov) indicates the presence of a CTR-type transporter (PID 137060) homologous to *Chlamydomonas* CTR1, although again having a slightly shorter N-terminal domain containing four, rather than six, Cys-Met motifs. The P80 protein of the social amoeba *D. discoideum* also contains Cys-Met motifs. P80 has been identified as a copper transporter solely on the basis of homology (in the membrane-integral region) to hCTR1 (Ravanel et al., 2001). P80 has a large

N-terminal domain containing eight motifs similar or identical to the Cys-Met motifs identified in CTR1 and CTR2 of *Chlamydomonas*. A gene encoding a P80 homolog is also present in the closely related *D. purpureum* (PID 17426; www.jgi.doe.gov). Cys-Met motifs are also present in the N-terminal domains of two CTR family copper transporters (PIDs 84890 and 36984) identified in public draft genome data (www.jgi.doe.gov) for *Batrachochytrium dendrobatidis*, a pathogenic fungus implicated in global amphibian declines. Additionally, EST data indicate the presence of CTR family copper transporters having N-terminal domains containing Cys-Met motifs in the free-living, opportunistically pathogenic amoeba *Acanthamoeba castellanii* and the entomopathogenic fungus *Zoophthora radicans*. EST data also indicate the presence of one or more proteins containing Cys-Met motifs in a second free-living amoeba, *Hartmannella vermiformis*, and a third social amoeba, *Polysphondylium pallidum*, although the sequence coverage is currently insufficient to distinguish whether these proteins are copper transporters.

Davis and O'Halloran (2008) have noted that in Cu(I) trafficking proteins, including copper transporters, copper binding motifs in domains exposed to the reducing environment of the cytoplasm tend to be Cys rich, while Met-rich motifs predominate in extracytoplasmic domains; they suggest that this is because while Cys binds copper more strongly than Met, Met is more resistant to oxidation. Species of the genus *Chlamydomonas* are found in such naturally hypoxic or even anoxic habitats as damp soil, bogs, stagnant water, and sewage lagoons (Harris, 2009). It has also been observed that in the absence of vigorous aeration, the respiratory activity of *Chlamydomonas* is sufficient to deplete cultures of oxygen within a day (Quinn et al., 2002). It is known that in microaerobic or anaerobic environments copper availability may be reduced by its precipitation as insoluble Cu(I) salts (e.g., sulfides; Osterberg, 1974), so that assimilatory transporters capable of high-affinity copper scavenging would likely confer a competitive advantage. Indeed, the measured affinity of the *Chlamydomonas* uptake system is 10- to 20-fold higher than that measured for *S. cerevisiae* (Lin and Kosman, 1990; Dancis et al., 1994; Hill et al., 1996). At the same time, low oxygen tensions would reduce oxidation of the Cys-Met motif Cys residues. Thus, the Cys-Met motif may represent a specific adaptation for copper assimilation in microaerobic environments. Other green algae and amoebae could be presumed to occupy similar natural habitats to *Chlamydomonas*, while *B. dendrobatidis* and *Z. radicans* might encounter microaerobic conditions within the bodies of their hosts. Or perhaps the Cys-Met motif is an adaptation for life in habitats where oxygen tension is variable, with the Cys residues offering the highest available affinity for Cu (I) under hypoxic or anoxic conditions and the Met residues offering lower, but still valuable, affinity under more oxidizing conditions. The Cys-Met motif is also interesting because while Cu(I) tends to form trigonal or tetrahedral complexes with sulfur ligands, the motif (CxxMxxMxxC-x<sub>5/6</sub>-C) contains five potential liganding residues. It is tempting to speculate that one of the Cys residues present may participate in rereduction of the other two in the event that they become oxidized and form a disulfide bond. We note also the presence of additional conserved Cys residues within the N-terminal domains of CTR1, CTR2, and related proteins. Clearly it will be important to characterize the Cys-

Met motif and to define its importance in copper uptake; a mutational analysis would reveal the importance of the Cys residues. COPT1, with its more typical Met and His-rich motifs, might provide a capacity for copper uptake when *Chlamydomonas* encounters more oxidic environmental conditions; alternatively, the lack of Cu regulation of the COPT1 mRNA may indicate that it functions in an intracellular compartment and has a distributive rather than assimilatory role. Its existence in *Chlamydomonas* may reflect the presence of both His/Met-based motifs and the Cys-Met motif in the earliest eukaryotic ancestor.

To understand the relative contribution of each CTR family gene to copper nutrition in *Chlamydomonas*, we determined the absolute number of CTR and COPT1 transcripts present in the cell under conditions of Cu repletion and deficiency. In cells grown under conditions of Cu sufficiency, the ratio of COPT1, CTR1, CTR2, and CTR3 transcripts was estimated at ~1:1:1:10 (Table 1). Under conditions of Cu deficiency, this ratio was changed to ~1:5:26:204. The values obtained are completely consistent with the levels of induction observed by real-time PCR and RNA gel blot hybridization (Figures 2 and 4) and suggest that while COPT1 may not be a quantitatively important player, CTR3 may be relevant in copper homeostasis.

### CTR3 Is Conserved in Other Organisms

Another intriguing finding from this work is the identification of the *Chlamydomonas* CTR3 gene. Like CTR1 and CTR2, the CTR3 protein has six Cys-Met motifs, but in other respects it differs significantly. Hydrophobicity analyses suggest that it does not contain any membrane-spanning  $\alpha$ -helices and is likely a soluble protein. We conclude that CTR3 is probably not a copper transporter in the conventional sense, but the observed upregulation of CTR3 under conditions of copper deficiency suggests

**Table 1.** Relative Copy Numbers of CTR1, CTR2, CTR3, and COPT1 Transcripts in *Chlamydomonas*

mRNA	+Cu	-Cu
COPT1	29	39
CTR1	43	200
CTR2	34	891
CTR3	324	6607

Figures represent the approximate number of mRNAs for each gene that are present in 1 ng of total RNA. *Chlamydomonas* strain 2137 was grown to  $3 \times 10^6$  cells/mL in TAP media with (+Cu; three cultures) and without (-Cu; three cultures) added copper and total RNA prepared from each culture used as template for cDNA synthesis (these RNA preparations were those analyzed in the experiment shown in Figure 2). The three +Cu and three -Cu cDNA preparations were combined to produce +Cu and -Cu cDNA mixtures, which were analyzed by real-time PCR. All C<sub>T</sub> values were the average of technical triplicates and were normalized to CBLP. Dilutions of cloned CTR1, CTR2, CTR3, and COPT1 cDNAs were amplified in triplicate under identical conditions to obtain standard curves relating C<sub>T</sub> values to the number of template copies present. Primers used are listed in Supplemental Table 1 online.

that CTR3 has some role in *Chlamydomonas* copper homeostasis. The FEA1 protein, an abundant soluble protein that is localized to the periplasm, has recently been shown to contribute to iron uptake by *Chlamydomonas*. Strains impaired in cell wall biosynthesis cannot retain FEA1 and exhibit poorer growth at low iron concentrations than wild-type strains, suggesting that FEA1 acts to concentrate iron for uptake via FOX1 and FTR1. (Allen et al., 2007b). CTR3 has a predicted N-terminal signal sequence, suggesting that it may also be translocated across the plasma membrane to the periplasmic space, where it could, by analogy with FEA1, act to concentrate copper ions for uptake by CTR1 and CTR2; the higher mRNA copy number for CTR3 suggests that the protein may be more abundant than CTR1 and CTR2 assuming there is no regulation at the protein level. A CTR3-like protein is found, along with CTR1 and CTR2 homologs, in *V. carteri*, while in *D. discoideum*, Ravel et al. (2001) noted, in addition to the CTR-type copper transporter P80 (with eight Cys-Met motifs), a P80-like protein (DDB0190088) that is predicted to be a soluble protein with four Cys-Met motifs and a cleavable N-terminal signal peptide. However, it must be stressed that the localization of *Chlamydomonas* CTR3 remains to be determined as we have so far been unsuccessful in raising an antibody that recognizes the protein in *Chlamydomonas* extracts.

### Regulation of *COPT1* and *CTRs*

In this work, we showed that each of the *CTR* genes, encoding proteins distinct from the *Arabidopsis*-like *COPT1*, is responsive to copper nutrition and dependent on CRR1 (Figure 4). Since CRR1 is a transcriptional activator that works on CuREs, we used reporter gene assays to test whether the *CTR* genes might be direct targets of CRR1. For *CTR1* and *CTR3*, the constructs recapitulated the magnitude of regulation of the respective endogenous genes. In the case of *CTR2*, the constructs, although responsive to copper nutrition, were only weakly so; therefore, we cannot rule out the possibility of additional layers of regulation on *CTR2*. In particular, since the *Arabidopsis* homolog of CRR1, SPL7, regulates target genes, including *COPT1* and *COPT2*, via microRNAs (miRNAs) (Yamasaki et al., 2007, 2009) and since *Chlamydomonas* also uses miRNAs for controlling gene expression (Molnár et al., 2007), regulation by miRNAs is a formal possibility. We expect that copper assimilation in *Chlamydomonas* is subject to multiple layers of regulation because it is unlikely that the fold difference in *CTR1* and *CTR2* RNA and protein abundance would be adequate for conferring the tight homeostasis noted in Figure 1. Therefore, posttranslational mechanisms that control the activity of the transporter or its location on the plasma membrane are a possibility. Another possibility is that there is a copper effluxer that is required in a situation of excess copper to maintain homeostasis. There are precedents for both types of regulation in other organisms (Odermatt et al., 1992; Ooi et al., 1996; Gitan et al., 1998; Kim et al., 2004)

We analyzed the 5' upstream region of *CTR1* by mutation of candidate CuREs and found that one of two GTACs (which forms the core of the CuRE and the SBP domain binding site) is indeed required for copper-responsive expression. This is true for the previously characterized *CYC6*, *CPX1*, and *CRD1*, where there

are several GTACs in the 5' upstream region, but the one that is part of the CuRE is distinguished only by mutation. Thus far, it has not been possible to delineate the sequence of the CuRE beyond the GTAC core.

We noted previously that CRR1 mediated not only copper-responsive regulation of its target genes, but it is also required for regulation of the same targets by hypoxia (Moseley et al., 2000; Quinn et al., 2000, 2002). The hypoxic response is physiologically relevant because *crr1* strains grow poorly in low O<sub>2</sub> (Eriksson et al., 2004). We found also that nickel ions serve as a pharmacological tool for stimulating the CRR1-dependent pathway (Quinn et al., 2003). When we tested the expression of the *CTR* genes in response to hypoxia or nickel treatment, we found that their expression followed the pattern of *CYC6*, *CPX1*, and *CRD1* regulation. This is consistent with the contention that the *CTRs* are part of the classical copper deficiency network in *Chlamydomonas*.

## METHODS

### Strains and Culture Conditions

The strains used in this work were obtained from the *Chlamydomonas reinhardtii* culture collection (Duke University). Growth of *Chlamydomonas* under conditions of copper deficiency has been described previously (Quinn and Merchant, 1998). Strain CC425 (*cw15 arg2*) was cultured in copper-free Tris-acetate-phosphate (TAP) medium supplemented with copper (2 μM; as CuSO<sub>4</sub>) and Arg (200 μg/mL). Transformants of strain CC425 were grown in TAP liquid medium shaken at 200 rpm or on TAP agar plates at 22 to 25°C and ~100 μmol/m<sup>2</sup>/s light intensity, with or without copper supplementation. Strains CC124, CC125, and 2137 were cultured under the same conditions without added Arg. Hypoxic conditions were achieved as described by Del Campo et al. (2004). For analysis of nickel-induced gene expression, 50 μM NiCl<sub>2</sub> was added to copper-supplemented cultures 5 h prior to harvest. For plasma membrane preparations, strain CC125 was grown in stirred 20-liter bottles containing 15 liters of +Cu or -Cu TAP at 24°C with ~200 μmol/m<sup>2</sup>/s surface light intensity. Inocula were 1-liter cultures grown in +Cu or -Cu TAP to cell densities of ~1 × 10<sup>7</sup> cells/mL.

### Metal Measurements

*Chlamydomonas* cells grown to 3 × 10<sup>6</sup> cells/mL at the indicated copper concentrations were collected by centrifugation and treated as described by Allen et al. (2007a), with the exception that nitric acid was used at a final concentration of 2.4%. Spent medium was transferred to acid washed tubes and any remaining cells removed by further centrifugation before nitric acid was added (to 2.4%). Total metal content was measured at the IRES (Institute for Integrated Research in Materials, Environments, and Society at California State University Long Beach) by the standard addition method.

### Nucleic Acid Analysis

Total *Chlamydomonas* RNA was prepared as described previously (Quinn and Merchant, 1998). *Chlamydomonas* DNA for colony PCR was isolated as described by Berthold et al. (1993). DNA sequencing was performed by Laragen and Agencourt Biosciences. Plasmids intended for transformation into *Chlamydomonas* were isolated using the Marligen High-Purity Plasmid Purification System.

### Real-Time PCR

cDNA preparation and real-time RT-PCR were performed as described by Allen et al. (2007a). Primers are listed in Supplemental Table 1 online. Data are presented as the fold change in mRNA abundance, normalized to the endogenous reference gene *CBLP*. The *CBLP* mRNA abundance does not change under the conditions tested. For the analysis of copper-dependent gene expression, a mixture of all six RNAs from three independent experiments was used as reference. Numbers of CTR transcripts in *Chlamydomonas* were estimated by relating the  $C_T$  values obtained for each RNA sample analyzed by real-time PCR to standard curves generated by amplification of serially diluted ( $10^9$  to  $10^2$  molecules per reaction) cloned cDNAs under identical reaction conditions.  $C_T$  values for amplifications of cDNAs fell within the ranges of the standard curves.

### RNA Gel Blot Hybridization

RNA was analyzed by hybridization as described previously (Quinn and Merchant, 1998). Total RNA (5  $\mu$ g) was loaded per lane. The probe used to detect *CTR1* transcripts was the *EcoRI-XhoI* insert from the cDNA clone 1031043E11 (GenBank accession number DQ021453). For *CTR2* transcripts, the probe was the 1071-bp 3'-RACE product cloned in pGEM-T Easy and excised with *EcoRI* (see Supplemental Methods online). For *CTR3* transcripts, the probe was a 559-bp *NsiI-XhoI* fragment from the 3' untranslated region excised from the cDNA clone 1031027G08 (GenBank accession numbers BI718490 and BI995501). For *COPT1* transcripts, the probe was a 974-bp PCR product comprising most of the 3' untranslated region (see Supplemental Methods online). For *CBLP* transcripts, the probe was a 915-bp *EcoRI* fragment from the cDNA cloned in pcf8-13 (Schloss, 1990). Specific activities of probes ranged from 3.3 to  $9.6 \times 10^8$  dpm  $\mu$ g<sup>-1</sup>. Blots were exposed to film (XRP-1; Eastman-Kodak) at  $-80^\circ\text{C}$  using two intensifying screens.

### Transcription-Translation

Transcription-translation was performed using the TNT T7 Quick system (Promega) with Redivue Pro-Mix L-<sup>[35S]</sup> label (GE Healthcare) according to the manufacturer's instructions except that reactions were scaled down to 12.5  $\mu$ L final volume. Reactions were primed with 0.2 to 0.3  $\mu$ g circular plasmid DNA. Reactions were stopped by addition of 50  $\mu$ L of 1.25 $\times$  Laemmli sample buffer and heating at  $65^\circ\text{C}$  for 15 min. Portions (0.3 to 5  $\mu$ L, depending on amount of label incorporated) of stopped reaction mixtures were separated by denaturing PAGE (7.5% monomer for CTR1, CTR2, and CTR3; 12% for COPT1). Gels were fixed by boiling for 2 to 3 min in 5% trichloroacetic acid and then neutralized for 5 to 10 min in 1 M Tris base and prepared for fluorography as described by Chamberlain (1979). Dried gels were exposed to film (XRP-1) for 20 to 40 h at  $-80^\circ\text{C}$ .

### Yeast Complementation Experiments

*C. reinhardtii* *CTR1* was amplified from cDNA clone 1031043E11 using primers CCTR1-F plus CCTR1-R. *S. cerevisiae* *CTR1* was cloned from genomic DNA using primers YCTR1-F and YCTR1-R (primers listed in Supplemental Table 4 online). Both products were isolated, digested with *HindIII* plus *XbaI*, and cloned in pYES2 (Invitrogen). The assembled *CTR2*, *CTR3*, and *COPT1* cDNAs were excised from their parent plasmids (see Supplemental Methods online) using *BamHI* plus *EcoRI*, *EcoRI* plus *XhoI*, and *EcoRI* plus *HindIII*, respectively, and cloned in pYES2. Plasmid DNA was transformed into the *S. cerevisiae*  $\Delta$ *ctr1* mutant strain (*MAT $\alpha$  trp1-63 leu2-3,112 gcn4-101 his3-609 ura3-52  $\Delta$ ctr1::LEU2*; Zhou and Gitschier, 1997) using the lithium acetate method (Gietz and Schiestl, 2007). Transformants were selected on synthetic dextrose plates lacking uracil and then tested for growth on YPE (1% yeast extract, 2% peptone, and

3% ethanol) containing 0.02% (w/v) galactose. This concentration of galactose is sufficient to stimulate transcription from the *GAL1* promoter (Sancenón et al., 2003) but did not support growth of the  $\Delta$ *ctr1* mutant or complemented strains. A number (6 to 12) of independent transformants were tested for each experiment.

### Plasma Membrane and Chloroplast Membrane Isolation

Membrane fractions were prepared from strain CC425 and separated into plasma membranes and a fraction containing all other membranes by partitioning in a two-phase aqueous polymer system as described by Herbig et al. (2002). Cells were grown to late log phase ( $1 \times 10^7$  cells/mL) in stirred 20-liter bottles as described above. Batches (220 mL) of resuspended cells were immersed in an ice-water bath and broken using a Fisher Scientific model 550 Sonic Dismembrator with microtip probe, amplitude setting 5, and six cycles of 3 min 20 s of sonication followed by 3 min 20 s of cooling. Chloroplasts were prepared from strain CC425 and lysed as described by Allen et al. (2008) and membranes recovered by centrifugation (110,000g, 1 h,  $4^\circ\text{C}$ ).

### Antibody Production and Immunoblotting

Antibodies to CTR1 and CTR2 were produced by AgriSera by immunization of rabbits with mixtures of synthetic peptides coupled to Keyhole Limpet hemocyanin. Peptides were NH<sub>2</sub>-CLKTLKGYLSLRWAHERA-COOH and NH<sub>2</sub>-CENYGAILHHERRVANAK-COOH (peptide 2) for CTR1, and NH<sub>2</sub>-CDHLAQSGYAVQRH DN-COOH and NH<sub>2</sub>-CRDAYAR-HIGRPP-COOH for CTR2. Peptides were designed by Environmental Proteomics. The antibodies to CTR1 were affinity purified by AgriSera using immobilized peptide 2. For immunoblot analysis, samples were solubilized by heating at  $65^\circ\text{C}$  for 15 min. Proteins were separated by denaturing PAGE (7.5 to 15% monomer) and transferred to nitrocellulose (0.45  $\mu$ M; Schleicher and Schuell) at a constant current of 1.5 mA/cm<sup>2</sup> for 60 to 90 min using a transfer buffer of 25 mM Tris, 192 mM glycine, 0.01% SDS, and 10% methanol. Membranes were blocked with 1% dried milk in PBS containing 0.3% (w/v) Tween 20 and 0.01% (w/v) sodium azide; this solution was used as the diluent for both primary and secondary antibodies and for washing. Primary antibodies were used at the following dilutions: anti-CTR1 (1:100), anti-CTR2 (1:100), antiplasma membrane H<sup>+</sup> ATPase (1:1000; Agrisera), anti-FOX1 ferroxidase (1:100; La Fontaine et al., 2002), anticytochrome *f* (1:1000), anti-CRD1 Mg-protoporphyrin monomethyl ester cyclase (1:3000; Moseley et al., 2002), anti-TOM20.3 (1:1000), and anti-COX2b (1:5000). The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) used at 1:3000. Bound antibody was detected using the alkaline phosphatase color reaction as described by Sambrook and Russell (2001).

### Reporter Constructs and Their Analysis

All primers are listed in Supplemental Table 5 online. DNA regions upstream of the *CTR1*, *CTR2*, and *CTR3* 5' ends were identified from *Chlamydomonas* genome data, and primers CTR1-pro-F2K plus CTR1-pro-R2K, CTR2-pro-GF1 plus CTR2-pro-GR1, and CTR3-pro-GF1 plus CTR3-pro-GR2 (positions relative to transcription start sites shown in Figure 9) designed to amplify these regions from *Chlamydomonas* genomic DNA (strain CC425). The *CTR1* promoter region amplification product was cloned in *EcoRV* cut pLITMUS28 to generate p5' CTR1 and verified by sequencing. The cloned segment was excised with *KpnI* and cloned into the *KpnI* site of pJD100 (ptubB2 $\Delta$ 3,2,1/ars; Davies and Grossman, 1994) to generate the construct pJD100-5' CTR1. The *CTR2* and *CTR3* promoter region amplification products were cloned via recombination in the Gateway entry vector pDONR221 (Invitrogen) and

verified by sequencing. The cloned segments were then transferred via recombination into the reporter vector pJD100GW (constructed by introduction of the Gateway *Cm<sup>r</sup>-ccdB* cassette into the Klenow-blunted *KpnI* site of pJD100) to generate the constructs pJD100GW-5'CTR2 and pJD100GW-5'CTR3.

The core GTAC regions of two potential CuREs in the *CTR1* promoter region were altered to CATG by mutagenic PCR as described by Allen et al. (2008). Plasmid p5'CTR1 (see above) was the template for amplification. The resulting plasmids were termed p5'CTR1-M1 (distal GTAC mutated) and p5'CTR1-M2 (proximal GTAC mutated), respectively. p5'CTR1-M1 was subjected to a second round of mutagenesis to generate p5'CTR1-M3 (both GTACs mutated). The mutated *CTR1* promoter region DNAs were excised with *KpnI* and cloned in pJD100 as described above to generate pJD100-5'CTR1-M1, pJD100-5'CTR1-M2, and pJD100-5'CTR1-M3.

Plasmid pJD100 and derivatives were linearized with *PsiI* or (pJD100GW based constructs) *BsaI* and cotransformed with pArg7.8 into strain CC425 as described previously (Quinn and Merchant, 1995). Arg prototrophs were picked and streaked to agar-solidified TAP with and without added copper (2  $\mu$ M). After two more rounds of restreaking to ensure copper deficiency of the cells growing on -Cu TAP, transformants were screened for arylsulfatase expression using 5-bromo-4-chloro-3-indolyl sulfate (X-sulfate; Sigma-Aldrich) as described by de Hostos et al. (1988).

Cotransformation frequency and intactness of integrated reporter constructs were assessed by colony PCR using primers pJD100-tub-f plus ARS1 (for the TUB2-ARS1a 5' region) and ARS-3'-F plus ARS-3'-R (for the ARS1a 3' end). Additionally, intactness of integrated reporter constructs containing the *CTR1* promoter region was tested using ARS1 plus the gene-specific primer CTR1-pro-F2.

Selected transformants were grown in liquid TAP medium with and without supplemental copper (2  $\mu$ M) and arylsulfatase activity in the culture medium measured after removal of the cells by centrifugation (de Hostos et al., 1988). In the case of pJD100-5'CTR1, pJD100-5'CTR1-M1, and pJD100GW-5'CTR3, the stronger arylsulfatase expressers were analyzed. In the case of pJD100, pJD100-5'CTR1-M2, and pJD100-5'CTR1-M3 (i.e., nonexpressers), transformants carrying full-length reporter constructs were analyzed.

Because nonhomologous recombination in *C. reinhardtii* is random, individual transformants carry integrated DNA at different locations in the genome and positional effects influence reporter construct expression. For this reason, a number of transformants was analyzed for each construct (Figure 9, pJD100,  $n = 8$  or 4 in C and D, respectively; pJD100-5'CTR1,  $n = 14$ ; pJD100GW-5'CTR3,  $n = 12$ ; Figure 10, pJD100,  $n = 3$ ; pJD100-5'CTR1,  $n = 3$ ; mutated constructs, six of each). All transformants were analyzed by immunoblotting for plastocyanin and cytochrome *c<sub>6</sub>* to test for copper repletion and depletion, respectively.

#### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ021453 (*CTR1*), DQ646486 (*CTR2*), DQ646487 (*COPT1*), and EU915720 (*CTR3*).

#### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Gene Models and Products for *CTR1*, *CTR2*, *CTR3*, and *COPT1*.

**Supplemental Figure 2.** Metal Content of *Chlamydomonas* Cells under Conditions of Copper Repletion and Deficiency.

**Supplemental Figure 3.** Immobilized Metal Affinity Chromatography of a TrxA-CTR1<sub>N</sub> Fusion Protein.

**Supplemental Table 1.** Primers Used for Real-Time PCR.

**Supplemental Table 2.** Summary of Reporter Construct Analyses.

**Supplemental Table 3.** Summary of Reporter Construct Analyses.

**Supplemental Table 4.** Primers Used for Generation of Gene Models and cDNA Construction.

**Supplemental Table 5.** Primers Used for Reporter Construct Generation and Analysis.

**Supplemental Methods.** Generation of Improved Gene Models, and Assembly of cDNAs, Expression in *E. coli*, and IMAC Chromatography of a TrxA-CTR1<sub>N</sub> Fusion Protein.

**Supplemental References.**

**Supplemental Data Set 1.** Text File Corresponding to Alignment in Figure 6.

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**Two *Chlamydomonas* CTR Copper Transporters with a Novel Cys-Met Motif Are Localized to the Plasma Membrane and Function in Copper Assimilation**

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