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Genetic Dissection of Nutritional Copper Signaling in Chlamydomonas Distinguishes Regulatory and Target Genes

Mats Eriksson,*[,1,2] Jeffrey L. Moseley,*[,†,1,3] Stephen Tottey,*[,1] Jose A. del Campo,*[,4] Jeanette Quinn,*[,5] Youngbae Kim* and Sabeeha Merchant*[,†]

**Department of Chemistry and Biochemistry, University of California, Los Angeles, California, 90095-1569 and †Molecular Biology Institute, University of California, Los Angeles, California 90095-1570*

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ABSTRACT

A genetic screen for *Chlamydomonas reinhardtii* mutants with copper-dependent growth or nonphotosynthetic phenotypes revealed three loci, *COPPER RESPONSE REGULATOR 1* (*CRR1*), *COPPER RESPONSE DEFECT 1* (*CRD1*), and *COPPER RESPONSE DEFECT 2* (*CRD2*), distinguished as regulatory or target genes on the basis of phenotype. *CRR1* was shown previously to be required for transcriptional activation of target genes like *CYC6*, *CPX1*, and *CRD1*, encoding, respectively, cytochrome c_6 (which is a heme-containing substitute for copper-containing plastocyanin), coproporphyrinogen III oxidase, and Mg-protoporphyrin IX monomethylester cyclase. We show here that *CRR1* is required also for normal accumulation of copper proteins like plastocyanin and ferroxidase in copper-replete medium and for apoplastocyanin degradation in copper-deficient medium, indicating that a single pathway controls nutritional copper homeostasis at multiple levels. *CRR1* is linked to the *SUPPRESSOR OF PCY1-AC208 13* (*SOP13*) locus, which corresponds to a gain-of-function mutation resulting in copper-independent expression of *CYC6*. *CRR1* is required also for hypoxic growth, pointing to a physiologically meaningful regulatory connection between copper deficiency and hypoxia. The growth phenotype of *crr1* strains results primarily from secondary iron deficiency owing to reduced ferroxidase abundance, suggesting a role for *CRR1* in copper distribution to a multicopper ferroxidase involved in iron assimilation. Mutations at the *CRD2* locus also result in copper-conditional iron deficiency, which is consistent with a function for *CRD2* in a pathway for copper delivery to the ferroxidase. Taken together, the observations argue for a specialized copper-deficiency adaptation for iron uptake in Chlamydomonas.

COPPER is usually utilized in organisms as a cofactor in enzymes or electron transfer proteins that catalyze redox reactions or oxygen chemistry. Therefore, it is an essential micronutrient. However, the potential for Cu ions to participate in Fenton chemistry resulting in the production of reactive oxygen species means that cells must balance the acquisition of the nutrient to saturate but not exceed intracellular copper binding sites. Studies of copper homeostasis have focused on the pathways of copper uptake from the environment, intracellular distribution via chaperones and distributive transporters, and mechanisms for sequestration and detoxification. These works have revealed a variety of assimilatory copper transporters whose function is regulated by copper availability (Dancis *et al.* 1994; Zhou and Gitschier 1997; Peña *et al.* 2000). In eukaryotes as well as in compartmentalized bacteria, there are distributive transporters that deliver copper to particular compartments, where it can be assembled into the active site of proteins like Fet3p, a ferroxidase required for iron assimilation, or plastocyanin, required for photosynthesis (Yuan *et al.* 1995; Askwith and Kaplan 1998; Tottey *et al.* 2001; Sancenón *et al.* 2003; Shikanai *et al.* 2003). Small proteins, called copper chaperones, bind intracellular copper and transfer it to appropriate compartments, in some cases via the distributive transporters (Glerum *et al.* 1996a,b; Culotta *et al.* 1997; Klomp *et al.* 1997; Lin *et al.* 1997; Valentine and Gralla 1997; Himelblau and Amasino 2000).

An immediate first line of defense to nutrient deficiency is the activation of assimilatory mechanisms, and this is well established for copper as well (Vulpe and Packman 1995; Yamaguchi-Iwai *et al.* 1997; Labbé and Thiele 1999). On the other hand, much less is known about how cellular metabolism is adjusted to deal with the situation where the amount of copper available is insufficient for the biosynthesis of a full complement of copper proteins. One well-established metabolic ad-

[1]These authors contributed equally to this work.

[2]*Present address:* Umeå Plant Science Center, Umeå University, SE-901 87 Umeå, Sweden.

[3]*Corresponding author:* Department of Plant Biology, Carnegie Institution of Washington, 260 Panama St., Stanford, CA 94305. E-mail: jeffrey.moseley@stanford.edu

[4]*Present address:* Instituto de Bioquímica Vegetal y Fotosíntesis (Universidad de Sevilla-CSIC). Centro de Investigaciones Científicas Isla de la Cartuja. Avda. Americo Vespucio s/n 41092 Sevilla, Spain.

[5]*Present address:* Stratagene, La Jolla, CA 92037.

aptation in photosynthetic microorganisms is the substitution under copper-deficient conditions of an iron-containing cytochrome for the abundant blue copper protein plastocyanin (Wood 1978; Sandmann *et al.* 1983; Merchant and Bogorad 1986). The molecular details of this adaptive switch are best understood in Chlamydomonas. In copper-replete medium, plastocyanin is normally present in Chlamydomonas chloroplasts at a stoichiometry of $\sim 8 \times 10^6$ molecules/cell, but when the cells are grown in copper-deficient medium, Chlamydomonas alters its requirement for copper (Merchant *et al.* 1991). The organism replaces this abundant copper protein with a catalytically equivalent heme-containing substitute, cytochrome c_6 (cyt c_6), and in this situation it requires much less copper for growth.

The mechanism by which the switch between plastocyanin and cyt c_6 is achieved involves copper-responsive modification of gene expression. The *PCY1* gene, encoding plastocyanin, is constitutively expressed irrespective of the copper status of the cell, but under copper-deficient conditions the apoprotein is actively degraded by an as yet unidentified protease in the thylakoid lumen (Li and Merchant 1995; Li *et al.* 1996). In contrast, the *CYC6* gene, encoding cyt c_6, is transcribed only in Cu-deficient cells. This occurs via transcriptional activation through copper-responsive elements containing a GTAC core (Quinn and Merchant 1995; Quinn *et al.* 2000). As Cu availability falls below the amount needed to saturate intracellular copper binding sites ($\sim 9 \times 10^6$/cell), a number of cellular responses besides changes in plastocyanin and cyt c_6 abundance are coordinately induced, suggesting that a common signal transduction pathway is responsible for handling nutritional copper deficiency.

For instance, a high-affinity copper-uptake system involving a transporter and a cell surface reductase is induced in copper deficiency. On the basis of the fact that the velocity of transport increases 20-fold but the K_m remains the same, Hill *et al.* (1996) concluded that Chlamydomonas must upregulate the same transporter that operates in Cu-replete cells. Proteolysis of apoplastocyanin is observed only in Cu-deficient cells, suggesting that a specific protease may be expressed (Li *et al.* 1996). Another change is the transcriptional activation of the *CPX1* gene, encoding coproporphyrinogen oxidase in the tetrapyrrole biosynthetic pathway (Hill and Merchant 1995; Quinn *et al.* 1999). The function of upregulation of *CPX1* is not known; it has been suggested that it provides for increased flux for heme biosynthesis, perhaps to provide the cofactor for cyt c_6. However, the recent discovery of copper-regulated differential accumulation of isoforms Crd1 *vs.* Cth1 of a chlorophyll biosynthetic enzyme (Moseley *et al.* 2002b; Tottey *et al.* 2003) suggests a more fundamental and broader-based connection between Cu and the tetrapyrrole pathway.

In this work we sought to discover components of copper-responsive signal transduction through isolation and genetic classification of mutants that require copper supplementation for growth. We established (i) a specialized copper-deficiency adaptation for high-affinity iron uptake, (ii) that a single pathway controls all known responses to copper deficiency, (iii) that the pathway is required also for copper homeostasis under copper-replete conditions, and (iv) that the previously identified genetic and molecular connection between copper nutrition and hypoxia is physiologically significant.

MATERIALS AND METHODS

Growth conditions: *Chlamydomonas reinhardtii* strains were grown in copper-supplemented (6 μM added $CuSO_4$) or copper-deficient Tris-acetate-phosphate (TAP) media as described by Quinn and Merchant (1998). Liquid cultures were grown in an orbital shaker (200 rpm) at 25° under continuous light at an intensity of 50–150 μmol/m^2/sec. Agar plates were grown at 22°–25° and 50–100 μmol/m^2/sec. For arginine-requiring strains the medium was supplemented with 250 μg/ml arginine. For growth at low oxygen concentrations, cell cultures were bubbled with gas mixtures as follows: 99.8% air and 0.2% CO_2 (control) or 98% N_2, 1.8% air, and 0.2% CO_2 (low oxygen conditions). The output mixture had a total flow of 2.5 liter/min/liter of culture and was filtered through a sterile 0.45-μm syringe filter. Cultures were maintained for 4 days at room temperature (22°–24°) with normal room lighting (5–15 μmol/m^2/sec) on a shaker at 100 rpm. The oxygen content of the cultures was measured with a standardized oxygen electrode (Orion Research, Beverly, MA).

Protein analysis: Soluble and pellet fractions of cells were prepared as described previously (Quinn and Merchant 1998; Quinn *et al.* 1999). Proteins were separated by discontinuous SDS-PAGE and transferred to polyvinylidene difluoride membranes for immunodetection of plastocyanin, cyt c_6, coprogen oxidase, Crd1, Cth1, Fox1, and OEE1. The amounts of samples loaded were normalized on the basis of either cell counts or chlorophyll. Antibody dilutions were 1:2000 for α-plastocyanin, 1:1000 for α-cyt c_6, 1:3000 for α-coprogen oxidase, and 1:1000 for α-Crd1, α-Fox1, and α-OEE1.

Analysis of RNA: RNA isolation and analysis by hybridization was performed essentially as described previously (Quinn *et al.* 1999; Moseley *et al.* 2000; La Fontaine *et al.* 2002). Three to 5 μg of total RNA was loaded in each lane. Hybridization was detected by exposure to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA). For quantitative real-time PCR, genomic DNA was removed from the total RNA preparation by treatment with RQ1 DNAse (Promega, Madison, WI) according to the manufacturer's instructions. Complementary DNA, primed with oligo(dT), was generated with reverse transcriptase (GIBCO BRL, Gaithersburg, MD) also according to the manufacturer's instructions, and used in the amplification reaction directly after dilution. The amplification reaction was carried out with reagents from the DyNAmo HS Sybr green qPCR kit (MJ Research, Watertown, MA). Each reaction contained 10 μl 2× master mix, 0.3 μM primers, and cDNA corresponding to 5 ng/μl input RNA in the reverse transcriptase reaction. For each gene, the primers were as follows:

CBLP: 5′-GCCACACCGAGTGGGTGTCGTGCG-3′ and 5′-CCTTGCCGCCCGAGGCGCACAGCG-3′;
ATX1: 5′-GGCATCCAGACGGTGAAAAGCG-3′ and 5′-TCACTGCGCCAGCTTTGCCTC-3′;
CTP1: 5′-GGTGTTGACCTGGCTACGCTGTG-3′ and 5′-GCCACCGGTGCCAAGGGAGGAG-3′;

CTP2: 5′-AGCAGCAGCAGCGGGGTGCAG-3′ and 5′-GTCGC TGCCGGCGATGCCGAC-3′.

The reaction conditions were: 95° for 15 min, followed by cycles of 95° for 10 sec, 65° for 10 sec, 72° for 30 sec up to a total of 35 cycles. The fluorescence was measured at each cycle at 72° and 83°. The $2^{-\Delta\Delta C_T}$ method was used to analyze the data (Livak and Schmittgen 2001). For visualizing the relative abundance of each transcript, product was removed from each set of reactions for a particular primer pair at a cycle where all samples showed product increase in the exponential phase and was separated by electrophoresis (2% agarose). The size of the product was estimated against markers (λDNA digested with *Pst*I).

Strains and genetic analysis: The wild-type strain used was CC-125. Insertional mutants were generated in the CC-425-pCU4 background (*arg2 cw15 mt+*; Quinn and Merchant 1995) and were backcrossed at least twice to wild-type or arginine auxotrophic strains (*arg2* derived from CC-425 or *arg7* derived from CC-1931). The suppressor of *pcy1-ac208* strain (*sop13*) was isolated by growing the parental *pcy1-ac208* strain on Cu-supplemented minimal medium plates in continuous light. Fast-growing colonies were chosen and screened by immunoblot analysis for cyt c_6 expression in +Cu medium. Mating of strains of opposite mating types and dissection of zygotes was performed as described by Harris (1989). Complementation analysis was performed by generating vegetative diploids according to the method of Harris (1989), using *arg2* and *arg7* double mutants with *crd1*, *crd2*, *crr1*, and *pcy1-ac208sop13* strains.

Measurements of fluorescence kinetics: Room temperature chlorophyll fluorescence induction kinetics were measured using an open FluorCam detector (Photon Systems Instruments, Brno, Czech Republic). Fluorescence emissions were recorded from either liquid cultures or colonies of cells on plates after dark adaptation periods of at least 5 min, using an actinic light intensity of ~60 μE/m²/sec for 2–3 sec.

Insertional mutagenesis: To mutagenize cells, the glass bead method (Kindle 1990) was used to insert the plasmid pSP109 containing the *ble* gene that confers resistance to Zeocin (Stevens *et al.* 1996). The strain used for transformation was CC-425 transformed with pCU4 (Quinn and Merchant 1995). In the pCU4 construct the copper responsive *CYC6* promoter drives the arylsulfatase encoding gene *ARS2*. [This strain was used because we had initially intended to use the reporter for screening among the transformants for regulatory mutants (no reporter gene expression in −Cu medium), but arylsulfatase expression varied too much post-transformation, and the strategy had to be abandoned.] After transformation, the cells were plated on +Cu TAP plates. After 5–7 days transformants were transferred to a new +Cu plate (50 transformants/plate) and after an additional 5–7 days these colonies were transferred to two sets of new plates, one set with and one set without copper. After two more transfers to +Cu or −Cu plates the transformants were scored for differences in appearance, growth, and fluorescence induction kinetics.

RESULTS

Three loci, *CRR1*, *CRD1*, and *CRD2*, are involved in copper nutrition signaling: In previous work we had defined a nutritional copper signaling pathway on the basis of the differential accumulation of cyt c_6 and plastocyanin that function in photosynthetic electron transfer (Merchant and Bogorad 1987a; Quinn and Merchant 1995). To identify additional players in the pathway, we screened colonies of insertionally mutagenized cells (see materials and methods) for copper nutrition-dependent growth phenotypes and photosynthetic function (see Moseley *et al.* 2000). Of 7568 Zeocin-resistant colonies tested, we identified 11 strains that displayed copper-conditional phenotypes, and these were subsequently categorized as carrying mutations in regulatory or target genes on the basis of phenotypic analysis (described below) and named *copper response regulator* (*crr*) or *copper response defect* (*crd*) strains, respectively. Genetic complementation and recombination analyses separated the mutants into three complementation groups: *CRR1*, represented by allelic strains *crr1-1* and *crr1-2*; *CRD1*, represented by 8 strains (of 8 *crd1* mutants identified in this screen plus another 4 from a separate screen, 9 were analyzed by recombination and complementation tests and shown to be allelic; Moseley *et al.* 2000); and *CRD2*, represented by a single strain, *crd2-1* (Table 1).

CRR1: Copper-deficient *crr1* cultures grow poorly compared to wild-type cells (Figure 1, A–C). A normal growth rate of *crr1* is evident only upon provision of excess copper (see 6 μM sample, Figure 1A), and severely copper-depleted *crr1* cells never reach the cell densities of wild-type cultures, indicating that the strain is copper limited for growth (see 0 and 25 nM samples, Figure 1B). In previous work we noted that complete repression of the *CYC6* gene, even in cultures with high cell density (1–2 × 10^7 cells/ml), occurred at 400 nM medium copper (Hill *et al.* 1991; Merchant *et al.* 1991), so the higher requirement for medium copper in *crr1* suggests that the *CRR1* locus controls aspects of copper homeostasis besides transcriptional activation of *CYC6*. Fluorescence induction and decay kinetics indicate a defect in photosynthetic electron transfer in −Cu *crr1* cells, but when *crr1* strains are grown in excess copper, photosynthesis is normal (Figure 1D). This can be explained if −Cu *crr1* strains lack cyt c_6 and if +Cu *crr1* strains can accumulate plastocyanin, and indeed this is the case (Figure 2B).

CRD1: The *CRD1* gene is now known to encode one of two isoforms of a component of the aerobic Mg protoporphyrin IX monomethylester cyclase in chlorophyll biosynthesis: the second isoform was named *CTH1* for copper target homolog (Moseley *et al.* 2002b; Pinta *et al.* 2002; Tottey *et al.* 2003). *CTH1* is expressed in +Cu cells, while in −Cu cells *CRD1* is expressed coordinately with *CYC6* and *CPX1* (Figure 2, A and B; Moseley *et al.* 2000, 2002b). The *crd1* mutants are accordingly chlorotic in copper-deficient medium, but are rescued by provision of nutritionally relevant amounts of copper with complete rescue occurring at 400 nM copper (Figure 1A). We note that this is the same amount of copper that is required to turn off the *CYC6* and *CPX1* genes and to support holoplastocyanin biosynthesis to its maximum abundance, which is consistent with *CRD1* being a target in the same pathway. The loss of chlorophyll

TABLE 1

Complementation and recombination analysis of *crr* and *crd* strains

	crr1-1	*crr1-2*	*crd1-1*	*crd1-5*	*crd2-1*
crr1-1	−	−[a]	ND	+	+
crr1-2	0/57 (17)[b]	−	ND	ND	ND
crd1-1	12/24 (6)	ND	−	ND	ND
crd1-5	80/200 (random spores)	ND		−	+
crd2-1	15[c]/83 (19)	ND	27/56 (16)	ND	−

ND, not done.

[a] The results of complementation tests are shown above the diagonal (top and right). A plus sign indicates that the vegetative diploids displayed wild-type growth and fluorescence induction kinetics on −Cu TAP medium, *i.e.*, positive complementation, whereas a minus sign indicates that the diploids displayed mutant growth and photosynthesis phenotypes, *i.e.*, negative complementation.

[b] The results of recombination tests are shown below the diagonal (bottom and left). The numerator refers to the number of recombinant progeny, while the denominator indicates the total progeny scored. The number in parentheses indicates the number of zygotes from which the spores originated.

[c] Only wild-type spores were scored as recombinants, since it was not possible to distinguish *crd2-1crr1-1* double mutants from *crr1-1* single mutants using growth and fluorescence analysis.

proteins in *crd1* in −Cu medium contributes to loss of photosynthetic capacity, evident from comparison of the fluorescence rise and decay kinetics of *crd1* strains to those of the wild type (Figure 1D), and hence at high light intensity the *crd1* strains are growth compromised in −Cu medium.

CRD2: The *crd2* strain initially showed a more severe growth phenotype in copper-deficient medium compared to *crr1* (Figure 1, A and C), but was clearly distinct from *crr1* because all cultures eventually reached a high cell density (Figure 1B) and photosynthetic function was independent of copper nutrition status. On this basis, we concluded that copper deficiency limits the growth *rate* of the *crd2* strain, but for *crr1* strains, copper deficiency limits the *capacity* for growth, and the *crd2* mutation affects an aspect of metabolism other than photosynthesis.

The *CRR1* locus encodes a copper homeostasis regulator required not only for the expression of *CYC6*, but also for degradation of apoplastocyanin and the biogenesis of plastocyanin and a plasma membrane ferroxidase: *Transcriptional activation:* To distinguish *CRR1* as a regulatory locus *vs. CRD1* and *CRD2* as target genes on the basis of biochemical criteria, we analyzed the expression of the *CYC6* gene, the prototypical copper-deficiency target in Chlamydomonas, and also other copper-deficiency targets like *CPX1* and *CRD1* (Quinn *et al.* 2002) by RNA blot hybridization (Figure 2A) and immunoblotting (Figure 2B). We noted that both *crr1* strains failed to turn on *CYC6* transcription under −Cu conditions, and they also fail to activate *CPX1* and *CRD1* beyond the basal level maintained under copper-replete conditions (Figure 2A) resulting in no cyt c_6 in extracts from −Cu cells and only basal abundance of coprogen oxidase (Figure 2B; Hill and Merchant 1995; Moseley *et al.* 2002b), suggesting that *CRR1* is a regulatory locus. Nevertheless both *crr1-1* and *crr1-2* do respond to other nutrient deficiencies (Quinn *et al.* 2002; data not shown), indicating that they are capable of gene expression even though they are severely growth compromised. On the other hand, *CYC6* is expressed normally in *crd2-1* and in all *crd1* strains tested, and the *CRD1* gene is expressed normally in *crd2-1* (Figure 2A; data not shown). These data are compatible with a previously proposed regulatory role for *CRR1* in determining the copper-deficiency-induced transcriptional activation of *CYC6*, *CPX1*, and *CRD1*.

Apoplastocyanin degradation: Immunoblot analysis revealed that the *crr1* strains also fail to downregulate plastocyanin abundance in copper deficiency, and most of this protein is found in the apoform (Figure 2B). Li *et al.* had suggested that the degradation of the plastocyanin polypeptide in −Cu cells required a −Cu-induced protease (Li and Merchant 1995; Li *et al.* 1996): the accumulation of plastocyanin in *crr1* indicates that this protease is yet another target of the *CRR1* locus. Plastocyanin regulation occurred normally in *crd1* and *crd2* mutants.

Holoplastocyanin formation: Interestingly, in the fully copper-supplemented situation as well, the *crr1* strains accumulate more apoplastocyanin relative to wild type. If copper is titrated into copper-deficient medium, we note that the plastocyanin biosynthetic pathway is saturated at ~400 nM Cu for wild-type cells but requires much more for the *crr1-1* strain (Figure 2C), suggesting that copper delivery to the site of holoplastocyanin biosynthesis—either uptake from the medium or distribution to the chloroplast—is dependent on *CRR1*.

Plasma membrane ferroxidase function is compromised: We wondered whether the *crr1* mutation affected the accumulation of other copper-containing proteins in Chlamydomonas, such as a plasma membrane ferroxidase, encoded by *FOX1* (Herbik *et al.* 2002; La Fontaine *et al.* 2002). Immunoblot analysis showed that ferroxidase accumulation was also compromised in the *crr1* strain (Figure 3C). Loss of ferroxidase occurs probably at the

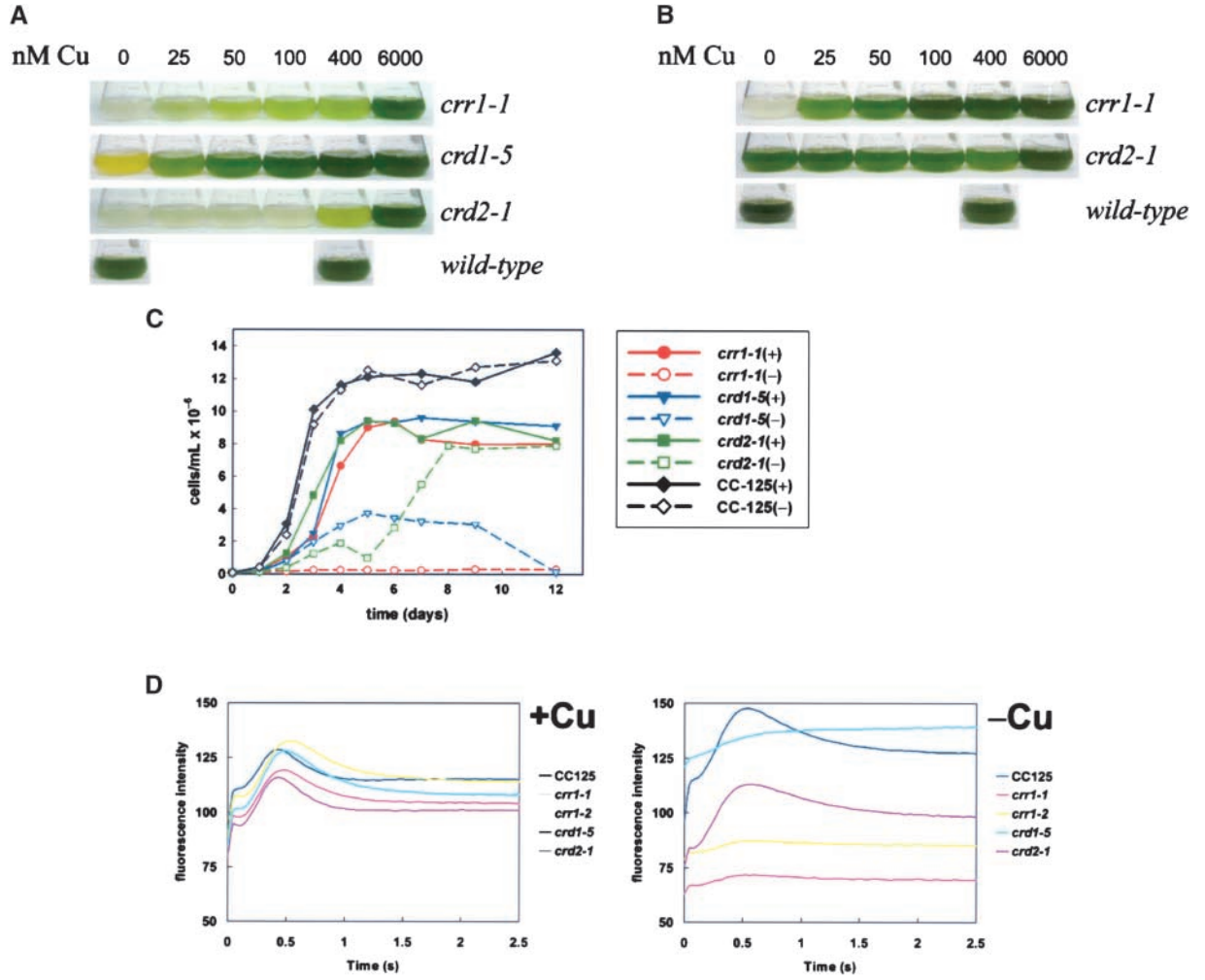

FIGURE 1.—Growth phenotypes of candidate mutants. (A) Appearance of cultures after growth for 5 days [wild type (CC125), *crr1-1*, and *crd2-1*] or 6 days (*crd1-5*), in TAP medium supplemented with the indicated Cu concentrations. (B) Appearance of the same wild-type, *crr1-1*, and *crd2-1* cultures after 13 days of growth. (C) Plot of cell density over time for wild-type (CC125), *crr1-1*, *crd1-5*, and *crd2-1* cultures over 12 days of growth in TAP medium supplemented with 6 μM (+) or 0 μM (−) Cu. (D) Chlorophyll fluorescence induction curves from wild-type *vs.* mutant strains grown on solid TAP medium, with (+, 6 μM) or without (−, 0 μM) Cu.

level of protein synthesis or protein stability because the abundance of *FOX1* mRNA is not reduced in −Cu *vs.* +Cu *crr1* (Figure 3B). Since the ferroxidase is proposed to function in a high-affinity iron-uptake pathway, we wondered whether compromised ferroxidase biosynthesis would result in poor iron nutrition.

Cells of *crr1* were transferred from Cu-enriched, iron-replete (18 μM) medium to copper-enriched *vs.* copper-depleted, iron-deficient (1 μM) medium. The wild-type culture maintains growth and chlorophyll accumulation, but the *crr1* strain displays classic iron-deficiency symptoms (MOSELEY *et al.* 2002a). Growth of *crr1* is reduced, and the strain is chlorotic (Figure 3A; 1.29 pg chlorophyll/cell in −Cu *crr1* relative to 2.31 pg/cell in +Cu *crr1* in one representative experiment). Conversely, if *crr1* is transferred to copper-deficient medium containing excess iron (200 μM) where the cellular requirement for the ferroxidase is greatly reduced (Figure 3C, compare lanes 1 and 2 *vs.* 7 and 8), its copper-conditional growth phenotype is suppressed (Figure 3D). We conclude that poor iron nutrition contributes to the growth phenotype of *crr1* strains in −Cu medium, possibly because of malfunction of the ferroxidase biosynthetic pathway.

We conclude that *CRR1* is a key regulator of nutritional copper homeostasis: it is required in −Cu cells for (i) upregulation of the accumulation of cytochrome c_6, coprogen oxidase, and the Crd1 cyclase isoform as indicated previously and (ii) downregulation of plastocyanin via activation of a protease and the Cth1 isoform of the cyclase by transcriptional occlusion of the proximal *CTH1* promoter (CULLEN *et al.* 1984; MOSELEY *et al.* 2002b). *CRR1* is also required in +Cu cells for maintenance of holoplastocyanin and in −Cu cells for maintenance of holoferroxidase.

***CRR1* is required for growth under hypoxic conditions:** We noted previously (MOSELEY *et al.* 2000, 2002b; QUINN *et al.* 2000, 2002) that each of the Cu-responsive

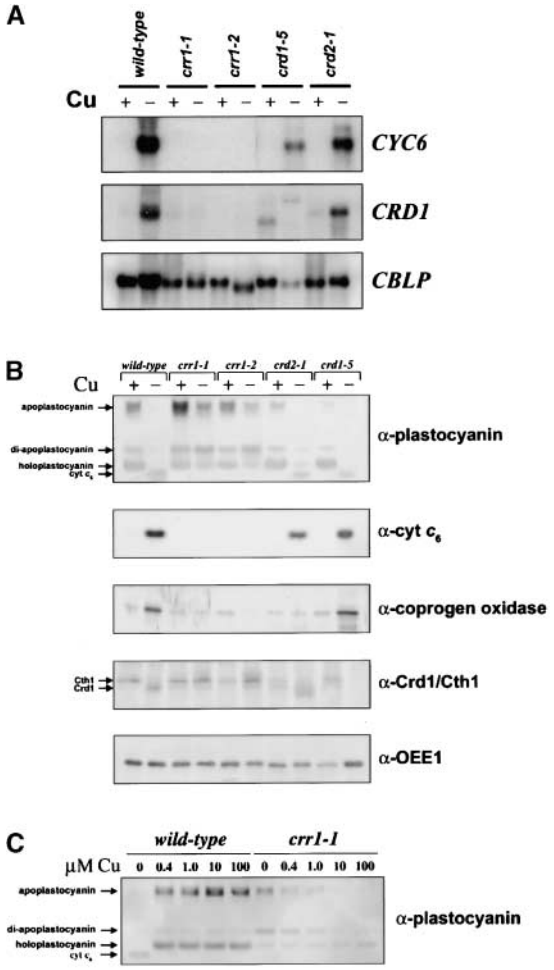

FIGURE 2.—Biochemical phenotypes of *crr* and *crd* strains. (A) RNA blot analysis comparing expression of Cu-responsive genes in wild-type *vs.* mutant cells. Three micrograms of total RNA was loaded in each lane. RNA was prepared from cultures grown in TAP medium supplemented with 6 μM (+) or 0 μM (−) Cu. −Cu *crr1-1* and *crr1-2* cultures were transferred to Cu-free medium once; all other −Cu cultures were transferred at least twice. *CYC6* encodes cyt c_6; *CRD1* encodes a component of the protoporphyrin IX monomethylester cyclase, formerly known as the *COPPER RESPONSE DEFECT 1* gene; and *CBLP* encodes a Chlamydomonas G-protein β-subunit-like protein, used here as a loading control. (B) Immunoblots to compare accumulation of Cu-responsive proteins in wild-type *vs.* mutant cells. The migration positions of apo-, di-apo-, and holoforms of plastocyanin are indicated with arrows in the first panel, as is the position of cyt c_6, which cross-reacts with the anti-plastocyanin antibody. In the fourth panel, arrows indicate the positions of the Cth1 and Crd1 gene products, which are both recognized by the anti-Crd1 antiserum. (C) Immunoblot showing the accumulation of cyt c_6, apo-, di-apo-, and holoplastocyanin in wild-type cells compared to *crr1-1* cells grown in TAP medium with the indicated concentrations (micromolar) of Cu.

genes is regulated also by hypoxia—*CYC6*, *CPX1*, and *CRD1* show increased expression as oxygen is depleted and, in the case of *CTH1*, the extended 3-kb nonfunctional (with respect to synthesis of the cyclase isoform) transcript is expressed at the expense of the functional 2-kb version. As little as a 50% reduction in the O_2 content of the medium is sufficient to upregulate *CPX1* and *CRD1* expression, and this is mediated via the Crr1 pathway (QUINN *et al.* 2002). To assess the physiological significance of the hypoxic activation of Crr1 target genes, the impact of the *crr1* mutation was tested by assessing growth under oxygen limitation (Figure 4A). Indeed the *crr1* mutants are growth compromised in low O_2 relative to air. We know that the growth defect is not a secondary effect of hypoxia on copper uptake because hypoxic cells show normal accumulation of holoplastocyanin (Figure 4B), which requires copper delivery to the thylakoid lumen.

Iron-deficiency symptoms and impaired ferroxidase accumulation in *crd2* strains: In *Saccharomyces cerevisiae* and also in mammalian systems, copper deficiency results in secondary iron deficiency owing to the involvement of a multicopper oxidase in iron assimilation (ASKWITH and KAPLAN 1998). Copper-deficient wild-type Chlamydomonas cells show no symptoms of iron deficiency even though they accumulate much less ferroxidase relative to copper-replete cells (LA FONTAINE *et al.* 2002). Therefore, we proposed that copper-deficient Chlamydomonas must use an alternate or modified mechanism for iron uptake relative to copper-replete cells so that its growth is not compromised by reduced ferroxidase abundance, and this idea is supported by the increased iron requirement of the *crr1* mutant strains (Figure 3D). Therefore, iron metabolism was a pathway of interest in the context of deciphering the basis of copper-conditional growth phenotypes.

crd2 strains are iron deficient: We noted during biochemical characterization of our mutant strains that the pattern of migration of iron-containing enzymes Crd1 and Cth1 in the *crd2-1* mutant was not normal (Figure 2B, compare *crd2* to wild type), but resembled the migration pattern seen in −Fe wild-type cells (J. L. MOSELEY, unpublished observations). This suggested to us that *CRD2* might indeed be involved in an iron-uptake pathway in copper-deficient cells.

To test this idea, we assessed the effect of iron nutrition on the copper-conditional growth phenotype of *crd2-1*. As noted previously, copper nutrition status does not change the threshold for establishment of iron-deficiency symptoms in the wild-type strain (Figure 5A). In copper-replete medium, the iron-dependent growth of *crd2-1* is indistinguishable from wild type, but in copper-deficient medium, *crd2-1* is growth compromised at 18 μM medium iron, which is considered iron replete for the wild type (MOSELEY *et al.* 2002a). The slow growth phenotype of *crd2-1* in −Cu medium can be further exacerbated by decreasing iron supplementation to 1 μM or can be suppressed completely by provision of excess iron to 200 μM (Figure 5A). To test whether *crd2-1* cells were internally iron deficient, we analyzed the expression of *FOX1* and *FTR1* that encode components of the high-affinity iron-uptake pathway (LA FONTAINE *et al.*

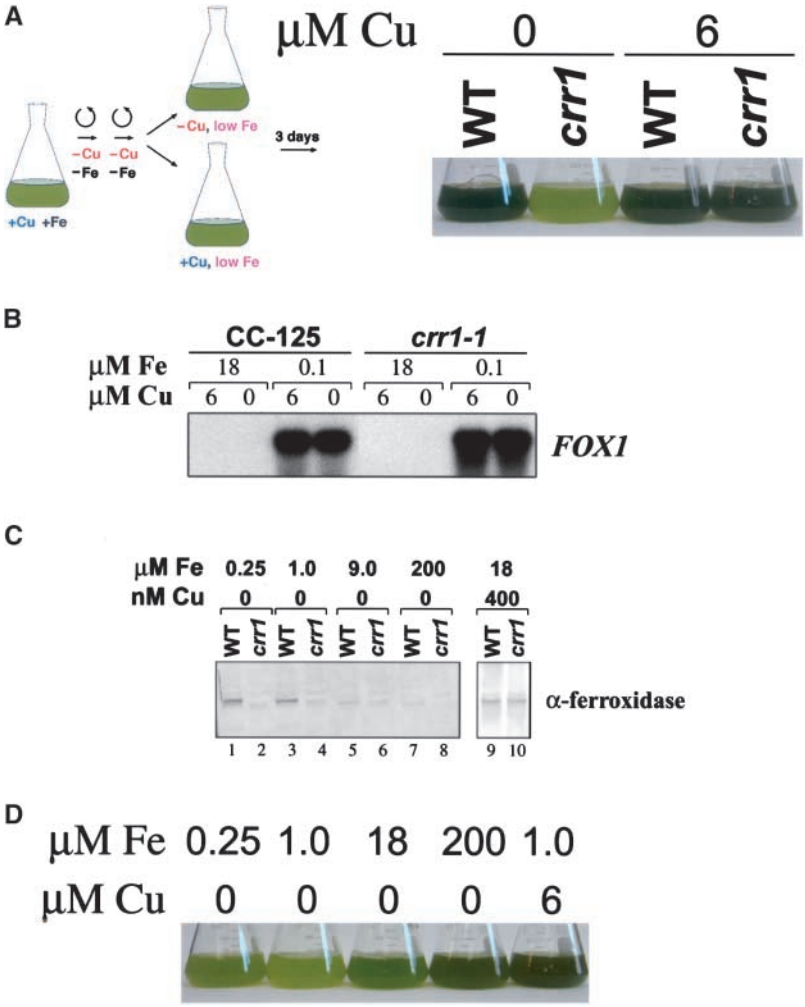

FIGURE 3.—Acclimation responses to Fe deficiency in wild-type *vs. crr1* strains. (A) Appearance of wild-type and *crr1-1* cultures grown in +Fe (18 μM) and +Cu (6 μM), washed with −Fe −Cu TAP and grown for 3 days in 1 μM Fe TAP with or without 6 μM added Cu, as indicated. *crr1-1* cells grown without copper contained ~50% of wild-type chlorophyll. (B) RNA blot analysis comparing *FOX1* mRNA expression in CC-125 (wild type) and *crr1-1* mutant strains. CC-125 or *crr1-1* cells were grown in TAP medium containing the indicated concentrations of Fe and Cu. Five micrograms of total RNA was loaded per lane. (C) Immunoblots demonstrating accumulation of ferroxidase in wild-type and *crr1-1* cells, grown in TAP medium with the indicated concentrations of Fe and Cu. (D) Appearance of *crr1-1* cultures grown for 6 days in TAP medium with the indicated concentrations of Fe and Cu, demonstrating partial rescue of the −Cu growth phenotype with excess Fe.

2002) and *FEA1* that encodes a periplasmic iron-uptake component (RUBINELLI *et al.* 2002; M. ALLEN and S. MERCHANT, unpublished observations). In the wild-type strain, the expression of these genes is independent of copper (Figure 5B) but is dependent on iron nutrition (LA FONTAINE *et al.* 2002; J. A. DEL CAMPO and S. MERCHANT, unpublished observations). We found that in *crd2-1*, *FOX1*, *FTR1*, and *FEA1* are all upregulated relative to wild type, and the magnitude of the upregulation is dependent on the degree of copper deficiency (Figure 5B), suggesting that *crd2-1* cells are experiencing iron deficiency. This is compatible with the slow growth phenotype of *crd2-1*, *i.e.*, as if the strain were nutrient limited.

Compromised ferroxidase biosynthesis: Since *crr1* showed impaired ferroxidase accumulation (Figure 3C), we wondered whether *crd2-1* might also be affected in ferroxidase function. Copper- and iron-replete *crd2-1* or wild-type cells were transferred to iron-deficient medium to induce ferroxidase accumulation and tested after 24 hr. Copper-supplemented wild-type and *crd2-1* cells accumulated ferroxidase to comparable levels but copper-deficient *crd2-1* cells did not (Figure 5D). Since we know that the abundance of *FOX1* and *FTR1* mRNA is high in *crd2-1* cells, the lower amount of ferroxidase in −Cu *crd2-1* must result from slower synthesis or decreased stability. We conclude that the delayed growth of −Cu *crd2-1* cultures results from slower assimilation of iron because of reduced function of Fox1/Ftr1, and we suggest that *CRD2* encodes a function required for high-affinity iron uptake especially in a situation where copper is limiting the operation of the multicopper oxidase pathway. Note that the effect of *crd2* is specific for ferroxidase biogenesis whereas *crr1* strains are affected in the accumulation of plastocyanin and also ferroxidase (Figures 2B and 3C).

One possibility is that *CRD2* might encode a component that facilitates Fox1/Ftr1 biogenesis, and its function is more important when copper, which is required for Fox1 biogenesis in the secretory pathway, is scarce within this compartment (LA FONTAINE *et al.* 2002). This would be analogous to the increased expression of *CPX1* in −Cu cells. Another possibility is that Crd2 is functionally redundant with another molecule whose expression is decreased in copper deficiency. This would be analogous to the Crd1/Cth1 situation.

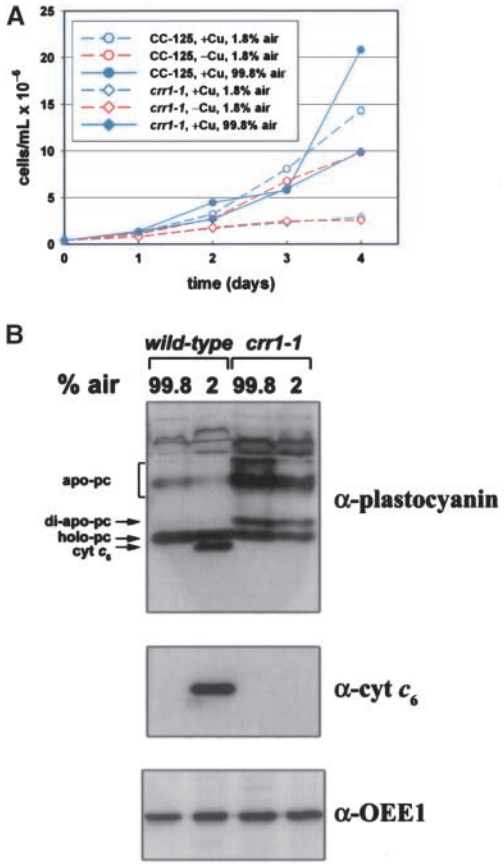

FIGURE 4.—Hypoxic growth of wild type *vs.* the *crr1* strain. (A) Wild-type (CC-125) and *crr1-1* strains were grown in TAP medium with 6 μM Cu (blue lines) or without Cu (red lines), bubbled with gas mixtures containing the indicated amounts of air (99.8% *vs.* 1.8%). All mixtures contained 0.2% CO_2, and the balance of the 1.8% air mixture was made up with N_2 (98% N_2). (B) Immunoblot analysis of plastocyanin and cyt c_6 accumulation in soluble protein fractions from wild-type and *crr1-1* strains grown for 4 days in 6 μM Cu with the indicated amounts of air. Detection of OEE1 was used to demonstrate equal loading.

The biogenesis of a plasma membrane ferroxidase, Fet3p, in *S. cerevisiae* is proposed to require a cytoplasmic copper chaperone, Atx1, to deliver copper from an assimilatory transporter to a distributive P-type ATPase in the secretory pathway (YUAN *et al.* 1995; LIN *et al.* 1997). The phenotype of *crd2* was strikingly similar to that of yeast mutants affected in copper loading of Fet3p. For example, the yeast *atx1* and *ccc2* strains are iron deficient and can be rescued for growth by either copper or iron supplementation. The release of a draft Chlamydomonas genome (http://www.jgi-doe.gov) allowed us to identify two candidate distributive transporters, *CTP1* [related to Arabidopsis *AHM7* and *RAN1* (HIRAYAMA *et al.* 1999) and mammalian *ATP7A/WND* (BULL *et al.* 1993; CHELLY *et al.* 1993; MERCER *et al.* 1993; TANZI *et al.* 1993; VULPE *et al.* 1993)] and *CTP2* (related to Arabidopsis *PAA1*; SHIKANAI *et al.* 2003). We tested the expression of *ATX1*, *CTP1*, and *CTP2* by quantitative RT-PCR but could not account for the phenotype on the basis of loss of expression of one of these genes in *crd2* relative to the wild type (Figure 5C). The expression of *CTP1* was increased slightly in proportion to the decrease in medium copper, which is expected because the gene is regulated by iron deficiency in Chlamydomonas (S. TOTTEY and S. MERCHANT, unpublished observations) like the homologous yeast gene (YAMAGUCHI-IWAI *et al.* 1996; LIN *et al.* 1997). The organization of the *CTP1* and *CTP2* genes was also intact in *crd2* as assessed by amplification with gene-specific primers (data not shown).

sop13* may be a weak gain-of-function allele of *CRR1*:** The function of cyt c_6 as a substitute for plastocyanin was established definitively when it was shown that the nonphotosynthetic, acetate-requiring phenotype of *pcy1-ac208* could be suppressed in copper-deficient medium where *CYC6* is expressed (WOOD 1978). On this basis, we had selected spontaneous second site suppressors of *pcy1-ac208* in which *CYC6* was misexpressed in copper-supplemented medium (MERCHANT and BOGORAD 1987b; LI *et al.* 1996). We considered the possibility that one suppression mechanism might involve mutations at the *CRR1* locus that allow copper-insensitive expression of *CYC6*. To test this model, we isolated new *pcy1* suppressor strains that grew photosynthetically on +Cu minimal medium at rates comparable to wild type (see MATERIALS AND METHODS). One of these, *sop13* (s**uppressor **o**f **p**cy1-ac208 **13***) was chosen for further analysis. Strains *pcy1* and *pcy1sop13* lack plastocyanin, but in standard TAP medium containing 6 μM copper salts, *pcy1sop13* accumulates ~5–10% of the amount of cyt c_6 that is found normally only in the −Cu situation (Figure 6A). This amount can be reduced by provision of excess copper, as noted previously for other suppressed strains (MERCHANT and BOGORAD 1987b).

To test whether the *sop13* mutation is linked to the *CRR1* locus, we crossed *pcy1-ac208sop13* with *crr1-1PCY1-SOP13* and analyzed the phenotypes of 85 spores from 25 different zygotes, including 8 complete tetrads, and 21 tetrads in total for which the genotype of each spore could be determined. The *pcy1-ac208* and *sop13* phenotypes were scored by immunodetection of plastocyanin and cyt c_6, respectively, in +Cu whole cell extracts (Figure 6B for two representative tetrads) and the *crr1* phenotype was scored on the basis of growth on −Cu TAP and fluorescence induction and decay kinetics (not shown). Recombination between *CRR1* and *SOP13* was not observed (χ^2 probability <1% for independent assortment), whereas recombination between *CRR1* and *PCY1* and *PCY1* and *SOP13* was. In vegetative diploids that are heterozygous for *sop13*, we noted that misexpression of cyt c_6 is maintained (Figure 6B), indicating that *sop13* is a dominant gain-of-function mutation. These data preserve the possibility that *sop13* may be a weakly constitutive allele of *crr1*, although we cannot

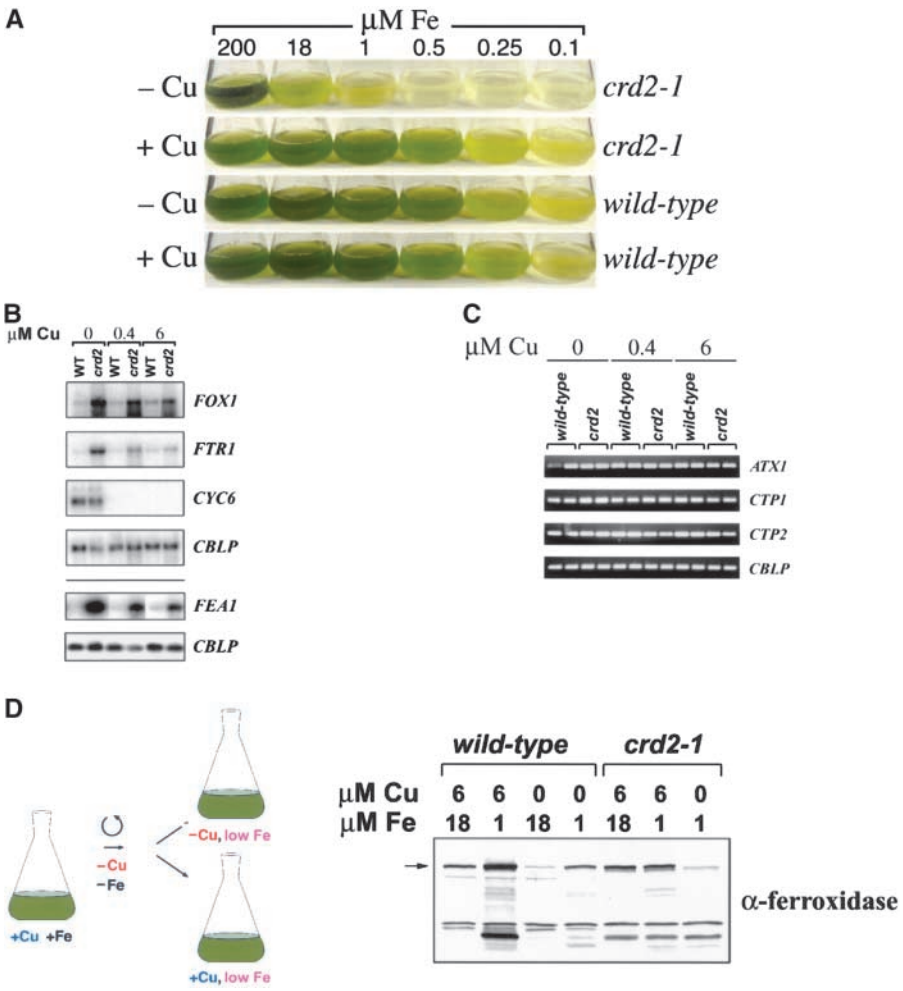

FIGURE 5.—Acclimation responses to Fe deficiency in wild-type *vs. crd2* strains. (A) Growth of *crd2-1 vs.* wild type in +Cu (6 μM) and −Cu (0 μM) TAP with the indicated amounts of added Fe. (B) RNA blot analysis of genes involved in Fe uptake and distribution in wild type and *crd2-1* grown in TAP medium with normal Fe supplementation (18 μM) and with the indicated concentration of added Cu. The *FEA1* panel and the bottom *CBLP* panel are separated from the other panels by a line to indicate that these RNA blots were performed with different RNA samples. Three micrograms of total RNA was loaded per lane. *CBLP* expression is used to demonstrate equal loading of the samples, and *CYC6* expression is used as a marker for copper deficiency. (C) Quantitative RT-PCR analysis of the samples described in B. Ten microliters of product was removed from the amplification reaction and analyzed by electrophoresis to authenticate the product on the basis of size and to visualize the difference in the abundance of the specific mRNA in the input RNA sample. Samples were removed at the following cycles: *CTP1*, 27; *CTP2*, 27; *ATX1*, 30; and *CBLP*, 19. For the experiment shown, *CTP1* is twofold induced in the low copper *crd2* samples relative to the saturating copper sample. (D) Immunoblot analysis of ferroxidase accumulation in wild-type and *crd2-1* cells grown in +Fe (18 μM) and +Cu (6 μM), washed with −Fe −Cu TAP, and grown for 24 hr in 1 μM Fe TAP with or without 6 μM added Cu, as indicated. The position of the ~140-kD ferroxidase protein is indicated with an arrow. Higher mobility bands, which are stoichiometrically more prevalent in −Cu wild-type cells or in *crd2* mutant cells, are likely to be degradation products. Degradation may be more pronounced under these conditions because of a different conformation of the protein when the copper sites are not fully loaded (HELLMAN *et al.* 2002).

rule out alternative models such as the possibility that the mutation lies in another component in the pathway that is linked to *CRR1*. For instance, *sop13* could be a *cis*-acting mutation in the *CYC6* promoter, (if *CYC6* is linked to *CRR1*) or it may be a mutation in a linked modifier of *CRR1* or in a linked assimilatory copper transporter.

DISCUSSION

***CRR1* encodes a regulator of Cu homeostasis under both nutritional deficiency and nutritional sufficiency:** *Cytochrome c_6 and plastocyanin:* Previously, we showed that Chlamydomonas responds to copper deficiency by changes in gene expression resulting in increased production of an alternate electron transfer catalyst, cyt c_6, to take the place of an abundant copper protein, and coprogen oxidase, an oxygen-dependent enzyme in the tetrapyrrole pathway, by transcriptional activation through CuREs associated with the corresponding genes. At the same time, plastocyanin is degraded, presumably to allow redistribution of copper to other copper-containing enzymes. In this work, we define *CRR1* as a central regulator of each of these well-characterized responses. The nonphotosynthetic phenotype is attributed to loss of cyt c_6, which is essential for the Z-scheme when plastocyanin function is compromised by lack of its cofactor, copper (Figures 1D and 2, A and B).

Heterotrophic growth (Figure 1, A and C) is also affected in −Cu *crr1*, perhaps because of the requirement for a copper enzyme, cytochrome oxidase, in respiration. Previously (LI and MERCHANT 1995), we had suggested that plastocyanin degradation was regulated. Plastocyanin is the most abundant copper protein in a photosynthetic cell, and its degradation would remove a major copper sink and in so doing allow copper reallocation to other copper enzyme-containing compartments in the cell. The accumulation of apoplastocyanin in *crr1* strains (Figure 2C) confirms that the decreased thermodynamic stability of apo- *vs.* holoplastocyanin

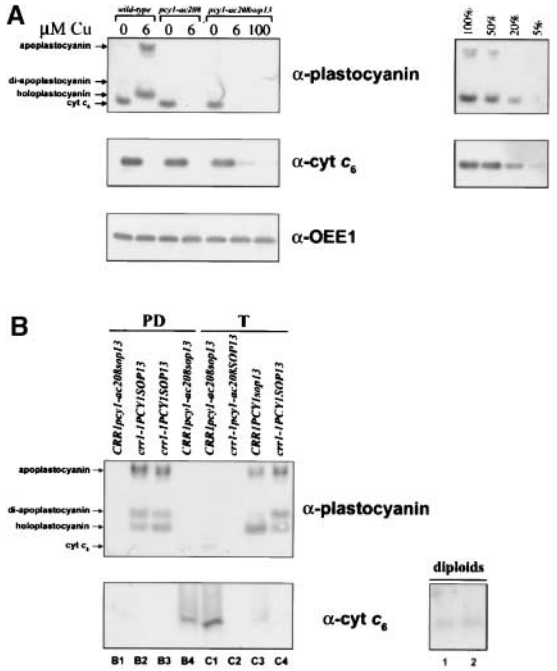

FIGURE 6.—Biochemical and genetic characterization of a *pcy1-ac208* suppressor strain. (A) Immunoblot analysis of soluble extracts to compare expression of plastocyanin and cyt c_6 in wild type, *pcy1-ac208*, and a *pcy1-ac208* suppressor strain, (*pcy1-ac208sop13*), grown in TAP medium supplemented with the indicated concentrations of Cu (micromolars). Dilution series of wild-type extracts enables estimation of plastocyanin and cyt c_6 abundance. Samples corresponding to equal amounts of chlorophyll were loaded in each lane, and immunodetection of OEE1 was used to demonstrate equal loading. (B) Anti-plastocyanin and cyt c_6 immunoblots of +Cu whole cell extracts illustrating phenotypic analysis of two representative tetrads from a cross of *crr1-1* and *pcy1-ac208sop13*. Cyt c_6 expression in +Cu cells indicates the presence of the *sop13* mutation. Accumulation of cyt c_6 in two vegetative diploid strains that are heterozygous for *sop13* is also shown.

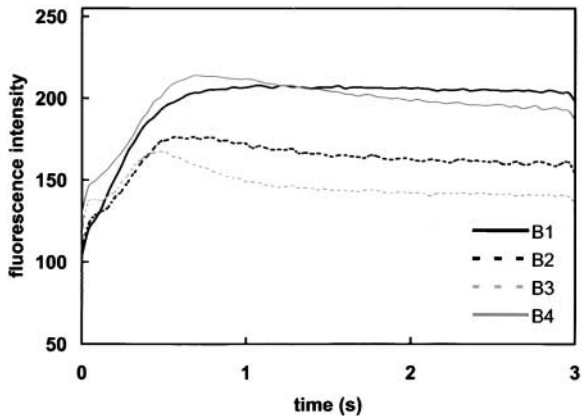

FIGURE 7.—Photosynthesis phenotypes of *crr1* and *sop13* strains. Shown are fluorescence rise and decay kinetics of +Cu cells from strains B1–B4 (shown in Figure 6B). B1, *CRR1pcy1-ac208sop13*; B2, *crr1-1PCY1SOP13*; B3, *crr1-1PCY1SOP13*; and B4, *CRR1pcy1-ac208sop13*.

and the increased protease susceptibility (LI and MERCHANT 1995) is not in itself sufficient for degradation *in vivo* and that activation of a proteolytic mechanism in −Cu cells is a necessary component of copper homeostasis.

Tetrapyrrole pathway: The (10- to 20-fold) increased expression of *CPX1* in −Cu cells was previously rationalized on the basis of an increased demand for heme owing to production of an abundant ($>10^6$ molecules/cell) cytochrome. [Coprogen oxidase is rate limiting in the synthesis of heme from δ-aminolevulinic acid (ANDREW *et al.* 1990)]. In a photosynthetic cell, however, the pathway intermediates downstream of coprogen oxidase are required also for the biosynthesis of chlorophyll, which is a much more abundant tetrapyrrole. The discovery of *CRD1* and *CTH1* as new Crr1 targets in the chlorophyll biosynthetic pathway suggests a link between copper and tetrapyrrole metabolism whose molecular basis remains to be discovered. The fact that Cth1 cannot fully cover loss of Crd1 function (MOSELEY *et al.* 2002b) suggests also additional complexity in the chlorophyll biosynthetic pathway.

Iron metabolism: We found also that Cu-deficient *crr1* strains display pleiotropic symptoms of Fe deficiency (Figure 3A), which we attribute to their reduced capacity for production of a multicopper ferroxidase involved in high-affinity Fe uptake (Figure 3C). Fe deficiency appears to be a major cause of the poor growth of Cu-deficient *crr1* strains, since addition of an excess of Fe to the medium improves the growth rate and increases the final cell density of −Cu *crr1* cultures (Figure 3D). In *S. cerevisiae*, nutritional or genetic Cu deficiency results in Fe deficiency owing to the involvement of Fet3p, a multicopper oxidase, in high-affinity Fe uptake (ASKWITH *et al.* 1994). However, we find it intriguing that wild-type −Cu Chlamydomonas cells are not Fe deficient to a greater degree than are +Cu cells (as established by monitoring gene expression and chlorosis; LA FONTAINE *et al.* 2002). One model is that cellular metabolism might be altered in −Cu cells in a Crr1-dependent pathway to reduce the internal demand for Fe; hence, *crr1* strains require more iron compared to the wild type. Another model is that Crr1 may play a role in maintaining an adequate Fe-uptake capacity in Cu-deficient cells. The discovery of *CRD2* as a target of nutritional copper signaling and the phenotype of *crd2* strains is compatible with this idea. A third possibility is that the reduced capacity for holoferroxidase production in −Cu *crr1* strains is simply another consequence of the failure to degrade apoplastocyanin, the presence of which could prevent reallocation of copper from the chloroplast compartment to the secretory pathway where ferroxidase is synthesized. Regardless of the molecular basis, Crr1 clearly plays a role in iron nutrition under copper-deficient growth.

Hypoxia: Since the same genes were found to be regu-

lated by copper and also oxygen deficiency, one might conclude that low oxygen, perhaps by modification of intracellular steady-state abundance of redox compounds, resulted in coincidental conversion of a thiol containing copper-pathway signaling component from the nonactivating to the activating form. The finding that loss of Crr1 function affects the growth of copper-replete cells under hypoxic relative to well-aerated cells suggests that hypoxic activation of Crr1 targets is physiologically meaningful. We propose that Crr1 function and Crr1 target genes are required for proper adaptation to hypoxic conditions. It is worth noting in this context that *HEM13* in *S. cerevisiae* (encoding coprogen oxidase) is also induced by hypoxia (Zagorec *et al.* 1988).

Summary: We have identified a central component of the signal transduction pathway for Cu and O_2 sensing in Chlamydomonas. This factor is implicated in the metabolism of at least three "nutrients" (Cu, Fe, and O_2) and reveals a hitherto unanticipated level of integration between nutrient stress acclimation responses.

***SOP13* is linked to *CRR1* and may be a weakly constitutive *crr1* allele:** Genetic analysis demonstrated that the *sop13* suppressor of *pcy1-ac208* mutation is linked to *CRR1*. This dominant mutation confers a gain of function, enabling partial induction of *CYC6* in +Cu cells, and allowing the accumulation of sufficient cyt c_6 to rescue the photosynthesis-minus phenotype of the *pcy1-ac208* strain (Figure 6). However, no induction of coprogen oxidase or Crd1 expression or decrease in Cth1 accumulation is observed (data not shown). Unlike cyt c_6, which is completely absent in wild-type strains, each of these other proteins is expressed at a significant level in +Cu (Hill and Merchant 1995; Quinn *et al.* 1999; Moseley *et al.* 2000, 2002b), and consequently a 5–10% increase or decrease in its abundance would not be discerned in these experiments. Therefore, we retain the model that *sop13* is a weak *crr1-up* allele, which enables leaky expression of the Cu-deficiency responsive gene expression pathway. However, we cannot rule out the model that the *sop13* mutation may affect a gene that is tightly linked to *CRR1*. The possibilities are several: a *cis*-acting mutation in the *CYC6* promoter, a weak loss of function mutation affecting a copper transporter resulting in internal copper deficiency despite plentiful supply in the medium, a weak gain of function of a Crr1-related factor, or a weak gain of function in an upstream or downstream component in the signaling pathway.

Interestingly, we noted that some *pcy1-ac208sop13* spores (16 spores out of 16 different zygotes) did not exhibit wild-type fluorescence on +Cu medium (Figure 7 for a representative tetrad) even though they did express cyt c_6, suggesting that the selected phenotype (*i.e.*, photosynthetic growth of *pcy1* on +Cu medium) results from a combination of more than one mutation. If misexpression of *CYC6* were sufficient to allow photosynthetic growth, all *pcy1sop13* strains should be photosynthesis plus. The initial premise that misexpression of *CYC6* is sufficient to restore photosynthetic growth to a plastocyanin-minus strain is therefore called into question. We wonder whether a second mutation that changes the composition of PSI or its acceptor side conformation (*e.g.*, at the plastocyanin binding site) may be required for good interaction with cyt c_6, which becomes more important when the abundance of cyt c_6 is substoichiometric (as is the case in *sop13* strains).

***CRD2* is required for stable accumulation of ferroxidase in −Cu:** The *crd2* mutant was isolated as a strain that grew slowly on −Cu medium, but unlike the *crr1* strains it did not have a photosynthesis defect (Figure 1). Instead, we have established that the growth phenotype of the *crd2* mutant is due to Cu-conditional Fe deficiency (Figure 5). Genes involved in Fe assimilation, such as *FOX1*, *FTR1*, and *FEA1*, are highly induced in −Cu *crd2* cells, even at Fe concentrations where the wild type or +Cu *crd2* is Fe replete. Like −Cu *crr1* strains, the *crd2* mutant is unable to synthesize or accumulate normal amounts of ferroxidase under low Fe conditions, suggesting that the defect affects delivery of Cu to apoferroxidase. La Fontaine *et al.* (2002) showed that at any given Fe concentration, Cu-deficient wild-type cells accumulate only ~50% of the amount of ferroxidase that is observed in Cu-replete cells; nevertheless, growth rates and the induction of Fe-assimilation-related genes are comparable between +Cu and −Cu. How then are −Cu cells able to keep up with +Cu cells?

One possibility is that ferroxidase is synthesized in excess of the requirement for maximum Fe uptake, and consequently the reduced amount of ferroxidase in −Cu cells is still sufficient to efficiently catalyze Fe assimilation. However, this idea is contrary to the well-accepted dogma that Fe assimilation is very tightly regulated (Eide 1998). An alternative explanation is that there is a Cu-independent "backup" pathway for Fe uptake that is functional in Cu-deficient cells and supplements Fe uptake through the Cu-dependent (ferroxidase-requiring) pathway. Such backup pathways for coping with nutrient deficiencies are prevalent in nature—the replacement of plastocyanin with cyt c_6 (Wood 1978), ferredoxin with flavodoxin (Hutber *et al.* 1977), and Cu- *vs.* Fe-containing forms of methane monooxygenase (Nielsen *et al.* 1997) are relevant examples. A third possibility, which is best supported by the evidence presented here, is that the ferroxidase/Ftr1 pathway operates in both +Cu and −Cu, but intracellular Cu distribution must be altered in Cu-deficient cells to ensure that sufficient holoferroxidase is produced to supply the Fe requirements of the cell. *CRD2* therefore is likely to encode a Cu-conditional modulator of the Fe-assimilation pathway. A number of candidate genes encoding components of copper homeostasis, including an assimilatory transporter *CPT1* and chaperones and distributive transporters *CTP1* and *CTP2*, have been identified in the EST database and the draft genome sequence of

Chlamydomonas (La Fontaine *et al.* 2002; Grossman *et al.* 2003). While we cannot rule out the possibility that *crd2* represents a point mutation or small deletion in one of these genes, we do know the *crd2* phenotype does not result from inappropriate expression of the known chaperones and transporters (Figure 5C).

If *CRD2* is a modifier of or a bypass mechanism for iron uptake whose function becomes essential in a copper-deficient situation when ferroxidase activity is compromised, it is likely that its expression might be regulated by *CRR1*. In this case, *crr1* mutants would be iron deficient because they fail to activate *CRD2* just as *crr1* strains are photosynthetically defective because they fail to express *CYC6* (Figure 1D). Although we attributed the iron-deficiency phenotype of *crr1* to poor copper uptake/delivery and hence loss of ferroxidase function, it is possible that loss of *CRD2* expression is another contributing factor to the iron-deficiency and ferroxidase-defective phenotype of *crr1* strains.

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