Chlamydomonas reinhardtii as a eukaryotic photosynthetic model for studies of heavy metal homeostasis and tolerance

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Summary

The green alga *Chlamydomonas reinhardtii* is a useful model of a photosynthetic cell. This unicellular eukaryote has been intensively used for studies of a number of physiological processes such as photosynthesis, respiration, nitrogen assimilation, flagella motility and basal body function. Its easy-to-manipulate and short life cycle make this organism a powerful tool for genetic analysis. Over the past 15 years, a dramatically increased number of molecular technologies (including nuclear and organelar transformation systems, cosmid, YAC and BAC libraries, reporter genes, RNA interference, DNA microarrays, …) have been applied to *Chlamydomonas*. Moreover, as parts of the *Chlamydomonas* genome project, molecular mapping, as well as whole genome and extended EST sequencing programs, are currently underway. These developments have allowed *Chlamydomonas* to become an extremely valuable model for molecular approaches of heavy metal homeostasis and tolerance in photosynthetic organisms.

Key words: *Chlamydomonas*, heavy metal homeostasis, heavy metal tolerance, copper, iron, cadmium

Abbreviation list: ABC (ATP-binding cassette), BAC (bacterial artificial chromosome), CuRE (copper-responsive element), EST (expressed sequence tag), GSH (glutathione), HMW (high molecular weight), HyRE (hypoxia-responsive element), LHC (light harvesting complex), LMW (low molecular weight), PC (phytochelatin), PSI (photosystem I), PSII (photosystem II), TRX (thioredoxin).
Introduction

Several heavy metals (such as copper, zinc and iron) are essential for many physiological processes but can be toxic at supraoptimal concentrations. Like other organisms, plants are able to maintain the homeostasis of essential metal ions in different cellular compartments. A regulated network of metal transport, chelation, trafficking and sequestration activities functions to provide the uptake and distribution of these metal ions. Other heavy metals (such as cadmium, lead and mercury) are not physiologically essential and are generally toxic at low concentrations in both animal and plant cells (Clemens, 2001).

The development of human activities and industrialization has led to an increased accumulation of heavy metals in the environment. The principal sources of heavy metal pollution are combustion of fossil fuels, mining and smelting activities, release of wastes and sewage waters and the use of fertilizers and pesticides. At the cellular level, essential heavy metals at supraoptimal concentrations and non-essential heavy metals at toxic concentrations can displace endogenous metal cofactors from their cellular binding sites and cause oxidative stress (Stohs & Bagchi, 1995; Goyer, 1997), leading to cell poisoning or cancers (Warren, 1989). To cope with the deleterious effects of heavy metals, eukaryotic cells overproduce organic acids (malate, citrate), amino acids (histidine, methionine, proline) and (poly)peptides (glutathione, phytochelatins, metallothioneins). Different enzymatic systems involved in metal excretion and compartmentalization, as well as in oxidative stress responses, also participate in detoxification mechanisms (reviewed by Rauser, 1999; Cobbett, 2000; Cobbett & Goldsbrough, 2002). A better understanding of the heavy metal detoxification mechanisms will provide new strategies for environmental cleaning by phytoremediation (Meagher, 2000; Clemens et al., 2002).
In this article, we present a short review of the possibilities offered by
Chlamydomonas as a model plant system and describe recent findings dealing with heavy
metal homeostasis and tolerance in this unicellular organism.

*Chlamydomonas reinhardtii* as a model photosynthetic organism

The haploid green alga *Chlamydomonas reinhardtii* is a useful model of a photosynthetic cell
(Harris, 1989; Harris, 2001). For more than 40 years, this unicellular eukaryote has been
intensively used for studies of a number of physiological processes such as photosynthesis,
respiration, nitrogen assimilation, flagellar motility and basal body function (Rochaix *et al.*, 1998; Silflow & Lefebvre, 2001). Gametic differentiation, zygote production, induction of
meiosis, isolation of diploid strains and haploid cytoductants are easily controlled in
*Chlamydomonas*, making this organism a powerful tool for genetic analysis (Harris, 1989;
Remacle & Matagne, 1998; Harris, 2001).

Over the past 15 years, there has been a dramatic increase in the number of molecular
technologies that can be applied to *Chlamydomonas*, greatly enhancing its interest as a model
organism (Fuhrmann, 2002). Different methods (agitation with glass-beads, electroporation
and biolistics) are available for genetic transformation (Kindle, 1998) and *Chlamydomonas* is
the only organism where transformation of the three genomes (nuclear, chloroplastic and
mitochondrial) has been achieved (Randolph-Anderson *et al.*, 1993; Goldschmidt-Clermont,
1998; Kindle, 1998). Moreover, the chloroplastic and mitochondrial genomes are fully
sequenced (Remacle & Matagne, 1998; Maul *et al.*, 2002). To analyse regulation of gene
expression, reporter genes have been developed, including arylsulfatase (Davies *et al.*, 1994;
Ohresser *et al.*, 1997), *Chlamydomonas* codon use-adapted GFP (green fluorescent protein)
and luciferase genes (Fuhrmann *et al.*, 1999; Minko *et al.*, 1999). Although an efficient
system for disruption of nuclear genes by homologous recombination is lacking, RNA interference (RNAi) technology developed recently for *Chlamydomonas* allows the inactivation of genes of interest (Schroda *et al.*, 1999; Fuhrmann *et al.*, 2001).

Different strategies have been developed to clone nuclear genes whose mutations produce new phenotypes: (i) gene tagging by insertional mutagenesis, (ii) complementation and (iii) positional cloning. These three strategies will be shortly discussed below.

Nuclear transformation has been achieved first using a *Chlamydomonas* gene (Arg7 or Nia1) to complement biochemical mutants (arg7 or nia1) (Kindle, 1998). More recently, dominant heterologous selectable markers have been developed: aadA conferring spectinomycin resistance (Cerutti *et al.*, 1997), Ble conferring bleomycin resistance (Lumbreras *et al.*, 1998), AphVIII conferring paromomycin resistance (Sizova *et al.*, 2001) and a mutant form of acetolactate synthase gene conferring resistance to sulfometuron methyl (Kovar *et al.*, 2002). The random insertion of transforming plasmids by heterologous recombination has been used extensively to induce mutants by gene disruption (Adam *et al.*, 1993; Tam & Lefebvre, 1993; Gumpel & Purton, 1994; Kindle, 1998). When the mutation is tagged, the flanking DNA can be isolated either by plasmid rescue in *E. coli* (Tam & Lefebvre, 1993), inverse PCR (Moseley *et al.*, 2000) or LMS (ligation-mediated suppression) PCR (Strauss *et al.*, 2001). Unfortunately, insertional mutagenesis very often results in untagged mutations through plasmid rearrangements or multiple insertions, or in the deletion of large genomic DNA fragments (up to 20 kb). Such events make identification of the gene responsible for the mutant phenotype more difficult. However, the insertional mutagenesis strategy has been used successfully by several groups and allowed the identification of genes involved in photosynthesis, motility, phototaxis, sulfur and nitrate assimilation (reviewed by Kindle, 1998) or heavy metal tolerance (M. Hanikenne, unpublished results).
For untagged or point mutations that create a counterselectable phenotype, the corresponding gene can be cloned by complementation. Indexed cosmid libraries and YAC (yeast artificial chromosome) libraries have been constructed and used to rescue mutant phenotypes by transformation (Purton & Rochaix, 1994; Zhang et al., 1994; Vashishtha et al., 1996; Randolph-Anderson et al., 1998).

Positional cloning represents a third possibility to identify a gene corresponding to a mutation. Hundreds of mutations and more than 240 molecular markers (including cloned genes, random cDNAs and small genomic fragments) have been located on the 17 linkage groups of the *Chlamydomonas* genetic map (Harris, 1989; Silflow, 1998; Lefebvre & Silflow, 1999). An indexed BAC (bacterial artificial chromosome) library composed of more than 15,000 clones with an average insert size of 70 kb and representing a 10-12 fold coverage of the nuclear genome has been constructed (Lefebvre & Silflow, 1999). As a part of the genome project (see below), the construction of a physical map linked to the genetic map is underway. This work includes BAC ends sequencing and construction of contigs of overlapping BAC clones anchored to the molecular markers (Davies & Grossman, 1998; Lefebvre & Silflow, 1999). The use of both genetic and physical maps will greatly facilitate the positional cloning of genes resulting from untagged or point mutations. Thanks to the rapid life-cycle of *Chlamydomonas*, it will be possible to complete map-based cloning within 6-8 weeks, while this procedure can often take more than a year in *Arabidopsis thaliana* (Grossman, 2000; Dent et al., 2001).

With the genome project initiated in 1999, *Chlamydomonas* is now entering in the era of genomics (Davies & Grossman, 1998). All data are available at the *Chlamydomonas* Ressource Center website (http://www.biology.duke.edu/chlamy_genome/). This project includes: (i) the generation of linked physical and genetic maps (see above), (ii) a whole genome sequencing (a rough draft of the *Chlamydomonas* nuclear genome sequence at 6-8
fold coverage is available since the end of January 2003 at the US Department of Energy Joint Genome Institute website http://www.jgi.doe.gov/, (iii) an extended EST (expressed sequence tag) sequencing program with the goal to identify, analyze, and catalog protein coding sequences. cDNA libraries have been constructed using mRNAs isolated from deflagellated or differentiated (gametes and zygotes) cells and from cells exposed to different environmental conditions including light, dark, low and high CO₂, hypoxia, nutrient starvation (for nitrogen, sulfur, phosphorous, copper and iron), nitrogen source change (nitrate to ammonium and ammonium to nitrate) and stress (hydrogen peroxide, sorbitol and cadmium) (Asamizu et al., 1999; Asamizu et al., 2000; Shrager et al., 2003; http://www.biology.duke.edu/chlamy_genome/libraries.html, http://www.kazusa.or.jp/en/plant/chlamy/EST/). More than 190,000 EST reads have been sequenced and are currently assembled and annotated. Moreover, the data can be used in connection with the microarray technology to investigate global pattern of gene expression. A first ‘chip’ including about 3000 genes is already available. Six thousand other genes will be soon added and the new ‘chips’ should be available in early summer 2003.

The completion of the genome project will make Chlamydomonas an even more attractive organism for cell and molecular investigations in the near future. A global approach of heavy metal homeostasis or tolerance using functional genomics in Chlamydomonas (as proposed for photosynthesis by Dent et al. (2001) will undoubtely enhance our understanding of these processes in plants.

Heavy metal homeostasis

Copper homeostasis
The impact of copper deficiency on both copper uptake and synthesis of metalloproteins involved in photosynthesis has been extensively investigated in *Chlamydomonas*. Its cells exhibit a very high capacity for copper uptake which is mediated by a high affinity copper transport system (with a $K_m$ of approximately 0.2 µM) active in both copper-supplemented and copper-depleted conditions (Hill et al., 1996). However, under copper starvation, the cells display up to 20-fold increased uptake capacity while the $K_m$ for copper is unchanged, which indicates that the expression or activity of the still unidentified copper transporter is induced in copper-depleted cells. Moreover, a cupric reductase activity, that may be associated to the transport activity, is increased 2-fold in copper-deficient cells. This activity is also induced under iron starvation, indicating that copper and iron reduction might be driven by the same enzyme (see below, Weger, 1999). The physiological characteristics of the cupric reductase and copper transport regulation are compatible with their involvement in the same uptake pathway (Hill et al., 1996).

Plastocyanin and cytochrome $c_6$ form a pair of interchangeable photosynthetic electron transfer catalysts responding to copper availability (reviewed by Merchant, 1998). Plastocyanin, encoded by *Pcy1* nuclear gene, is a 98 aa copper protein involved in electron transfer from cytochrome $b_6$ to the photosystem I (PSI). In copper-supplemented cells, the mRNA is translated and the pre-apoprotein is imported into the chloroplast then processed. Under copper deficiency, the apoprotein is degraded and cytochrome $c_6$ functionally substitutes to plastocyanin. This 90 aa heme-containing protein, encoded by *Cyc6*, is synthesized only under copper deficiency when the function of plastocyanin is compromised. The *Cyc6* gene expression is strictly regulated by copper availability, but not by iron status, at the transcriptional level. The accumulation of cytochrome $c_6$ however is dependent on heme availability. Moreover, heme or a tetrapyrrole pathway intermediate might serve to regulate the translation of the *Cyc6* mRNA (Merchant, 1998).
The synthesis of coproporphyrinogen III (coprogen) oxidase, an enzyme encoded by the \textit{Cpx1} nuclear gene and involved in heme biosynthesis is stimulated in copper-deficiency conditions (Hill \& Merchant, 1995). The increased synthesis of coprogen oxidase in copper-depleted cells is attributed to increased level of \textit{Cpx1} mRNA and is rationalized on the basis of a higher need for heme when cytochrome \textit{c} synthesis is induced (Hill \& Merchant, 1995).

The \textit{Cpx1} gene transcription produces 3 transcripts distinct in size (Quinn \textit{et al.}, 1999). The two longer forms are present in both copper-supplemented and copper-depleted cells whereas the shortest transcript is induced under copper deficiency conditions and represents up to 12 fold the amount of the two long transcripts. Transcriptional activation of \textit{Cpx1} occurs through a CuRE (copper-responsive element) containing region of the promoter and is coordinated with the expression of the \textit{Cyc6} gene (Quinn \textit{et al.}, 1999). The constitutive and induced \textit{Cpx1} transcripts have the same half-life \textit{in vivo} and encode the same polypeptide, but the shortest transcripts represent a 2-4 fold better template for translation. The induction of coprogen oxidase by copper deprivation appears to be specific, the transcript abundance of all the members of the tetrapyrrole pathway examined being not changed in response to the cellular copper status (Quinn \textit{et al.}, 1999). Further studies of the coordinated expression of \textit{Cpx1} and \textit{Cyc6} showed that CuREs of both genes contain a GTAC core essential for transcriptional regulation by copper (Quinn \textit{et al.}, 2000). Moreover, the two genes are also induced under hypoxic conditions and surprisingly this regulation also occurs, in part, through the CuRE sequences (Quinn \textit{et al.}, 2000).

Thus, a common regulatory pathway controls various copper-responsive processes under copper starvation: increased cupric reductase activity and copper transport, plastocyanin degradation and activation of \textit{Cpx1} and \textit{Cyc6} gene expression. Furthermore, the CuRE-dependent induction of \textit{Cpx1} and \textit{Cyc6} by hypoxia suggests the occurrence of a
crosstalk between the copper-responsive and the hypoxia signal transduction pathways (Hill et al., 1996; Merchant, 1998; Quinn et al., 1999; Quinn et al., 2000).

A search for new mutants displaying copper-conditional phenotypes led to the isolation of the crdl (copper response defect) mutant exhibiting copper-deficiency conditional chlorosis. The Crdl gene encodes a putative di-iron enzyme required for assembly of PSI and light-harvesting complex I (LHCI) under copper deficiency or hypoxia conditions (Moseley et al., 2000). Recently, it has been suggested that Crdl encodes an enzyme involved in chlorophyll biosynthesis and represents a key target of plastid iron deficiency (see below, Moseley et al., 2002a; Pinta et al., 2002). Crdl mRNA accumulates at a low level in copper- or oxygen-supplemented cells and is induced up to 20 fold in copper- and oxygen-deficient cells (Moseley et al., 2000). Similar amounts of copper are required to rescue the crd phenotype, to repress Cpxl and Cyc6 expression and to maintain the plastocyanin level, suggesting that Crdl is a target of the same signal transduction pathway. This hypothesis has been confirmed by the identification of the Crrl (copper response regulator 1) locus, which is required for adaptation to copper deficiency (Moseley et al., 2002b; Quinn et al., 2002). Under copper starvation, the crrl mutation determines a slow growth rate phenotype and prevents induction of the Cyc6, Cpxl, Crdl target genes (Quinn et al., 2002). In addition, as the GTAC core of the CuREs, the Crrl locus is also required for hypoxia response. However, oxygen-deficiency response requires, in addition to the CuREs, a second cis-element (HyRE), indicating that the two pathways are not identical (Quinn et al., 2002).

A search of the Chlamydomonas EST database allowed the identification of a Crdl paralog which has been named Cthl (copper target homolog) (Moseley et al., 2000). Cthl encodes a 407 aa protein sharing 66% identity with Crdl (Moseley et al., 2002b). The accumulation pattern of Crdl and Cthl is reciprocal: Crdl abundance is increased under copper- or oxygen deficiency while Cthl accumulates in copper-supplemented or oxygenated...
conditions. Fluorescence analyses showed that copper-responsive adjustment of the Cth1/Crd1 ratio results in modification of the interactions between PSI and associated LHCs (Moseley et al., 2002b).

Iron homeostasis

The adaptation of *Chlamydomonas* cells to iron deficiency has been analysed by several groups. Iron starvation leads to a rapid and large increase of cell surface ferric-chelate reductase and ferricyanide reductase activities, both being likely mediated by the same enzyme (Eckhardt & Buckhout, 1998; Lynnes et al., 1998; Weger, 1999). The increase in reductase activities is inversely correlated with iron availability in the medium. Iron (Fe^{2+}) uptake, only detected in iron-deficient cells, is inhibited by 87% when 100 fold excess Cu^{2+} is added to the medium, while it is stimulated by cadmium and calcium. This result suggests that the same enzyme might be responsible for both cupric and ferric reductase activities (Eckhardt & Buckhout, 1998). Comparison of the iron reduction and iron uptake rates indicates that uptake represents the limiting-step in iron assimilation (Eckhardt & Buckhout, 1998).

A multicopper ferroxidase (encoded by the *Fox1* gene) involved in high affinity iron uptake has been identified recently (Herbik et al., 2002; La Fontaine et al., 2002). *Fox1* expression is induced under iron deficiency both at mRNA and protein levels. While the *Fox1* mRNA induction in iron starvation conditions is not affected by copper deficiency, the protein accumulation is strongly dependent on copper availability (La Fontaine et al., 2002). Whether a copper deficiency affects iron uptake remains unclear. Indeed, copper-depleted cells display no sign (such as chlorosis) of iron deficiency (Hill et al., 1996; La Fontaine et al., 2002), but a reduction of iron uptake has been recorded in these conditions (Herbik et al., 2002). It has
been proposed recently that there may be a copper-independent enzymatic pathway regulated by copper availability for iron assimilation (La Fontaine et al., 2002).

Moreover, genes encoding an iron permease (Ftr1), a copper chaperone (Atx1), and a copper-transporting ATPase (Ccc2 homolog) were identified in the Chlamydomonas EST database (La Fontaine et al., 2002). Fox1 and Ftr1 are coordinately induced (up to $10^2$ fold) by iron deficiency, suggesting the occurrence of a ferroxidase/iron permease complex involved in iron uptake similar to that described in the yeast Saccharomyces cerevisiae (La Fontaine et al., 2002). Atx1 is also induced by iron deficiency although to a lesser extent than Fox1 and Ftr1, and is related to the yeast copper chaperone as demonstrated by functional complementation. Together with a copper-transporting ATPase, Atx1 might function in the Fox1 protein biosynthesis and more generally in copper delivery to the secretory pathway (La Fontaine et al., 2002). Altogether, these results reveal, for the first time in a photosynthetic organism, the role of copper in iron assimilation and the occurrence of an iron assimilation pathway related to the high affinity iron uptake pathway of S. cerevisiae (Herbik et al., 2002; La Fontaine et al., 2002).

As iron uptake involves both ferric-chelate reductase (Eckhardt & Buckhout, 1998; Lynnes et al., 1998; Weger, 1999) and multicopper ferroxidase activities (Herbik et al., 2002; La Fontaine et al., 2002), the question arises as to why a combined reduction of ferric-chelates and reoxidation of Fe$^{2+}$ is required for iron uptake. It has been proposed that the ferroxidase confers selectivity and specificity to high affinity iron uptake and that reoxidation of Fe$^{2+}$ avoids the production of reactive oxygen species (Askwith & Kaplan, 1998; Herbik et al., 2002).

A Fer1 cDNA encoding ferritin, a key protein for iron storage and homeostasis in the cell, was also identified in Chlamydomonas EST database (La Fontaine et al., 2002). The abundance of Fer1 mRNA increases up to 10 fold under iron starvation. This induction might
be rationalized as a part of a mechanism to anticipate iron overload: this transient overload
might result from either iron resupplying to iron-starved cells after induction of the uptake
pathway or iron released from degrading PSI (La Fontaine et al., 2002; Moseley et al.,
2002a).

Recently, the impact of iron starvation on photosynthesis has been investigated in
Chlamydomonas (Moseley et al., 2002a). Iron deficiency leads to chlorosis owing to a
sequential adaptation of the photosynthetic apparatus. The first response which occurs before
the manifestation of chlorosis is the disconnection of LHCI antenna from PSI. This initial
uncoupling of the LHCI from PSI seems to be regulated via the K subunit of PSI in response
to a change in plastid iron content, which is sensed through the occupancy, and thus activity,
of the iron-containing active site in Crd1 (see above). This first adaptation is followed by a
specific degradation of existing LHCs and induction of new complexes leading to a
remodeling of the antenna. The authors suggest that these adaptations allow to by-pass the
light sensitivity resulting from PSI loss in iron-depleted cells.

Heavy metal tolerance in Chlamydomonas

Cell responses to heavy metal exposure

The effects of heavy metals (copper, zinc, iron, mercury, lead, cadmium) have been studied
for decades in Chlamydomonas. Mercury (0.25-5 mg l-1) significantly reduces growth (Ben-
Bassat et al., 1972; Weiss-Magasic et al., 1997) while lead (1-20 µM) causes a marked
reduction of photosynthesis and induces severe ultrastructural changes, notably alteration of
the thylakoidal, mitochondrial and nuclear structures (Irmer et al., 1986). The growth,
photosynthetic activity and chlorophyll content are also affected by cadmium and copper
(Collard & Matagne, 1990; Nagel & Voigt, 1995; Prasad et al., 1998; Boswell et al., 2002). In addition, cadmium, copper and zinc inhibit nitrate uptake by the cells (Devriese et al., 2001).

The *Chlamydomonas* cell wall displays a high affinity for metallic cations (Collard & Matagne, 1990) and represents the first protection barrier against heavy metals. Wall-less strains are consistently more sensitive to cadmium, copper, nickel and cobalt than are walled strains (Collard & Matagne, 1990; MacFie et al., 1994; Prasad et al., 1998).

Gekeler et al. (1989) first demonstrated the occurrence of phytochelatins (PC) in *Chlamydomonas*. More recent works showed that these metal-binding peptides are the major intracellular metal-chelators induced upon cadmium treatments, PC complexes sequestering up to 70% of the total cadmium found in cadmium-treated cells (Howe & Merchant, 1992; Hu et al., 2001). Two types of PC-Cd complexes have been identified: the acid labile sulfide-containing high molecular weight (HMW) complexes and the low molecular weight (LMW) complexes. LMW complexes are rapidly converted in HMW complexes that accumulate into the cells and contribute to a stable cadmium sequestration. LMW complexes only accumulate after prolonged cadmium exposures and appear to be an early sign of metal stress (Hu et al., 2001).

Thioredoxins (TRXs) also appear to contribute to heavy metal detoxification in *Chlamydomonas* (Lemaire et al., 1999; Lemaire et al., 2002). Two TRX genes (encoding isoforms *m* and *h* located in the chloroplast and the cytosol, respectively) have been characterized (Jacquot et al., 1998). The transcriptional expression of both genes is stimulated by cadmium and mercury, but in a different manner. Relevant cis-acting elements and protein accumulation are only observed for TRX *h*. Moreover, heavy metals inactivate TRXs, presumably by binding to their dithiol active site. The data of Lemaire et al. (1999) suggest a possible implication of TRXs in heavy metal detoxification with a different regulation pattern for each TRX.
Finally, glutathione (GSH) was shown to be the principal compound induced after exposure of cells to mercury (Howe & Merchant, 1992).

Expression of foreign genes and heavy metal tolerance

The expression in *Chlamydomonas* of a chicken class II metallothionein (MT-II) gene enhances tolerance of the algal cells to cadmium toxic concentrations (Hua *et al.*, 1999). Moreover, cells expressing the MT-II gene have a two-fold higher cadmium binding capacity relative to wild-type cells when exposed to cadmium concentrations (5 µM) that do not induce PC expression. When MT-II cells are exposed to cadmium concentration (40 µM) that induces PC synthesis, there is however no increase in their cadmium-binding capacity relative to wild type (Hua *et al.*, 1999).

In order to investigate the role of proline (Pro) in heavy metal tolerance, a mothbean (*Vigna aconitifolia*) gene (*P5CS*) encoding Δ¹-pyrroline-5-carboxylate synthetase, has been introduced in *Chlamydomonas* (Siripornadulsil *et al.*, 2002). *P5CS* is involved in the first step of Pro biosynthesis from glutamate. Transgenic algae expressing the *P5CS* gene have nearly 2-fold higher free Pro level, are more tolerant to cadmium and have 4-fold higher cadmium level per cell than wild-type cells. Extended X-ray absorption fine structure (EXAFS) spectroscopy analyses have shown that cadmium is sequestered by phytochelatins, and not by Pro in transgenic clones. Measurements of reduced/oxidized GSH ratios and free-radical lipid damages suggest that the free Pro acts as an antioxidant in the cadmium-treated cells, resulting in a more reducing cellular environment. The higher GSH level in turn facilitates PC synthesis and sequestration of PC-Cd complexes in vacuoles (Siripornadulsil *et al.*, 2002).

Mutants resistant or sensitive to heavy metals
Metal-resistant mutants have been isolated by different groups (Collard & Matagne, 1990; Collard & Matagne, 1994; Fujiwara et al., 2000; Hu et al., 2001) but to our knowledge the corresponding genes have never been identified. As expected, the screening for resistance mutations essentially led to the isolation of permeability mutants, probably resulting from an alteration of the metal transport across the plasma membrane. The 13 arsenate-resistant mutants isolated by Fujiwara et al. (2000) accumulate lower level of arsenic than the wild type. In the cadA<sup>R</sup> and cadB<sup>R</sup> cadmium-resistant mutants, as well as in a cadA<sup>R</sup> cadB<sup>R</sup> double mutant, the cadmium tolerance was also associated with a lower metal accumulation (Collard & Matagne, 1990; Collard & Matagne, 1994). Two cadmium-resistant mutants (KL19 and KL23) isolated by Hu et al. (2001) produce higher levels of HMW PC-Cd complex, reduced GSH and cysteine than the wild type. Two other mutants (KL16 and KL20), displaying lower levels of PC-Cd complexes, are probably permeability mutants (Hu et al., 2001). Finally, another resistant mutant, displaying an unaffected cadmium uptake and cadmium sequestration by PC, was shown to be impaired in photosynthetic activity, as revealed by a reduced growth under photoautotrophic conditions, a decreased – but cadmium resistant – photosynthetic oxygen evolution, a reduced PSII activity and an altered chlorophyll fluorescence induction in dark-adapted cells (Nagel & Voigt, 1995; Voigt et al., 1998). The acetate inhibition of the water-splitting complex of PSII observed in the wild type is suppressed in the resistant mutant suggesting that the donor side of PSII is impaired in this strain (Voigt & Nagel, 2002). Whether cadmium-resistance phenotype and impaired PSII activity are related to the mutation of one or several genes remains to be determined. It can be hypothesized that the resistance mutation results from reduced affinity for cadmium of a PSII polypeptide.
In order to identify genes involved in heavy metal tolerance, insertional mutagenesis has been used to induce heavy metal hypersensitive mutants. Cadmium-sensitive mutants have been isolated by Pfeifer-McHugh et al. (1994) but to our knowledge, these mutant strains have never been further characterized.

In our laboratory, more than 7500 transformants induced by insertional mutagenesis (using Arg7 or Ble as selectable marker) were screened for cadmium and copper hypersensitivity (Hanikenne et al., 2001; M. Hanikenne, unpublished results). Out of 28 mutants isolated, six are only sensitive to cadmium while five are only sensitive to copper. The seventeen other mutants are pleiotropic and display sensitivity to several (2 to 7) agents (cadmium, copper, lead, hydrogen peroxide, tert-butylhydroperoxide, paraquat, UVC and light). Further analyses have shown that five (Cd30, Cd34, Cd41, Cd43 and Cd135) of the six mutants exclusively sensitive to cadmium are allelic (Cds1 gene) whereas the sixth mutation (Cd47), conferring a lower sensitivity to cadmium, affects another gene (Cds2). The mutation is tagged (insertion of a single and intact plasmid copy linked to the mutant phenotype) in five mutants (Cd34, Cd61, Cu109, Cu141, Cu145) whereas several intact or truncated plasmid copies are integrated in the genome of the other mutants (untagged mutations). Cloning of the Cds1 gene has been undertaken using plasmid rescue in E. coli followed by a screening of the Chlamydomonas BAC library with the rescued probe. The Cds1 gene encodes a protein sharing strong similarities with ABC (ATP binding cassette) transporters (M. Hanikenne, to be published). In yeasts, two ABC transporters involved in cadmium detoxification – both vacuolar – were previously described: Hmt1 in Schizosaccharomyces pombe (Ortiz et al., 1992; Ortiz et al., 1995) and Ycf1 in S. cerevisiae (Wemmie et al., 1994; Li et al., 1997). To our knowledge, this is the first time that a gene encoding an ABC transporter involved in cadmium tolerance is identified in plants.
The search for genes differentially expressed upon metal treatments was also performed in *Chlamydomonas*. mRNA differential display has been used to analyse changes in transcript levels after a short (2 h) exposure to 25 µM cadmium. Thirteen non-redundant differentially expressed mRNAs were found which allowed the identification of four genes (Table 1) of known function induced by cadmium treatment (Rubinelli et al., 2002).

*Crd1* encodes a putative di-iron enzyme possibly involved in chlorophyll biosynthesis and in remodeling of PSI under iron deficiency (see above, Moseley et al., 2002a; Pinta et al., 2002). The Crd1 protein is also required for assembly of PSI and light-harvesting complex LHCl under copper deficiency and hypoxia conditions (Moseley et al., 2000; Moseley et al., 2002b). The 2 fold induction of *Crd1* by cadmium could result from a cadmium interference with either uptake or cofactor function of copper or iron.

*CHLL* encodes the regulatory subunit of the light-independent protochlorophyllide reductase and is regulated by the chloroplast redox state. The weak induction (2 fold) of this gene could be related to oxidizing conditions resulting from cadmium exposure or to the susceptibility of its thiol groups to cadmium poisoning.

*CHRSAMS* encodes an S-adenosylmethionine (SAM) synthetase, SAM being a precursor of cysteine. An increase in *CHRSAMS* transcript abundance (2 fold) could thus support an enhanced GSH synthesis and in turn PC synthesis.

*H43* encodes a high-CO$_2$-inducible protein localized in the periplasmic space (Kobayashi, 1997). This gene is related to the high CO$_2$-inducible and iron-deficiency inducible *HCR1* gene of *Chlorococcum littorale* (Sasaki et al., 1998). *H43* is induced 20 fold upon cadmium exposure, but also after iron deficiency, and is able to partially complement the Fe-uptake double-mutant *fet3fet4* of *S. cerevisiae*. Cadmium is assumed to compete with
iron for uptake, resulting in iron deficiency and in the induction of the H43 gene (Rubinelli et al., 2002). However, H43, which is not related to A. thaliana Irt1 and Nramp3 iron transporters, plasma membrane proton-ATPases or ferric reductases, might represent a novel alga-specific protein iron transporter (Rubinelli et al., 2002).

The availability of DNA microarrays for Chlamydomonas (see above) will allow to investigate global pattern of gene expression upon heavy metal treatments as it has recently been done for cadmium response in the yeast S. cerevisiae (Momose & Iwahashi, 2001).

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### Table 1. Genes induced upon cadmium treatment in *C. reinhardtii*.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genbank Accession No.</th>
<th>Product</th>
<th>Function</th>
<th>Fold induction</th>
</tr>
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<tbody>
<tr>
<td>Crd1</td>
<td>AF236101</td>
<td>putative di-iron enzyme</td>
<td>involved in chlorophyll biosynthesis and in remodeling of PSI under iron deficiency; required for PSI and LHCI assembly under copper deficiency and hypoxia</td>
<td>2</td>
</tr>
<tr>
<td>CHLL</td>
<td>X60490</td>
<td>regulatory subunit of the light-independent protochlorophyllide reductase</td>
<td>chlorophyll biosynthesis</td>
<td>2</td>
</tr>
<tr>
<td>CHRSAMS</td>
<td>AF008568</td>
<td>SAM synthetase</td>
<td>S-adenosylmethionine (SAM) synthesis</td>
<td>2</td>
</tr>
<tr>
<td>H43</td>
<td>AB042098</td>
<td>periplasmic protein inducible by high CO₂ and iron-deficiency</td>
<td>might be involved in iron uptake</td>
<td>20</td>
</tr>
</tbody>
</table>