A mitochondrial half-size ABC transporter is involved in cadmium tolerance in *Chlamydomonas reinhardtii*

Running title: Mitochondrial ABC transporter and cadmium tolerance

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Abstract

Five cadmium-sensitive insertional mutants, all affected at the *CDS1* ("*cadmium-sensitive 1*") locus, have been previously isolated in the unicellular green alga *Chlamydomonas reinhardtii*. We here describe the cloning of the *Cds1* gene (8314 bp with 26 introns) and the corresponding cDNA. The *Cds1* gene, strongly induced by cadmium, encodes a putative protein (CrCds1) of 1062 amino acid residues that belongs to the ATM/HMT subfamily of half-size ABC transporters. This subfamily includes both vacuolar HMT-type proteins transporting phytochelatin-cadmium complexes from the cytoplasm to the vacuole and mitochondrial ATM-type proteins involved in the maturation of cytosolic Fe/S proteins. Unlike the *Δsphmt1* cadmium-sensitive mutant of *Schizosaccharomyces pombe* that lacks a vacuolar HMT-type transporter, the *cds1* mutant accumulates high amount of phytochelatin-cadmium complexes. By epitope tagging, the CrCds1 protein was localized in the mitochondria. Even though mitochondria of *cds1* do not accumulate important amounts of "free" iron, the mutants cells are hypersensitive to high iron concentrations. Our data show for the first time that a mitochondrial ATM-like transporter play a major role in tolerance to cadmium.

Key-words: *Chlamydomonas*, ABC transporters, cadmium tolerance, mitochondria, iron homeostasis

Introduction

Cadmium is one of the major heavy metal pollutants originating from metal working industries, power stations, use of fertilizers and waste incineration (Warren 1989; Satarug, Baker, Urbenjapol, Haswell-Elkins, Reilly, Williams & Moore 2003). Its toxicity to cells occurs at very low concentrations and chronic exposure to the metal is known to cause various pathological disorders in humans (Warren 1989; Satarug *et al*. 2003). In plants, cadmium affects the water balance, damages several components of the photosynthetic apparatus and inhibits the oxidative phosphorylation in mitochondria, thus causing oxidative stress and lipid peroxidation (Ouariti, Boussama, Zarrouk, Cherif & Ghorbal 1997; Sanita di Toppi & Gabbrielli 1999; Watanabe, Henmi, Ogawa & Suzuki 2003). As a rule, cadmium strongly interacts with sulfhydryl groups and displaces endogenous metal cofactors from their enzymatic and cellular binding sites (Goyer 1997).

To cope with the deleterious effects of cadmium, eukaryotic cells overproduce organic acids (malate, citrate, oxalate), amino acids (proline) and/or sulfhydryl group-containing (poly)peptides (glutathione, phytochelatins, metallothioneins, thioredoxins), all involved in detoxification mechanisms. Cadmium also induces different enzymatic systems participating in metal excretion or compartmentalization and in oxidative stress responses (Rauser 1999; Cobbett & Goldsbrough 2002; Clemens & Simm 2003). In plants, algae and some fungi, glutathione (GSH) and phytochelatins (PC) play a prominent role in cadmium detoxification and mutants of *Arabidopsis thaliana* and *Schizosaccharomyces pombe* deficient in these cystein-containing compounds are hypersensitive to cadmium (Cobbett & Goldsbrough 2002).

Some ABC (“ATP-binding cassette”) transporters are also implicated in cadmium tolerance. These ubiquitous transporters that are involved in a large number of physiological processes,

constitute one of the largest protein families. Typical ABC transporters (the so-called full-size transporters) possess two conserved nucleotide binding folds (NBF) responsible for ATP hydrolysis alternating with two highly hydrophobic transmembrane domains that specify the substrates to be transported. The half-size ABC transporters possess a single copy of each domain and are assumed to function as homo- or heterodimers (Higgins 1992; Holland, Cole, Kuchler & Higgins 2003).

The ScYCF1 protein of the yeast *Saccharomyces cerevisae*, a full-size ABC transporter of the MRP ("multidrug-resistance-related protein") subfamily, ensures the transport of bis(glutathionato)cadmium complexes from cytoplasm to vacuoles. The lack of the transporter determines hypersensitivity to cadmium, arsenite and mercury (Szczypka, Wemmie, Moye-Rowley & Thiele 1994; Li, Lu, Zhen, Szczypka, Thiele & Rea 1997; Ghosh, Shen & Rosen 1999; Gueldry, Lazard, Delort, Dauplais, Grigoras, Blanquet & Plateau 2003). The AtMRP3 transporter of *A. thaliana*, a homolog of the yeast ScYCF1 protein, is also probably implicated in cadmium detoxification and transport. The *AtMrp3* gene is up-regulated by cadmium and this induction is apparently related to the accumulation of cadmium within the plant organs (Bovet, Eggmann, Meylan-Bettex, Polier, Kammer, Marin, Feller & Martinoia 2003). Moreover, *AtMrp3* complements the cadmium sensitivity of a *Δscycf1* yeast mutant (Tommasini, Vogt, Fromenteau, Hortensteiner, Matile, Amrhein & Martinoia 1998) and conversely the overexpression of ScYCF1 in *A. thaliana* enhances the tolerance of the plant to Pb^{2+} and Cd^{2+} (Song, Ju Sohn, Martinoia, Jik Lee, Yang, Jasinski, Forestier, Hwang & Lee 2003).

In the fission yeast *S. pombe*, the half-size ABC transporter SpHMT1 is involved in the transport of phytochelatin-cadmium complexes from the cytoplasm to the vacuoles. A mutant strain lacking this transporter is unable to accumulate high molecular weight (HMW)

phytochelatin-cadmium complexes in the vacuoles and displays hypersensitivity to cadmium (Ortiz, Kreppel, Speiser, Scheel, McDonald & Ow 1992; Ortiz, Ruscitti, McCue & Ow 1995). We here describe the cloning of *Cds1* ("*cadmium-sensitive 1*"), a gene responsible for cadmium tolerance in the unicellular alga *Chlamydomonas reinhardtii* (Hanikenne, Matagne & Loppes 2001; Hanikenne 2003). The gene that is induced by cadmium encodes a mitochondrial ABC protein related to the ATM subfamily of half-size transporters. To our knowledge, a role in heavy metal tolerance constitutes a novel property for a mitochondrial ABC transporter.

Materials and methods

C. reinhardtii strains and growth conditions

Strains C1, Cd30, Cd34, Cd41, Cd43 and Cd47 of *C. reinhardtii* have been previously described (Hanikenne *et al.* 2001). All are derived from an *arg7 cw15* mt^+ strain by transformation with plasmid pASL, harbouring the *Arg7* gene (Adam & Loppes 1998). Except C1 (control), all strains are hypersensitive to cadmium. Mutations from strains Cd30, Cd34, Cd41 and Cd43 all map at the *CDS1* locus whereas Cd47 is affected at the unlinked *CDS2* locus (Hanikenne *et al.* 2001). Strain Cd135 is another cadmium-sensitive insertional mutant obtained by transformation with plasmid pSP124s, harbouring the *Ble* marker (Lumbreras, Stevens & Purton 1998). It was shown to be allelic to the Cd34 mutant (Nieberding C. and Hanikenne M., unpublished). Strain Cd34-nit_1 is a arg^+ *nit1 cw15 cds1* product obtained from the backcross *cw15 arg7 nit1 nit2* mt^- x Cd34. This strain allows co-transformation with the *Nit1* marker. All strains were grown on TRIS-acetate-phosphate (TAP) agar medium (Harris 1989). For cadmium and phytochelatin-cadmium complex

accumulation experiments, cells were grown in TAgP medium, in which inorganic phosphates were replaced by glycerophosphate (Collard & Matagne 1990; Hu, Lau & Wu 2001). Iron sensitivity was determined in liquid TAP medium supplemented with $FeCl_2$. Growth rate was determined after 40 h of culture by measuring the protein content of the cell suspension.

Transformation and complementation experiments

Cell wall-less *C. reinhardtii* strains were transformed by the glass-bead method (Kindle 1990) with minor modifications as previously described (Adam, Lentz & Loppes 1993). For complementation experiments, Cd34-nit_1 cells were co-transformed with 1 µg of *Eco*RI-restricted pAD35 plasmid and 3 µg of genomic DNA. Cadmium-resistant clones were directly selected on TAP_{NO3} + 100 µM cadmium. Plasmid pAD35 (pBluescript II KS^+ + 8.8-kb *Xho*I-*Eco*RI fragment bearing the *Nit1* gene coding for nitrate reductase) was a gift from Dr E. Fernández (University of Córdoba, Spain).

Isolation of the *Cds1* gene

Total DNA from strain Cd34 was extracted as previously described (Hanikenne *et al.* 2001) and digested with *Sal*I to release a fragment including $pBCKS^+$ (3.4-kb) and a genomic sequence flanking the bacterial sequence of the plasmid. The DNA fragments were ligated at low DNA concentration (1 µg/ml) and transformed into *E. coli* (Adam & Loppes 1998). The isolated chloramphenicol-resistant clones harboured a 7.6-kb plasmid (named pC34) showing a single *Sal*I site. Restriction of pC34 with *Sal*I and *Xba*I released a 4.2-kb sequence flanking one of the insertion sites. This fragment hybridized to a single wild-type genomic fragment as shown by Southern Blot (data not shown) and was used to screen a *Chlamydomonas* BAC

library (Lefebvre & Silflow 1999) following the manufacturer's instructions (Incyte Genomics Inc.). The single clone isolated (4G03 that we renamed pBAC1) was able to complement the *cds1* mutation. A pBAC1 sub-library was constructed by *Sau*3AI partial digestion, purification and subcloning of 8 to 15-kb fragments into pBluescript II KS^+ (Stratagene, La Jolla, CA, USA). Among the seventeen clones isolated, two (p5 and p46) complemented the *cds1* mutation as did the complete pBAC1. The genomic sequence of p46 plasmid (14.1-kb) was determined on one strand, as well as a 8.5-kb region spanning the *Cds1* gene on the second strand. Three gene prediction sofwares were used to search for open reading frames: (i) GreenGenie for *C. reinhardtii* genes (http://www.cse.ucsc.edu/%7Edkulp/cgi-bin/greenGenie), (ii) GeneMark used with *C. reinhardtii* parameters (http://opal.biology.gatech.edu/GeneMark/eukhmm.cgi), and (iii) GENSCAN used with *A. thaliana* parameters (http://genes.mit.edu/GENSCAN.html).

Isolation of the *Cds1* cDNA

The cDNA was isolated from a *C. reinhardtii* λgt10 cDNA library (kindly provided by Dr L.G. Franzén, University of Göteborg) by plaque hybridization. The *Cds1* probe used is a 522-bp *Kpn*I fragment located in the last exon of the *Cds1* gene (from 7565 to 8086 positions). It appeared that none of the 4 isolated cDNA were full-length clones, but were lacking both 5' and 3' ends. The 5' end of the transcript (1576-bp) was isolated by reverse transcription-PCR with forward primer 5'-TGCAAACAATCAGTGCAACTTTCG-3' and reverse primer 5'-TCATCTCGCGACGCAGCTTG-3'. The transcription initiation site was determined by primer extension as previously described (Loppes & Radoux 2001) using primer 5'-CTGCGTTATGCAGATGATGTC-3'. The 3' end of the transcript (1355-bp) was isolated by reverse transcription-PCR with forward primer 5'-

TGCACAACATCCGCTACGGCA-3' and reverse primer 5'-TTACGGTACATTAACTCCGGCGT-3'. Alignment of this sequence with ACE20021010.567 from *Chlamydomonas* EST database (Shrager, Hauser, Chang, Harris, Davies, McDermott, Tamse, Zhang & Grossman 2003) showed that the RTPCR product was 13-bp shorter than the EST contig. Altogether, the complete cDNA is 3690-bp long. Reverse transcription was carried out using the Powerscript reverse transcriptase (Clontech Laboratories Inc.) and 1 µg of total RNA extracted from C1 strain cells after 4 h of exposure to 200 µM cadmium.

Construction of a *Cds1* gene tagged with 3 copies of a Hemagglutinin (HA) epitope

A 145-bp fragment harbouring 3 copies of a HA epitope was amplified by PCR from the plasmid p3xHA (Silflow, LaVoie, Tam, Tousey, Sanders, Wu, Borodovsky & Lefebvre 2001) using forward primer 5'-**GGGTCCC**CGGGGAGGCCTGTCGCGATAC-3' and reverse primer 5'-**GGGACCC**CGGCGAGTACTGCTAGCGGC-3' (*Ppu*MI restriction sites indicated in boldface letters have been added at the end of both primers). This fragment was digested with *Ppu*MI and inserted in-frame (at position 7663) into the *Cds1* genomic sequence. Correct cloning was confirmed by sequencing.

Immunocytochemistry

C. reinhardtii cells expressing *Cds1* or *Cds1::HA* genes were grown for 16 hrs in TAP + 100 µM cadmium. The cells were washed 3 times, then incubated for 30 minutes in TAP medium added with 3 µM MitoTracker Orange CMTMRos (Molecular Probes, Leiden, The Netherlands). After fixation for 10 min in 100% methanol at -20°C, the cells were allowed to

adhere to Starfrost slides (VWR, Westchester, PA, USA). The cells were then permeabilized 2 min in acetone at -20°C, air-dried and rehydrated in blocking buffer (PBS + 1 % BSA + 5 % normal goat serum) for 1 h. The slides were incubated for 16 h at 4°C with a rat anti-HA high affinity antibody (Roche Diagnostics, Mannheim, Germany), diluted at 1 µg/ml in blocking buffer, washed 3 times with PBS buffer and incubated for 2 h at 4°C with an Alexa-488-conjugated goat anti-rat IgG (Molecular Probes, Leiden, The Netherlands) diluted 1:500 in blocking buffer. The spatial distribution of fluorescence was imaged by confocal microscopy using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Germany) equipped with one argon and two helium-neon lasers, and an acousto-optical tunable filter (AOTF) for excitation intensity. Digitized images were acquired using a 63 x NA 1.5 Plan-Apo water-immersion objective at 1024 x 1024 pixel resolution. The diameter of the pinhole was always set up equal to the Airy unit. For each cell, serial optical sections were recorded with a Z-step of ~ 0.2 µm. Images were acquired under identical conditions and we ensured that the maximal fluorescence signal was not saturating the PMTs. For multicolour imaging, Alexa488 was visualized by using an excitation wavelength of 488 nm and the emission light was dispersed and recorded at 500 to 550 nm. MitoTracker was detected by using an excitation wavelength of 543 and the 488/543 dichroic mirror, and the fluorescence emission was dispersed and recorded at 570 to 605 nm. To avoid crosstalk of the fluorescence emissions, we performed sequential image recording using Leica software (version 2.5). Captured images were exported as TIFF format files and further processed using Adobe Photoshop 7.0 only for figure mounting and labelling purpose.

Other methods

Total *C. reinhardtii* RNA was extracted according to Loppes & Radoux (2001). Northern blot analyses were performed as described by Dinant, Baurain, Coosemans, Joris & Matagne (2001) using the *Cds1* probe (*see* above). Cadmium accumulation in *C. reinhardtii* cells was followed by atomic absorption spectrophotometry and phytochelatin-cadmium accumulation was analyzed by gel filtration chromatography as described previously (Hu *et al.* 2001). Whole-cell respiration measurements, as well as mitochondria purifications (EDTA was omitted in extraction buffers for iron content dosage) in *C. reinhardtii* were achieved according to published procedures (Duby & Matagne 1999; Cardol, Matagne & Remacle 2002; Baurain, Dinant, Coosemans & Matagne 2003). SOD activities were determined on non-denaturing polyacrylamide gel by negative coloration (Flohe & Otting 1984). Free iron content of mitochondria was determined using the bathophenanthroline method (Tangeras, Flatmark, Backstrom & Ehrenberg 1980).

Results

Cloning of the *Cds*1 gene and cDNA from *C. reinhardtii*

We have previously isolated six insertional mutants of *C. reinhardtii* displaying hypersensitivity to cadmium. Five mutations (strain Cd30, Cd34, Cd41, Cd43 and Cd135) were found to be allelic and located at the *CDS1* locus, while the sixth one (strain Cd47) affected another locus (*CDS2*) (*see* Materials and Methods). We also showed that in the Cd34 mutant, the *CDS1* locus was tagged by the pASL plasmid (Hanikenne *et al.* 2001).

In the present study, a 4.2-kb genomic DNA fragment flanking the bacterial sequence of the tagging plasmid was isolated by plasmid rescue in *E. coli* and used to screen a *Chlamydomonas* BAC library. A single BAC clone (pBAC1) with an insert size of about 100-kb was isolated. When transformed into the Cd34 mutant background, pBAC1 complemented

the *cds1* mutation and restored resistance to cadmium. To identify the *Cds1* gene present on the 100-kb insert, a pBAC1 sub-library was constructed and among the 17 subclones isolated, two (p5 and p46) were found to complement the sensitivity of the Cd34 mutant. The mutant allele present in the Cd41 strain was also complemented by the two subclones (data not shown). The 14.1-kb genomic sequence of the p46 sub-clone was determined and a single open reading frame containing numerous introns was identified using three gene prediction softwares (GreenGenie, GeneMark and GENSCAN, data not shown). The corresponding sequence was found in a search of the *C. reinhardtii* nuclear genome (available at the US Department of Energy Joint Genome Institute website, http://www.jgi.doe.gov/). It falls within the scaffold 122 and is represented by genewise.50.44.1 and genie.50.0 predictions. We found that the p46 sub-clone shared no sequence homology with the 4.2-kb rescued probe (data not shown) suggesting that a large deletion (of at least 20-kb, data not shown) occurred during the mutagenic insertion of the plasmid into the nuclear genome of Cd34. Such important rearrangments are commonly observed in mutants obtained by insertional mutagenesis in *C. reinhardtii* (Tam & Lefebvre 1993; Adam & Loppes 1998; Kindle 1998).

Using a 522-bp probe located in the last exon of *Cds1* (see Figure 1A), we isolated four partial cDNAs from a *Chlamydomonas* cDNA library. The remainder of the cDNA was isolated by reverse transcription-PCR. The complete sequence of the cDNA (3,690-pb, GenBank accession number AY327516) was determined and the origin of transcription was identified by primer extension (data not shown).

Cds1 encodes a protein of the ATM/HMT subfamily of half-size ABC transporters

Alignment of cDNA and genomic sequences allowed to determine the intron/exon structure of the *Cds1* gene. With a size of 8314-bp (GenBank accession number AY327517), the *Cds1*

gene contains 26 introns, the first one being located in the 5' untranslated region of the sequence (Figure 1A). A possible TATA box (TATTTAT) is located at position -26 from the initiation site of transcription and a putative polyadenylation signal (TGTAC) is found 19-bp upstream the polyadenylation site (data not shown). Two in-frame possible initiation codons (at positions +281 and +299) have been identified. Only the first one (CGCCATGGCC) almost perfectly fits with the consensus motif (A/C) A (A/C) (A/C) ATG (G/C) C (C/G) defined by Silflow (1998) for a start codon in *C. reinhardtii*.

As deduced from the cDNA sequence, the CrCds1 protein is a 1062-amino acid residue polypeptide (Figure 1B) with a predicted molecular mass of 111.3 kDa. A BLASTP analysis revealed that CrCds1 belongs to the ABC transporter superfamily and shares 40 to 50% sequence identity and 62 to 70% sequence similarity with half-size ABC transporters from a wide range of organisms including yeasts, plants, worms, insects and humans (data not shown). The most conserved region is found in the central part of the protein (from amino acid 250 to amino acid 870). CrCds1 possesses a single transmembrane domain including 11 putative membrane-spanning regions as determined using the TMHMM program (Krogh, Larsson, von Heijne & Sonnhammer 2001), as well as a single nucleotide binding fold (NBF) containing the Walker A and B motifs and the ABC signature characteristic of this family of transporters (Walker, Saraste, Runswick & Gay 1982; Higgins 1992) (Figure 1B). A C-terminal extension of about 128 amino-acid residues showing no homology to the other half-size ABC transporters accounts for the particularly large size of the CrCds1 protein (Figure 1B). Whether this extension is associated with a specific function remains unknown.

Applying the PAUP ("Phylogenetic Analysis Using Parsimony") program to the protein sequences of representative ABC transporters showed that CrCds1 belongs to the ATM ("ABC Transporter of the Mitochondria")/HMT ("Heavy Metal Tolerance") subfamily of half-size ABC transporters (Sanchez-Fernandez, Davies, Coleman & Rea 2001) (Figure 1C).

This subfamily includes mitochondrial transporters (HsMTABC3, HsABC7, AtATM3/STA1, ScATM1, SpATM) involved in the maturation of cytosolic Fe/S proteins (Lill & Kispal 2003) as well as vacuolar transporters (SpHMT1, CeHMT1) involved in the transport of phytochelatin-cadmium complexes from the cytoplasm into the vacuoles (Ortiz *et al.* 1992; Ortiz *et al.* 1995). CrCds1 is much more distant from TAP, MDR and MRP subfamilies. In particular, the protein clearly does not cluster with ScYCF1 (Figure 1C). Similarly, CrCds1 is poorly related to the *A. thaliana* ABC transporters (full-size, half-size and soluble ones, (Sanchez-Fernandez *et al.* 2001) other than the AtATM proteins.

The phylogenetic tree presented in Figure 1C moreover shows that two sub-clusters can be distinguished within the ATM/HMT subfamily. Sub-cluster I includes only mitochondrial transporters, all possessing five or six conserved transmembrane-spanning regions as determined with the TMHMM software. Sub-cluster II, to which belongs CrCds1, includes both mitochondrial (HsMTABC3) and vacuolar transporters (SpHMT1, CeHMT1) possessing five additional transmembrane-spanning segments at the N-terminal end of the protein.

The expression of the *Cds1* gene is induced by cadmium

A search of the *C. reinhardtii* sequence database led to the identification of six expressed sequence tags (EST) homologous to the *Cds1* gene. These EST have been assembled in a single contig (ACE20021010.567) of 1614-bp (Shrager *et al.* 2003) corresponding to the 3' end of *Cds1*. Four of these EST were identified in the stress II cDNA library notably prepared from cells incubated in the presence of cadmium, whereas the two others originated from the gamete library (Shrager *et al.* 2003).

We have analyzed the possible regulation of *Cds1* by cadmium. Total RNAs were extracted from wild-type cells (C1 strain) exposed for different times (0-16h) to various cadmium

concentrations (0-400 µM) then submitted to Northern blot analysis using the *Cds1* probe (Figure 1A). In the absence of cadmium, a 3.7-kb transcript was present in low amount (Figure 2A). The transcript level was strongly increased in the presence of cadmium and displayed a peak of accumulation after 4 h of exposure (Figure 2A). The induction of *Cds1* by cadmium, particularly high at 200 to 400 µM concentrations (Figure 2A), suggests an increased requirement for the corresponding protein under toxic conditions, which is consistent with the cadmium-sensitive phenotype of the *cds1* mutant.

Total RNA were also extracted from wild type (C1), five different *cds*1 allelic mutants (Cd34, Cd30, Cd41, Cd43, Cd135), the *cds*2 non-allelic mutant (Cd47) and strain p46 (the Cd34 mutant complemented with the p46 genomic clone) after 4 h of exposure to 100 µM Cd. The *Cds1* mRNA was modified in size in Cd30, lacking in the four other *cds1* alleles and present in strain p46 (Figure 2B). The *cds*2 mutation did not affect the expression of *Cds*1 (Figure 2B).

The *cds1* cells produce high molecular weight phytochelatin-cadmium complexes

We have established above that the CrCds1 protein of *C. reinhardtii* belongs to the ATM/HMT subfamily of half-size ABC transporters. As mentioned in the introduction, the SpHMT1 transporter from the yeast *S. pombe* is involved in the transport of phytochelatin-cadmium (PC-Cd) complexes from the cytoplasm to the vacuoles and confers cadmium tolerance to the cells. The *Δsphmt1* mutant displaying cadmium hypersensitivity is unable to accumulate high molecular weight (HMW) PC-Cd complexes (Ortiz *et al.* 1992; Ortiz *et al.* 1995). As phytochelatins are the main intracellular chelators for cadmium in *C. reinhardtii* (Howe & Merchant 1992; Hu *et al.* 2001), a mutation affecting the PC metabolism is likely to result in cadmium hypersensitivity.

To determine whether the *cds1* mutation results in the loss of a vacuolar PC-Cd complex transporter, we have analyzed the accumulation of PC-Cd complexes in C1 wild-type and Cd34 mutant strains. Extracts were prepared from cells untreated or exposed to 50 μM cadmium for 3 days, and loaded on a Sephadex G-50 column. The absorbance at 254 nm was recorded during elution. After cadmium treatment, wild-type (C1) cells produced an additional peak of small peptides (Figure 3A). With an apparent molecular mass of ca 5-kDa, these polypeptides corresponds to the HMW PC-Cd complexes (Hu *et al.* 2001). This peak contained the major part of the cadmium detected in the extract (Figure 3B). The peak centred at fraction 24 results from non-specific binding of cadmium to cellular components (data not shown). HMW PC-Cd complexes also accumulated in *cds1* cells, the peak presenting a shoulder which most probably corresponds to the low molecular weight (LMW) complexes (ca 3.2-kDa, Hu *et al.* 2001) (Figures 3C and 3D). The amounts of HMW PC-Cd complexes and of cadmium associated to these complexes were even higher (about 30 %) in the mutant. Cadmium content measurements confirmed that the mutant was accumulating about one third more cadmium than the wild type (data not shown). It thus appears that unlike the *Δsphmt1* mutant of *S. pombe*, the Cd34 mutant is able to accumulate high amounts of PC-Cd HMW complexes. Therefore the *cds1* mutation does not determine the lack of a vacuolar PC-Cd complex ABC transporter. The particularly high content of PC-Cd complexes observed in the Cd34 mutant might originate from the loss of another detoxification mechanism.

CrCds1 is a mitochondrial protein and the *cds1* mutant is sensitive to high iron concentrations

To define the subcellular localization of the CrCds1 protein, we inserted three copies of a hemagglutinin (HA) peptide into the 3' end of the *Cds1* genomic coding sequence (*Cds1::HA*). We first demonstrated that the tagged gene was still able to complement the *cds1*

mutation indicating that the CrCds1::HA protein is functional (data not shown). Transformed cells resistant to cadmium and thus expressing the CrCds1::HA protein were then incubated with MitoTracker Orange, a specific stain for mitochondria. After fixation and permeabilization, the cells were incubated with an anti-HA antibody, then a secondary antibody conjugated with the Alexa-488 fluorophore and observed under laser confocal microscope. Green fluorescence (corresponding to the Alexa) was only detected in cells expressing the tagged CrCds1 and co-localized with the MitoTracker Orange in mitochondria (Figure 4). The CrCds1 protein is thus a mitochondrial half-size ABC transporter of the ATM subfamily. One has however to mention that in a transformant expressing the tagged protein at a particularly high level, a diffuse labeling was also detected in the whole cell in addition to the mitochondrial green signal (data not shown). The overexpression of the gene could lead to a mislocalization of the tagged protein as already observed for other proteins (Cooper & Bussey 1992; Nantel, Huber & Thomas 1999).

In several cases, mutations in *Atm-like* genes lead to hyperaccumulation of "free" iron (eg non heme and non Fe/S protein bound iron) within mitochondria (Lill & Kispal 2003). However, the accumulation of mitochondrial “free” iron considerably varies according to the organism. In the *atatm3* mutant of *A. thaliana*, the “free” iron content of mitochondria is only 1.5 to 1.8 times higher than the wild-type level (Kushnir, Babiychuk, Storozhenko, Davey, Papenbrock, De Rycke, Engler, Stephan, Lange, Kispal, Lill & Van Montagu 2001). In contrast, the inactivation of the *ScAtm1* gene from *S. cerevisiae* leads to a 30-fold increase of "free" iron within the organelle (Kispal, Csere, Guiard & Lill 1997). This high iron content induces oxidative stress which in turn determines the degradation of respiratory heme-containing proteins in mitochondria (Leighton & Schatz 1995; Kispal *et al.* 1997). We found that Cd34 mutant cells had wild-type levels of chloroplastic iron superoxide dismutase (FeSOD) and mitochondrial manganese superoxide dismutase (MnSOD) activities. Similarly, total

respiration, as well as activities of respiratory complex I, complex IV and alternative oxidase, were not altered in the mutant (data not shown). Moreover, the "free" iron content of partially purified mitochondria was apparently similar in mutant and in wild-type cells (data not shown). If a slight iron accumulation occured in Cd34, it might remain undetected owing to the presence of some chloroplastic contaminants in our mitochondrial extracts.

In the same line of experiments, we tested the sensitivity of Cd34 cells to iron by supplementing the growth medium with various amounts of $FeCl_2$. Compared to the wild type, the Cd34 strain showed an increased sensitivity to iron at concentrations of 500 and 600 µM (Figure 5). However, the mutant did not display any cross-sensitivity to any other metal tested, including copper, lead, mercury, zinc, arsenate, and nickel (Hanikenne *et al.* 2001).

Discussion

We report here the cloning of the *Cds1* gene whose inactivation determines cadmium sensitivity in *C. reinhardtii*. The *Cds1* gene (8.3-kb) has the typical structure of the *C. reinhardtii* nuclear genes with many short introns and a long 3'UTR (376-bp) (reviewed by Silflow 1998). The first intron is located in the 5' UTR, a situation already described for a few genes of *C. reinhardtii* (Silflow 1998). This intron might be involved in the regulation of *Cds1* as it is the case for the *Oda6* gene (encoding a dynein of the flagella in *C. reinhardtii*) which is under the control of an enhancer element located in the intron of the 5'UTR (Kang & Mitchell 1998).

The *Cds1* gene is up-regulated by cadmium, with a peak of expression after four hours of metal exposure. Rubinelli, Siripornadulsil, Gao-Rubinelli & Sayre (2002) used a mRNA differential display strategy to identify genes induced by cadmium in *C. reinhardtii* but the *Cds1* transcript was not present among the thirteen upregulated mRNAs detected. Based on

our observations, it is likely that under the experimental conditions (2 h exposure to 25 µM cadmium) used by Rubinelli *et al.* (2002), the transcript levels of *Cds1* were too low to be detected.

The *Cds1* gene encodes a protein, here named CrCds1, belonging to the ubiquitous ABC transporter superfamily. To our knowledge, only one ABC transporter gene (*HLA3*) has been identified in *C. reinhardtii*. Regulated by light intensity and CO_2 level, it encodes a chloroplastic protein of the MRP subfamily which might be involved in bicarbonate uptake (Im & Grossman 2002).

Among the numerous ABC transporters characterized to date, only a few vacuolar proteins seem to be involved in cadmium tolerance (*see* Introduction). Our sequence and phylogenetic analyses showed that CrCds1 belongs to the ATM/HMT subfamily of half-size transporters including both mitochondrial (ATM-type) and vacuolar (HMT-type) proteins. Using epitope tagging and immunodetection, we localized the CrCds1 protein within mitochondria, thus demonstrating that the protein is a ATM-like ABC transporter. The iPSORT software (Bannai, Tamada, Maruyama, Nakai & Miyano 2002) suggested a chloroplastic or a mitochondrial localization for the CrCds1 protein (data not shown). Moreover, an *in silico* analysis (data not shown) revealed that its N-terminal part is related to mitochondrial targeting signals (reviewed by Chaumont & Boutry 1995), notably by its amino acid composition and by the presence of a putative cleavage site (Arg-Gly-Val↓Ser) at position 66 of the protein. It is however surprising that the first transmembrane segment of the protein is included within this sequence. A fusion of the N-terminal part of CrCds1 (162 amino acid residues) to the CrGFP reporter protein (Fuhrmann, Oertel & Hegemann 1999) could not be detected in the cells (data not shown), suggesting that the targeting of CrCds1 to the mitochondria might require internal signal segments distributed throughout the entire

399 polypeptidic sequence, as it is the case for many proteins inserted in the mitochondrial inner
400 membrane (Chacinska, Pfanner & Meisinger 2002).
401 A number of mitochondrial ABC transporters have been shown to play a role in the
402 maturation of cytosolic Fe/S proteins. Mutations in *Atm-like* genes lead to more or less altered
403 phenotypes in *S. cerevisiae*, *A. thaliana* and human and compared to the wild type, the
404 mutants accumulate variable amounts of "free" iron within mitochondria (Lill & Kispal 2003).
405 In contrast to these mutants, the *cds1* mutant displays no altered phenotype in the absence of
406 cadmium and does not accumulate high amount of iron in mitochondria. This suggests that
407 the lack of the CrCds1 protein might be compensated by another transporter. Interestingly, a
408 search of the *C. reinhardtii* sequence database allowed us to identify, in addition to *Cds1*, two
409 other genes encoding ATM/HMT proteins in the algal genome (data not shown). One of these
410 genes might be partially redundant, as it has been shown for the AtATM proteins in *A.*
411 *thaliana* (Kushnir *et al.* 2001). Although Cd34 cells do not accumulate high amount of iron in
412 the mitochondria, we have shown that the mutant is hypersensitive to high concentrations of
413 Fe^{2+} suggesting that iron homeostasis might be affected in the *cds1* mutants. Whether the
414 CrCds1 mitochondrial ABC transporter is involved in the maturation of cytosolic Fe/S
415 proteins like its close relatives in yeast, *A. thaliana* and human, remains to be determined. In
416 this respect, it is interesting to note that homologs of Nfs1p, Isup, Nfu1p, Isap, Yah1p, Yfh1p
417 and Erv1p, proteins which are involved in the mitochondrial synthesis and maturation of Fe/S
418 clusters in yeast and *A. thaliana* (Kushnir *et al.* 2001; Lill & Kispal 2003), have been found in
419 the *C. reinhardtii* sequence databases (data not shown). This suggests that a biosynthetic
420 pathway for Fe/S clusters exists in the *C. reinhardtii* mitochondria.
421 The involvement of a mitochondrial ATM-like protein in cadmium tolerance has never been
422 described previously, but might represent a new property of this family of ABC transporters.
423 Several hypotheses can be proposed concerning the molecular mechanisms underlying the

cadmium-sensitive phenotype of the *cds1* mutant. First, the CrCds1 protein could be directly involved in the export of cadmium outside the mitochondrial matrix, thereby protecting the mitochondrial function from cadmium toxicity. Our observation that total respiration is more altered by cadmium in the mutant than in the wild type supports this idea (data not shown). In that context, an interesting point to consider in the future will be to determine the nature of the substrate transported by the mitochondrial protein in the process of cadmium detoxification. A possible candidate might be chelates of glutathione and cadmium which would be transported from the mitochondrial matrix to the cytosol. In this respect, it is worth mentioning that (i) SpHMT1, a close homolog in *S. pombe,* is involved in the transport of PC-Cd complexes, PC being a derivative of glutathione (Ortiz *et al.* 1992; Ortiz *et al.* 1995), and (ii) ScYCF1 in *S. cerevisiae* (Li *et al.* 1997) and AtMRP3 in *A. thaliana* (Tommasini *et al.* 1998) belonging to the MRP subfamily of ABC transporters are both involved in the transport of glutathione-cadmium chelates. Alternatively, the cadmium- and iron-sensitive phenotype of the *cds1* mutant could be an indirect consequence of a modification of iron homeostasis in the algal cells. The lack of the CrCds1 mitochondrial transporter could indeed results in cytosolic iron deficiency, as suggested previously for the *atm1* yeast mutant (Schueck, Woontner & Koeller 2001). It has been shown recently that in several organisms, iron deficiency can lead to an increased uptake of cadmium, due to the induction of the iron uptake systems, thus often resulting in sensitivity to cadmium (Thomine, Wang, Ward, Crawford & Schroeder 2000; Connolly, Fett & Guerinot 2002; Lombi, Tearall, Howarth, Zhao, Hawkesford & McGrath 2002; Thomine, Lelievre, Debarbieux, Schroeder & Barbier-Brygoo 2003; Bressler, Olivi, Cheong, Kim & Bannona 2004). Further work will be necessary to determine if one of these hypotheses allows to explain the cadmium-sensitive phenotype of the *cds1* mutant.

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Figure 1. Structure of *Cds1* gene and cDNA, predicted amino acid sequence of CrCds1 and CrCds1 phylogenetic tree. (a) Structure of *Cds1* gene and cDNA. cDNA sequences (exons, 5' and 3' untranslated regions) and introns are indicated by black rectangles and continuous lines, respectively. The putative inititation codon (ATG) and the in-frame stop codon (TAA), as well as the *Cds1* probe (522-bp), are shown at their respective positions. (b) Predicted amino acid sequence of the CrCds1 protein. The two possible initiation Methionine residues are indicated in boldface letters. The eleven putative membrane-spanning regions are underlined. The Walker A and B motifs (GATGSGKST and MLVLDE, respectively) and the ABC signature motif (LSGGEKQRVAFA) are indicated in italic boldface letters. The C-terminal extension of 128 amino acid residues is indicated in italic letters. (c) CrCds1 protein sequence was aligned with representative ABC transporters using ClustalX (version 1.83). The alignment (1777 positions) was used to produce a phylogenetic tree by the TBR heuristic method using PAUP 4.0. Gaps were treated as missing and AtTAP1 was chosen as outgroup. Bootstrap values for 1000 replicates are given above corresponding branches. The ABC transporters used in this analysis are: half-size ABC transporter DmCG4225 from drosophila (NP650503), mitochondrial half-size ABC transporters HsMTABC3 and HsABC7 from human (Q9NP58 and O75027, respectively), AtATM3 from *A. thaliana* (AAG09829), ScATM1 from yeast *S. cerevisiae* (P40416) and SpATM from yeast *S. pombe* (NP594288), heavy metal tolerance protein 1 from yeast *S. pombe* (SpHMT1) and *C. elegans* (CeHMT1) (S25198 and AAM33380), multidrug resistance AtMDR1 from *A. thaliana* (AAN28720) and HsMDR1 from humans (NP_000918), transporter associated with antigen processing-like protein AtTAP1 from *A. thaliana* (AAL85485), multidrug-resistance-related protein AtMRP3 from *A. thaliana* (AAC49791) and yeast cadmium factor protein 1 (ScYCF1) from *S. cerevisiae* (P39109). (m) mitochondrial and (v) vacuolar.

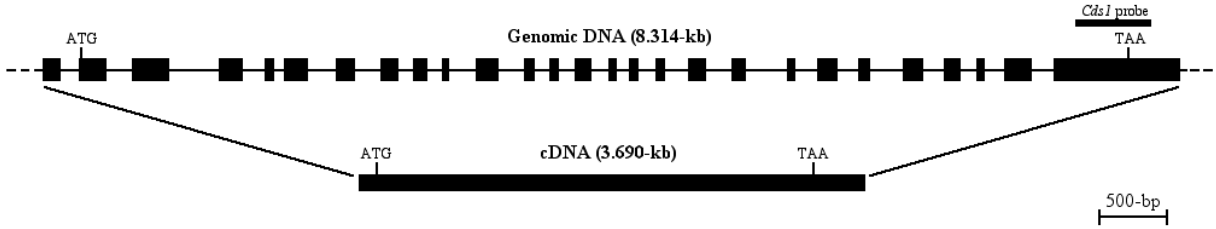
Figure 2. Northern blot analyses of the *Cds1* transcript expression. (a) Expression of *Cds1* transcripts in C1 strain (WT) after different times of exposure (0-16 h) to increasing Cd concentrations (0-400 µM). (b) Expression of *Cds1* transcripts in C1 strain (WT), *cds1* allelic mutants (Cd34, Cd30, Cd41, Cd43, Cd135), *cds2* non-allelic mutant (Cd47) and strain p46 (Cd34 mutant upon transformation with p46 genomic clone and restauration of cadmium resistance) after 4 h of exposure to 100 µM $CdCl_2$. In all case, total *C. reinhardtii* RNAs were extracted, loaded on agarose gel (15 µg per lane), blotted and hybridized with the *Cds1* ^{32}P-labelled probe (located in the last exon of the *Cds1* gene, *see* Figure 1A). rRNA abundance was used as a loading control.

Figure 3. Phytochelatin-cadmium complex accumulation in wild-type (C1 strain) and *cds1* mutant (Cd34 strain) cells. Soluble extracts (10 mg) from cells exposed or not to 50 µM cadmium for 3 days were analyzed by gel filtration chromatography. The cadmium content of the collected fractions (5 ml) was determined by atomic absorption spectrophotometry. (a & c) Gel filtration profiles (A 254 nm) of soluble extracts from wild-type and *cds1* cells treated or not with 50 µM Cd, respectively. (b & d) Cadmium distribution in the fractions collected after gel filtration of the extracts from wild-type and *cds1* cells treated with 50 µM Cd, respectively.

Figure 4. Immunolocalization of CrCds1::HA. *C. reinhardtii* cells expressing *Cds1* or *Cds1::HA* genes were stained with rat anti-HA antibody, followed by Alexa-488-conjugated goat anti-rat IgG (green). The mitochondria were labeled with MitoTracker Orange (Orange). Upper panel: Localization of CrCds1::HA (green, left), MitoTracker Orange (orange, center) and both CrCds1::HA and MitoTracker Orange (yellow is coincident localization, right). Lower panel: Same detection in cells expressing an untagged CrCds1 protein.

Figure 5. Iron hypersensitivity of the *cds1* mutant (strain Cd34) compaired to the wild type (strain C1). Relative growth rate (expressed as percentage of the growth in TAP medium) was determined after 40 h of culture in TAP medium added with 250, 500 and 600 μM $FeCl_2$ by measuring the protein content of the cell suspension (n=2).

(a)



(b)

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1     MAANGLMWCGGIQDVGWLGPLLSPCTFESGASALLLVATLVSVLAQGGRLGLIHQLKLQG
61    RLRRGVSGLSGAFIACCLFMVGTHLLHFSVGLAILRRFPFHVTYHACLALTWGTMLGFAL
121   YTCRVAATVDFRFVTGPAVAVYCISLYSFYHLYFDAHNFPMSYIKASIWTAMLQSAMAAV
181   TTWLGARRAAKNPSLQTMQAQLGFGYAPLSGDDAAGSRAGGSSSKPGGSGDAGPSGASAG
241   GGNAGDGEGDGRTWISLFGDACAYVWPTELHLQLRAIACLLLLVAMRFINLAVPILYKRV
301   VDTLAAASAKHPAAAAGEPPRGLGLDLGIGRLVSAWLKGGGDDADPSAVNFGALVWPWII
361   LYLAAVFFQGGAGGGIVGFINNMRSYLWIPVSQDAYRRISLRVFDHVMDLDLTFHLRKKT
421   GEVTKVVDRGTNAMQNILSTILFNVLPQIFDVLAAATYLAQALEPTIAIIVFIAVGSYIP
481   LTVIITEWRGKLRREMNATDQVKSARATDALLNYETVKYFTNERYESVEYAKAIDAYQDA
541   EFRSMSSINVLNVTQSAIMFIGIASGLLVCAAGVADHSLTVGDSVLFLSLMAQLYGPLNF
601   FGSYYRTIQQYMIDMENLLELLGRQPVVADTTTSRDLVVSTGELVFDDVSFQYEAGQTVL
661   RNVSFRVPGGQTIALV***GATGSGKST***ITRLIFRFYDVSSGAVRVDGQDVRNVSQTSLRRAI
721   GMVPQDTVLFNDTIMHNIRYGNLSASDEQVQEAARLACIHDTIVNRFPKGYSTVVGERGL
781   R***LSGGEKQRVAFA***RALLKNPA***MLVLDE***ATSALDTITEKKIQGSLAELRNNRTTVIVAHRL
841   STIADADIIVVMATGRVVEQGSHSELLARGGLYAEMWSRQAQKAQNGEGMDPPSGEPSSK
901   SGSALDLRKLDDGGSGSSGAVVSHVAPTLQVSSV*SDRSGTSASNLTAAVPGASGAGTAPA*
961   *DAAGSAVSARAGPSSSGPSAGSAAAPSAAAPLPPAATAPAPSVAGLAGGTEPSAATAAAG*
1021  *AEGSVEAKGEADETEGGSAVDAPGAGEAAGAAGKSKKGKKKK*
```

(c)

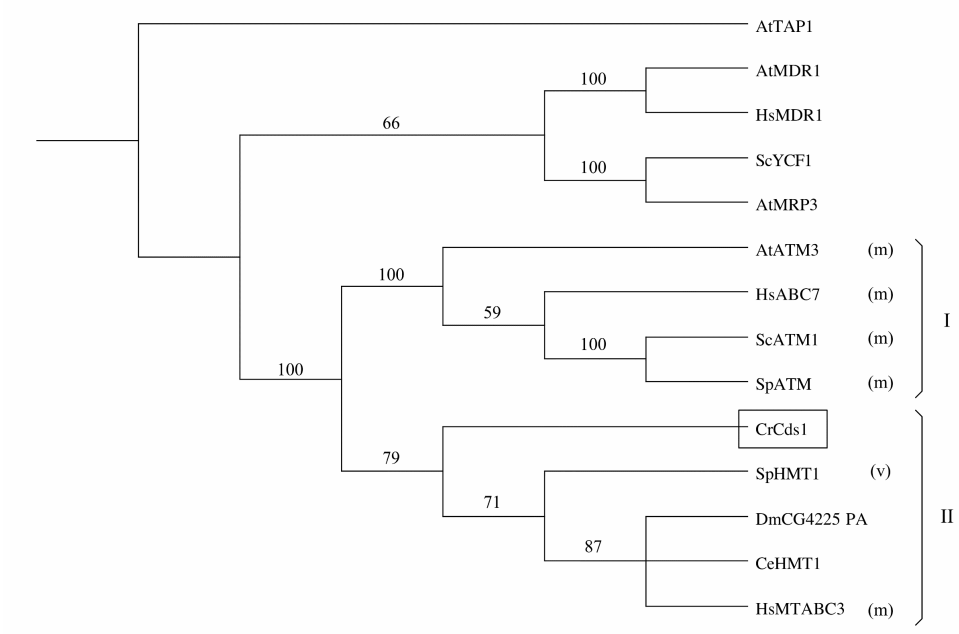

Figure 1.

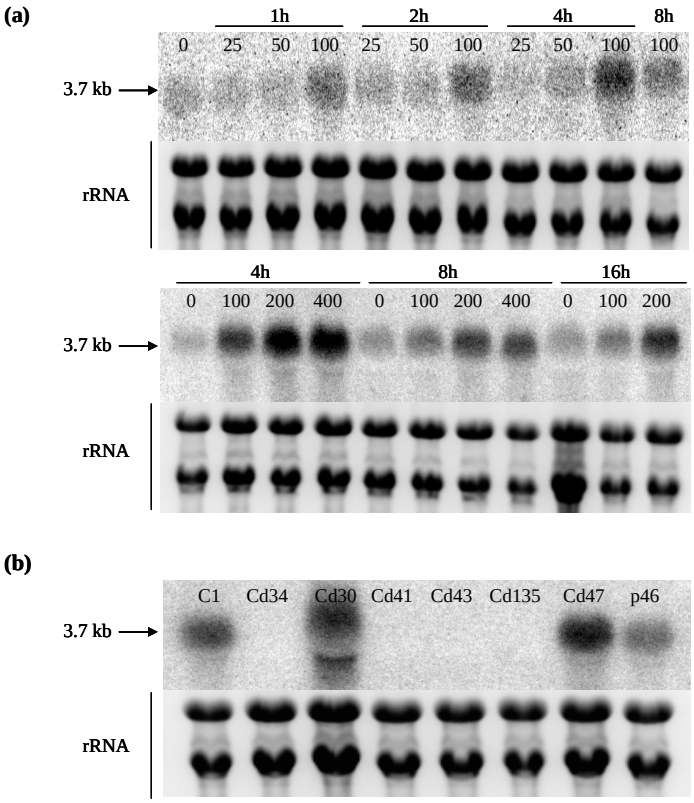
(a)
1h
2h
4h
8h
0 25 50 100 25 50 100 25 50 100 100
3.7 kb
rRNA
4h
8h
16h
0 100 200 400 0 100 200 400 0 100 200
3.7 kb
rRNA
(b)
C1 Cd34 Cd30 Cd41 Cd43 Cd135 Cd47 p46
3.7 kb
rRNA

Figure 2

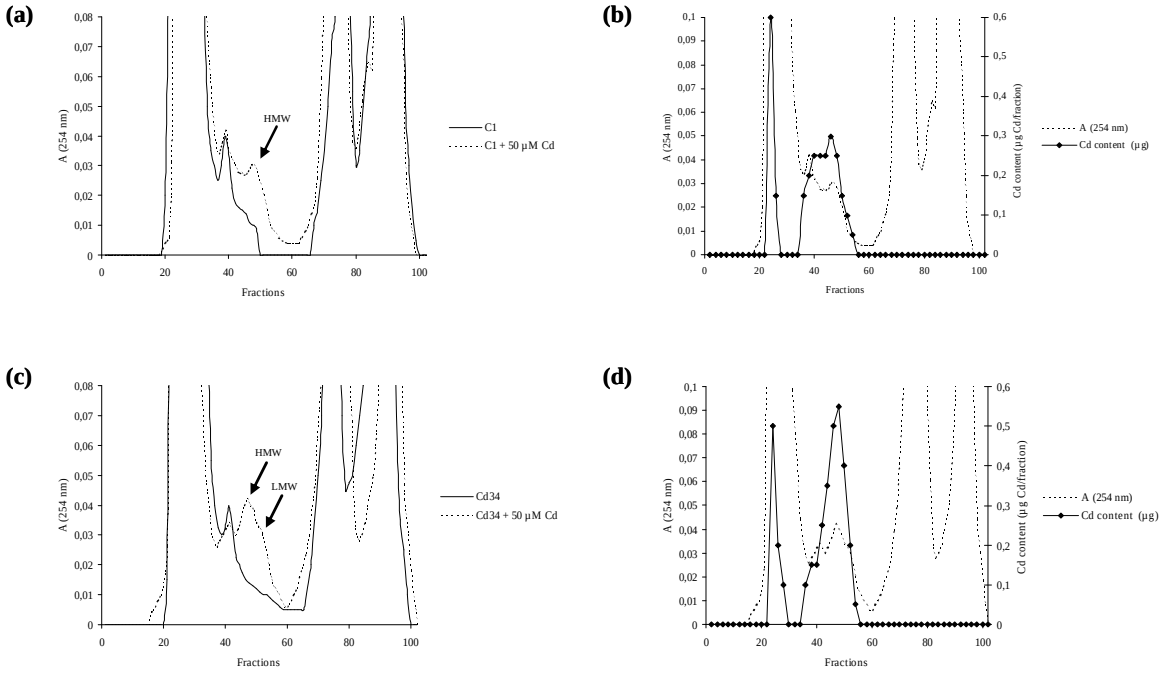
(a)
A (254 nm)
HMW
C1
C1 + 50 µM Cd
Fractions
(b)
A (254 nm)
Cd content (µg Cd/fraction)
A (254 nm)
Cd content (µg)
Fractions
(c)
A (254 nm)
HMW
LMW
Cd34
Cd34 + 50 µM Cd
Fractions
(d)
A (254 nm)
Cd content (µg Cd/fraction)
A (254 nm)
Cd content (µg)
Fractions

Figure 3

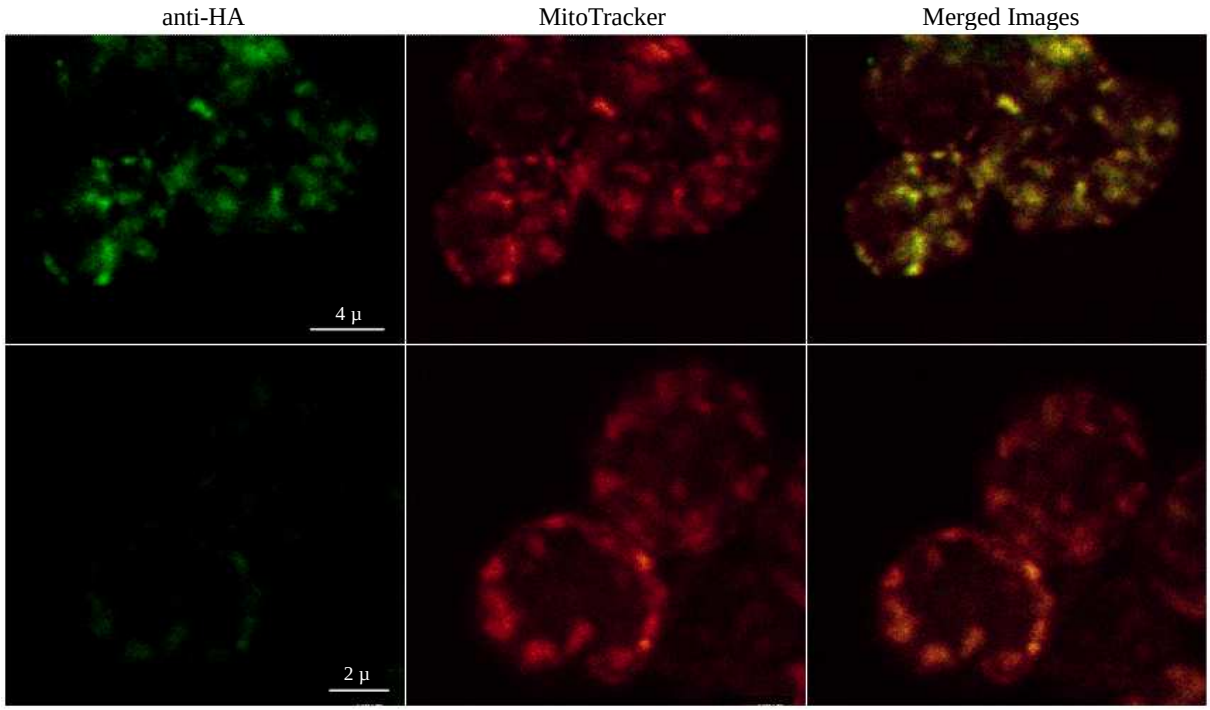

Figure 4

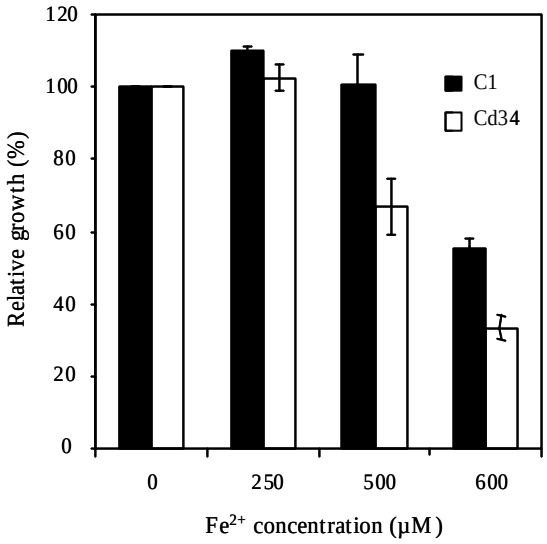
120
100
80
60
40
20
0
Relative growth (%)
C1
Cd34
0
250
500
600
Fe2+ concentration (µM)

Figure 5