

# 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<sup>1</sup>

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

<sup>1</sup>Corresponding author  
e-mail: merchant@chem.ucla.edu

*Chlamydomonas reinhardtii* adapts to copper deficiency by degrading apoplastocyanin and inducing *Cyc6* and *Cpx1* encoding cytochrome *c*<sub>6</sub> and coproporphyrinogen oxidase, respectively. To identify other components in this pathway, colonies resulting from insertional mutagenesis were screened for copper-conditional 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, lysyl 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 Olivares 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  $\sim 8 \times 10^6$  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*<sub>6</sub>*l**f* 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 ( $< 9 \times 10^6$  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*<sub>6</sub> (Wood, 1978; Ho *et al.*, 1979).

The processes induced in copper-deficient *C.reinhardtii* cells are: transcription of *Cyc6* (encoding cyt *c*<sub>6</sub>) and *Cpx1* (encoding the tetrapyrrole biosynthesis enzyme 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 c<sub>6</sub>*, 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*.

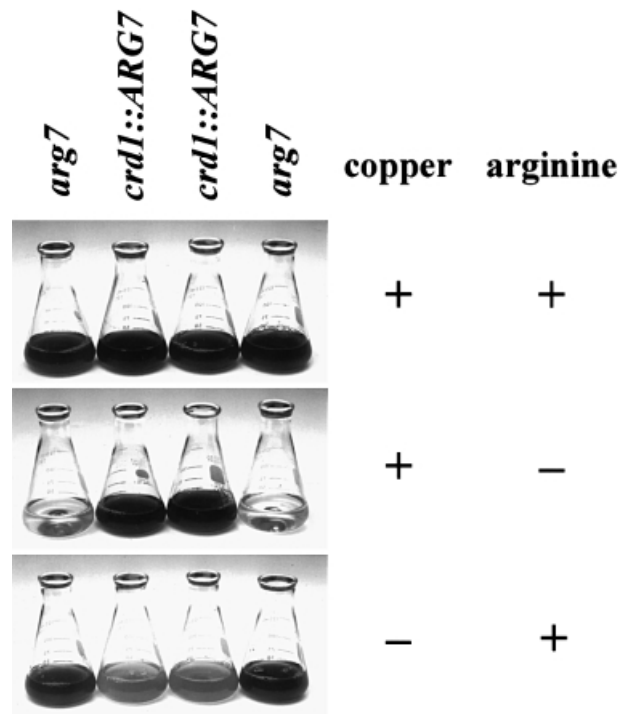
## Results

### 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  $7.5 \times 10^3$  zeocin-resistant and  $4 \times 10^3$  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  $1 \times 10^3$  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, *crd1-1::ARG7* (Table I); 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 *crd1* phenotype with *Arg7* or *ble* as appropriate, *crd1-1* was crossed to *arg7*, and the other eight *crd1-2*–*crd1-9* strains were crossed to the wild-type strain CC124. All 19 *crd1* progeny from the *crd1-1* × *arg7* cross were arginine prototrophs, indicating that *crd1-1* is tightly linked to *ARG7*. Similarly, all progeny from crosses of *crd1-2*, *crd1-5*, *crd1-6* and *crd1-7* with CC124 that displayed the mutant phenotype were also resistant to 10 µg/ml zeocin, demonstrating that *crd1* is tightly linked to *ble* in these four strains. Thus, the *CRD1* locus is tagged in five out of nine *crd1* strains.

The chlorophyll content in copper-deficient *crd1* strains was only 30–40% of that of equivalently grown wild-type cells, but the chlorophyll *a:b* ratio was unchanged in -Cu *crd1* cells (2.51) versus +Cu cells (2.47). Restoration of chlorophyll content to wild-type abundance required



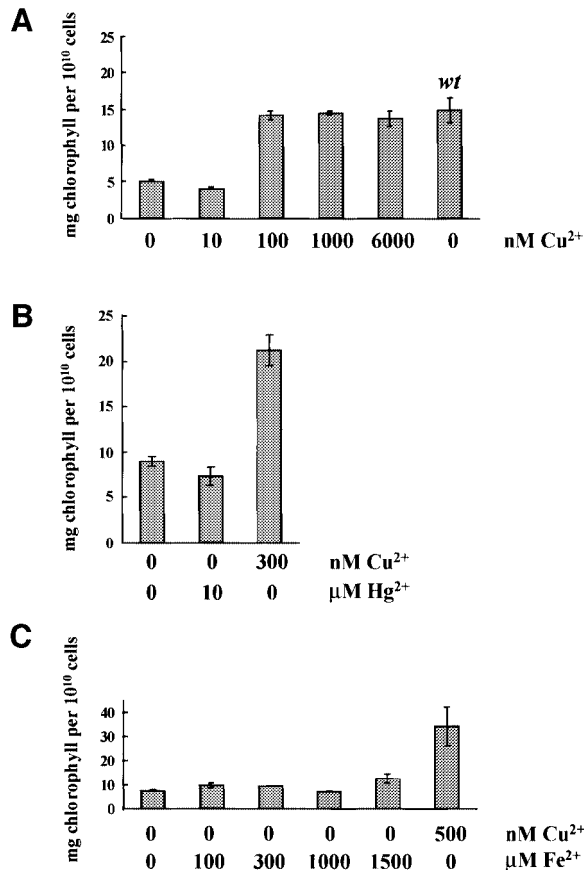
**Fig. 1.** Phenotype of *crd1* strains. A representative tetrad from a cross of *crd1-1::ARG7* to *arg7* shows that progeny displaying the *crd1* phenotype are arginine prototrophs. Cultures were grown for 8 days (21°C, 50 µmol/m<sup>2</sup>s constant light, 200 r.p.m.). Arginine supplementation (250 µg/ml) is indicated.

100 nM CuSO<sub>4</sub> (Figure 2A). At this cell density (~ $1.5 \times 10^7$  cells/ml), a concentration of 100 nM corresponds to  $4.1 \times 10^6$  Cu ions/cell, which is in the range required to repress *Cyc6* and *Cpx1* expression by 50% ( $4.5 \times 10^6$  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 HgCl<sub>2</sub>, which is enough to repress the *Cyc6* and *Cpx1* genes completely, did not restore chlorophyll accumulation in -Cu *crd1* 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



**Fig. 2.** Copper but not mercuric or iron salts rescues the chlorophyll deficiency in *-Cu crd1* cells. (A) Erlenmeyer flasks (125 ml) containing 50 ml of copper-free TAP medium supplemented with CuSO<sub>4</sub> as indicated were inoculated to an initial density of  $1 \times 10^5$  cells/ml with copper-deficient *crd1* cells, and grown for 5–9 days (21°C, 50 μmol/m<sup>2</sup>s constant light, 200 r.p.m.). The parental strain cc425 (*arg7cw15*), wild-type for *CRD1*, was grown the same way except that the culture was supplemented with 250 μg/ml arginine. Cultures were grown to a density of  $\sim 5$ – $10 \times 10^6$  cells/ml. (B) Fifty milliliter cultures of a cell-walled *crd1-1* spore were grown for 7 days (*-Cu* TAP medium, 21°C, 50 μmol/m<sup>2</sup>s constant light, 200 r.p.m.). Cu or Hg salts were added as indicated and the cultures were returned to the same conditions for another 7 days, after which time cell density and chlorophyll concentrations were determined. (C) One hundred milliliter *crd1-1* cultures were grown for 9 days at 24°C,  $\sim 100$  μmol/m<sup>2</sup>s, 250 r.p.m. with shaking, in copper-free TAP medium containing the indicated concentrations of additional FeSO<sub>4</sub>-EDTA or 500 nM CuSO<sub>4</sub>. The average of two samples from the culture is shown for each experiment. Copper-free TAP medium contains 18 μM FeSO<sub>4</sub>-EDTA, and  $\sim 1$  μM chelated iron is adequate for growth of wild-type copper-deficient cells. Thus, 1.5 mM represents a 10<sup>3</sup>-fold excess.

up to 1.5 mM FeSO<sub>4</sub> to *-Cu* cultures of *crd1* cells does not restore chlorophyll accumulation, indicating that the loss of chlorophyll in *crd1* strains is not a consequence of defective iron uptake (Figure 2C). The phenotype of *crd1* strains was also tested in dim light (5 μmol/m<sup>2</sup>s) and in darkness. Under these conditions, both copper-deficient and copper-supplemented *crd1* cells grew at equal rates, but the *-Cu* cultures still accumulated less chlorophyll (data not shown). At light intensities of 100 μmol/m<sup>2</sup>s, *crd1* 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 *crd1* strains in either copper- or oxygen-deficient medium

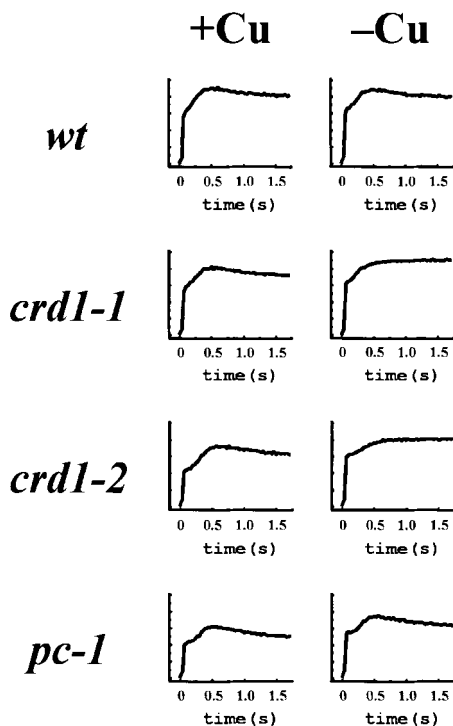
Photosynthetic function was examined by monitoring the fluorescence induction and decay kinetics of *crd1* strains grown on +Cu versus *-Cu* medium (Figure 3). In the wild type, the transient rise phase reflects the reduction of a particular plastoquinone, Q<sub>A</sub>, in PSII, while the decline phase 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 *crd1* 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 *pc-1* mutant that has lost the light-dependent protochlorophyllide reductase has a chlorophyll content similar to that of copper-deficient *crd1* strains,  $\sim 40\%$  of the amount in wild type (Li and Timko, 1996). However, *pc-1* displays wild-type fluorescence in both copper-supplemented and copper-deficient cells. The photosynthesis defect in *-Cu crd1* 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 *Crd1* is a target of the same pathway, one might expect to induce the *crd1* 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, *crd1* strains become chlorophyll deficient, and when such hypoxic cells are transferred to aerated medium, chlorophyll accumulation is restored, indicating that the wild-type *Crd1* gene is also required in oxygen-deprived cells (Figure 4A). Induction of the *crd1* phenotype was verified as being due to O<sub>2</sub> depletion (as opposed to

**Table I.** Tight linkage of independently generated *crd1* mutants

	<i>crd1-1</i>	<i>crd1-2</i>	<i>crd1-3</i>	<i>crd1-4</i>	<i>crd1-5</i>	<i>crd1-6</i>	<i>crd1-7</i>	<i>crd1-8</i>	<i>crd1-9</i>
Recombinants/complete tetrads or octets	0/8	0/11	0/12	0/6	0/10	0/6	0/2	0/7	0/6
Recombinants/total progeny (zygotes)	0/36 (8)	0/44 (11)	0/48 (12)	0/42 (12)	0/70 (20)	0/46 (14)	0/33 (8)	0/53 (10)	0/44 (12)

A mating type minus *crd1-1* reference strain was crossed to mating type plus *crd1* test strains. Zygotes were germinated and spores were transferred three times to *-Cu* TAP plates. Segregation of the *crd1* 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.

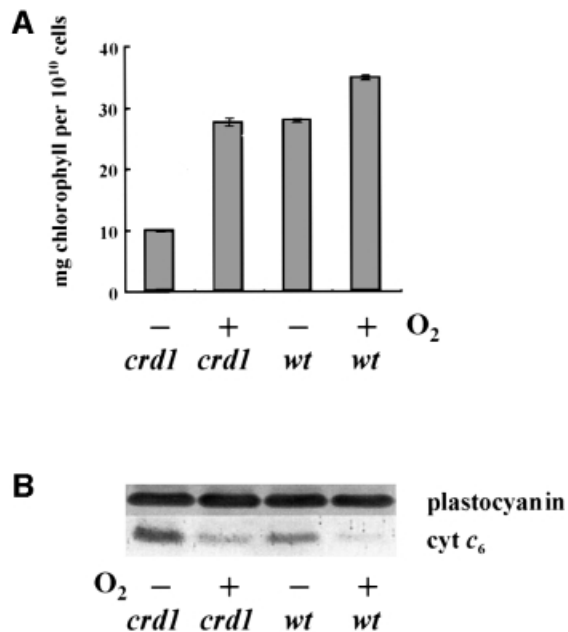


**Fig. 3.** *crd1* strains display mutant fluorescence induction and decay kinetics. Fluorescence transients of cells grown on  $\pm$ Cu TAP plates. Representative traces are shown from *crd1-1* and *crd1-2*. *wt* = CC124. The *pc-1* mutant lacks the light-dependent protochlorophyllide reductase.

depletion of another gas such as CO<sub>2</sub>) by showing that bubbling with a mixture of 2% air, 2% CO<sub>2</sub> in N<sub>2</sub> also induces chlorosis (J.Quinn, unpublished).

The copper-conditional block in electron transfer in *crd1* cells suggested that cyt *c*<sub>6</sub> 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 *c*<sub>6</sub> and coprogen oxidase is normal in *crd1* cells (Figures 4B and 5A). We conclude that the *crd1* 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 *crd1* 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 *crd1* 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  $\sim$ 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  $\alpha$ - and  $\beta$ -subunits of ATP synthase). We conclude that the primary electron

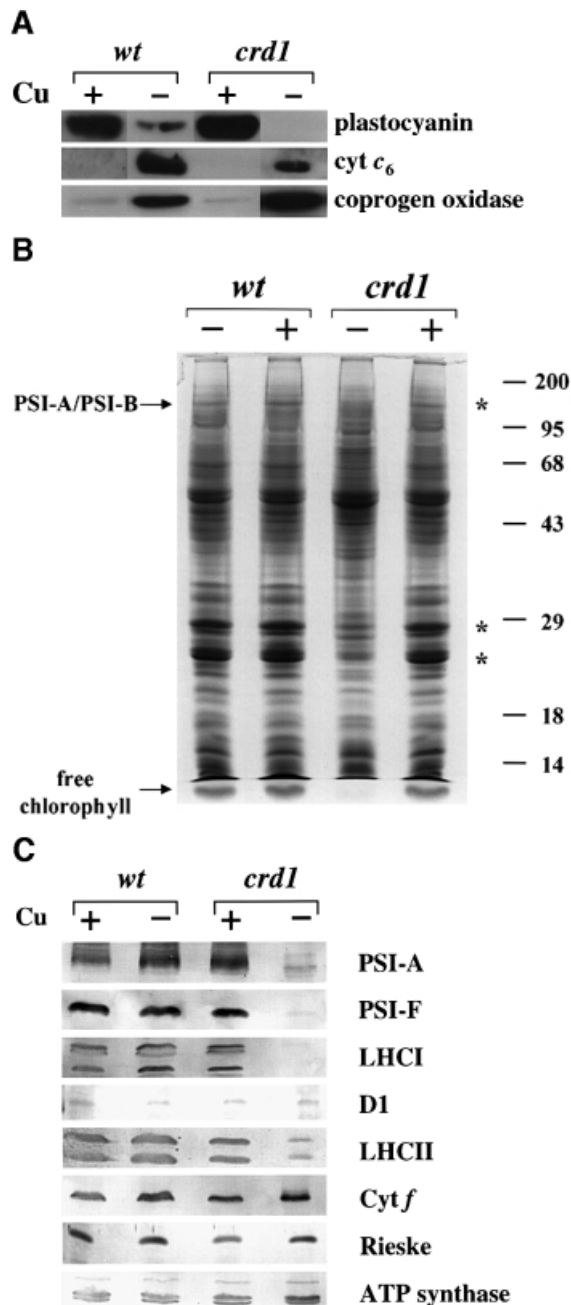


**Fig. 4.** Hypoxic conditions mimic the *crd1* phenotype. (A) Chlorophyll deficiency in oxygen-deprived *crd1* cultures. +Cu TAP cultures (200 ml) of the *arg7* (*wt*) strain and *crd1-1* were grown on the benchtop in 250 ml Erlenmeyer flasks with a magnetic stir-bar revolving at  $<60$  r.p.m. Cultures were inoculated to an initial density of  $1 \times 10^6$  cells per ml, grown for 3 days and then re-inoculated into new medium. After another 3 days, cells from this second transfer were used to inoculate either a third round of cultures that were grown for 3 days with low basal stirring ( $-$ O<sub>2</sub>) or 100 ml cultures that were agitated vigorously at 250 r.p.m. ( $+$ O<sub>2</sub>). Cells were counted and chlorophyll content was determined. Similar results were obtained when cultures were O<sub>2</sub> depleted by bubbling with 2% air, 2% CO<sub>2</sub> in N<sub>2</sub>. (B) Immunoblot analysis for plastocyanin and cyt *c*<sub>6</sub> accumulation.

transfer defect in copper-deficient *crd1* cells is attributable to a PSI deficiency. The unusual phenotype of *crd1* strains points to a hitherto unrecognized role for copper in PSI maintenance. Since both nucleus- and plastid-encoded proteins are affected in  $-$ Cu *crd1* 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 *crd1* relative to the wild type; in fact, for *psaB* and *CabII*, it is enhanced in the mutant (Figure 6).

#### ***Crd1* is a previously unknown protein found in photosynthetic organisms**

Since five of the nine well characterized *crd1* strains appeared to be tagged, we sought to clone *Crd1* based on its linkage to the marker genes. Southern analysis of genomic DNA from *crd1-7* revealed that the *ble* gene is present on a 6.5 kb *Bam*HI 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 *crd1* alleles, confirming that the amplified DNA represented sequences that closely flanked the *Crd1* gene. A 3 kb DNA fragment of the amplified product was used to probe a wild-type genomic library. Three overlapping  $\lambda$  clones were identified, and each of these complemented the *crd1*



**Fig. 5.** The *crd1* mutant shows (A) normal regulation of plastocyanin, cytochrome *c*<sub>6</sub> and coprogen oxidase abundance but lacks PSI polypeptides (B and C). (A) Immunoblot analysis of *cyt c*<sub>6</sub>, plastocyanin and coprogen oxidase abundance. Liquid cultures in  $\pm$ Cu TAP of a wild-type (*cc425::ARG7*) strain and *crd1-1::ARG7* were grown for 8 days at 21°C, 200 r.p.m.,  $\sim$ 50  $\mu$ mol/m<sup>2</sup>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 *crd1* 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 *crd1*. Proteins in whole-cell extracts of a wild-type (*arg7,Crd1*) strain and a *crd1-1* 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.

phenotype when introduced into the *crd1-6* background (Figure 7B). A 5.8 kb *SalI* fragment of  $\lambda$  clone C1 was subcloned (pCrd1-5) and shown to be adequate for rescue of both the chlorophyll deficiency and the non-photosynthetic phenotype of *crd1-6* (data not shown). This fragment therefore contained a functional *Crd1* gene. A 1.2 kb *PstI*-*XhoI* fragment of pCrd1-5 was subsequently used to isolate two cDNA clones containing the entire *Crd1* coding region from a -Cu cDNA library (Merchant and Bogorad, 1987a).

The *C.reinhardtii* *Crd1* 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 phytochrome-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).

#### *Crd1* expression is activated in -Cu cells

Is *Crd1* expressed coordinately with *Cyc6* and *Cpx1*? RNA blot analysis reveals that in +Cu wild-type cells, *Crd1* mRNA accumulates at a low, basal level and is induced 15- to 20-fold in -Cu cells (Figure 10). *Crd1* expression is activated similarly in oxygen-deficient cells (data not shown). As expected, *Crd1* mRNA is not detected in the *crd1* mutant. This induction of *Crd1* in Cu- and O<sub>2</sub>-deficient cells suggests an increased requirement for the gene product under these conditions, consistent with the *crd1* mutant phenotype.

#### Discussion

Copper-deficient cells require a new protein, Crd1, for normal photosynthesis. The unexpected phenotype of the *crd1* strains reveals: (i) an unrecognized role for copper in photosynthetic metabolism; and (ii) an unrecognized biochemical function in maintenance of PSI and LHCS.

#### *Crd1* and PSI

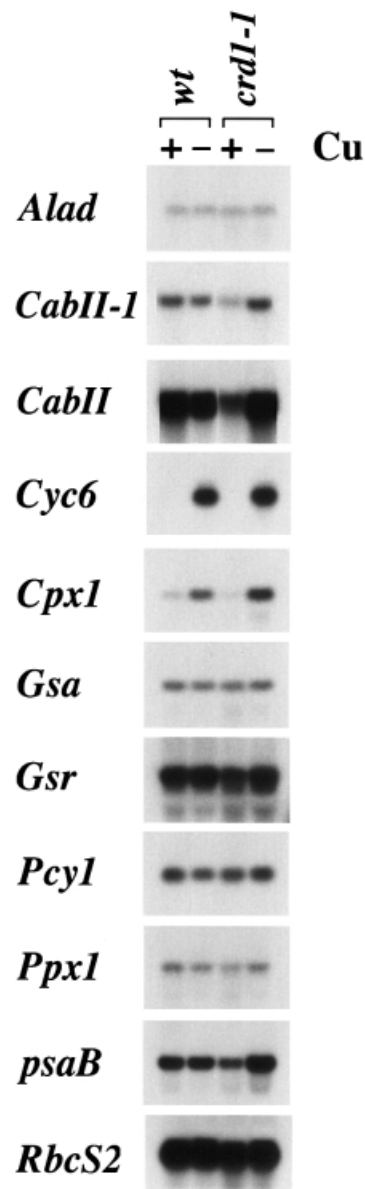
The phenotype of *crd1* strains, namely a pleiotropic deficiency in PSI and light-harvesting proteins, should provide a clue to Crd1 function. For instance, Crd1 could be a novel subunit in a photosynthetic complex that is modified in response to copper and oxygen availability. This is preceded by the occurrence of alternative isoforms of respiratory complex subunits in *Saccharomyces* mitochondria (reviewed by Kwast *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 Straus, 1988; Burnap *et al.*, 1993; Leonhardt and Straus, 1994; Vinnemeier *et al.*, 1998). Interestingly, the organellar and prokaryotic *Crd1*

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  $-Cu$  *crd1* 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 *crd1* phenotype are dependent on the growth conditions. For example, growth of  $-Cu$  *crd1* 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 *crd1* cells do grow photoautotrophically (at 50–100  $\mu\text{mol}/\text{m}^2\text{s}$ ), 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 the difference in 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 *crd1*. Perhaps the rate of cofactor production becomes more limiting when cell division is occurring more rapidly.

### Crd1 function

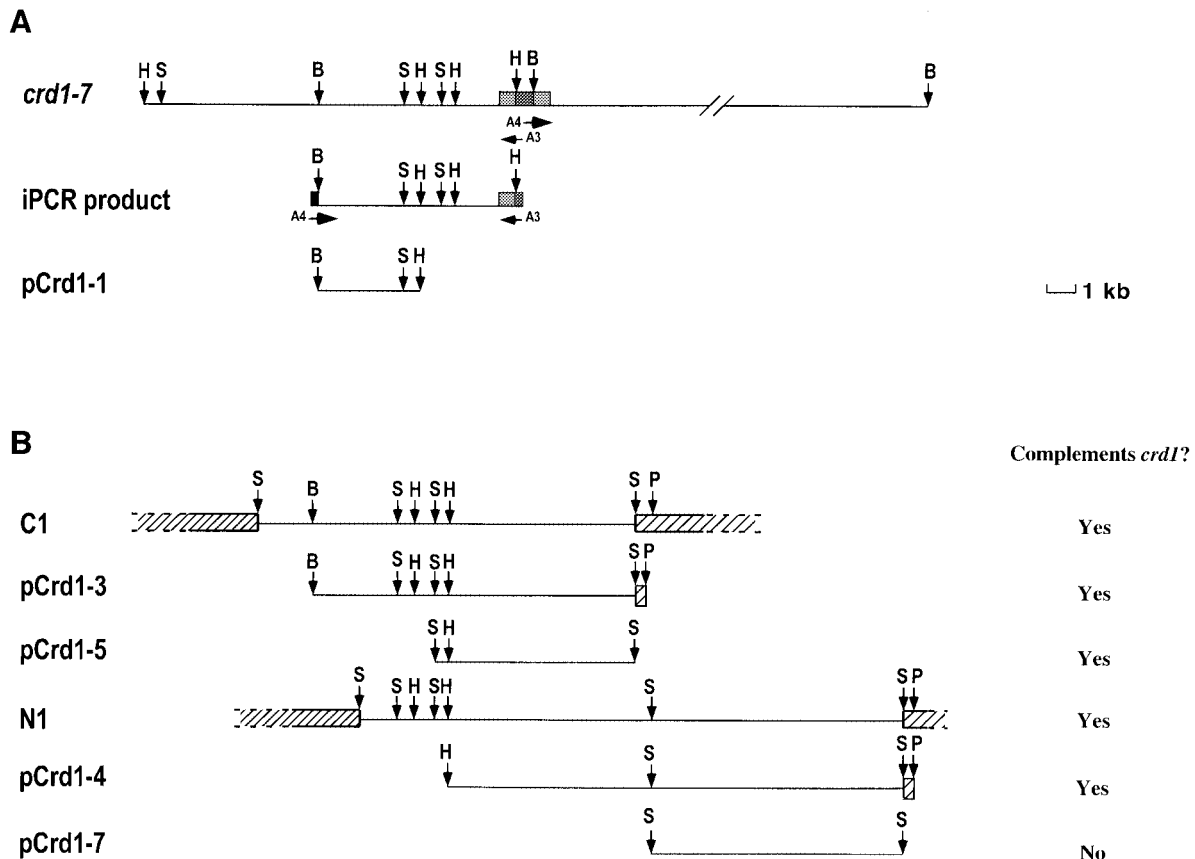
What does the Crd1 sequence reveal about its function? Two of the most highly conserved sequences, WCQDENRHGD and SRDEARHAG, separated by ~80 amino acids in the Crd1 proteins, are also present in plastid stearoyl-ACP desaturases (reviewed by Los and Murata, 1998; Shanklin and Cahoon, 1998) and a stearoyl-ACP desaturase from *Burkholderia cepacia* (Kang *et al.*, 1998), respectively. These sequences contain consensus  $^D/E\text{ExxH}$  motifs for the carboxylate-liganded 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 monooxygenase, and the characteristic feature of these enzymes is that the Fe ions are bridged by carboxylates and oxide or hydroxide ions. Two  $^D/E\text{ExxH}$  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



**Fig. 6.** RNA blot analysis. Total RNA was isolated from wild-type or *crd1* cells in medium containing copper or not (as indicated). A 10  $\mu\text{g}$  aliquot of total RNA was loaded in each lane. *Alad* encodes aminolevulinic acid dehydratase, *CabII* encodes chlorophyll *alb*-binding protein II; the *CabII-1* probe is specific for the *CabII-1* gene, while the *CabII* probe recognizes a family of similarly sized mRNAs; *Cyc6* encodes *cyt c6*; *Cpx1* encodes coprogen oxidase; *Gsa* encodes glutamyl-1-semialdehyde aminotransferase; *Gsr* encodes glutamyl-1-semialdehyde reductase; *Pcy1* encodes plastocyanin; *Ppx1* encodes protogen oxidase; *psaB* encodes photosystem I P700 apoprotein Ib; and *RbcS2* encodes the Rubisco small subunit.

the plastid desaturases, reduced ferredoxin provides the electrons. In all Crd1 homologs, the  $^D/E\text{ExxH}$  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-liganded 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 pSP109 were used to map restriction digests of genomic DNA from *crd1-7*. The divergent oligonucleotide primers A3 and A4 were used to amplify an ~6.5 kb *Bam*HI fragment containing the inserted *ble* gene. This fragment was digested with *Bam*HI and *Hind*III and a 3 kb fragment was cloned into pBluescript KS+ to create plasmid pCrd1-1. B, *Bam*HI; H, *Hind*III; S, *Sal*I; medium gray boxes, *RbcS2* sequences; dark gray boxes, *ble* sequences. (B) Genomic clones corresponding to *Crd1*. The 3 kb *Bam*HI-*Hind*III insert in pCrd1-1 was used to probe a genomic DNA library. DNA from overlapping  $\lambda$  clones C1, I1 (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, *Spl*I. Hatched boxes, *EMBL3* left and right arm sequences.  $\lambda$  clones C1, I1 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 (Kindle, 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 those 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 post-transcriptional level (Figures 5 and 6).

#### Response to copper and oxygen deficiency

The induction of cyt *c*<sub>6</sub> in copper-deficient algal cells has been understood for over two decades as a back

-696 AAGCTTGCATAGCAAAACGCTGGTCTAGGTAATTCGCGGAAGAAGCGCACGGCGCTGGCTTCATGAAATGCTTCTTTGAAGCTGTCTT  
-606 CTTGCACCGCAACCGCTTTCGTAAGCAGCGACGAAAGTGCAAGCGTAGGAAAGGGCTTGACAGCACGAAGGCCCGCGCTTTGATTTTG  
-516 TTGGTCTTGCATTTGGGCTTACCGGTTACGCTAAACAGCTTTGGCTATGCGGTCCTTTCGGCCGCGCGCACAGCGGCACTCCGGTGGCTCG  
-426 TCGCCAGAGAGCCACAGTAGCAGTAAACTGGGCTGACATCATTACATTGTAGTGTGGGGCTGAACATGGTTCGGGAAAACGTAAGGGGG  
-336 CGCTTTTACGACGAGACTCAAGCTAATTTGCGTCTTTGCTTTCGCGCTCCACTTTTTCGGGTATGTAGTGTAAAACGGTGTCTGGT  
-246 TGCTGATTTGGCTTTTCGGCTGTACAGCAGGAGCCTCGGCGAGCCGGATCTAGGCGAGGGGCTCTGGTACCGCGCGAGGGGGCCGGCT  
-156 GGCCTCAGGTACAGCGCGCGTCCCCCGCATGATCTCGCTCAATCTTGGCTCTGCTCCTCGGTGAGGCTTCGCGACTACAAATAC

-66 TCCC CGCTTTCGAGGCCAACCATACCAGTTGCTTCTCTCAGTGACCAGTTCGGCTGAAGTTTCGGTGGTCTTCTCTTCCGAGCGGC  
+25 CTTCTTCTTGTTCGACCTGCAAGCCCTGATCCAAGGGTGCCTGAGAGCGTTAGCAACCAAGTTAATCTGCAAGCCGTAGTCTCAT  
+115 CACCGACCTCGTTGCTCTGCTTCTTCGAAAAGAGCAATCATGCGAGCCACCTTGAAGCAGCAGCGCTTCCGGCCCGCTCTCGGCC  
M Q T T L K Q Q R A S G R V S A

↓

+205 CGCCAGCCTTTCCGGTGGAGCAATGGGCCCCGAAGACACTTCCGAGCCTTGTGCTTGGCGTGTTCATGCGGTTCGCGACCAATCGGA  
R Q P F R  
+295 CTCCGTGCGCGCCGACCGAGTTGTTCTTCTTAGCGGCCAAGCCTTCCCTTCTTGATCTTGCCCGTACTCTTGACGCTGGTACTAATACCA

↓

+385 ACGAATTTGCGCTGCGTTGATCTGCGCGCAGCTCGGCAGCCGTGGCGCGCCCTCGCCGAGCACCGTTCGCGTGCAGGCGAGCGCTGCC  
S A A V A R P R R S T V R V Q A S A A P  
+475 CCCTGAATGATGGCCTGGGCTTCGAGACCATGCGCGATGGCATCAAGTGGCTGCCAAGGAGACCCTGCTGACCCCGCTTCTACACCA  
L N D G L G F E T M R D G I K V A A K E T L L T P R F Y T T  
↓

+565 CCGGTGAGCGCCCAAGCCCTCCGGTGTGCAACACCCTGTACCGCCCGGAGCCACGCGCTGCAAGCCCACTCCGCTTCCGCT  
D  
+655 CTGGTCTACGTACGCATTAGTTCGCAACACTCGTCAATCCCTGGCTCTGGCCGAACCCACCTCACCTTGGCCATGCCATCCGCTCT  
↓

+745 GCCTTCTCCATCGCCCTGCGCTGCCGTTTCTCACATCCCTTACCGCCCGTTACAATTCCCTCCCTCCCTCACAGACTTTCGACGAG  
F D E  
+835 ATGGAGCAGCTGTTTCAGCAAGGAGATTAACCCCACTGGACATGGAGGAGCTGAACGCTGCCTGAACGAGTTCGCAACGACTACAAC  
M E Q L F S K E I N P N L D M E E L N A C L N E F R N D Y N  
↓

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K V H F V R N E T F K  
↓

+1015 TGTGAAGTACGAAAATGAGCCCGTGTGCTACGCATCACTCATTGCTCTCTTGCCTCCTCTGCGGAGCCGTTCCAGGCCCGCC  
A A  
+1105 GCCGACAAGGTGACTGGCGAGACCCCGCATCTTTCATTGAGTTCCTGGAGCGCTCTTGACCCGCGAGTTCAGCGGCTTCTGCTGTAC  
A D K V T G E T R R I F I E F L E R S C T A E F S G F L L Y  
↓

+1195 AAGGAGCTGGCGCGCCGATGAAGCCCTCCAGCCCGAGGTGGCCGAGATGTGAGTATTGCGCGTSCGAGCGCGGCTGGCCGGCTG  
K E L A R R M K A S S P E V A E M  
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↓

+1465 CGCCTTGGCCCACTCACCCCAACCCAGCCTCACCTCCACCGCTCAACCTCACTCCCGGCAACGCAACCAACAGGTTCTCTGCTCA  
F L L M  
+1555 TGTCTCGGATGAGGCCCGCACGCGGCTTCTGAACAAGCCCTGTGGACTTCAACCTGGCCCTGGACCTGGGCTTCTGACCAAGA  
S R D E A R H A G F L N K A L S D F N L A L D L G F L T K N  
+1645 ACCGCACCTACACCTTCAAGCCCAAGTTCATCTACGCCACCTTCTGTGCGGAGAAGATTGGTACTGGCGCTACATCACCTCT  
R T Y T Y F K P K F I I Y A T F L S E K I G Y W R Y I T I Y  
↓

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R H L Q R N P D N Q F Y P L F E Y F E N W C Q  
+1825 GGGAGAAGCGTGGGGAGGGGAGGCGAGGGGCGGCTGGCCCTGGGTGTAGCCGGGCGCGCATGGGGTGGGGACGGGTAGGG  
+1915 ATGGGTGTCGTGATGCCCTGCCGCTTGTGTCACACATGCCCTGCCCTGTCGCCACGACTGCTCCTGATGCTTCCCGCTCACCCG  
↓

+2005 CCTGCCTCCCAACAGGACGAGAACCAGCCCGGACTTCTGGCCGCTCCTGAAGGCCAAGCCCGAGCTGCTCAACACCTTTGAGG  
D E N R H G D F L A A C L K A K P E L L N T F E A  
+2095 CCAAGCTCTGGTCCAAGTCTCTGCTGCTCGGCTACATCACCATTGACCTGAACGACCCAGCGCACCAAGTTCACGATCCCTG  
K L W S K F F C L S V Y I T M Y L N D H Q R T K F Y E S L G  
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L N T R Q F N Q H V I I E T N R A T E R L F P V P D E D  
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↓

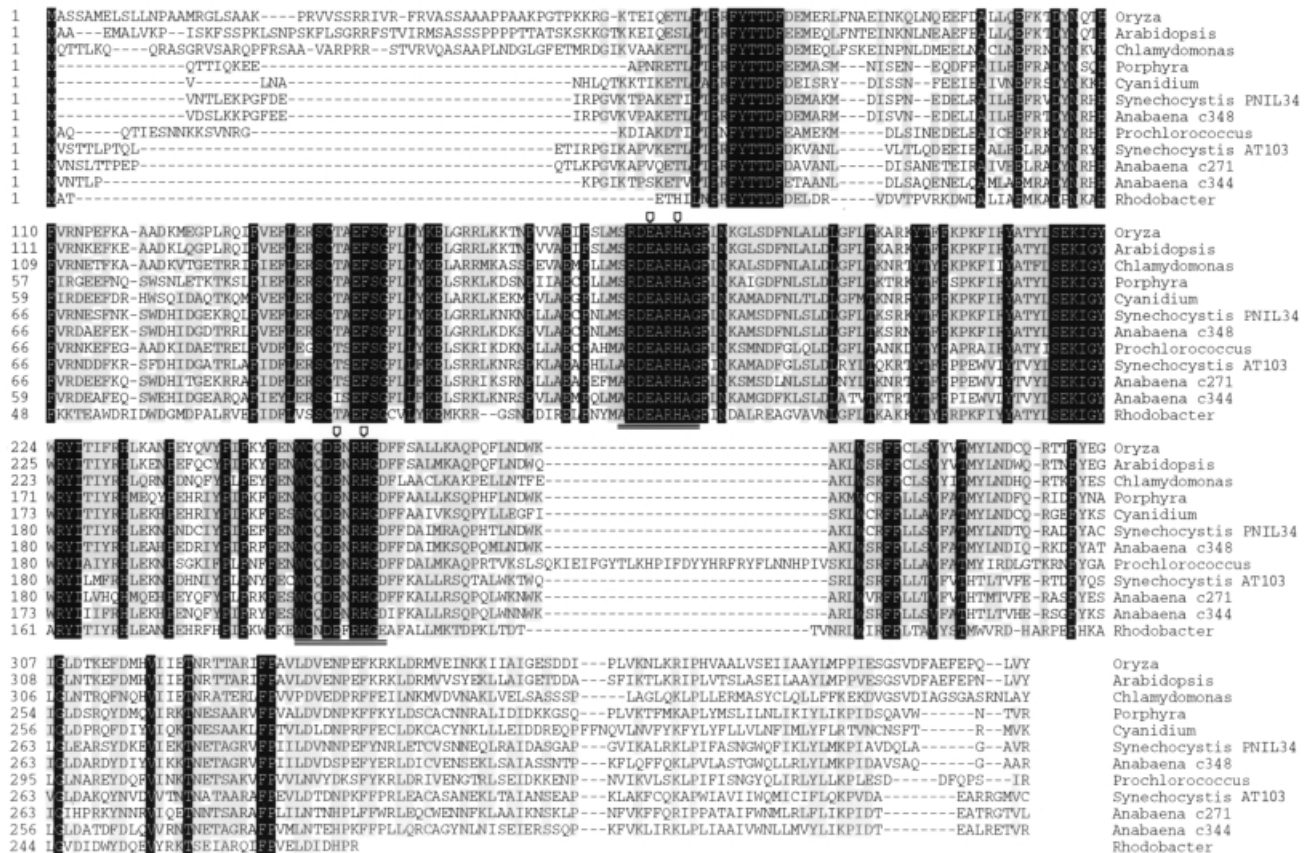
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Q K L P L L  
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E R M A S Y C  
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L Q L L F F K E K D V G S V D I A G S G A Y S R N L A Y \*  
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+3445 GTACTGTGGCCGCTGCTCAAAGCGGACCTGTAATGAGCCCTGCCATAACTTCTCTTTGCTT

**Fig. 8.** *Crd1* genomic sequence. The genomic and cDNA *Crd1* sequences have been submitted to the DDBJ/EMBL/GenBank database under accession Nos AF226628 and AF237671, respectively. A possible TATA box is indicated (single underline). A putative transcription start site (→) is suggested based on the 5' UTR end of a *Crd1* cDNA clone. Intron-exon boundaries are indicated by (↓). An asterisk denotes the stop codon, and the TGTA polyadenylation signal (double underline) was found 15 bp upstream of the *Crd1* cDNA poly(A) sequence.



A



B

1 MCTTLKQQRASGRVRSARQPPRSAAVA---RRPRRSVVRVQASAAPLNDGLGFETMRDGIKVAAKETLLTPRFYTTDF Crd1  
 1 MATMLNK-RVIGAFGKQAPVRSARVAAVRPSRSSVRVAALAAP-QEVEGKVMRDGIKVAASDETLLTPRFYTTDF Cth1

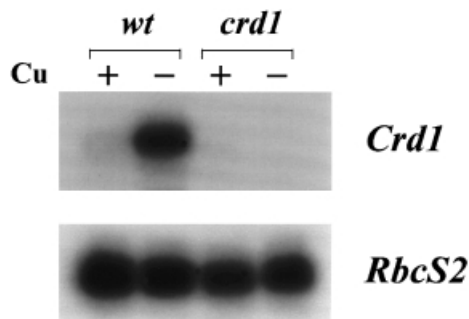
74 DEMEQLFKEINPNLDMEELNAALNEFRNDYNNKVFVRNETFKAAADKVGETRIIFIEFLERSCTAEFSGFLLY Crd1  
 74 DEMERLLESLLELNKNQDMEEFDAALNEFKLDYNNRHFVRNETFKAAEKIQPTRKISLSFGALVPAESRASALTE Cth1

149 KELARRMKASSPEVAEMFLL Crd1  
 149 LVALKPTRVRDLSLFRERRGL Cth1

**Fig. 9. (A)** Conservation of Crd1. 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 WCQENRHGD 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 PNIL34, *Synechocystis* sp. 6803 AT103 and *Rhodobacter sphaeroides*, are AP000815, AF236101, U38804, AF022186, D90899, D90912 and AF195122 respectively. *Anabaena* sp. PCC 7120 sequences were retrieved from the Cyanobase database at <http://www.kazusa.or.jp/cyano/anabaenal>. The *Prochlorococcus marinus* homolog was found at the Prochlorococcus sequencing homepage at <http://bbp.lnl.gov/jgi/microbial/prochlorococcushomepage.html>. The homolog from *Pharbitis nil* 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 *Cth1*, a partial *Chlamydomonas* EST (DDBJ/EMBL/GenBank accession No. AV391947), is similar to the N-terminal region of Crd1.

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 all 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



**Fig. 10.** *Crd1* expression is induced in  $-Cu$ . Total RNA was isolated from wild-type and *crd1* +Cu and  $-Cu$  cells as indicated. An 8  $\mu$ g aliquot of total RNA was loaded in each lane. A probe made from a *Crd1* genomic sequence containing the 3' UTR was used to detect the *Crd1* message. The *RbcS2* mRNA encoding the small subunit of Rubisco was used as a loading control.

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; J.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  $O_2$  deprivation, whereas the induction of the *Cyc6* 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  $c_6$  replaces copper-containing plastocyanin). This idea is preceded by the reciprocal copper-responsive expression of iron- versus copper-containing methane monooxygenase in methanotrophic 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  $c_6$  (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, while 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 photoautotrophic, 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*, *cw15*) and crossed as described below. The preparation of

copper-deficient (-Cu) media for culturing *C.reinhardtii* strains is described in Quinn 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 FeSO<sub>4</sub> that was prepared by boiling 100 mM FeSO<sub>4</sub> (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/m<sup>2</sup>s. Strains were cultured on plates at either 18 or 25°C at light intensities between 5 and 100 μmol/m<sup>2</sup>s. To generate O<sub>2</sub>-deprived cultures, 250 ml Erlenmeyer flasks filled with 200 ml of copper-supplemented TAP medium were inoculated to an initial cell density of 1 × 10<sup>6</sup> 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/m<sup>2</sup>s. 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 Institute of Biological Chemistry (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/m<sup>2</sup>s and dark adapted for at least 5 min. The colonies were illuminated for 1.73 s with 30 μmol/m<sup>2</sup>s of red light (640 nm) from light-emitting diodes (HLMP C116, Hewlett Packard) and the emitted fluorescence was captured immediately by a CCD camera (Cohu, 2122–1000) in conjunction with a PIXCI-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 *c<sub>6</sub>* and coprogen oxidase as described previously (Quinn and Merchant, 1998; Quinn *et al.*, 1999). Proteins were usually transferred to PVDF membranes for 1 h at 100 V in blotting buffer containing 20% methanol. The following solutions were used for processing 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 dilutions: anti-plastocyanin 1:10 000, anti-cyt *c<sub>6</sub>* 1:5000, anti-coprogen oxidase 1:3000 and horseradish peroxidase-conjugated goat-anti-rabbit 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-PSI/LHCI, 1:5000 anti-D1, 1:800 anti-LHCII, 1:1000 anti-cytochrome *f*, 1:1000 anti-Reiske iron-sulfur protein and 1:5000 anti-CF<sub>1</sub>. 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 *psaB*, a 1 kb *Bam*HI fragment from the *Eco*RI fragment of chloroplast DNA in plasmid p67A (*Chlamydomonas* Culture

Collection) was used as the probe. The *CabII* coding probe and the *CabII-1* 3'-untranslated region (UTR) probe are described in Jacobshagen and Johnson (1994). For *Cpx1*, the probe was made from the 102 bp *Hind*III fragment from pCpx1.0 (Quinn *et al.*, 1999). The *Crd1* probe was made from a 1.2 kb *Pst*I-*Xho*I fragment of pCrd1-5. The other probes are described in Quinn *et al.* (1999). The specific activities ranged from 5 × 10<sup>8</sup> to 7 × 10<sup>8</sup> 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*, *Cyc6*, *Cpx1*, *Crd1*, *Gsa*, *Gsr* and *Ppx1*) or at room temperature without screens (*psaB* and *RbcS2*). Exposure times (Figure 6) were 2.5 h for *Alad*, *Cyc6*, *Cpx1* and *Gsa*, 24 h for *Gsr* and *Ppx1*, 1 h for *psaB* and *RbcS2*, and (Figure 10) 16 h for *Crd1* 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 tetrads or 12 *crd1* progeny were scored for segregation of the mutant phenotype with arginine prototrophy (*crd1-1* × *arg7*) or resistance to 10 μg/ml zeocin (Stevens *et al.*, 1996) (*crd1-2*–*crd1-9* × CC124). Alleles *crd1-2*–*crd1-9* were each crossed to *crd1-1* to test for recombination.

#### Inverse PCR

Genomic DNA from strain *crd1-7* was prepared by a modified miniprep procedure and 5 μg of DNA was digested [6–10 h, 37°C, 100 U of *Bam*HI (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 mM ATP and 2 U 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 (GAAGTCGTCCTCCACGAAGT) and A4 (GACGTGACCCTGTT-CATCAG) complementary to sequences within the *ble* gene (Stevens *et al.*, 1996) and *Pfx* polymerase from Gibco-BRL.

#### Cloning and sequencing of *Crd1*

A 3 kb *Bam*HI-*Hind*III 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 *crd1* phenotype of an *arg2crd1-6* 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 pCrd1-3, pCrd1-4, pCrd1-5 and pCrd1-7 (Figure 7B), and the smallest complementing fragment was identified (pCrd1-5). The presence of introduced copies of *Crd1* in rescued strains was verified by PCR amplification using primers P9 (CCGTTA-CAATTCCTCC) and P20 (AGCTCCTTGTACAGCAGG) within the *Crd1* sequence that flank the site of *ble* insertion. The sequence of a 4.2 kb region within the 5.8 kb genomic DNA in pCrd1-5 spanning the *Crd1* gene was determined on both strands, except for the last 75 bp, which were sequenced twice on one strand. A 1.2 kb *Pst*I-*Xho*I fragment of pCrd1-5 was used to screen a *Chlamydomonas* -Cu cDNA library in λgt11 by plaque hybridization, and two *Crd1* 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 *Crd1* 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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