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Cadmium- and iron-stress-inducible gene expression in the green alga *Chlamydomonas reinhardtii*: evidence for H43 protein function in iron assimilation

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Abstract Early transcriptional responses of a cell wall-deficient mutant of the green alga *Chlamydomonas reinhardtii* to heavy-metal stress have been investigated using the method of mRNA differential display. We have identified, sequenced, and quantified the induction of a number of transcripts that are up-regulated by a brief (2-h) exposure to 25 µM cadmium chloride, including one transcript which is also highly responsive to iron (Fe) deficiency. These transcripts represent both nuclear- and chloroplast-encoded genes, and include both novel genes and genes with known or suspected functions. Among these is a gene with significant homology to *HCR1*, a high-CO₂- and Fe-deficiency-inducible gene from *Chlorococcum littorale*. We further characterized the regulation of the *HCR1*-like gene (*H43*) and found that this transcript is also induced by Fe-depletion of the medium. Heterologous expression of *H43* in the Fe-uptake mutant *fet3fet4* of *Saccharomyces cerevisiae* resulted in partial suppression of the slow-growth phenotype of this mutant in minimal medium, and resulted in a 2-fold increase in Fe accumulation per cell. Our results demonstrate the utility of *Chlamydomonas cw⁻* strains for functional genomics studies of metal stress. The magnitudes of induction and functional analyses suggest possible utility for these genes in the study of metal stress sensing in green plants and development of novel Fe acquisition and phytoremediation strategies.

Keywords Cadmium · *Chlamydomonas* (Cd, Fe) · Iron · Metabolism · Phytoremediation · Stress (heavy metals)

Abbreviations EST: expressed sequence tag · PCR: polymerase chain reaction · PSI, PSII: photosystem I, II · RT: reverse transcription · SAM: *S*-adenosylmethionine

Introduction

Pollution of ground water and soil with toxic heavy metals, particularly mercury, lead, arsenic and cadmium (Cd), presents potential health risks. Metals are non-degradable and can accumulate and concentrate as they move up the food chain. Cadmium has become of particular concern in recent years due to its increasing use in plastics manufacturing, electroplating of steel, Ni-Cd batteries, and in pigments. Whereas lead, mercury, and arsenic production have decreased through the substitution of other agents for these metals and through recycling, Cd production has increased (Dudka and Adriano 1997). Cadmium is readily taken up by plants (Kloke et al. 1994), does not form volatile organic species, and thus can accumulate in crops. A number of adverse health effects have been linked to chronic Cd exposure, such as kidney dysfunction (Elinder et al. 1987), osteomalacia (Nogawa and Kido 1993), and developmental defects (Desi et al. 1998).

Methods of controlling heavy-metal pollution include adsorption and recycling from industrial waste streams and remediation of polluted sites. Conventional methods of heavy-metal remediation, such as excavation of contaminated soil, can be very expensive (Salt et al. 1998). An attractive alternative technology is the use of hyperaccumulating plants such as *Brassica juncea* and *Thlaspi caerulescens* for in situ bioremediation (phytoremediation or phytoextraction). Several successful field trials using phytoremediation have been reported (Salt et al. 1998). Current limitations of this technology are

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that naturally hyperaccumulating plant species have relatively low biomass compared to many crops and grow more slowly. In addition, some naturally hyperaccumulating plants will be poorly adapted to the particular climates and/or soil conditions at polluted sites. These limitations, however, might be circumvented through the application of molecular biology techniques to transfer genes conferring improved uptake, translocation, sequestration and repair of damage from heavy metals to suitable plant species.

With these issues in mind, we have investigated the use of *Chlamydomonas reinhardtii*, a unicellular green alga, as a model system for the identification and characterization of genes involved in the adaptation to heavy-metal stress. *Chlamydomonas* has several advantages as a model system for stress responses. Growth is rapid, reaching logarithmic phase in 3–4 days. *Chlamydomonas* cell wall-deficient mutants are sensitive to heavy metals (Cai 1996) and are competent for transformation with DNA. Genes encoded by the chloroplast genome can be studied using homologous recombination. Its unicellular nature and growth in liquid culture ensures uniform exposure of all cells in the culture to the heavy metal, resulting in rapid and potentially well-synchronized responses. Genes of interest found in *Chlamydomonas* may encode conserved amino acid motifs that would allow the identification of homologs in other plants. In addition, *Chlamydomonas* genes with novel sequences may reveal new mechanisms of tolerance to toxic trace metals that could be engineered into plants.

In order to specifically investigate early transcriptional responses of cells to Cd we made use of the mRNA differential display technique to analyze changes in mRNA from a *Chlamydomonas cw⁻* strain after a brief (2-h) exposure to 25 μ M CdCl₂. Here we present the verification and quantification of the induction of these transcripts, as well as sequence analysis, and functional analysis of one of these genes, called *H43*. Through expression analysis and heterologous expression in an iron (Fe)-uptake mutant of *Saccharomyces cerevisiae*, we provide evidence that *H43* functions in Fe assimilation. The apparent interaction of Cd and Fe during Fe assimilation in *Chlamydomonas* will be discussed.

Materials and methods

Strains and culture conditions

The *Chlamydomonas reinhardtii* strains used in this study are strain 2137 (CC-1021) (*nit1*, *nit2*, *mt*⁺) and CC-406 (*cw15*, *mt*⁻). Axenic cultures were grown until mid logarithmic phase with shaking (160 rpm) under continuous illumination with 40 μ mol photons m⁻² s⁻¹ of white light in 100–500 ml of a modified TAP medium (Harris 1989). 1 mM glycerophosphate was substituted for 1 mM KPO₄ to prevent precipitation of Cd as the phosphate salt. Cadmium (as CdCl₂; J.T. Baker, ACS grade) or ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine; Sigma) was added to culture medium from filter-sterilized 100 mM stock solutions to a final concentration of 25 μ M or 1 mM, respectively. All media were prepared using deionized water. Cultures exposed to

Cd, ferrozine, or neither treatment were then harvested by centrifugation after 2 h of exposure to these reagents, and the cell pellets were frozen rapidly by immersion in liquid nitrogen and immediately stored at –80 °C until RNA extraction. For molecular cloning, *Escherichia coli* strain DH5 α was grown using standard methods (Sambrook et al. 1989). Bacteriophage lambda strain 1149 was propagated in *E. coli* strain BHB2600 using standard methods (Sambrook et al. 1989). For yeast growth-curve experiments, *fet3fet4* mutant yeast strain (DEY1453, genotype: *ade2*⁺, *ura3*/*ura3*, *trp1*/*trp1*, *leu2*/*leu2*, *his3*/*his3*, *can1*/*can1*, *fet3-2::HIS3*/*fet3-2::HIS3*, *fet4-1::LEU2*/*fet4-1::LEU2*) and the control strain WAT11 (*FET3 FET4 ura3*) were transformed with empty vector pYES2 or pYES2 harboring the Flag epitope-*H43* gene fusion as described below, and grown in SC-URA/galactose [0.67% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories), 2% galactose, 0.5% ammonium sulfate, and 0.2% SC-URA mix (Qbiogene, Carlsbad, Calif., USA)] with or without addition of sterile FeSO₄ to a final concentration of 100 μ M. Culture density was measured as spectrophotometric absorbance at 600 nm relative to sterile medium as a blank.

Preparation of total RNA

Total RNA was extracted from the frozen harvested cultures using Trizol reagent (Gibco-BRL) as described by the manufacturer. All glassware was baked for 16 h at 180 °C prior to use. Total RNA was then stored at –80 °C prior to use.

mRNA differential display

Differential display reactions were performed using the RNAimage kit #5 (GenHunter Corp., Nashville, Tenn., USA) as recommended by the manufacturer, except that we used 4.71 \times 10⁵ Bq per reaction of α -[³⁵S]dATP (specific activity 4.71 \times 10¹³ Bq/mmol; New England Nuclear) instead of α -[³²P]dATP. The differential display technique has been described by Liang and Pardee (1992). The primers of kit #5 consist of eight arbitrary 13-mer primers and three primers consisting of poly(T)₁₁ followed by a 3' end A, C, or G. Through the combination of reverse transcription (RT) and subsequent polymerase chain reaction (PCR) protocols, these primers were used in all possible combinations (24) for both Cd-treated and control RNA, yielding a total of 48 differential display reactions. The differential display reactions were loaded onto 6% polyacrylamide/urea sequencing gels. The gels were run at a constant voltage of 1,500 V and vacuum-dried onto Whatman 3MM filter paper. Autoradiographs of the dried gels were obtained by exposure of Kodak MR film to the dried gels for 3 days.

Cloning of differential display bands

The bands of interest were excised from the sequencing gels, using a new razor blade for each one. The DNA was eluted from the gel slices as described by the manufacturer (GenHunter). Eluted DNA was amplified by PCR using the same primer set used in the original differential display reaction. The PCR products were then subcloned using T4 DNA ligase into either the pGEM-T vector (Promega) or into a PCR cloning vector (pJMTC) developed in our laboratory.

cDNA library screening

The *Chlamydomonas reinhardtii* cDNA library used in this study was a gift from Dr. Michel Goldschmidt-Clermont (Department of Molecular Biology, University of Geneva, Switzerland). This library was constructed in the bacteriophage lambda cloning vector, λ 1149, using *Eco*R1/*Not*I adapters, from cDNA prepared from mixotrophic, exponential-phase vegetative *C. reinhardtii* cells. Approximately 300,000 plaques from the library were propagated on

agar plates using *E. coli* host strain BHB2600, and screened by standard methods (Sambrook et al. 1989). Positively hybridizing plaques were purified by secondary screening and amplified by the low multiplicity of infection method (Sambrook et al. 1989). The bacteriophage clone insert DNA was excised using *Eco*R1 and ligated into the plasmid vector pBluescript KS+ (Stratagene).

Northern blotting

Approximately 5- μ g samples of total RNA from Cd-treated, ferrozine-treated, and control cultures were fractionated on formaldehyde-agarose electrophoresis gels using standard methods (Ausubel 1994). The RNA gels were transferred to Hybond-N nylon membranes (Amersham-Pharmacia) using standard methods (Sambrook et al. 1989). The complete *rbcs2* cDNA (Goldschmidt-Clermont and Rahire 1986), representing one of two *Chlamydomonas* ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small-subunit genes, was used as a loading-control hybridization probe. A 0.9-kb *Eco*R1 fragment representing a partial *H43* cDNA corresponding to nucleotides 617 through 1560 of the *Chlamydomonas H43* mRNA sequence (GenBank accession number AB042098) was used as the experimental hybridization probe. Probe DNAs were random-primer-labeled using the RTS Rad-Prime random octamer system (Gibco-BRL) and 1.89×10^6 Bq α - 32 P]dCTP (specific activity 1.13×10^{14} Bq/mmol; Amersham-Pharmacia). Probes were hybridized to Northern blots at a concentration of 1×10^6 – 2×10^6 dpm/ml and a temperature of 65 °C in Church and Gilbert solution (Church and Gilbert 1984).

Reverse Northern slot-blot analysis

The cloned inserts were amplified by PCR in 50- μ l reactions consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 2 μ M each of T7 and SP6 promoter primers (Integrated DNA Technologies, Coralville, Iowa, USA), 0.2 mM of each of the four dNTPs and 2.5 units Taq DNA polymerase (Gibco-BRL). 230 ng of each PCR product was diluted with TE buffer [10 mM Tris-HCl (pH 7.5), 1.0 mM EDTA] to 1 ng/ μ l. Then 100- μ l (100-ng) aliquots of these dilutions were blotted on each of two duplicate Hybond-N nylon slot blots using the protocol of Anderson (1999) and a slot-blot vacuum manifold (Miniflow II; Schleicher and Schuell). As a positive control, and for controlling for any difference in specific activity of the probes, 100 ng of the 0.8-kb *rbcs2* cDNA was also blotted. As a negative control, two slots were loaded with an equal volume of TE buffer. The immobilized DNA was alkali-denatured, neutralized and fixed to the blots by baking for 2 h at 80 °C.

First-strand cDNA probes were prepared in 60- μ l radioactive RT reactions consisting of 7.5 μ g CC-406 control or CC-406 Cd-exposed total RNAs, 25 ng/ μ l poly d(T)₁₈ primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 33 μ M each dNTP (except dCTP), 0.33 μ M α - 32 P]dCTP, and 400 units (2 μ l) Superscript II reverse transcriptase (Gibco-BRL) at 42 °C for 50 min. Reactions were terminated by heating at 70 °C for 15 min followed by gel filtration on Centri-sep spin columns as directed by the manufacturer (Princeton Separations, Adelphia, N.J., USA). Each probe was hybridized to one of the two duplicate slot blots with both probes at a concentration of 1.1×10^6 dpm/ml. Hybridizations were performed at 65 °C in 10 ml of Church and Gilbert solution (Church and Gilbert 1984) for 16 h. The blots were washed at high stringency in pre-warmed 0.2 \times SSC (30 mM NaCl, 3 mM Na₃-citrate, pH 7.0), 0.1% SDS at 65 °C for 20 min. The washed blots were wrapped in Saran wrap and placed in a film cassette for exposure of Kodak MR film for 5 days. The blots were then removed from the film cassette and placed on a phosphor screen (Molecular Dynamics) for 2 days. Phosphor signals in each slot were quantified using a constant area.

Since the first-strand cDNA probe of the Cd-exposed cells had a higher specific activity, all signals on the Cd-exposed probe blot were normalized by multiplying by the ratio of the positive control signal on the no-Cd blot over the positive-control signal on the

Cd-exposed probe blot (*rbcs2* signal_{0 Cd}/*rbcs2* signal_{25 Cd}). The presented data represent the mean and standard error of either two or three independent experiments after normalization (i.e., in some cases, one of the three independent clones was a false positive (e.g., see G37-1, Fig. 2) and was disregarded).

DNA sequence analyses

DNA sequencing of the differential display and other clones described below was performed at the Neurobiotechnology Center and Plant-Microbe Genomics Facility of Ohio State University using cycle-sequencing and ABI-Prism dye terminators (Applied Biosystems). The DNA sequences were entered as queries into the BLASTN program, and translated sequences containing open reading frames consistent with the orientation of the poly(A)-tails were entered as query sequences into the BLASTP program (Altschul et al. 1997). The DNA sequences were also aligned to each other using the BLAST2 program to determine the overlap between the differential display products. Other sequence analyses used were the PROSITE program for identifying conserved amino acid motifs, SCIPROTEIN for analysis of hydrophobic character, CLUSTALW for multiple sequence alignments (Thompson et al. 1994), and a neural network algorithm (Nielsen et al. 1997) for analysis of signal peptides. All of the programs except SCIPROTEIN are publicly available on WWW servers.

Heterologous expression of H43 in yeast

The missing 5' end of the *H43* cDNA was obtained using RT-PCR. Five micrograms of total RNA purified from *Chlamydomonas* strain CC406 exposed to 25 μ M CdCl₂ for 2 h was used as the template in an RT reaction. Oligo(dT)-primed first-strand cDNA was generated using the Superscript First Strand Synthesis System for RT-PCR kit (Gibco-BRL). One-tenth of this reaction was then used as the template in a PCR reaction specifically amplifying the 5' half of the *H43* cDNA. Gene-specific primers used for the amplification were H43S (5'-CCGAACCTCGAGAAGCCCGTCGCACAGTTA) and H43AS (5'-TAGCTCAGCTTGGAGGCG), which comprise nucleotides 26–43 and 999–982, respectively, of the *H43* coding sequence (GenBank accession number AB042098). The PCR product was then ligated into the pGEM-T plasmid (Promega). A portion of this cloned product from the internal *Pst*I site (position 807 of the GenBank sequence) to an *Xho*I site engineered into the H43S primer was then ligated into another plasmid (L2-8-9-2) harboring a partially overlapping 3' half of the *H43* cDNA as an *Eco*R1 insert in pGEM11Z. The correctness of the resulting full-length cDNA was confirmed by DNA sequencing as described above. This construct was designated I(9-18)1.

The Flag epitope was engineered into the *H43* coding sequence by the method of overlap extension PCR (Ho et al. 1989). Two primers complementary and encoding the Flag epitope at their 5' ends, primer c (5'-GACTACAAGGACGACGACGAC AAG-CAGCCACGACGACTGGC) and primer b (5'-CTTGTCTGTC GTCGTCCCTGTAGTCCGCCGTCGCCACGACAAG), were used in conjunction with primers a (5'-CGCAGCCGAACGACC-GAG) and d (5'-GTCACGGCGGAGGCCTTG), which comprise nucleotides 397–380 of pGEM11Z and 960–943 of the *H43* coding sequence. Each primer was used in the PCR reactions at 0.2 μ M with 1×10^4 copies of construct I(9-18)1 as template. The locations of these primers are illustrated in Fig. 6A. The correctness of the location and sequence of the Flag epitope fused to the *H43* sequence was confirmed by DNA sequencing.

The Flag-*H43* gene fusion was then ligated into the yeast expression vector, pYES2, as a *Bam*H1/*Eco*R1 fragment. Two isolates of this construct, designated L2-3-13-1 and L2-3-13-2, as well as the empty vector, were transformed into both WAT11 and *fet3fet4* mutant *Saccharomyces cerevisiae* by the method of Adams et al. (1998). Transformants were selected on SC-URA/glucose agar medium (0.67% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories), 2% glucose, 0.5% ammonium sulfate, 0.2% SC-URA mix (Bio 101), and 2% agar).

Western blotting was performed by extraction of protein from 2.0 O.D._{600nm} units of yeast transformant cells (Adams et al. 1998) followed by SDS-PAGE of 10 µg of extract per lane on a 10% acrylamide resolving gel [0.38 M Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulfate, and 0.06% TEMED]. The SDS-PAGE gel was transferred to an Immobilon P membrane (Millipore) using a semidry transfer apparatus (Sartorius) and a power supply set at 1.2 mA/cm² current for 1 h according to White et al. (1998). The membrane was then air-dried for 15 min. followed by wetting in phosphate-buffered saline/1% Tween 20 (PBST). The membrane was blocked overnight at 4 °C with shaking in 20 ml of 3% casein/PBST. Anti-Flag M2 monoclonal antibody (Sigma) was bound to the membrane at a concentration of 4 mg/ml in 0.1 ml PBST/1% bovine serum albumin (BSA) per cm² of membrane (5 ml). The primary antibody was detected by binding rabbit anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Bio-Rad) diluted 1/3,000 in 5 ml of PBST/1% BSA, and by use of the Opti-4CN chemiluminescence detection kit (Bio-Rad) as directed by the manufacturer.

Elemental analysis of transgenic yeast

For elemental analysis of Fe and Cd, cultures were grown in SC-URA/galactose with or without 50 µM CdCl₂. Metal precipitates were removed by collecting the cells on an 80% sucrose cushion following centrifugation of the culture at 3,000 g, and by washing the cells from above the cushion three times in sterile medium without added metals. The final washed pellets were resuspended in 2.0 ml medium and the cell density of the resuspended pellets was measured by absorbance at 600 nm. The resuspended cells were then wet-ashed by acidification with 4 vol. of concentrated nitric acid and heating at 110 °C for 2–3 h until the volume was reduced to about 2 ml, followed by refluxing at 140 °C for several hours until the samples were colorless. The volumes of all samples were adjusted to 3.0 ml with 2% nitric acid. Elemental analysis of the samples was performed at the Ohio State University Microscopic and Chemical Analysis Research Center by inductively coupled plasma-mass spectrometry (ICP-MS).

Results

Identification and cloning of 21 candidate Cd-induced mRNA transcripts

We applied the differential display technique to identify and clone cDNA fragments representing mRNA transcripts that are up-regulated by a brief (2-h) exposure to 25 µM CdCl₂. We chose 25 µM because this concentration was previously shown to be sufficient to induce the synthesis of phytochelatins in *Chlamydomonas*, yet this concentration is low enough not to inhibit the normal functioning of signal transduction pathways

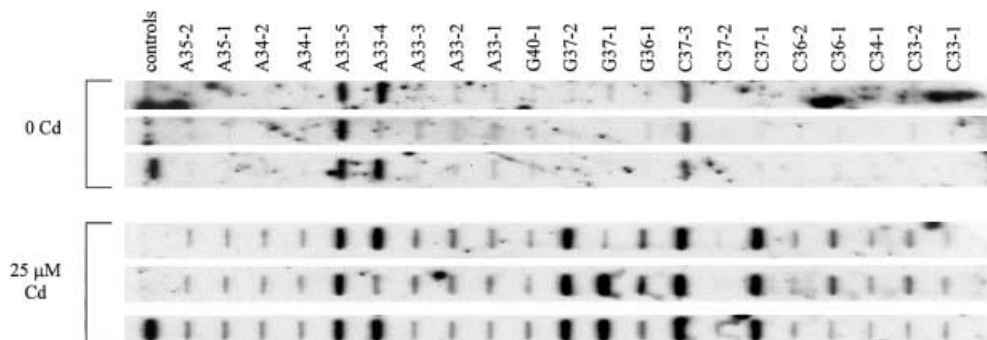
(Howe and Merchant 1992). The percent GC content of the arbitrary primers used in this study (71% GC) is similar to the GC content of known *C. reinhardtii* nuclear genome coding sequences (65% GC). Using this kit we found 21 Cd-induced bands from 24 differential display reactions (data not shown).

The 21 differential bands were excised from the sequencing gel, eluted, and PCR-amplified. The relative sizes of these PCR products as observed by ethidium bromide staining of agarose electrophoresis gels were consistent with their relative positions on the sequencing gel (data not shown). The PCR products were then ligated into PCR cloning vectors, and between 6 and 11 independent clones of each PCR product were identified by miniprep plasmid isolation and restriction endonuclease digestion.

Confirmation of differential expression by reverse Northern blotting

In order to confirm that the cloned PCR products represent Cd-responsive genes, three independent clones of each PCR product were immobilized on duplicate slot-blot arrays. These arrays were then hybridized with radiolabeled first-strand cDNA prepared from the same RNA samples used for differential display (i.e., total RNA from control cells and cells exposed for 2 h to 25 µM CdCl₂) (Fig. 1). The slot-blot hybridization signals were then quantified by phosphorimaging and normalized for the difference in specific activity (see figure legend, Fig. 1) of the two probes. Figure 2 shows

Fig. 1 Reverse Northern slot-blot analysis confirming Cd-induced transcripts in *Chlamydomonas reinhardtii*. Three clones of each differential display band were tested for Cd induction. The differential bands were named after the differential display primer set used to amplify them. The three clones were arrayed vertically on the same blot and on a duplicate blot. Each blot (indicated by *brackets* to the left of the blots) was hybridized with a different first-strand cDNA population as radiolabeled probe. The specific activities of the CC-406 control and CC-406 Cd-exposed probes were estimated to be 1.8×10^5 and 1.9×10^5 Bq/µg RNA, respectively, based on scintillation counting of the probes and on measurements of the RNA concentration. The positive control is located in the lower left slot of each blot, and contains 100 ng *rbcS2* cDNA. The negative-control slots are located in the leftmost slots of the first and second row of each blot, and contain 100 µl of TE buffer. The results shown are from a single experiment



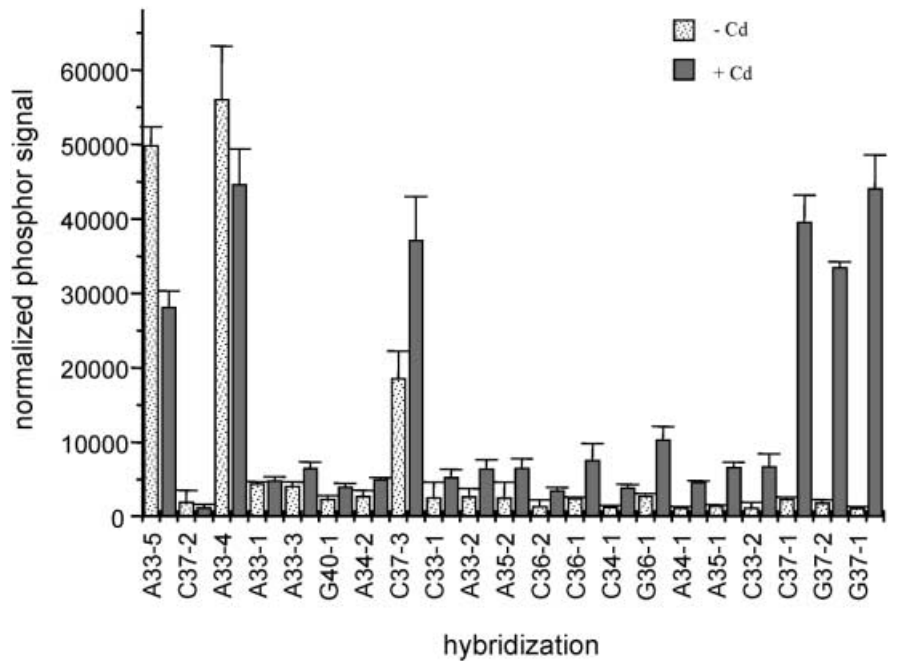


Fig. 2 Phosphorimager quantitation of reverse Northern blot hybridization signals for Cd-induced transcripts in *C. reinhardtii*. The y-axis represents the normalized phosphorimager quantitation of the reverse Northern blot signals. The x-axis represents each hybridization of first-strand cDNA probe (indicated in bar-graph legend) to slot-blotted clone insert DNA. Hybridizations are arranged from left to right in order of increasing magnitude of Cd induction, in the same order as shown in Table 1. The bars and error brackets represent the mean and standard error of the three independent *C. reinhardtii* clones tested, except in those cases where one of the three clones was a false positive (e.g., A33-4 and G37-1)

the normalized phosphor signals of each slot-blot hybridization arranged from left to right in order of increasing magnitude of Cd induction (ratio of normalized +Cd over -Cd signals). Of the 21 differential bands found, 4 appear to be false positives based on the phosphorimager quantification. These are A33-5, C37-2, A33-4, and A33-1. The remaining 17 clones are classified in Table 1 into three groups: low induction (≈ 2 -fold), medium induction (≈ 3 -to 6-fold), and high induction (≈ 20 -fold). Application of an analysis of variance (ANOVA) test to the normalized phosphorimager data allowed us to confirm that at least 8 of the 17 clones exhibiting Cd-inducible expression had significantly higher mean levels of expression in the presence of Cd than in its absence (Table 1).

DNA sequence analysis of Cd-inducible transcripts

All 21 clones were sequenced and the sequences were analyzed using the BLAST programs (Altschul et al. 1997). Translations of the sequences that were consistent with their orientation, as evident from the presence of poly(A)-tails, were analyzed using the BLASTP program. In addition, each cloned insert DNA sequence was analyzed using the BLASTN program aligned to all non-redundant sequence databases and, separately, to

the *C. reinhardtii* expressed sequence tag (EST) database (Asamizu et al. 1999). Table 1 summarizes the results of these analyses, along with the fold induction and size of each cloned insert. A pairwise comparison of all of the 21 sequences using the BLAST2 program indicated that there are 16 non-overlapping sequences (Table 1 footnotes). Of the 16 non-overlapping sequences, 10 have fold induction values of 2.0 or higher, while one had a fold induction of ≈ 20 . The latter sequence was found to be highly similar to the *H43* gene of *Chlamydomonas*, encoding a high-CO₂-inducible protein reported to be localized to the periplasmic space (Kobayashi 1997). The *H43* coding sequence is in turn significantly similar to a high-CO₂- and Fe-deficiency-inducible cDNA (*HCRI*), from a marine green alga, *Chlorococcum littorale* (Fig. 3; Sasaki et al. 1998).

In order to further investigate the degree of homology between *H43* and the highly induced (≈ 20 -fold) differential display products, we screened a *C. reinhardtii* cDNA library with one of these products, C37-1, as the hybridization probe. Several positively hybridizing plaques were identified from $\approx 300,000$ plaques screened. An ≈ 0.9 -kb *EcoRI* insert was subcloned from one of the hybridizing phage isolates and was sequenced. The sequence was found to be essentially identical [936/943 (99%) identical nucleotides], over its entire length to the reported *H43* mRNA sequence (accession number AB042098). This partial cDNA was subsequently used as the probe of Northern blots to investigate *H43* expression.

H43 gene expression under Cd and Fe stress

In addition to high-CO₂-induced expression, the *HCRI* transcript of *Chlorococcum littorale* is also induced by Fe depletion (Sasaki et al. 1998). In order to test the

Table 1 Summary of sequence and expression analyses of *Chlamydomonas reinhardtii* putative Cd-responsive cDNAs

Clone name	Size of cloned insert (bp)	Fold induction	Significant ^a GenBank database alignment (BLASTP) ^b	<i>C. reinhardtii</i> EST database BLASTN alignment ^b	Significant GenBank database alignment (BLASTN) ^b
A33-5 ^d	134	0.564	c	AV398005 Score = 260 bits. Expect = 9e-70	<i>C. reinhardtii</i> protein P1 Score = 119 bits. Expect = 3e-25
C37-2	272	0.616	c	c	Human DNA sequence Score = 46.1 bits. Expect = 0.009
A33-4 ^d	140	0.797	c	AV394461 score = 278 bits. Expect = 4e-75	<i>C. reinhardtii</i> protein P1 Score = 119 bits. Expect = 3e-25
A33-1	225	1.09	psbA (<i>C. reinhardtii</i>) Score = 158 bits. Expect = 4e-38	c	<i>C. reinhardtii psbA</i> Score = 428 bits. Expect = 1e-118
A33-3	168	1.58	frxC (<i>C. reinhardtii</i>) Score = 110 bits. Expect = 3e-24	c	<i>C. reinhardtii CHLL</i> gene Score = 315 bits. Expect = 4e-84
G40-1	386	1.68	c	c	c
A34-2 ^h	87	1.80	c	c	c
C37-3	236	2.00	c	AV622411 score = 452 bits. Expect = e-127	<i>C. reinhardtii</i> SAM synthetase (<i>CHRSAMS</i> gene) Score = 385 bits. Expect = 1e-105
C33-1 ^e	178	2.05	c	AV393546 score = 139 bits. Expect = 3e-33	<i>C. reinhardtii</i> copper-response (<i>CRD1</i>) gene Score = 119 bits. Expect = 5e-25
A33-2 ^e	195	2.38	c	AV393546 score = 139 bits. Expect = 3e-33	<i>C. reinhardtii</i> copper response (<i>CRD1</i>) gene Score = 119 bits. Expect = 5e-25
A35-2	98	2.59	c	c	c
C36-2	135	2.63	c	c	c
C36-1 ^f	274	3.08	c	AV391528 score = 402 bits. Expect = e-112	c
C34-1 ^h	70	3.13	c	AV640479 score = 139bits. Expect = 1e-33	c
G36-1 ^{f, h}	273	3.72	c	AV391528 score = 400 bits. Expect = e-112	c
A34-1 ^h	122	4.29	c	c	c
A35-1 ^h	400	4.79	c	c	c
C33-2	124	6.03	c	c	c
C37-1 ^{g, h}	395	16.9	c	c	<i>C. reinhardtii</i> h43 protein Score = 755 bits. Expect = 0.0
G37-2 ^{g, h}	390	18.9	c	c	<i>C. reinhardtii</i> h43 protein Score = 757 bits. Expect = 0.0
G37-1 ^{g, h}	579	41.9	c	c	<i>C. reinhardtii</i> h43 protein Score = 765 bits. Expect = 0.0

^aOnly alignments with an expectation threshold of ≤ 0.01 are reported in this table

^bOnly the highest scoring alignment is listed

^cNo significant similarity found

^{d, e, f, g}Clones with same superscript letter have complete or partial DNA sequence overlap

^hSignificantly higher mean values of normalized phosphorimager signal in the presence of Cd than in the absence of Cd (based on one-way ANOVA at 95% confidence level)

hypothesis that the *H43* transcript might also be responsive to Fe depletion, a *C. reinhardtii* culture was treated with 1 mM ferrozine, an Fe-specific Fe chelator. Total RNA from the ferrozine-treated culture was analyzed by

RNA gel blot hybridization to an *H43* 0.9-kb partial cDNA fragment and compared with a control RNA sample from the same cell wall-deficient strain (CC406) and with RNA from a fully walled strain (strain 2137). As

Fig. 3 Amino acid sequence alignment of *C. reinhardtii* H43 and *Chlorococcum littorale* HCR1. CLUSTALW (1.81) multiple sequence alignment of the H43 and HCR1 protein sequences. Numbers to the right of the sequence indicate the amino acid position in the sequence. Asterisks below the sequence indicate amino acid identities. Conservative substitutions are indicated by two vertical dots. Gaps are indicated by hyphens

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H43 -----MSVGFVLVLAGALVVATAQPTTTGTRFEGFSYAGNVIGYVNMMDYCDIKAAM 53
HCR1 MMNRKIAVASVLLAFVAAGSVAGSVFQDITGLMLGPFKFSGNVAGYVNMFLDYCDIQAL 60
      :: :* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
H43 AAGNFTEALSIYSTGKNSFSGLARRTFFRFASYITANGSVEPLHDSILAGKDTSSSLDAAI 113
HCR1 YAGDTATALEIYNFGKNSERGAAPRSFALWAEANHTG---QIFYDALGLPSETYLTDFV 116
      ** : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * : * :
H43 RAALADGKATLAAGLIQVGTLLKYHLHEVDEAYNKIKTYLADGTGNLTNLVSDASGAPHNV 173
HCR1 SGAAGDGDATMAMAVIDAIIKIKYMFHEWDTAVSRTASGNPD-----PSYGAPHNL 166
      * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * : * :
H43 DEAWALWAGGAANNCGTSLGWASSLG-AAMGTTFLGKSYVNTAMINTVNEMLAAARLSTL 232
HCR1 DEAAAMYFAGTEGQCGASVGLHMANQAAMLKGMLYFGSSPSNTLMSLSFINAQDATLTN 226
      *** * : * : * : * * * * * * * * * * * * * * * * * * * * * * * * * * * *
H43 NIQAYDAARTNEVRLLTLLGLQGVSVAAAYTADAAAACKRPAAEVEDAKTMIAVHWAYLEP 292
HCR1 NTAKIESAYATFLNQMQVLQQLRQLYTDAALLSYCKCG---NTTISSETILVSWMLTHQ 282
      * : * * : * : * * * * * * * * * * * * * * * * * * * * * * * * * * *
H43 MLKLRNFKASAVTELHHQLTASKLSYKKVAAAVKGVLSAMGRRSSELGAPQSAIIAA-- 349
HCR1 MLQMPQLTGMTVNHSTIETIDFI IENTECLMSQAIEIALKDVVMELGVEWADIFGNGID 342
      *** : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * :
H43 -----NWKCSSKTLRSIA----- 362
HCR1 YEIMDESMGLECGGETGWVLKGNRSN 368
      : * : * :

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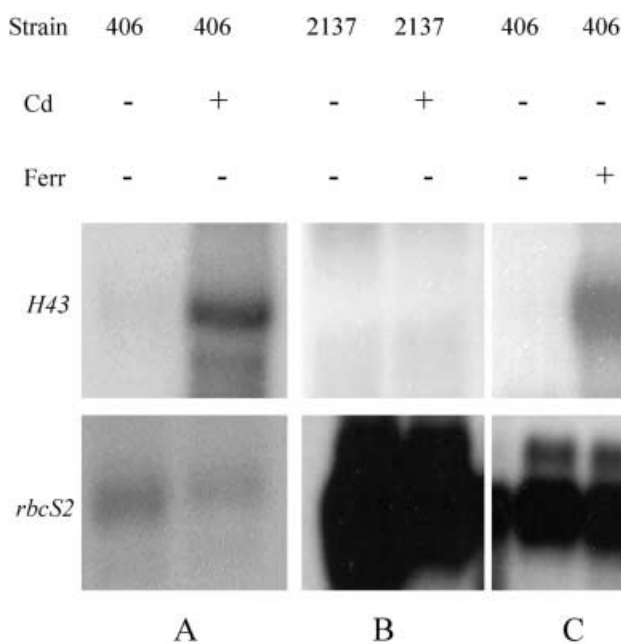


Fig. 4A–C Effect of Cd and the Fe chelator ferrozine on steady-state abundance of *H43* *C. reinhardtii* transcripts. Northern blot hybridizations using a partial *H43* cDNA probe (0.9-kb *Eco*R1 fragment from the λ library-derived partial cDNA) and complete *rbcS2* cDNA loading control probe (0.8 kb). **A** Strain CC406 (*cw*). Cd treatments “–” and “+” represent 0 and 25 μ M Cd, respectively. **B** Strain 2137 (wild type) with Cd treatments as in **A**. **C** Strain CC406. Ferrozine treatments “–” and “+” represent 0 and 1 mM ferrozine, respectively. The results shown are a composite from three separate Northern blots

shown in Fig. 4, ferrozine treatment greatly increased the steady-state level of the *H43* transcript (Fig. 4C) to a level similar to that in the Cd-treated cells (Fig. 4A). Interestingly, the *H43* transcript was not detectable and showed no induction in Cd-treated 2137 cells (Fig. 4B), suggesting that walled cells are less responsive to the Cd

concentration used in these experiments, thus demonstrating the utility of the wall-deficient strain for the identification of new Cd-inducible genes.

H43 sequence analysis

The complete coding sequence of *H43* is known, and encodes a 362-amino-acid protein with an apparent molecular mass of 43 kDa as estimated by SDS-PAGE. *H43* is reported to be a secreted protein induced by high (3%) CO_2 concentrations. It is believed to contain asparagine-linked oligosaccharides, as evident from lectin binding (Kobayashi 1997). The coding sequence was examined for conserved protein domains in order to gain clues about its function (Fig. 5). A hydropathy plot was constructed using the SciProtein program, and indicated a strongly hydrophobic N-terminus, consistent with the presence of a secretory signal peptide (Fig. 5A). The N-terminus of *H43* has a typical signal peptide composition, in terms of both length and amino acid composition, compared with known signal peptides. The output of the signal-peptide cleavage-site prediction algorithm (Nielsen et al. 1997) indicates cleavage likely occurs between residues 18 and 19 of the *H43* protein sequence (Fig. 5B). This site is also consistent with the “-3,-1” rule (von Heijne 1983). Other characteristics of the sequence are four consensus (N-X-S/T) asparagine-linked glycosylation sites, a “Walker A” nucleotide binding domain (P-loop) that conforms to the consensus sequence (A/G-X₄-G-K-S/T), and a large percentage of alanine residues.

Cadmium-inducible genes with known functions

Four genes with known functions were identified in this study as being Cd-inducible (Table 1). The *psbA* gene of

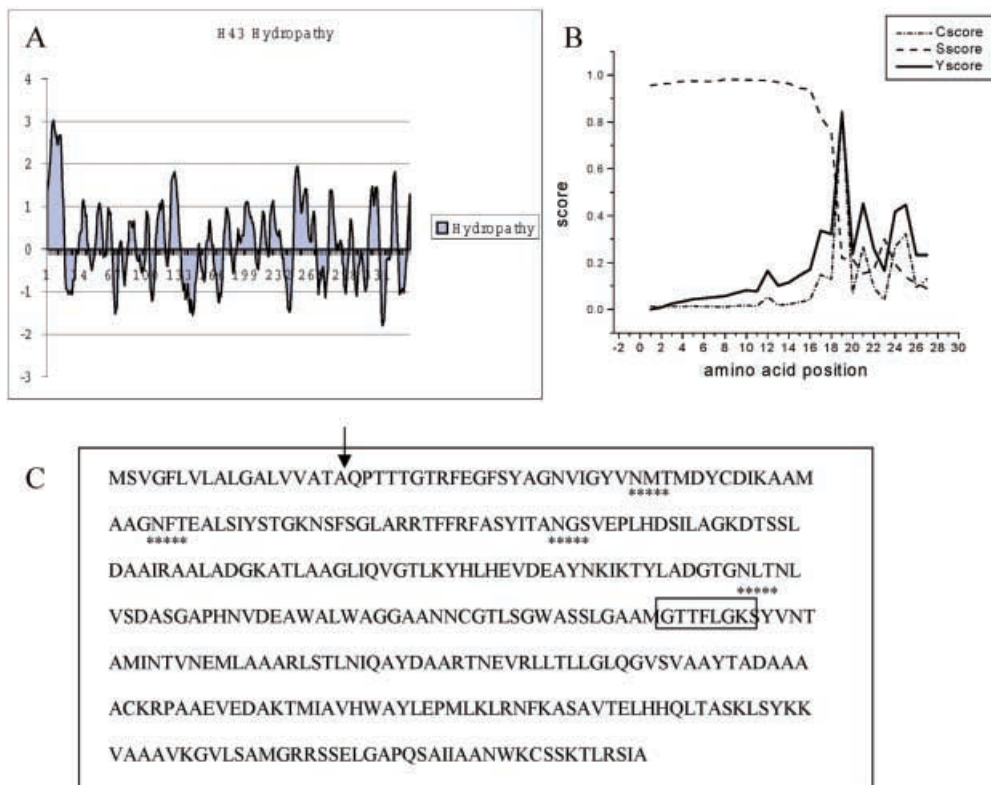


Fig. 5A–C Amino acid sequence motifs of *C. reinhardtii* H43. **A** Hydropathy plot showing presence of the putative signal sequence from amino acid positions 1–18. **B** Predicted signal-peptide-processing site based on the algorithm of Nielsen et al. (1997). The S- and C-scores represent estimates of the probability of the residue belonging to the signal peptide and being the first residue of the mature protein, respectively. The Y-score is the geometric average of the C-score and a smoothed derivative of the S-score. **C** Other sequence motifs and their location within the derived amino acid sequence. The putative N-linked glycosylation sites are indicated by *asterisks*, and the predicted nucleotide-binding site is *boxed*. The *downward arrow* indicates the predicted signal-peptide cleavage site

Chlamydomonas, encoding the D1 protein component of the photosystem II (PSII) reaction center, was found as one of the cloned putative differential display products. However, the reverse Northern analysis (Figs. 1, 2) suggests that this clone may be a false positive, since its fold-induction value is only 1.09. The *CRD1* gene of *Chlamydomonas* encodes a putative di-Fe enzyme required for assembly of PSI and the light-harvesting complex LHC I under copper deficiency and hypoxia conditions (Moseley et al. 2000), however, recent evidence suggests that the *CRD1* gene may encode an enzyme involved in chlorophyll synthesis (S. Merchant, personal communication). The *CHLL* gene of *Chlamydomonas* encodes the regulatory subunit of the light-independent protochlorophyllide reductase, and has been shown to be regulated at the posttranscriptional level by the redox state of the chloroplast (Cahoon and Timko 2000). The *CHRSAMS* gene of *Chlamydomonas* encodes an *S*-adenosylmethionine (SAM) synthetase. SAM can act as a precursor in

cysteine biosynthesis. Thus an increase in SAM might contribute to an increase in the synthesis of glutathione, which in turn may provide for increased synthesis of the Cd-detoxifying peptide, phytochelatin. The *CRD1*, *CHLL*, and *CHRSAMS* transcript levels were induced ≈ 2 -fold by Cd exposure.

Heterologous expression of *H43* suppresses the mutant yeast *fet3fet4* phenotype

We investigated the possibility of a function for the *Chlamydomonas* H43 protein in Fe assimilation by heterologous expression of the full-length *H43* cDNA in the Fe-uptake mutant *fet3fet4* of *Saccharomyces cerevisiae*. This mutant strain is defective in the high- and low-affinity Fe transporters, Fet3 and Fet4, and is more sensitive to Fe depletion relative to wild-type *Saccharomyces* (Dix et al. 1994). The full-length *H43* cDNA was obtained through RT-PCR based on the GenBank sequence (accession number AB042098). We confirmed expression of *Chlamydomonas* *H43* in *Saccharomyces* by first engineering a sequence encoding the eight-amino acid Flag epitope (DYKDDDDK) into the *H43* cDNA, in between the signal peptide and the N-terminus of the mature protein (Fig. 6A), using the method of overlap extension PCR (Ho et al. 1989). This modified *H43* gene was then placed under the transcriptional control of the *GAL1* galactose-derepressible promoter in the yeast expression vector pYES2. The correctness of the sequence of the Flag-*H43* gene fusion was confirmed by DNA sequencing. This sequence includes a Kozak

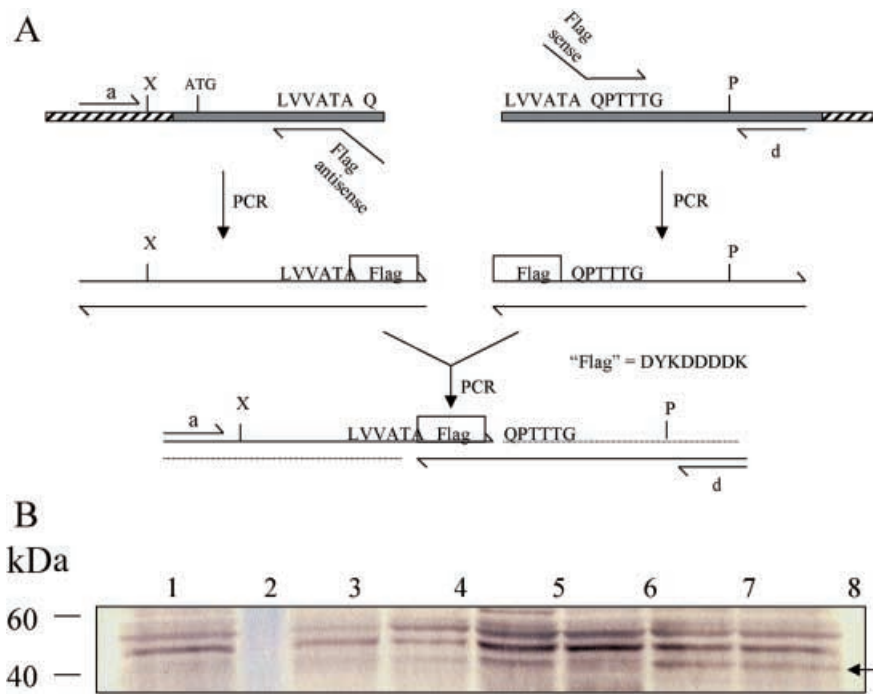


Fig. 6 **A** Construction of the Flag epitope-*H43* gene fusion by overlap extension PCR. The bars represent a portion of the 1(9-18)1 plasmid harboring the full-length *H43* cDNA (dark-grey region) inserted into the multiple cloning site of pGEM11Z (striped region). Both bars represent the same plasmid being used as template with different primer sets. The products of the first round of PCR are gel purified and annealed through the complementary flag epitope sequence and the strands are extended during the initial cycles of the second round of PCR. The outer primer set (primers *a* and *d*) completes the amplification. **B** Western blot using anti-Flag M2 antibody applied to extracts from transgenic yeast harboring the Flag epitope-*H43* gene fusion in yeast expression vector pYES2. Lanes: 1, *fet3fet4*-transformed strain harboring pYES2 empty vector control; 2, protein standards; 3, *fet3fet4* strain harboring one of two isolates (pH43-1) of the Flag epitope-*H43* gene fusion in the expression vector pYES2 grown in glucose; 4, *fet3fet4* strain harboring a second isolate of the *H43*-expression plasmid (pH43-2) grown in glucose; 5, *fet3fet4* with pH43-1, galactose (Gal)-grown cells; 6, *fet3fet4* with pH43-2, Gal-grown; 7, *FET3FET4*, *ura*⁻ control strain with pH43-1, Gal-grown; and 8, *FET3FET4*, *ura*⁻ strain with pH43-2, Gal-grown

consensus sequence preceding the initiating methionine codon for proper initiation of translation. Immunoblot analysis of yeast transformants indicated that an immunoreactive band of approximately 43 kDa was present in the extracts from galactose-induced cultures but was absent from both the empty-vector control strain and strains containing the modified *H43*-containing expression vector grown under the non-inductive conditions (Fig. 6B). This analysis confirmed that the modified *H43* protein is expressed in both *fet3fet4* and a *FET3 FET4 ura*⁻ control strain of *Saccharomyces* under the inductive conditions.

We then investigated the effect of expression of the modified *H43* on the growth of *fet3fet4* yeast cultures when sufficient Fe for normal growth of this mutant was present or withheld. Figure 7 shows *fet3fet4* yeast transformant growth curves in SC-URA/galactose

minimal medium in the absence (“-Fe”) and presence (“+ Fe”) of added Fe. The amount of Fe in SC-URA/galactose medium is about 1 μ M, and this is not sufficient for normal growth of the *fet3fet4* double mutant, as can be seen by greatly delayed growth of the pYES2-Fe culture (harboring the empty expression vector plasmid, pYES2). When *H43* was expressed in the absence of added Fe (pH43-1 and pH43-2) the growth arrest was partially suppressed. When Fe was added to the medium as FeSO₄ at 100 μ M final concentration, both empty-vector- and *H43*-expressing strains showed a normal growth pattern. This indicates that the *fet3fet4* cells used in these experiments can be rescued by addition of Fe to the medium, as expected, and that the different degrees of growth arrest of empty-vector- and *H43*-expressing transformants are not due to unrelated insertional mutation events. The results shown in Figs. 6 and 7 suggest that heterologous expression of *H43* can partially suppress the *fet3fet4* Fe-uptake mutations.

If *H43* functions in Fe assimilation, we reasoned that *fet3fet4* mutant cells expressing *H43* in the Fe-limiting condition of SC-URA/galactose medium might have an increased level of intracellular Fe compared to the empty vector control. We therefore quantified the Fe content of the same isolates of *fet3fet4* transformants harboring the empty vector and *H43* expression plasmids analyzed above. Cultures were grown to mid-logarithmic phase, washed, measured for cell density, and then were wet-ashed by refluxing in nitric acid. The samples were then analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) to quantify total Fe in each sample. The *H43*-expressing strain in the *fet3fet4* background (pH43-1) had almost 2-fold higher Fe content after normalizing for cell number than the pYES2 strain (Fig. 8). Interestingly, yeast expressing the

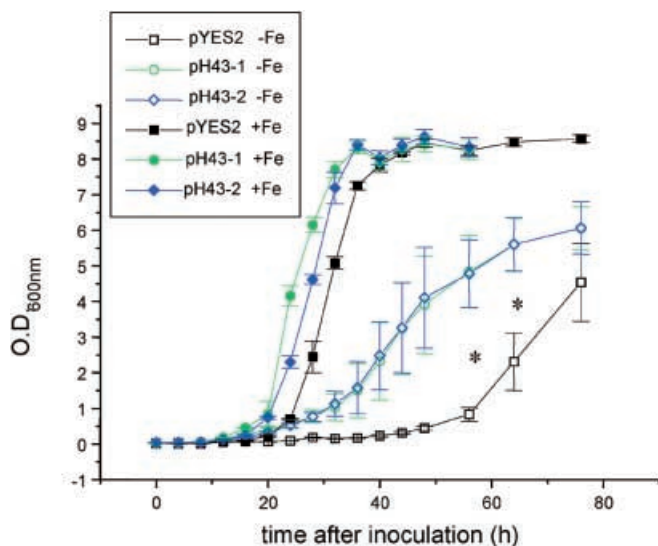


Fig. 7 Growth curves of *fet3fet4*-transformed strains of *Saccharomyces cerevisiae* harboring the Flag epitope-*H43* gene fusion or pYES2 expression plasmids. *Open symbols* represent cultures grown in SC-URA/galactose without added Fe. *Closed symbols* represent cultures grown in SC-URA/galactose with 100 μ M FeSO₄. Data are means \pm SE of three replicate experiments. The *asterisks* indicate data points at which the mean values of pH43 and pYES2 transformant growth (without added Fe) were confirmed as being significantly different at the 95% confidence level (one-way ANOVA test)

H43 protein had the same levels of Cd/cell as yeast lacking the H43 protein (data not shown). These results indicate that the H43 protein does not facilitate Cd uptake in yeast.

Discussion

Thirteen non-redundant Cd-induced cDNAs identified

We have identified and cloned 21 differential display products that were induced by a brief exposure of *Chlamydomonas* wall-deficient cells to CdCl₂ (Table 1). Pairwise analysis of the DNA sequences of these clones demonstrates that they can be grouped into 16 non-redundant, non-overlapping sequences (data not shown). Quantification of the fold induction of each corresponding transcript via reverse Northern blot analysis and phosphorimaging (Figs. 1, 2) revealed that 4 of the 21 clones (3 out of 16 non-overlapping sequences) are probably false positives based on having a fold induction of ≈ 1 , after rounding the fold-induction value to the nearest whole number (Table 1). Of the remaining 13 non-overlapping cDNAs, 5 have a fold induction of ≈ 2 , 7 have fold-induction values ranging from ≈ 3 to 6, and 1 cDNA had ≈ 20 -fold induction. In short, there are 13 non-redundant mRNAs identified in this study that are at least doubled in abundance by the CdCl₂ treatment applied.

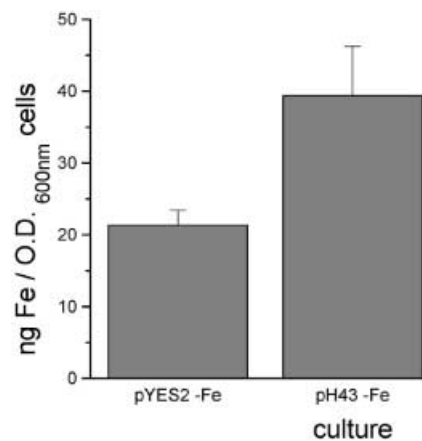


Fig. 8 Inductively coupled plasma-mass spectrometry (ICP-MS) analysis of cellular Fe content of *S. cerevisiae*. Elemental analyses of wet-ashed cultures were normalized for cell number by measuring the total number of O.D._{600 nm} units of cells in the sample prior to wet-ashing. Data are means \pm SE of three replicate experiments

Sequence analysis of Cd-inducible transcripts: novel genes

In some cases the differential display products may consist predominantly or exclusively of 3' untranslated sequence, and these sequences will not generate significant alignments unless a highly related or completely homologous sequence at the DNA or RNA level is present in the databases. This may explain the lack of significant alignments of several clones using the BLASTP program, whereas the same sequences did have significant alignments using the BLASTN and *Chlamydomonas* EST database searches (e.g., clones A33-2 and C33-1, encoding *Chlamydomonas* CRD1). Other clones, such as C33-2, for which no significant homology was found using all three search strategies may represent novel genes that were not expressed under the conditions used to generate the *Chlamydomonas* ESTs. It is also possible, however, that in these cases the partial sequence obtained in this study was insufficient to detect homologies that would be revealed by longer cDNA fragments. Experiments utilizing RACE PCR are underway to further evaluate possible homologies of these putatively novel transcripts.

Two of the differential display products with novel sequences, C34-1 and C36-1, had very extensive sequence similarity over their entire lengths to *Chlamydomonas* ESTs (Table 1). In the case of C36-1, the sequence identity with the highest scoring EST (accession number AV391528) is 259/277 nucleotides (93%) and this homology extends from about the middle of the EST to the 3' ends of both sequences, where the C36-1 sequence ends in a poly(A)-tail. Therefore, AV391528 appears to extend the C36-1 cDNA sequence considerably (253 nucleotides in the 5' direction). However, BLASTN and BLASTP analyses of the AV391528 sequence failed to identify any significant similarities. The

EST AV640479 has 100% sequence identity with C34-1 (70/70 nucleotides). This EST, however, extends in the 5' direction only 37 nucleotides beyond the 5' end of C34-1. It extends mainly in the 3' direction, 393 nucleotides beyond the point at which C34-1 ends in a poly(A)-tail. Thus AV640479 probably represents an alternatively spliced form of the same pre-mRNA. BLASTN and BLASTP analyses of AV640479 using the entire EST sequence did not indicate significant homology to any known gene.

Sequences with homology to known genes

The present study has revealed the possible involvement of several known genes in response to Cd exposure for which there was no previous indication of such a role. These are the *CRD1*, *H43*, and *Chlamydomonas* SAM synthetase (*CHRSAMS*) genes. The possible functions of these and the other known genes identified as responsive to Cd are discussed below.

Crd1

The *CRD1* gene of *Chlamydomonas* is required for PSI and LHC I accumulation under copper-deficient conditions, and the *crd1* mutant exhibits copper-deficiency-conditional chlorosis (Moseley et al. 2000). The *CRD1* mRNA is markedly induced by copper deficiency. Although the exact function of *CRD1* is not yet clear, it has significant sequence similarity to the stearyl-ACP desaturase gene family. Recent evidence suggests, however, that *CRD1* protein may be involved in ring V formation in chlorophyll (S. Merchant, personal communication). The observed induction of *CRD1* mRNA by Cd exposure might be explained if Cd interferes with copper cofactor function or the supply of copper to the chloroplast through, for example, competition for uptake. Recent evidence suggests that, in *Chlamydomonas*, Fe uptake is significantly reduced in the presence of a 100-fold molar excess of copper, but not reduced in the presence of several other metals at the same molar excess (Eckhardt and Buckhout 1998). Thus copper and Fe may compete for uptake, but it is not known if copper and Cd compete for uptake in *Chlamydomonas*. Importantly, Fe uptake is increased by approximately 80% when *Chlamydomonas* is grown in the presence of excess Cd (Eckhardt and Buckhout 1998). These results suggest that Cd may selectively affect Fe uptake in *Chlamydomonas*.

CHLL

There is pharmacological evidence that expression of *CHLL*, encoding the regulatory subunit of the light-independent protochlorophyllide reductase, is regulated by the redox state of the chloroplast. Although not normally expressed in the light, *CHLL* was up-regulated

at the post-transcriptional level in the light when wild-type *Chlamydomonas* cells were treated with 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU) and dibromothymoquinone (DBMIB), inhibitors of electron transport from quinone to plastoquinone and from plastoquinone to cytochrome *b₆f*, respectively (Cahoon and Timko 2000). This suggests that either an oxidized state or a low-energy state within the chloroplast is sufficient for the synthesis of *CHLL*. Our results suggest that a small increase in *CHLL* transcript abundance can also result from oxidizing conditions such as Cd exposure. *CHLL* also has significant similarity to the Fe protein NifH, the dinitrogenase reductase component of nitrogenase. This protein serves as the electron donor to the Mo-Fe protein component of nitrogenase (Ljones and Burris 1972). It is noteworthy that this reduction is mediated by a thiol-bound Fe-sulfur cluster on NifH that is exposed on its surface (Hausinger and Howard 1983). These thiols may be susceptible to Cd poisoning.

CHRSAMS

In *Chlamydomonas*, SAM synthetase is encoded by the gene, *CHRSAMS*. In addition to its role as a donor of methyl groups, SAM can also serve as a precursor in cysteine biosynthesis (Voet and Voet 1990). An increase in available cysteine through increased SAM synthetase gene expression could represent an adaptive response to Cd toxicity, since cysteine plays a central role in detoxification through its incorporation into glutathione.

psbA

The D1 protein of the PSII reaction center, encoded by the *psbA* gene in the *Chlamydomonas* chloroplast, has a higher light-dependent turnover rate than other chloroplast proteins under physiological conditions (Mattoo et al. 1989), and oxygen evolution, in which D1 plays a central role, is also well documented to be one of the primary sites of Cd-induced inhibition of photosynthesis (Sheoran et al. 1990). We initially cloned the differential display product designated A33-1 under the assumption that it represented a Cd-inducible transcript, but subsequent reverse Northern blot analysis indicated that it was a false positive. Despite our negative result, it is worth noting that there is some evidence for D1 accumulation in at least some plant species shortly after Cd exposure. A 2-fold increase in D1 protein after 5 h of Cd exposure was observed in cut petioles of *Vicia faba* seedlings by pulse labeling with [³⁵S]methionine. This increase, however, was short lived, and D1 subsequently was found to decrease upon longer (≥24 h) Cd exposure times (Franco et al. 1999).

H43

The *H43* gene of *Chlamydomonas* encodes a high-CO₂-inducible protein that is reported to be localized in the

periplasmic space (Kobayashi 1997). H43 is significantly similar to the predicted protein HCR1. The BLASTP alignment using H43 as the query has a bit score of 107 and an E value of 2×10^{-22} . There are 90 amino acid identities between the two proteins, and the best alignment from BLASTP indicates a 32% amino acid identity over the aligned region. Overall, the two proteins have 25% amino acid identity when aligned by the CLUSTALW program (Fig. 3). An analysis of the significance of the CLUSTALW alignment based on the "normalized alignment score" method of Doolittle (1986) indicates that the two proteins are probably significantly related to each other, since they have a normalized alignment score that is between 5 and 10 standard deviations above the mean score of randomized sequences of the same amino acid length (data not shown).

HCR1 is encoded by a high- CO_2 -inducible and Fe-deficiency-inducible transcript from a marine green alga, *Chlorococcum littorale*. We found that the steady-state level of H43 mRNA is greatly increased (about 20-fold) in the cell wall-deficient strain CC406 by both Cd exposure and by the presence of the Fe chelator ferrozine. However, H43 gene expression was not induced by Cd exposure in the wild-type strain CC2137 (Fig. 4). Previous studies in our laboratory (Cai 1996) have shown that the growth rate of cell wall-deficient strains of *C. reinhardtii* is inhibited about 50% when exposed to 40 μM Cd, whereas growth of strain CC2137 was unaffected by the same concentration of Cd. This suggests that the cell wall may somehow block the toxic effects of Cd, possibly by acting as an effective exclusionary barrier to Cd entry into the cytosol. Thus, Cd-inducible genes are not up-regulated in fully walled cells, possibly due to a lower cytosolic Cd concentration.

The connection between Cd and Fe depletion can be clarified in light of recent studies of Fe uptake by pea and *Arabidopsis*. In pea seedlings, a high-affinity, saturable Cd-influx mechanism was detected in lateral roots and the rate of this influx increased 7-fold when seedlings were grown in Fe-deficient medium rather than Fe-replete medium. The high-affinity Cd influx was abolished by the addition of the metabolic inhibitors carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and KCN, further suggesting that the influx is mediated by an active transport process (Cohen et al. 1998). In *Arabidopsis*, two Fe transporters, IRT1 and NRAMP3 have been identified by their ability to complement an *S. cerevisiae* double mutant defective in both low- and high-affinity Fe uptake (*fet3fet4*) and sensitive to Fe limitation (Eide et al. 1996; Thomine et al. 2000). IRT1 and NRAMP3 both show an increase of their steady-state mRNA when the plants are grown in Fe-depleted medium. Both genes confer Fe(II) uptake on *fet3fet4*, and this uptake is significantly inhibited by Cd in the case of IRT1. NRAMP3 also confers Cd sensitivity on wild-type yeast. IRT1 also confers saturable uptake of manganese and zinc when expressed in a yeast mutant defective in manganese uptake (*smf1*) (Korshunova et al. 1999), and NRAMP3 also complements this mutant

(Thomine et al. 2000). These results strongly suggest that these Fe transporters mediate the uptake of a broad spectrum of metals, including Cd. Our results in *Chlamydomonas* are consistent with a model in which Cd uptake occurs via a broad-spectrum metal transporter, wherein Cd inhibits Fe(II) transport by saturating or inhibiting the transporter, reducing the amount of Fe entering the cell and triggering an Fe-stress response that includes H43 gene expression. Interestingly, a recent study of Fe uptake in *C. reinhardtii* reported a 1.8-fold increase in Fe uptake by wild-type cells [from 19.3 to ≈ 35 pmol $(10^6 \text{ cells})^{-1} \text{ h}^{-1}$] in the presence of a 100-fold molar excess of Cd (Eckhardt and Buckhout 1998), a result consistent with the above model.

H43 bears no significant similarity to any of the previously reported proteins with a role in Fe acquisition under Fe stress, such as *Arabidopsis* IRT1 and NRAMP3, plasma-membrane proton-ATPases, or ferric reductases. Likewise HCR1 is novel, but in *C. littorale* a second gene (*HCR2*) was found to be coordinately regulated by high CO_2 and Fe deficiency. The predicted amino acid sequence of HCR2 has significant similarity to several ferric reductases. Immunoblot analysis indicates that HCR2 is membrane-localized and is also regulated at the protein level by high CO_2 and Fe deficiency (Sasaki et al. 1998). Similarly, in *Chlamydomonas*, ferric chelate reductase is induced 6 h after transfer to Fe-deficient media (Eckhardt and Buckhout 1998). These results suggest that *C. littorale* and *Chlamydomonas* use similar strategies to facilitate Fe uptake in Fe-deficient media.

Analysis of the H43 sequence using the PROSITE database indicates the presence of the conserved ATP or GTP binding site known as the Walker A motif or P-loop (Fig. 5). Prediction of the secondary structure suggests that in H43 this motif is present in the expected surrounding context, namely that the P-loop often connects a hydrophobic beta strand with an alpha helix (Saraste et al. 1990). However, the sequence alignment with HCR1 (Fig. 3) shows that the highly conserved lysine residue of the P-loop (Saraste et al. 1990) is not present in HCR1. Confirmation of binding of ATP or GTP by H43 will require biochemical analysis of the purified protein and will be addressed in future experiments. It is also interesting to note that both H43 and HCR1 contain four cysteine residues, the number of cysteines that typically coordinate 4Fe-4S clusters, and the positions of these four residues are conserved (Fig. 3).

No known or predicted protein with significant similarity to H43 was found at the time of writing in the currently available annotation of the recently completed *Arabidopsis* genome. The only homologue of H43 so far identified (HCR1) is from the marine green alga, *Chlorococcum littorale*, a member of the same taxonomic class as *Chlamydomonas*, the Chlorophyceae. Thus, H43 and HCR1 may represent novel, algal-specific proteins that may facilitate Fe transfer from the ferric reductase (both in *Chlamydomonas* and complimented Fe-uptake yeast mutants) to the Fe-transporters.

The probable functions of the known genes identified in this study, as discussed above, suggest responses to various kinds of metabolic damage from Cd. Serendipitously, the further investigation of the *H43* gene may also contribute to an understanding of Fe homeostasis and Fe-responsive gene expression.

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