The Crd1 gene encodes a putative di-iron enzyme required for photosystem I accumulation in copper deficiency and hypoxia in Chlamydomonas reinhardtii

Jeffrey Moseley, Jeanette Quinn, Mats Eriksson and Sabeeha Merchant

Department of Chemistry and Biochemistry and Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90095-1569, USA

1Corresponding author
e-mail: merchant@chem.ucla.edu

Chlamydomonas reinhardtii adapts to copper deficiency by degrading apoplastocyanin and inducing Cyc6 and Cpx1 encoding cytochrome c_6_ and coproporphyrinogen oxidase, respectively. To identify other components in this pathway, colonies resulting from insertional mutagenesis were screened for copper-sensitive phenotypes. Twelve crd (copper response defect) strains were identified. In copper-deficient conditions, the crd strains fail to accumulate photosystem I and light-harvesting complex I, and they contain reduced amounts of light-harvesting complex II. Cyc6, Cpx1 expression and plastocyanin accumulation remain copper responsive. The crd phenotype is rescued by a similar amount of copper as is required for repression of Cyc6 and Cpx1, and for maintenance of plastocyanin at its usual stoichiometry, suggesting that the affected gene is a target of the same signal transduction pathway. The crd strains represent alleles at a single locus, CRD1, which encodes a 47 kDa, hydrophilic protein with a consensus carboxylate-bridged di-iron binding site. Crd1 homologs are present in the genomes of photosynthetic organisms. In Chlamydomonas, Crd1 expression is activated in copper- or oxygen-deficient cells, and Crd1 function is required for adaptation to these conditions.

Keywords: di-iron enzyme/light-harvesting complex/oxygen/photosynthesis/plastocyanin

Introduction

Copper is an essential micronutrient because it functions as a cofactor in enzymes and electron transfer proteins that catalyze oxygen chemistry or redox reactions (e.g. cytochrome oxidase, llsyl oxidase and plastocyanin). Nevertheless, its reactivity in these types of reactions requires that its intracellular abundance be controlled by regulated uptake and distribution so that toxicity is avoided. Copper metabolism has been studied extensively in Saccharomyces cerevisiae, where copper resistance mechanisms involving metallothioneins, copper uptake mechanisms involving reductases and transporters, and intracellular copper delivery pathways to cytosolic Cu/Zn superoxide dismutase, the Fet3p multicopper oxidase in the plasma membrane and cytochrome oxidase in the mitochondrion, have been identified (reviewed by Zhou and Thiele, 1993; Askwith et al., 1994; Eide, 1998; Winge et al., 1998; Pena et al., 1999). In S.cerevisiae, genes encoding the high-affinity copper transporters Ctr1p and Ctr3p, and the copper reductases Fre1p and Fre7p are induced by the transcriptional activator Mac1p in response to copper deficiency (Jungmann et al., 1993; Georgatsou et al., 1997; Labbe et al., 1997; Martins et al., 1998).

Conversely, when copper levels are high, a related protein, Ace1p, stimulates transcription of CUP1 and CRS5 encoding metallothioneins that sequester and detoxify excess copper (Thiele, 1988; Welch et al., 1989; Culotta et al., 1994). Copper homeostasis therefore involves a balance between the expression and function of the target genes of two transcription factors, Ace1p and Mac1p, and its purpose is to ensure an adequate but not excessive supply of intracellular copper for metabolic pathways requiring copper.

Much less is understood about the adaptation of copper-requiring metabolic pathways when intracellular copper is inadequate to saturate the biosynthesis of all copper proteins. Such a situation can occur when the nutritional supply of copper is too low (reviewed by Olives and Uauy, 1996) or when a genetic defect prevents normal copper assimilation, as in Menkes’ disease (reviewed by Danks, 1995). An excellent model system for the study of this aspect of copper biology is Chlamydomonas reinhardtii. This organism, like many green algae and cyanobacteria, displays a highly regulated adaptive response to copper deficiency that includes compensatory modification of a major metabolic pathway (reviewed by Merchant, 1998b). Plastocyanin, present at a stoichiometry of ~8 x 10^6 molecules per cell, is the most abundant copper protein in Chlamydomonas and in photosynthetic tissues of vascular plants (Merchant et al., 1991). Plastocyanin transfers electrons from the cytochrome (cyt) b(hf) complex to photosystem I (PSI) and hence is an obligatory catalyst in photosynthesis (Gorman and Levine, 1965). Nevertheless, when C.reinhardtii and other algae/cyanobacteria face copper deficiency (~9 x 10^6 Cu ions/cell for Chlamydomonas) in nature or in the laboratory, they remain photosynthetically competent by replacing plastocyanin function with the heme protein cyt c_6_ (Wood, 1978; Ho et al., 1979).

The processes induced in copper-deficient C.reinhardtii cells are: transcription of Cyc6 (encoding cyt c_6_) and Cpx1 (encoding the tetrahyrrole biosynthetic cytochrome coproporphyrinogen oxidase), degradation of apoplastocyanin and copper assimilation through a pathway involving a transporter and a cupric reductase (Hill and Merchant, 1995; Li and Merchant, 1995; Quinn and Merchant, 1995; Hill et al., 1996; Quinn et al., 1999). The induction of a transport pathway is preceded by the Mac1p-dependent induction of Ctr1p, Ctr3p, Fre1p and Fre7p in S.cerevisiae. The degradation of apoplastocyanin can be rationalized as a
mechanism for ensuring re-distribution of copper from the photosynthetic apparatus to the respiratory apparatus in the mitochondrion. Activation of Cpx1 expression has been attributed to an increased demand for heme, the cofactor of cyt e₉, in copper-deficient cells. The Cyc6 and Cpx1 genes are likely to be targets of the same copper-responsive signal transduction pathway (Quinn et al., 2000) and, based on the pattern of induction of the assimilatory pathway, it is proposed that so are the genes for the transporter and reductase (Hill et al., 1996). To identify these candidate target genes, and also to identify additional metabolic changes in copper-deficient cells, we sought a genetic approach to the study of copper deficiency in *C. reinhardtii*.

**Results**

**The CRD1 locus is defined by a novel phenotype**

To identify copper response regulators and additional copper-responsive genes besides Cpx1 and Cyc6, insertionally mutagenized cells were screened for copper-conditional phenotypes with the expectation that growth phenotypes might correspond to strains carrying either regulatory mutations or mutations in copper assimilation, while a photosynthesis defect might correspond to a strain in which Cyc6 was disrupted. Either ble (Stevens et al., 1996) or Arg7 (Debuchy et al., 1989) DNA was used for transformation, and 7.5 x 10⁶ zeocin-resistant and 4 x 10⁶ arginine prototrophs were screened after three transfers to +Cu versus –Cu plates. Colonies displaying growth defects were identified as expected, but an unexpected phenotype was also noted at high frequency regardless of whether ble or Arg7 DNA was used for transformation. Approximately 1 in 1 x 10³ transformants was yellow/pale-green in copper-deficient conditions but showed wild-type coloration under copper-supplemented conditions (Figure 1), suggesting that chlorophyll content was reduced. Indeed the strains contained less chlorophyll/cell when grown in copper-deficient conditions but as much as wild type when grown in the usual copper-supplemented medium (Figure 2A).

Nine independently generated strains were tested for: (i) allelism with the reference allele, crdl-1::ARG7 (Table 1); and (ii) linkage with the marker used for transformation. As no recombinants were found among the nine strains tested, we conclude that the mutants represent alleles at a single locus, named CRD1 for copper response defect. To examine linkage of the crdl phenotype with Arg7 or ble as appropriate, crdl-1 was crossed to arg7, and the other eight crdl-2–crdl-9 strains were crossed to the wild-type strain CC124. All 19 crdl progeny from the crdl-1 x arg7 cross were arginine prototrophs, indicating that crdl-1 is tightly linked to ARG7. Similarly, all progeny from crosses of crdl-1, crdl-5, crdl-6 and crdl-7 with CC124 that displayed the mutant phenotype were also resistant to 10 μg/ml zeocin, demonstrating that crdl is tightly linked to ble in these four strains. Thus, the CRD1 locus is tagged in five out of nine crdl strains.

The chlorophyll content in copper-deficient crdl strains was only 30–40% of that of equivalently grown wild-type cells, but the chlorophyll a/b ratio was unchanged in –Cu crdl cells (2.51) versus +Cu cells (2.47). Restoration of chlorophyll content to wild-type abundance required 100 nM CuSO₄ (Figure 2A). At this cell density (~1.5 x 10⁷ cells/ml), a concentration of 100 nM corresponds to 4.1 x 10⁶ Cu ions/cell, which is in the range required to repress Cyc6 and Cpx1 expression by 50% (4.5 x 10⁶ Cu ions/cell) (Merchant et al., 1991; Hill and Merchant, 1995). This result indicated that the CRD1 gene product was required only when Cyc6 and Cpx1 were expressed, i.e., when the cells perceived a copper deficiency, and suggested that the expression of wild-type CRD1 might be regulated by copper—specifically, that it might be a target of the same signal transduction pathway as the one controlling Cyc6 and Cpx1 expression.

Mercuric ions at high concentrations can mimic copper ions in turning off the Cyc6 and Cpx1 genes (Hill et al., 1991; Quinn et al., 2000). If the phenotype resulted from a gain of function in copper deficiency or if expression of the phenotype required the activation of the copper deficiency pathway, it might be rescued by the addition of mercuric ions. However, addition of 10 μM HgCl₂, which is enough to repress the Cyc6 and Cpx1 genes completely, did not restore chlorophyll accumulation in –Cu crdl cultures (Figure 2B). It is rather more likely that the phenotype results from loss of a biochemical function that is required or becomes important only in copper-deficient cells.

Is the chlorotic phenotype a direct or indirect consequence of copper deficiency? We considered two possibilities: first, that the phenotype resulted from a secondary iron deficiency because iron-deficient *C. reinhardtii* cells are chlorotic (Weger, 1999); and, secondly, that the phenotype resulted from bleaching as a consequence of a photosynthesis defect (Spreitzer and Mets, 1981). In *S. cerevisiae* and also in humans, copper is required for
iron assimilation because it is a cofactor in a multi-copper oxidase involved in iron transport and distribution (Askwith et al., 1994; Harris et al., 1995). Addition of up to 1.5 mM FeSO₄ to –Cu cultures of crdl1 cells does not restore chlorophyll accumulation, indicating that the loss of chlorophyll in crdl1 strains is not a consequence of defective iron uptake (Figure 2C). The phenotype of crdl1 strains was also tested in dim light (5 μmol/m²/s) and in darkness. Under these conditions, both copper-deficient and copper-supplemented crdl1 cells grew at equal rates, but the –Cu cultures still accumulated less chlorophyll (data not shown). At light intensities of 100 μmol/m²/s, crdl1 strains also showed a growth phenotype, indicating that the growth phenotype, but not the chlorophyll accumulation phenotype, is a consequence of photosensitivity.

A photosynthesis defect in crdl1 strains in either copper- or oxygen-deficient medium

Photosynthetic function was examined by monitoring the fluorescence induction and decay kinetics of crdl1 strains grown on +Cu versus –Cu medium (Figure 3). In the wild type, the transient rise phase reflects the reduction of a particular plastoquinone, QA, in PSII, while the decline phase reflects its reoxidation by downstream electron carriers. Wild-type cells display similar fluorescence in both +Cu and –Cu, but the decay phase is not observed in crdl1 strains when they are grown on –Cu medium, indicating a defect downstream of PSII. Is the photosynthetic block attributable to a defect in chlorophyll accumulation? The pe-1 mutant that has lost the light-dependent protochlorophyllide reductase has a chlorophyll content similar to that of copper-deficient crdl1 strains, ~40% of the amount in wild type (Li and Timko, 1996). However, pe-1 displays wild-type fluorescence in both copper-supplemented and copper-deficient cells. The photosynthesis defect in –Cu crdl1 cells is therefore a specific defect rather than a consequence of reduced chlorophyll content. We also did not find any alterations in the expression of a number of genes functioning in the tetrapyrrole biosynthetic pathway (Figure 6).

The Cyc6 and Cpx1 genes respond not only to copper deprivation but also to oxygen deprivation (Quinn et al., 2000). If Crdl1 is a target of the same pathway, one might expect to induce the crdl1 phenotype in oxygen-depleted cultures. Chlamydomonas reinhardtii cells grown in low light become oxygen depleted if they are kept suspended by low basal stirring instead of by vigorous aeration (Wood, 1978; J.Quinn, unpublished). Under these conditions, crdl1 strains become chlorophyll deficient, and when such hypoxic cells are transferred to aerated medium, chlorophyll accumulation is restored, indicating that the wild-type Crdl1 gene is also required in oxygen-deprived cells (Figure 4A). Induction of the crdl1 phenotype was verified as being due to O₂ depletion (as opposed to

Table I. Tight linkage of independently generated crdl1 mutants

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<td>0/6</td>
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<tr>
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<td>0/44</td>
<td>0/48</td>
<td>0/42</td>
<td>0/70</td>
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A mating type minus crdl1-1 reference strain was crossed to mating type plus crdl1 test strains. Zygotes were germinated and spores were transferred three times to –Cu TAP plates. Segregation of the crdl1 phenotype was scored visually (chlorophyll deficiency) and by fluorescence. The first row shows the number of complete tetrads analyzed, while the second row shows the recombinants out of the total number of spores analyzed. The number of zygotes dissected is shown in parentheses.
Fig. 3. crdl strains display mutant fluorescence induction and decay kinetics. Fluorescence transients of cells grown on ±Cu TAP plates. Representative traces are shown from crdl-1 and crdl-2. wt = CC124. The pc-1 mutant lacks the light-dependent protochlorophyllide reductase.

depletion of another gas such as CO₂) by showing that bubbling with a mixture of 2% air, 2% CO₂ in N₂ also induces chlorosis (J. Quinn, unpublished).

The copper-conditional block in electron transfer in crdl cells suggested that cyt c₅₅ synthesis or accumulation might be affected, although loss of function of the Cyc6 gene seemed unlikely because mutations affecting this step in photosynthetic electron transfer (as in pcy1 or ccs strains) do not result in such drastic reductions in chlorophyll content (Howe and Merchant, 1992; Li et al., 1996). Indeed the abundance and expression of cyt c₅₅ and coprogen oxidase is normal in crdl cells (Figures 4B and 5A). We conclude that the crdl phenotype does not result from disruption of the Cyc6 gene or a putative regulator of copper-responsive expression.

Gel electrophoretic analysis of total cellular protein showed that copper-deficient crdl cells do not accumulate the PSI-A/PSI-B polypeptides of PSI and contain reduced amounts of light-harvesting complex I (LHCI) and LHCII polypeptides (Figure 5B). Immunoblot analysis confirmed that the abundance of PSI-A and PSI-F is drastically reduced in –Cu crdl cells, as is also the case for the LHCI polypeptides between 20 and 30 kDa (Figure 5C). The major polypeptides of the LHCII trimeric complex accumulate to ~10% of wild-type levels, accounting for the severe chlorophyll deficiency. On the other hand, the abundance of the D1 protein of PSII is only slightly reduced, and the accumulation of other photosynthetic complexes is normal (as gauged by the abundance of cyt f, Rieske iron–sulfur protein and the α- and β-subunits of ATP synthase). We conclude that the primary electron transfer defect in copper-deficient crdl cells is attributable to a PSI deficiency. The unusual phenotype of crdl strains points to a hitherto unrecognized role for copper in PSI maintenance. Since both nucleus- and plastid-encoded proteins are affected in –Cu crdl cells, we suspect that the mutation affects photosynthetic complex accumulation at a post-transcriptional level. RNA blot analysis indicates that the expression of genes encoding LHC and PSI subunits is not reduced in –Cu crdl relative to the wild type; in fact, for psaB and CabII, it is enhanced in the mutant (Figure 6).

Crdl1 is a previously unknown protein found in photosynthetic organisms

Since five of the nine well characterized crdl strains appeared to be tagged, we sought to clone Crdl based on its linkage to the marker genes. Southern analysis of genomic DNA from crdl-7 revealed that the ble gene is present on a 6.5 kb BamHI fragment in this strain (Figure 7A). This fragment was amplified by inverse PCR using divergent primers complementary to sequences within ble. Southern blots with the PCR product as a probe revealed restriction fragment length polymorphisms (RFLPs) between wild-type strains and several crdl alleles, confirming that the amplified DNA represented sequences that closely flanked the Crdl1 gene. A 3 kb DNA fragment of the amplified product was used to probe a wild-type genomic library. Three overlapping λ clones were identified, and each of these complemented the crdl
phenotype when introduced into the crdl-6 background (Figure 7B). A 5.8 kb SalI fragment of λ clone C1 was subcloned (pCrd1-5) and shown to be adequate for rescue of both the chlorophyll deficiency and the non-photosynthetic phenotype of crdl-6 (data not shown). This fragment therefore contained a functional Crdl gene. A 1.2 kb PstI-HindIII fragment of pCrd1-5 was subsequently used to isolate two cDNA clones containing the entire Crdl coding region from a −Cu cDNA library (Merchant and Bogorad, 1987a).

The C. reinhardtii Crdl gene encodes a 47.2 kDa soluble polypeptide in a 2.6 kb sequence (Figure 8). The protein occurs in the genomes of plants [>80 expressed sequence tags (ESTs) in >9 species], chromophytic algae, cyanobacteria, a prochlorophyte and a purple photosynthetic bacteria, but not so far in non-photosynthetic organisms (up to March 2000). Its function is not known. A homolog was identified originally as a phytocrome-regulated gene in Pharbitis nil (Zheng et al., 1998). A multiple alignment of the deduced Chlamydomonas protein and the candidate homologs demonstrates that the proteins are highly conserved, with 79% identity between the rice and Arabidopsis sequences, and 40–70% identity between all other pairs (Figure 9A).

Crdl expression is activated in −Cu cells
Is Crdl expressed coordinately with Cyt6 and Cpx1? RNA blot analysis reveals that in +Cu wild-type cells, Crdl mRNA accumulates at a low, basal level and is induced 15- to 20-fold in −Cu cells (Figure 10). Crdl expression is activated similarly in oxygen-deficient cells (data not shown). As expected, Crdl mRNA is not detected in the crdl mutant. This induction of Crdl in Cu- and O₂-deficient cells suggests an increased requirement for the gene product under these conditions, consistent with the crdl mutant phenotype.

Discussion
Copper-deficient cells require a new protein, Crdl, for normal photosynthesis. The unexpected phenotype of the crdl strains reveals: (i) an unrecognized role for copper in photosynthesis; and (ii) an unrecognized biochemical function in maintenance of PSI and LHCs.

Crdl and PSI
The phenotype of crdl strains, namely a pleiotropic deficiency in PSI and light-harvesting proteins, should provide a clue to Crdl function. For instance, Crdl could be a novel subunit in a photosynthetic complex that is modified in response to copper and oxygen availability. This is preceded by the occurrence of alternative isoforms of respiratory complex subunits in Saccharomyces mitochondria (reviewed by Kwart et al., 1998). Different isoforms of cytochrome oxidase subunits (encoded by COX5a/COX5b) and cytochrome c (encoded by CYC1/CYC7) are expressed in oxygen-replete versus oxygen-deficient S. cerevisiae cells. A similar phenomenon occurs in response to iron deficiency in cyanobacteria, where the CP43 polypeptide encoded by isiA is induced to high levels (Laudenbach and Stras, 1988; Burnap et al., 1993; Leonhardt and Stras, 1994; Vinnemeier et al., 1998). Interestingly, the organellar and prokaryotic Crdl

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Fig. 5. The crdl mutant shows (A) normal regulation of plastocyanin, cytochrome c₅₅ and coprogen oxidase abundance but lacks PSI polypeptides (B and C). (A) Immunoblot analysis of cyt c₅₅, plastocyanin and coprogen oxidase abundance. Liquid cultures in −Cu TAP of a wild-type (cc425:ARG7) strain and crdl-1::ARG7 were grown for 8 days at 21°C, 200 r.p.m., 150 μmol/m/s constant light. Ten microliters of soluble protein extract from equal numbers of cells were separated by denaturing electrophoresis (15% acrylamide) and analyzed by immunoblotting. (B) Accumulation of chlorophyll-containing proteins in crdl versus wild type. Total cell proteins were separated by denaturing electrophoresis (12% acrylamide) and stained with Coomassie Blue R250. Whole-cell extracts were made from equal numbers of cells, and samples were loaded without heating prior to electrophoresis to prevent dissociation of the PSI-A and PSI-B polypeptides. Asterisks indicate the positions of the green PSI and LHC subcomplexes prior to staining. Molecular weights (in kDA) are indicated. (C) Abundance of complex-specific proteins in crdl. Proteins in whole-cell extracts of a wild-type (arg7:Crdl) strain and a crdl-1 spore were separated by denaturing electrophoresis (12% acrylamide) and analyzed by immunoblotting. Whole-cell extracts were made from equal numbers of cells, and samples were boiled for 45 s prior to loading.
homologs are found in clusters of genes encoding reaction center proteins. Thus, Crd1 could be an alternative copper and oxygen deficiency version of a PSI or LHC subunit, and hence its absence in –Cu crdl cells could result in destabilization of these complexes. There are numerous examples of multisubunit complexes that are degraded if one of the constituent polypeptides is not synthesized (reviewed by Wollman et al., 1999). However, we do not think that this is the situation here. The biosynthesis and maintenance of C. reinhardtii PSI is well characterized (reviewed by Webber and Bingham, 1998), and LHC deficiency has not been observed in strains carrying deletion mutations in the core polypeptides of PSI (Girard-Bascou et al., 1987; Takahashi et al., 1991). Likewise, mutants affected in LHC abundance do not show a linked PSI defect (Plumley and Schmidt, 1995). Finally, the protein and pigment compositions of the reaction center and light-harvesting apparatus differ significantly between purple photosynthetic bacteria and PSI in the oxygenic organisms (cyanobacteria, algae and plants), precluding a specific structural function for Crd1. Instead, it is more likely that Crd1 and its homologs function in a metabolic pathway that is common to each organism and is important for photosynthetic function.

The nature and severity of the crdl phenotype are dependent on the growth conditions. For example, growth of –Cu crdl strains at lower temperatures (18°C) partially relieves the chlorophyll deficiency, but does not improve PSI accumulation. In minimal medium the defect is also less pronounced; indeed crdl cells do grow photoautotrophically (at 50–100 μmol/m²s), indicating that they must contain some PSI. Conversely, cells grown at higher temperatures (25°C) display chlorosis more readily, and the fluorescence transients indicate a reduction in PSII function as well (data not shown). The variation in phenotype can be attributed to a difference in the growth rate, as if faster growth exacerbates the phenotype and slower growth ameliorates it. This result is compatible with a defect in cofactor metabolism in crdl. Perhaps the rate of cofactor production becomes more limiting when cell division is occurring more rapidly.

Crd1 function

What does the Crd1 sequence reveal about its function? Two of the most highly conserved sequences, WCQDENRHGD and SRDEARHAG, separated by ~80 amino acids in the Crd1 proteins, are also present in plastid stearoyl-ACP desaturases (reviewed by Los and Murata, 1998; Shanklin and Cahoon, 1998) and a stearoyl-ACP desaturase from Burkholderia cepacia (Kang et al., 1998), respectively. These sequences contain consensus D₁ExxH motifs for the carboxylate-ligated di-iron-binding class of enzyme. The prototypical members of this class are the acyl-ACP desaturases, the R2 subunit of ribonucleotide reductase and methane monoxygenase, and the characteristic feature of these enzymes is that the Fe ions are bridged by carboxylates and oxide or hydroxide ions. Two D₁ExxH motifs, usually separated by ~100 amino acids, provide two carboxylate and two imidazole ligands for the di-iron cluster. Di-iron enzymes catalyze oxidation of a substrate by activation of molecular oxygen at the di-iron cluster. They also require a source of electrons for reducing at least one of the oxygen atoms to water. For the plastid desaturases, reduced ferredoxin provides the electrons. In all Crd1 homologs, the D₁ExxH motifs are separated by the right distance and occur in highly conserved regions of the proteins (Figure 9A). Therefore, we propose that Crd1 is a new member of the di-iron-carboxylate-ligated class of oxygen-dependent oxidases.

What might its substrate be? The best characterized plant di-iron enzymes are the plastid acyl-ACP desaturases, but we doubt that Crd1 is a fatty acid desaturase.
Fig. 7. (A) Physical map of the ble insertion in crd1-7 and diagram of the cloned flanking DNA. Probes from the ble gene and the RbcS2 3’-UTR from pSPI09 were used to map restriction digests of genomic DNA from crd1-7. The divergent oligonucleotide primers A3 and A4 were used to amplify an ~0.6 kb BamHI fragment containing the inserted ble gene. This fragment was digested with BamHI and HindIII and a 3 kb fragment was cloned into pBluescript KS+ to create plasmid pCrd1-1. B. BamHI; H, HindIII; S, SalI; medium gray boxes, RbcS2 sequences; dark gray boxes, ble sequences. (B) Genomic clones corresponding to Crd1. The 3 kb BamHI-HindIII insert in pCrd1-1 was used to probe a genomic DNA library. DNA from overlapping λ clones C1, 11 (not shown) and N1 that hybridized to the probe were isolated and subcloned into pBluescript KS+ to create plasmids pCrd1-3, pCrd1-4, pCrd1-5 and pCrd1-7. P. SspI. Hatched boxes, EMBL3 left and right arm sequences. λ clones C1, 11 and N1, and plasmids pCrd1-3, pCrd1-4, pCrd1-5 and pCrd1-7 were co-transformed by the glass bead method with pARG7.8 into an arg2crd1-6 strain and arginine prototrophic colonies were selected on –Cu TAP plates (Kiddle, 1990). Colonies in which the crd1 phenotype was rescued were chosen visually and by fluorescence. The column on the right indicates whether or not the DNA fragment shown rescues the crd1 phenotype. ‘Yes’ indicates positive complementation, ‘No’ indicates that the fragment did not rescue.

because there is no similarity between Crd1 and four different Chlamydomonas desaturases. Another possibility is that it might be involved in the biosynthesis of an as yet unidentified xanthophyll. This model is attractive because of the structural roles of carotenoids and xanthophylls in the photosystems and LHCs. However, a lor1 npq2 double mutant, which is blocked in the production of the most abundant xanthophylls (presumed precursors for other xanthophylls) (Niyogi et al., 1997, 1998), does not display –Cu induced chlorosis. Hence, the phenotype is unlikely to be caused by a xanthophyll deficiency. A third option is that Crd1 might be involved in some aspect of quinone modification or in generation of a radical in PSI. While we cannot rule out these models, they are inconsistent with the occurrence of Crd1 in Rhodobacter sphaeroides (which has a completely different complement of quinone cofactor and a different type of photosynthetic reaction center). A fourth possibility is that Crd1 function lies in metabolism of a metal cofactor. We favor this model and suggest further that this metal cofactor might be iron because the phenotype of crd1 strains is strikingly similar to that of iron-deficient cells (J. Moseley, unpublished).

Although we could not rescue the crd1 strains by provision of extracellular iron, it is possible that the defect is compartment specific (i.e. in the plastid). Alternatively, the defect may not lie in supply but in mobilizing iron for biosynthesis of iron-containing proteins.

In this context, we propose that Crd1 and its homologs are plastid localized. In at least two eukaryotes, the homologous gene is plastid encoded, indicating that in these organisms the Crd1-like protein functions in the plastid. Examination of the C. reinhardtii and vascular plant sequences reveals an N-terminal extension relative to the cyanobacterial and plastid-encoded sequences, supporting the idea that the nucleus-encoded proteins may be targeted to an organelle. Characterization of the crd1 phenotype also suggests a plastid site of action. Specifically, we note reduced accumulation of both nucleus- and plastid-encoded gene products at a posttranscriptional level (Figures 5 and 6).

**Response to copper and oxygen deficiency**

The induction of cyt c6 in copper-deficient algal cells has been understood for over two decades as a back
Fig. 8. Crlf genomic and cDNA Crlf sequences have been submitted to the DDBJ/EMBL/GenBank database under accession Nos AF226628 and AF237617, respectively. A possible TATA box is indicated (single underline). A putative transcription start site (→) is suggested based on the 5' UTR end of a Crlf cDNA clone. Intron–exon boundaries are indicated by (⊥). An asterisk denotes the stop codon, and the TGTTA polyadenylation signal (double underline) was found 15 bp upstream of the Crlf cDNA poly(A) sequence.
A

up mechanism to ensure photosynthetic function when plastocyanin cannot be synthesized (Wood, 1978; Merchant and Bogorad, 1987b). Molecular characterization of copper-deficient C. reinhardtii cells revealed a number of other responses that occur coordinately with activation of the Cyc6 gene, and these have each been rationalized as mechanisms for adaptation to copper deficiency (see Introduction). Recently, we noted that two of the targets of the signal transduction pathway, Cyc6 and Cpx1, are also induced in hypoxic cells (Quinn et al., 2000). The significance of the response to hypoxia is not well understood. One model, proposed initially by Wood (1978), is that oxygen-deficient cells become copper deficient in nature because of the reduced solubility of Cu(I) relative to Cu(II), and we have argued that the response to oxygen might be a way for the organism to

B

Fig. 9. (A) Conservation of Crdl. The alignment was generated using the CLUSTALV algorithm and LaserGene software (DNASTAR). Residues that are similar in a majority (six) of sequences are shaded gray. Residues that are identical in all sequences are shaded black. The SRDEARHAG and WQENRHGD motifs that are conserved in stearoyl-ACP desaturases are double-underlined and the prospective Fe-binding ligands are indicated by a downward arrow. The DDBJ/EMBL/GenBank accession numbers of the sequences from Oryza sativa, Arabidopsis thaliana, Porphyra purpurea, Cyanidium caldarium, Synechocystis sp. 6803 PN1934, Synechocystis sp. 6803 AT103 and Rhodobacter sphaeroides are, AP000815, AF236101, U38804, AFO22186, D90899, D90912 and AF195122 respectively. Anabaena sp. PCC 7120 sequences were retrieved from the Cyanobase database at http://www.kazusa.or.jp/cyan/anabaenan. The Prochlorococcus marinus homolog was found at the Prochlorococcus sequencing homepage at http://www.bbrp.lnl.gov/ggi/microbial/prochlorococccushomepage.html. The homolog from Phaeodactilus nld is not shown as we believe that there is a frameshift error in the 5' end of the cDNA sequence (DDBJ/EMBL/GenBank accession No. U37437). This introduces a stop codon between the probable true initiator ATG and the next ATG in the sequence, resulting in elimination of the N-terminal leader sequence in the derived open reading frame. (B) The translated sequence of Cbl1, a partial Chlamydomonas EST (DDBJ/EMBL/GenBank accession No. AV391947), is similar to the N-terminal region of Crdl.

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anticipate and prepare for copper deficiency in the natural environment (Quinn et al., 2000). Nevertheless, in the laboratory, oxygen-deficient cultures are not copper deficient because they can accumulate holoplastocyanin and provision of excess copper does not repress the hypoxic response (Figure 4B; I. Quinn, unpublished), suggesting that the oxygen deficiency response pathway might operate in parallel to the copper deficiency response pathway rather than through it. The crd1 strains were identified on the basis of a copper-conditional phenotype, and this is phenocopied under hypoxia. This result provides the first indication of a physiological function of the oxygen-responsive signal transduction pathway. The fact that the crd1 mutant phenotype occurs in hypoxic cells tells us that the Crd1 gene product and hence gene expression is required for normal adaptation to O2 deprivation, whereas the induction of the Cyl6 and Cpx1 genes has not yet been shown to be necessary. Furthermore, it suggests a functional link between copper- and oxygen-responsive signal transduction.

How can we rationalize the requirement for Crd1 function in copper-deficient and oxygen-deficient cells? Crd1 expression resembles that of Cpx1, with a basal level of RNA accumulation in copper-supplemented cells and a 15- to 20-fold increase in copper and oxygen deficiency (Figure 10). One possibility is that there is an increased requirement for Crd1, an oxygen-utilizing enzyme, under oxygen-deficient conditions, as is the case for coprogen oxidase in S. cerevisiae (reviewed by Kwast et al., 1998). Alternatively, Crd1 could be the iron substitute for a copper enzyme (comparable to the situation where heme-containing cyt c6 replaces copper-containing plastocyanin). This idea is preceded by the reciprocal copper-responsive expression of iron- versus copper-containing methane monoxygenase in methanotropic bacteria (Nielsen et al., 1997). The former is a di-iron enzyme while the latter is a multi-copper oxidase.

Crd1 distribution and expression
Crd1 homologs are found exclusively in photosynthetic organisms—plants, cyanobacteria, photosynthetic bacteria and algae (March 2000 database) (Figure 9A). A vascular plant homolog of Crd1, PNZIP, was identified originally as a phytochrome-responsive target gene. Its expression is under circadian control and is restricted to photosynthetic tissue (Zheng et al., 1998), but its function was not tested experimentally. Based on the distribution and expression of Crd1 homologs, their highly related sequences and the phenotype of crd1 mutants, we would argue that they all have a similar biochemical function, probably in assembly of components of the photosynthetic apparatus.

Interestingly, multiple homologs of Crd1 are found in C. reinhardtii, Synechocystis sp. and Anabaena sp. (~50–65% identity for homologs in the same species); each of these organisms has a signal transduction pathway for responding to copper deficiency and each replaces plastocyanin with cyt c6 (reviewed by Merchant, 1998a). Possibly one copy of the gene has evolved a specialized pattern of expression that is important primarily for adaptation to copper deficiency, while the other has a so-called ‘housekeeping’ function similar to that of Crd1 homologs in vascular plants. A search of the CyanoMutants database for Synechocystis sp. knockouts indicates that one of the disrupted genes (DDBJ/EMBL/GenBank accession No. D90899) could not be segregated, which is consistent with the possibility that the gene might be essential. However, no phenotype is reported for the disruption of the other gene (DDBJ/EMBL/GenBank accession No. D90912). Perhaps the latter gene encodes the functional equivalent of C. reinhardtii Crd1—the strain might well show a phenotype if tested in copper-deficient medium.

In Chlamydomonas, Crd1 is expressed at a very low level in cells grown in copper-supplemented acetate medium, but is highly induced in copper deficiency, consistent with the model that its function is more important when copper is limiting. Accordingly, the cells show a strong phenotype in -Cu medium. A Crd1 homolog, which we have called Cth1 (for copper-target homolog) was found in the Chlamydomonas EST database (Figure 9B, 47% nucleotide identity, 599 bp and 51% amino acid identity), but ESTs corresponding to Crd1 were not found. The ESTs represent sequences expressed under photautotrophic, copper-supplemented conditions (Asamizu et al., 1999). Preliminary results indicate that expression of Cth1 is not activated in -Cu cells (J. Moseley, unpublished); perhaps Cth1 has the housekeeping function. The unique patterns of expression of Crd1 and Cth1 (which are suggestive of a different level of requirement for Crd1 and Cth1 function under various metabolic conditions) could well explain the variation in penetrance of the phenotype under different growth regimes. The Crd1/Cth1 gene pair in C. reinhardtii undoubtedly arose as a result of gene duplication, and disruption of Crd1 occurred at an unusually high frequency during our mutagenesis (1 per 1000 transformants), suggesting that recombination events are favored at the CRD1 locus. Perhaps in microorganisms faced with highly variable environments, possession of multiple Crd1 homologs has provided a means of adapting expression of the genes to different conditions.

Materials and methods
Strains and culture conditions
Mutants were generated in the background of Chlamydomonas strain CC425 (arg2, cwl15) and crossed as described below. The preparation of
copper-deficient (−Cu) media for culturing C. reinhardtii strains is described in Quin and Merchant (1998). Cultures were transferred three times to −Cu medium from a +Cu inoculum in order to eliminate residual intracellular copper. Copper-supplemented medium was either prepared by the standard method (Harris, 1989) or copper salts were added to −Cu medium at the concentrations indicated. Iron was added from a 100 mM copper-free stock solution of chelated FeSO₄ that was prepared by boiling 100 mM FeSO₄ (Aldrich) with 134 mM EDTA in an acid-washed bottle. The pH was adjusted to 6.5 with KOH while maintaining the temperature above 70°C, and the solution was left at −20°C for 2–7 days prior to use. Mercuric salts were added from a stock solution (10 mM) to the final concentration indicated. Liquid cultures were grown usually in 125 or 250 ml Erlenmeyer flasks at 21–24°C with agitation at 200–250 r.p.m. and light intensity between 50 and 100 µmol/m²s. Strains were cultured on plates at either 18 or 25°C at light intensities between 5 and 100 µmol/m²s. To generate O₂-deprived cultures, 250 ml Erlenmeyer flasks filled with 200 ml of copper-supplemented TAP medium were inoculated to an initial cell density of 1 × 10⁶ cells/ml and agitated by slow stirring on a magnetic stirring plate set at the lowest possible speed. Ambient temperature was 20–24°C and the light intensity was −10 µmol/m²s. Samples were collected for analysis after 3 days of growth.

Chlorophyll determination
Cells were collected from duplicate 1 ml aliquots of the culture by centrifugation (14 000 r.p.m. in a microcentrifuge), chlorophyll was extracted from the pellet into 80% acetone in methanol and its concentration was determined spectrophotometrically after removal of protein by centrifugation. Chlorophyll a/b ratios were determined according to the method of Porra et al. (1989).

Fluorescence measurements
A fluorescence imaging system built by Professor David Kramer at the Center for Advanced Biochemical Instrumentation at the University of Chicago (Washington State University, Pullman, WA) was used to measure the in vivo chlorophyll fluorescence induction kinetics of colonies. Prior to fluorescence measurements, cells grown on solid TAP medium were incubated overnight at 18°C at 10–25 µmol/m²s and dark adapted for at least 5 min. The colonies were illuminated for 1.73 s with 30 µmol/m²s of red light (640 nm) from a light-emitting diodes (HPML C116, Hewlett Packard) and the emitted fluorescence was captured immediately by a CCD camera (Coolcam, 2122–1000) in conjunction with a PEPIX-SV4 imaging board and XCIP software (EPIX Incorporated, Buffalo Grove, IL).

Protein analysis
Samples from equal numbers of cells were prepared and analyzed for accumulation of plastocyanin, cyt e and coproporphyrin oxide as described previously (Quin and Merchant, 1998; Pfeifer et al., 1999). The samples were usually transferred to PVDF membranes for 1 h at 100 V in blotting buffer containing 20% methanol. The following solutions were used for probed: the blots, wash, Tris-buffered saline with 0.2% Tween-20 (TBS-Tween); block, 30% calf serum in TBS-Tween, primary antibody in 10% calf serum in TBS-Tween. The antibodies were used at the following dilution: anti-plastocyanin 1:10 000, anti-cyt e 1:5000, anti-coproporphyrin oxide 1:3000 and horseradish peroxidase-conjugated goat-antibody IgG (Biorad) at 1:50 000. The blots were washed three times after each incubation. Bound antibody was detected with chemiluminescent reagents. For analysis of membrane proteins, samples were prepared similarly to the soluble proteins, except that whole cells were used and samples were only boiled for 45 s to avoid aggregation of the PSI core polypeptides. The separating gel contained 12% total acrylamide and the standard Tris-glycine blotting buffer contained 0.01% SDS in addition. Proteins were transferred to PVDF membranes for 2 h at 50 V. Blots were incubated overnight with primary antibodies as follows: 1:1000 anti-PSI-A, 1:1000 anti-PSI-F, 1:1000 anti-PS-1-LHCl, 1:5000 anti-D1, 1:800 anti-LHCII, 1:1000 anti-cytochrome f, 1:100 anti-Reiske iron–sulfur protein and 1:5000 anti-CP. A 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates) was used as the secondary antibody, and bound antibody was detected using the alkaline phosphatase color reaction as described (Sambrook et al., 1989).

RNA blot analysis
RNA abundance was estimated by blot analysis as described previously (Quinn et al., 1999). For psbA, a 1 kb BamHI fragment from the EcoRI fragment of chloroplast DNA in plasmid p67A (Chlamydomonas Culture Collection) was used as the probe. The CabI1 coding probe and the CabI1 3’-untranslated region (UTR) probe are described in Jacobshagen and Johnson (1994). For Cps1, the probe was made from the 102 bp HindIII fragment from pCpxl.10 (Quinn et al., 1999). The Crdl probe was made from a 1.2 kb PstI-XhoI fragment of pCrdl-1. The other probes are described in Quinn et al. (1999). The specific activities ranged from 5 × 10⁶ to 7 × 10⁶ c.p.m./µg DNA. Hybridization signals were visualized after exposure to Kodak X-OMAT Blue XB-1 film at −80°C with two intensifying screens (Alad, Cycb, Cpx1, Crdl1, Gsa, Gsr and Ppxl) or at room temperature without screens (psbA and RbcS2). Exposure times (Figure 6) were 2.5 h for Alad, Cycb, Cpx1 and Gsa, 24 h for Gsr and Ppxl. 1 h for psbA and RbcS2, and (Figure 10) 16 h for Crdl1 and 45 min for RbcS2.

Genetic analysis
Generation of gametes, matings and zygote dissections was performed as described by (Harris, 1989). For linkage analysis, at least six complete tetrad or 12 crdl progeny were scored for segregation of the mutant phenotype with arginine prototrophy (crdl1-1’argin7) or resistance to 10 µg/ml zeinocin (Stevens et al., 1996) (crdl1-2–crdl1-9’CC121). Alleles crdl1–2–crdl1-9 were each crossed to crdl1-1 to test for recombination.

Inverse PCR
Genomic DNA from strain crdl-7 was prepared by a modified miniprep procedure and 5 µg of DNA was digested [6–10 h, 37°C, 100 U of BanHI (NEB)]. The restriction enzyme was denatured (80°C, 20 min), and the DNA was ligated [overnight, 15°C, 450 µl volume containing 1× ligase buffer (Gibco-BRL), 1 mATP and 2 µU of T4 DNA ligase (Gibco-BRL)]. The DNA was extracted with phenol–chloroform–isoamyl alcohol (25:24:1), chloroform, recovered by precipitation with ethanol and dissolved in 10 µl of water. A 6.5 kb inverse PCR product was obtained by amplification of 2 µl of ligated DNA with divergent primers A3 (GAAGTCGTCTCCACCGAGAAGT) and A4 (GAGGTGACCTTTGATTCCATCAG) complementary to sequences within the ble gene (Stevens et al., 1996) and 7 psf polymerase from Gibco-BRL.

Cloning and sequencing of Crdl
A 3 kb BanHI–HindIII fragment of the inverse PCR product was cloned into pBluescript KS+ (Stratagene) and used to probe a Chlamydomonas genomic library in λEMBL3 by plaque hybridization. Three clones whose inserts contained overlapping DNA were obtained, each of which complemented the crdl phenotype of an arg2crdl-6 strain when introduced into the nuclear genome along with the Arg7 gene by glass bead transformation. The inserts were subcloned into pBluescript KS+ (Stratagene) to generate plasmids pCrdl-3, pCrdl-4, pCrdl-5 and pCrdl-7 (Figure 7B), and the smallest complementing fragment was identified (pCrdl-5). The presence of introduced copies of Crdl in rescued strains was verified by PCR amplification using primers p9 (CCGTGATCAATGCTCCCGC) and pCrdl (AGCTCCTGGTACAGG) within the Crdl sequence that flank the site of ble insertion. The sequence of a 4.2 kb region within the 5.8 kb genomic DNA in pCrdl-5 spanning the Crdl gene was determined on both strands, except for the last 75 bp, which were sequenced twice on one strand. A 1.2 kb PstI-XhoI fragment of pCrdl-5 was used to screen a Chlamydomonas –Cu cDNA library in KABAC by plaque hybridization, and two Crdl1 cDNA clones containing the entire coding sequence were isolated. The sequence of the gene product was deduced by alignment of the translated sequence with the sequences of candidate Crdl1 homologs in the database, by prediction of intron–exon boundaries based on the Chlamydomonas gene consensus (Silflow, 1998) and by comparison of the genomic and cDNA sequences. The BLAST algorithm was used to search the nucleotide and protein databases, and sequence alignments were generated with CLUSTAL V in the Lasergene software package (DNASTAR).

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