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 organellar 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 rearrangments 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 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 CO_2, 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 unindentified 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 involvment 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_6f 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 *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 *Cpx1* mRNA and is rationalized on the basis of a higher need for heme when cytochrome c_6 synthesis is induced (Hill & Merchant, 1995). The *Cpx1* gene transcription produces 3 transcripts distinct in size (Quinn *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 *Cpx1* occurs through a CuRE (copper-responsive element) containing region of the promoter and is coordinated with the expression of the *Cyc6* gene (Quinn *et al.*, 1999). The constitutive and induced *Cpx1* transcripts have the same half-life *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 *et al.*, 1999). Further studies of the coordinated expression of *Cpx1* and *Cyc6* showed that CuREs of both genes contain a GTAC core essential for transcriptional regulation by copper (Quinn *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 *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 *Cpx1* and *Cyc6* gene expression. Furthermore, the CuRE-dependent induction of *Cpx1* and *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 *crd1* (copper response defect) mutant exhibiting copper-deficiency conditional chlorosis. The *Crd1* 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 *Crd1* 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). *Crd1* 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 *Cpx1* and *Cyc6* expression and to maintain the plastocyanin level, suggesting that *Crd1* is a target of the same signal transduction pathway. This hypothesis has been confirmed by the identification of the *Crr1* (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 *crr1* mutation determines a slow growth rate phenotype and prevents induction of the *Cyc6*, *Cpx1*, *Crd1* target genes (Quinn *et al.*, 2002). In addition, as the GTAC core of the CuREs, the *Crr1* 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 *Crd1* paralog which has been named *Cth1* (copper target homolog) (Moseley *et al.*, 2000). *Cth1* encodes a 407 aa protein sharing 66% indentity with Crd1 (Moseley *et al.*, 2002b). The accumulation pattern of Crd1 and Cth1 is reciprocal: Crd1 abundance is increased under copper- or oxygen deficiency while Cth1 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; Weger, 1999). 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 occurence 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 occurence 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 results 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 Δ^1-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 differents 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 $cadA^R$ and $cadB^R$ cadmium-resistant mutants, as well as in a $cadA^R$ $cadB^R$ 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 an other 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.

Search for genes differentially expressed upon metal treatments

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 LHCI 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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734 **Table 1.** Genes induced upon cadmium treatment in *C. reinhardtii*.

Gene name	Genbank Accession No.	Product	Function	Fold induction
Crd1	AF236101	putative di-iron enzyme	involved in chlorophyll biosynthesis and in remodeling of PSI under iron deficiency; required for PSI and LHCI assembly under copper deficiency and hypoxia	2
CHLL	X60490	regulatory subunit of the light-independent protochlorophyllide reductase	chlorophyll biosynthesis	2
CHRSAMS	AF008568	SAM synthetase	S-adenosylmethionine (SAM) synthesis	2
H43	AB042098	periplasmic protein inducible by high $C0_2$ and iron-deficiency	might be involved in iron uptake	20

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