

FEA1, *FEA2* and *FRE1*, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with *FOX1* and *FTR1* in iron-deficient *Chlamydomonas reinhardtii*

Running title: Iron assimilation in *Chlamydomonas*

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ABSTRACT

Previously, we had identified FOX1 and FTR1 as iron-deficiency inducible components of a high affinity copper-dependent iron uptake pathway in *Chlamydomonas*. In this work, we survey the Version 3.0 draft genome to identify a ferrireductase, FRE1, and two ZIP family proteins, IRT1 and IRT2, as candidate ferrous transporters based on their increased expression in iron-deficient vs. iron-replete cells. In a parallel proteomic approach, we identified FEA1 and FEA2 as the major proteins secreted by iron-deficient *Chlamydomonas reinhardtii*. The recovery of FEA1 and FEA2 from the medium of *Chlamydomonas* strain CC425 cultures is strictly correlated with iron nutrition status, and the accumulation of the corresponding mRNAs parallels that of the *Chlamydomonas* FOX1 and FTR1 mRNAs, although the magnitude of regulation is more dramatic for the FEA genes. Like the FOX1 and FTR1 genes, the FEA genes do not respond to copper-, zinc- or manganese-deficiency. The 5' flanking untranscribed sequences from the FEA1, FTR1 and FOX1 genes confer iron-deficiency dependent expression of ARS2, suggesting that the iron assimilation pathway is under transcriptional control by iron nutrition. Genetic analysis suggests that the secreted proteins FEA1 and FEA2 facilitate high affinity iron uptake, perhaps by concentrating iron in the vicinity of the cell. Homologues of FEA1 and FRE1 were identified previously as high CO₂-responsive genes, HCR1 and HCR2, in *Chlorococcum littorale*, suggesting that components of the iron assimilation pathway are responsive to carbon nutrition. These iron response components are placed in a proposed iron assimilation pathway for *Chlamydomonas*.

INTRODUCTION

Although iron is abundant in soil, its bioavailability in the aerobic world is poor because of the relative insolubility of Fe(III) and the tight binding of iron to organic chelators (reviewed in 33, 44, 56). Iron is therefore one of the essential nutrients that limits virtually all forms of life, and its assimilation involves mechanisms for iron capture from otherwise inaccessible forms plus mechanisms for transport. Many organisms, especially microbes, use multiple pathways for iron uptake because of the varied forms and amounts of iron supply in nature. At the same time, because of its propensity for redox chemistry, especially in an aerobic environment, iron can be toxic to cells. Therefore, the metabolism of iron is very tightly regulated (e.g. reviewed in 9, 13, 22, 41, 75).

Fungi: Because of the importance of iron nutrition to life, the mechanisms of iron assimilation and distribution have been studied in many eukaryotic organisms. One well-studied pathway for iron assimilation was discovered initially through studies of iron metabolism in *Saccharomyces cerevisiae*. In this pathway, iron is first solubilized by reduction of ferric to ferrous, and is then available for uptake by high affinity transporters like the Fet3p/Ftr1p complex (3, 14, 27, 49, 52, 72, 81, 92). There are several transcriptionally-regulated *FRE* genes in *S. cerevisiae* with different substrate selectivity. Fre1p and Fre2p are the major cell surface iron reductases that reduce ferric citrate and siderophore-bound iron, Fre3p shows selectivity for iron in complex with hydroxamate-type siderophores and Fre4p has low affinity activity on rhodotorulic acid. Fet3p is a plasma membrane-associated multi-copper oxidase that re-converts Fe²⁺ to Fe³⁺, which is a substrate for the associated trivalent cation-specific permease, Ftr1p. This type of pathway is broadly distributed in the fungi, and an analogous mechanism for iron distribution occurs also in mammals, and accordingly iron metabolism in these organisms shows a requirement for copper nutrition (e.g. 4, 23, 46, 48, 60). More recently, analysis of the iron regulon in *S. cerevisiae* revealed the importance of a ferrichrome transporter and also three mannoproteins located in the cell wall for utilization of iron bound to a subset of siderophores like ferrichrome (45, 66). These pathways allow the organism to utilize a range of iron sources.

Animals: Iron metabolism in multi-cellular organisms is much more complex. A di-heme ferrireductase, Dcytb, whose expression is increased at the RNA level during iron-deficiency, is involved in iron uptake in the duodenal brush border of animals (54). A divalent cation transporter, DCT1 or DMT1, related to the Nramp family of transporters, then takes up ferrous iron (29, 35). Dietary iron needs to be distributed systemically. Iron movement involves sequential redox chemistry – oxidation of ferrous to ferric by multicopper oxidases ceruloplasmin and hephaestin and reduction by ferrireductases – and membrane transport by ferroportin/Ireg1/MTP1 (1, 19, 39, 53, 86). Extracellular iron is circulated in a non-toxic form, tightly bound to transferrin, which is taken up by the transferrin receptor, and intracellular iron is stored in oxidized form in ferritin (reviewed in 41, 82).

Plants: Two iron assimilation mechanisms have been studied in plants, called Strategy I and Strategy II (reviewed in 13, 40). Both are copper-independent. In Strategy I, soil acidification followed by iron reduction by a root plasma membrane reductase releases Fe^{2+} , which can be taken up by one of the iron-deficiency induced plasma membrane IRT transporters (10, 21, 71, 84). The IRT transporters belong to the so-called ZIP family of transporters found in fungi, plants and animals. Members of the family function to transport divalent cations (reviewed in 34). In Strategy II, discovered in grasses, the plants secrete phytosiderophores that solubilize Fe^{3+} by chelation (56, 85). The iron-chelate complex is then taken up by specific high affinity YSL transporters (12). Functional homologues of the Nramp transporters are found in Strategy I and Strategy II plants but the role of individual gene products in iron assimilation vs. distribution has not been detailed yet (83). As for fungi and animals, inducible iron reductases are also key enzymes in iron utilization by plants. In Arabidopsis, a ferrireductase encoded by *FRO2* is involved in root iron uptake and an NADH-dependent ferrireductase encoded by *NFR* in maize is implicated in ferrireductase activity (5, 7, 71, 90).

Algae: Iron metabolism has been studied also in Chlamydomonas, a unicellular green alga that serves as an excellent model for classical, molecular and reverse genetic analysis of chloroplast function and of eukaryotic cilia. The involvement of inducible cell surface

reductases, which appears to be a general requirement for iron metabolism in the Eukarya, is well-documented in *Chlamydomonas* as well as in many other algae (20, 51, 77, 87), although the identity of the enzymes is not yet known. A major component in the iron assimilation pathway is a ferroxidase / ferric transporter system, analogous to the molecules found in fungi and animals (42, 47). The multicopper oxidase, encoded by *FOX1*, although related to the multicopper oxidases of fungi and animals, is clearly also distinct with respect to the arrangement of copper binding sites and is likely to have resulted from domain re-arrangement during evolution. The 959 residue protein is associated with the plasma membrane and is coordinately expressed with the *FTR1* gene encoding a putative ferric transporter related to those in fungi. However, iron utilization by wild-type *Chlamydomonas* does not show a requirement for copper, suggesting that an alternative pathway for iron uptake may be induced in copper-deficient cells (47). The components of the copper-independent iron uptake pathway of *Chlamydomonas* are not yet known. The availability of a draft genome for *Chlamydomonas* opened the door to the possibility of identifying additional components in iron homeostasis by testing candidate genes, homologous to those identified in other eukaryotes, for their pattern of expression relative to iron and copper nutrition.

A diversity of iron assimilation pathways operate in various algae, including siderophore mediated mechanisms in a *Scenedesmus* species and a transferrin-dependent pathway in *Dunaliella salina* (6, 28, 88). The *D. salina* extracellular, plasma membrane associated transferrin is proposed to bind ferric ion generated by a surface exposed plasma ferroxidase (64). This suggests that secreted molecules might be important in iron-acquisition pathways in green algae. Therefore, we compared the secretome of iron-deficient vs. iron-replete *Chlamydomonas* cells to identify two related proteins, FEA1 and FEA2.

MATERIALS AND METHODS

Strains and Culture

The strains used in this work were obtained from the *Chlamydomonas* culture collection (Duke University) and grown in the light ($60 - 80 \mu\text{mol m}^{-2} \text{s}^{-1}$) in tris-acetate-phosphate medium (TAP). For experiments involving iron nutrition, wild-type strains and transformants of strain CC425 were maintained in standard TAP medium and transferred to Fe-free TAP medium supplemented with Fe-EDTA from stock solution at the indicated concentrations (58). Previously, we had identified 3 stages of iron nutrition based on appearance of “chlorosis”, expression of marker genes and proteins (*FOX1*, ferroxidase) and rate of cell growth (55, 58). Iron-deficient cells ($1-3 \mu\text{M}$ supplementation) are not chlorotic, exhibit growth rates and growth density (at stationary phase) comparable to iron-replete cells ($20 \mu\text{M}$) but show increased expression of iron assimilation components. Iron-limited cells ($<0.5 \mu\text{M}$) are chlorotic (50% of the Chl content of replete cultures), are inhibited in the rate and extent of cell division, and show maximal up-regulation of iron assimilation genes. The iron concentrations used in specific experiments is indicated in each legend. In experiments involving iron limitation where it was necessary to avoid carry over of iron from the starter culture, log phase cells ($5 - 7 \times 10^6$ cells/ml) were collected by centrifugation ($1700 \times g$, 5 min), washed once in iron-free TAP medium, and inoculated into the desired iron-limited medium to a density of 5×10^4 cells/ml. Growth in copper-free medium has been described previously (68). For analysis of gene expression in response to manganese- or zinc-deficiency, strains were grown in standard TAP medium and transferred (1:1000 dilution of a log phase culture ($4-8 \times 10^6$ cells/ml) at each transfer) to TAP medium lacking the test nutrient. Cells were collected at densities as indicated in the legends.

Genetic analysis

Strains CC124 or CC125 (that can grow on medium containing low iron) were crossed to strains CC424 or CC425 (that require higher iron for growth). Opposite mating types were mated and dissected as described by Harris (38). Resulting progeny were scored for 2:2 segregation of

arginine auxotrophy, analyzed for retention of FEA proteins in washed cells, and for growth (assessed visually) in medium containing limiting (0.1 μM) iron (provided as ferrous sulfate). For testing FEA retention, cells grown in medium containing 1 μM iron were collected by centrifugation at 1000g, washed twice in 1 volume of 10 mM sodium phosphate, pH 7.0, and resuspended in a minimum volume.

Isolation of proteins secreted by Chlamydomonas

Cells of Chlamydomonas strain CC400 (*cw15*, *mt+*) were grown to mid-log phase ($\sim 4 \times 10^6$ cells/ml) in TAP (tris-acetate-phosphate) medium, and collected under sterile conditions by centrifugation (3800 g, 5 min). Cells were washed twice in sterile $-\text{Fe}$ TAP to remove traces of iron, and inoculated into fresh TAP medium containing 0.1, 1 or 18 μM FeSO_4 at 2.5×10^5 cells/mL. Cultures were grown for 4 days under constant light ($75 \mu\text{mol m}^{-2} \text{s}^{-1}$, 150 rpm). Cells were counted before collection, and the cultures were adjusted to 0.1M NaCl, 1mM ϵ -amino-n-caproic acid, and agitated (150 rpm) for 10 min before the cells were removed from the growth medium by centrifugation (4,000 g, 5 min, 4°C). The supernatant was collected and centrifuged again (8,000 g, 10 min, 4°C) to remove any remaining cells. Ammonium sulfate was added to 80% saturation and the solution was stirred (1h, 4°C). Precipitated proteins were collected by centrifugation (30,000 g, 90 min, 4°C), resuspended in 5 mL of a solution containing 50 mM Tris-Cl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM ϵ -amino-n-caproic acid, pH 8.0, and re-precipitated by adding 100% ice cold trichloroacetic acid (10% w/v final concentration), and mixing by gentle rocking (1h, 4°C). Precipitated proteins were collected by centrifugation (19,000 g, 30 min, 4°C) and resuspended in a solution containing 0.1 M Na_2CO_3 , 0.1 M DTT with volumes adjusted to reflect equal cell number per ml in each sample. Proteins in each sample were resolved on an SDS-containing polyacrylamide gel (12% monomer) and visualized by staining with Coomassie blue.

Immunoblot analysis

For immunoblot analysis, proteins were separated on an SDS-containing polyacrylamide gel (12% monomer) and transferred onto PVDF (0.45 μm , Millipore) for 1h at 4°C under constant

voltage (100 V) in 25 mM Tris, 192 mM glycine, 0.01% SDS, 20% methanol. Membranes were blocked with 5% dry milk in TBS (10 mM TrisHCl, 150 mM NaCl, pH 7.5) + Tween-20 (0.05% w/v). A 1:50,000 dilution of *Chlamydomonas* anti-FEA1 was used as the primary antibody, and 1:5000 dilution of goat anti-rabbit horseradish peroxidase was used as the secondary antibody. Signals were detected using Amersham ECL kit.

Identification of polypeptides

The 43 and 38 kDa bands were excised by a spot-excision robot (Proteome Works, BioRad) and deposited into 96-well plates. Gel spots were washed, digested with sequencing-grade trypsin (Promega, Madison, WI), and the resulting tryptic peptides were extracted using standard protocols (79). The trypsin digestion and extraction, and peptide spotting onto a matrix-assisted laser desorption ionization (MALDI) targets were accomplished by a robotic liquid handling workstation (MassPrep, Micromass-Waters, Beverly, MA). MALDI peptide fingerprint mass spectra were acquired with a MALDI time-of-flight (TOF) instrument (M@LDI-R, Micromass-Waters, Beverly, MA), using α -cyano-4-hydroxycinnamic acid (Sigma) as the matrix. The MS data from MALDI-MS peptide fingerprint mass spectra were searched against the non redundant protein sequence database at NCBI and also the gene models from Version 2 of the draft *Chlamydomonas* genome using the Mascot (Matrix Science, London, UK) search programs. Positive protein identification was based on standard Mascot criteria for statistical analysis of the MALDI peptide fingerprint mass spectra.

Nucleic acids analysis

Total *Chlamydomonas* RNA was prepared and analyzed by hybridization as described by (68). The insert in plasmid MXL014c06 from Kazusa DNA Research Institute, corresponding to EST BP093819, was sequenced on one strand by Agencourt Biosciences. There were no differences compared to the sequence available on the JGI browser.

5' end identification: The 5' end of *FRE1* mRNA was identified using the 5' RACE system (Invitrogen) as described by the manufacturer using the protocol for GC rich sequences (5% DMSO). The primers were 5' GTT GTC CAA GAC ACG CAT GG, 5' ACC ATG TCC

AGG ACG CTC AG, 5' TTA ATT GCG CGT CCC AGG and 5' TAT TGG GAG TGG GGG AAG AG. The product from the final nested PCR was cloned into pCR2.1 Topo (Invitrogen) and sequenced (Agencourt Biosciences). The sequence was assembled with the sequence of the insert in plasmid MXL014c06 and deposited under accession EF042874.

For *FEA2*, the automated gene model was truncated at the 5' end relative to the *FEA1* model. To verify the 5' end of the *FEA2* gene model, a primer (5' GCG CTG CGC CCC CAA GCGT) designed upstream of the predicted 5' end (and corresponding to the 5' end of the *FEA1* model) was used with an internal primer (5' AGG TTG GTC AGG GTG TTG TCC) to amplify a 511 bp fragment from cDNA, which was sequenced (deposited under accession DQ223113) and used to generate an improved gene model.

RNA blot hybridization: Five micrograms of RNA was loaded per lane. The probe used to detect *FOX1* transcripts was generated by EcoRI-XhoI digestion of clone HCL036d03 (Kazusa DNA Research Institute; GenBank accession no. AV641563). For the detection of *FEA1* transcripts, plasmid (9-18)1 (74) was digested with EcoRI and XhoI. The 1.0 Kb fragment was isolated and used as a probe. For *FEA2*, an amplification product generated by using the primers listed in Table I was used as the probe. For *CβLP*, a 915-bp *EcoRI* fragment from the cDNA insert in plasmid pcf8-13 was used (78). *CβLP* hybridization signal is used for normalization between samples because it does not vary as a function of metal nutrition. Specific activities of probes ranged from 5.7 to 8.4×10^8 cpm μg^{-1} for the experiments shown here. The blots were exposed at -80°C to film (XRP-1; Eastman-Kodak, Rochester, NY) with two intensifying screens, and were developed after a few hours exposure.

Quantitative Real-Time PCR: Genomic DNA was removed from the total RNA preparation by treatment with RQ1 DNase (Promega) according to the manufacturer's instructions. Complementary DNA, primed with oligoDT, was generated with reverse transcriptase (Gibco/BRL) also according to the manufacturer's instructions, and used in the amplification reaction directly after dilution. The amplification reaction was carried out with reagents from the iQ SYBR Green Supermix qPCR kit (Biorad Laboratories). Each reaction

contained the vendor's master mix, 0.3 μ M of each primer, and cDNA corresponding to 5 ng/ μ l input RNA in the reverse transcriptase reaction. Primers that are not described previously (2) are listed in Table I. The primers were tested for efficiency and the identity of the product verified by sequencing. The reaction conditions for the Opticon 2 from MJ Research were: 95°C 15 min, followed by cycles of 95°C 10 s, 65°C 30s, 72°C 30s up to a total of 40 cycles. The fluorescence was measured at each cycle at 72°C and 83°C. The $2^{-\Delta\Delta C_T}$ method was used to analyze the data based on the fluorescence at 83 °C (50). Melting curves were performed after the PCR reaction to assess the presence of a unique final product, and the product was sequenced from one reaction to verify that it represented the gene of interest. The data are presented as the fold change in mRNA abundance, normalized to the endogenous reference gene (*C β LP*), relative to the RNA sample from cells grown in 18 μ M Fe (considered Fe replete). A second reference gene (*UBQ2*) was also used in some experiments. Neither *UBQ2* nor *C β LP* had changed abundance under the conditions tested in this work.

Reporter construct and its analysis

Upstream regions of the *FEA1* (formerly known as *H43*), *FOX1* and *FTR1* genes were identified initially in the first draft of the *Chlamydomonas* genome (<http://genome.jgi-psf.org/chlre1/chlre1.home.html>). Primers (for *FEA1*, forward = 5'FEA1-fwd1: 5'-CGG GGT ACC AGG ACA GAG TGC GTG TGGC-3' and reverse = 5'FEA1-rev1: 5'-CGG GGT ACC TGG TTA ACT GTG CGA CGGG-3' corresponding to position -1157 to +43 relative to the 5' end of the transcript; for *FOX1*, forward = 5'FOX1-fwd1: 5'-CGG GGT ACC CAC GCG ACA TAT AGC CGG GAT-3' and reverse = 5'FOX1-rev1: 5'-CCG GGT ACC CTT TTC GGC TTT TGC CTG TT-3' corresponding to position -1130 to +10 relative to the 5' end of the transcript, for *FTR1*, forward = 5'FTR1-fwd1: 5'-CGG GGT ACC TGC TCT GGA ATC TTG TAC TCC-3' and reverse = 5'FTR1-rev1: 5'-CGG GGT ACC CGG CTT GCA AGT TAG AGT GCT-3' corresponding to position -1280 to -1 relative to the 5' end of the transcript) were designed to amplify the regions from *Chlamydomonas* genomic DNA (strain CC125). The resulting fragments were verified by restriction digest, cloned into the unique KpnI site of plasmid pJD100

containing the minimal promoter from the β -tubulin-encoding gene fused to a promoter-less *ARS2* (designated ptubB2 Δ 3,2,1/ars; (16). The resulting plasmids were linearized with PvuI (p5'*FEA1-ARS2*, p5'*FTR1-ARS2*) or BsaI (p5'*FOX1-ARS2*), and introduced into strain CC425 (*arg2*) by cotransformation with EcoRI-linearized pArg7.8 carrying the *ARG7* gene encoding argininosuccinyl lyase (18) according to (67). Transformants were selected for their ability to grow on TAP medium without arginine supplementation. To test the reporter expression in the transformants, the plates were sprayed with a freshly-diluted (10 mM frozen stock) solution of 2mM 5-bromo-4-chloro-3-indolyl sulfate (XSO₄; Sigma) as described by (15). Arylsulfatase activity was assayed quantitatively as described by (17). The presence of the test construct in the various strains was ascertained by amplification using construct specific primers, 5'*FEA1-fwd2* = 5'-CGG GGT ACC CAC ATT GAA AAA CGA GCGC-3', 5'*FOX1-fwd2* = 5'-CGG GGT ACC GTA TCG GCG AAG GTC AGA CG-3', and 5'*FTR1-fwd2* = 5'-CGG GGT ACC GGC AGG CGC GGG CGC CTG CT-3', with reporter specific primer Rev-*ARS2* = 5'-ATG GCA GGG GAG GAA CCG GTT-3', which produces an approximately 9×10^2 bp fragment corresponding to approximately 5×10^2 bp of the upstream region for each gene plus 1×10^2 bp of the *TUB2* minimal promoter plus 3×10^2 bp of the genomic *ARS2* sequence.

RESULTS

Test of candidate transporters and reductases for function in iron assimilation

We queried the draft genome of *Chlamydomonas* (initially version 2 and subsequently version 3) by BLAST to identify gene models corresponding to homologues of metal transporters and ferrireductases of other organisms, including fungi, plants and animals (55). Since the known components of iron assimilation, including *FOX1*, *FTR1*, *ATX1*, and *FER1*, were regulated through changes in RNA abundance (47), we sought to distinguish the relevance of candidates by screening their pattern of expression as a function of iron nutrition.

***FRE1*:** For the reductases, four good candidates were identified, corresponding to protein IDs 169091, 189216, 145439 and 163751 in the version 3.0 draft genome (Figure 1). The expression of one gene (representing model 169091), now named *FRE1*, is dramatically regulated by iron-deficiency (up to 10^3 fold in medium containing 1 μ M iron in different experiments with different wild-type strains) and shows the same pattern of regulation as do the *FOX1* and *FTR1* genes (see Figure 5A). Specifically, the *FRE1* mRNA is more highly induced in cells maintained in iron-deficient (1-3 μ M) compared to iron-limited (< 0.5 μ M) conditions (see methods). A full-length sequence for the mRNA was derived by assembling the sequence of the 5' RACE product with the sequence of the insert in plasmid MXL014c06. The *FRE1* gene product is similar to the *HCR2* gene product from *Chlorococcum* along its entire length, but its central portion, which includes the transmembrane ferric reductase motif (Pfam Accession PF01794), is most related to the corresponding domain of plant *FRO* genes. *Chlorococcum HCR2* was named for its increased expression in response to high CO_2 , but was shown also to be responsive also to iron nutrition (76, 77). The version 3.0 draft genome shows three other related genes: the expression of two linked genes (corresponding to 189216 and 145439) is not affected by either iron or copper. These genes were named *RBOL1* and *RBOL2* because of their relationship to plant respiratory burst oxidase proteins. We found also a good gene model (163751) for a cytochrome *b*₅-reductase related to the maize *NFR* gene product (5). However, this gene is also unresponsive to iron and copper nutrition on the basis of RNA abundance.

IRTs: Besides the reductases, we identified homologues of metal transporters belonging to various distinct families (37, 55). These include the CTR/COPT transporters that function in copper assimilation, the NRAMPs (also called DMT1 or DCT1) that are proton coupled divalent cation transporters, the heavy metal transporting P-type ATPases (referred to as HMAs in plants), the cation diffusion facilitators (also called MTPs in plants), and the ZIP transporters (30, 34, 63, 65, 80). We tested the abundance of mRNAs for *Chlamydomonas* homologues of these proteins as a function of iron nutrition to determine whether the pattern of RNA accumulation of any might indicate a significant role in iron assimilation. Both copper-supplemented and copper-deficient cells were analyzed, since a copper-independent pathway for iron uptake might be expressed only in a copper-deficient situation (25, 47). None of the candidate transporters showed regulation of the magnitude noted for *FRE1*, *FTR1* and *FOX1* expression (data not shown). Because of the potential toxicity of iron, high affinity iron uptake is very tightly regulated in most organisms. Therefore, it seemed unlikely that any of these molecules might contribute substantially to iron uptake in a situation of deficiency or limitation. On the other hand, the mRNAs for two ZIP family transporters, which might contribute to iron assimilation in a replete cell, show about a 10^2 -fold difference in abundance in response to iron but not zinc, copper or manganese nutrition (Figure 2, and data not shown). The corresponding genes were therefore named *IRT1* and *IRT2*.

FEA1 and FEA2 are secreted by iron-deficient cells

Extracellular molecules are components of iron assimilation in other chlorophyte algae, which prompted us to search for these in *Chlamydomonas*. A transferrin-like molecule is known in a related *Chlamydomonad* alga, *Dunaliella* (28), but we did not find homologues of the *Dunaliella* protein in the *Chlamydomonas* genome. Therefore, we used the approach of previous studies in which extracellular phosphatases, sulfatases and carbonic anhydrases that facilitate growth on low phosphate-, sulfate- or CO₂, respectively, were identified (e.g. (8, 17, 62, 69)). The secreted proteins are easily isolated from culture supernatants of cell-wall deficient *Chlamydomonas* strains because the proteins are not retained with the cells but are lost into the

medium.

We compared proteins secreted by cells grown in 0.1 μM vs. 1 μM vs. 18 μM Fe (the concentration of iron in the standard TAP medium). Two proteins with mobilities around 40 kDa appeared progressively more abundant as the iron content of the medium was reduced (Figure 3). The bands were excised, subjected to in-gel trypsin digestion, and the resulting tryptic fragments were analyzed by MALDI-TOF mass spectrometry. The 43 kDa protein was identified as a previously known protein, H43, and the 38 kDa one was identified as a previously unknown homologue by searching against the predicted gene models from the draft genome of *Chlamydomonas reinhardtii* (www.jgi.doe.gov). The homologue corresponds to protein ID 173281 that is immediately adjacent to H43 (protein ID 129929 in Version 3.0 of the genome). Sayre and co-workers had previously identified the *H43* gene in a screen for genes displaying differential expression under cadmium treatment (74). Nevertheless, they suggested that the gene encodes an iron assimilation component based on its increased expression in iron-deficient *Chlamydomonas* and its ability to stimulate the growth of a *fet3fet4* strain of *Saccharomyces cerevisiae*. *H43* was re-named *FEA1* (for Fe assimilation) in anticipation of such a function and the homologue was named *FEA2*. The identification of *FEA1* is based on nine peptides corresponding to coverage of 34% of the predicted protein with an RMS error of 66 ppm (Table II). For *FEA2*, 11 peptides, representing 43% coverage were matched with an RMS error of 22 ppm. Occasionally peptides corresponding to *FEA1* were found also in the 38 kDa band but the identification of *FEA2* in the 38 kDa band is unambiguous because of the presence of unique peptide masses. We attempted to identify the 60 kDa protein, apparently iron-deficiency inducible protein by mass spectrometry, but have been unsuccessful thus far.

The original gene model for *FEA2* was incomplete at the 5' end because of the absence of EST coverage. Therefore, we amplified the 5' end of the corresponding RNA isolated from iron-deficient cells. The sequence (Accession DQ223113) was used to generate an improved model.

FEA1 and *FEA2* share 43% sequence identity and the gene arrangement is a presumed consequence of gene duplication (Figure 4). *FEA1* is predicted to encode a 362 amino acid

protein while FEA2 is predicted to be 363 amino acids; both sequences include a predicted signal peptide. The difference in migration may result from different post-translational processing. Given that a peptide close to the predicted C-terminus of FEA1 was identified in the 38 kDa band, we attribute the presence of FEA1 in the 38 kDa band to N-terminal degradation of the 43 kDa species, but it is certainly possible that there may be differently modified forms of the protein (e.g. resulting from glycosylation or processing). FEA1 is several fold more abundant than FEA2.

A BLAST search of genome databases and sequences at NCBI (May 07) indicates that the FEA proteins are found in several Chlorophyte algae, including *Scenedesmus obliquus*, *Cc. littorale* and *Volvox carteri* and a dinoflagellate, *Heterocapsa triquetra*. *V. carteri* encodes three FEA-like proteins; two are orthologues of FEA1 and FEA2 based on synteny, and a third is unlinked. A multiple alignment identified several conserved residues, including some (Cys, Asp/Glu, Tyr, His) with functional groups that are suitable ligands for either Fe(II) or Fe(III) binding, but no other motifs that speak to function.

We noted that copper nutrition state did not modify the pattern of FEA1 and FEA2 accumulation (Figure 3), indicating that these molecules were not part of the putative copper-independent high affinity iron uptake pathway. Neither was the pattern of any other secreted protein modified by copper nutrition, arguing against a role for secreted proteins in copper assimilation.

FEA1* and *FEA2* are expressed coordinately with *FOX1* and *FTR1

A plasma membrane ferroxidase (encoded by *FOX1*) was the first iron assimilation component to be identified in *Chlamydomonas* (42, 47). As expected for an assimilatory component, *FOX1* expression is increased prior to the appearance of iron-deficiency symptoms in *Chlamydomonas* (58). We note that the same is true for *FEA1* (Figure 5). *FEA1* is induced coordinately with *FOX1*, and both genes are nearly fully activated at 1.0 μ M Fe in TAP medium at which point chlorosis is not yet evident. Given the relative low abundance of the FEA2 polypeptide recovered (Figure 3) and the absence of ESTs in the database, we were surprised to

note that *FEA2* mRNA is also increased substantially in iron-deficiency and its pattern of expression parallels that of *FEA1* (Figure 5). On the other hand, a second gene, *FOX2*, encoding a putative ferroxidase, although expressed, is not differentially regulated by iron nutrition status. Quantitative PCR indicates that the fold induction of *FEA1* and *FEA2* is much more dramatic than that of *FOX1*. *FOX1* mRNA is about 10^2 -fold induced in 0.1 vs. 18 μ M Fe while *FEA1* and *FEA2* are induced about 10^3 to 10^4 -fold. The exact fold induction varies, dependent upon the strain analyzed, and the cell density of the culture when it was sampled for RNA extraction. RNA blot hybridization experiments confirmed this dramatic up-regulation as well as the strain specific level of gene expression (Figure 5B). Absolute quantitation of *FEA1* vs. *FEA2* mRNAs indicates that *FEA1* is present at about 10 times the stoichiometry of *FEA2* in an iron-replete cell but this changes to a 100-fold difference in iron-deficiency. This is consistent with the occurrence of over 30 *FEA1* ESTs but no *FEA2* ESTs.

When we analyzed RNAs prepared from cells adapted to copper-, manganese- or zinc-deficiency, we noted that the iron assimilation components *FOX1*, *FTR1*, *FEA1* and *FEA2* are induced primarily by iron-deficiency (Figure 6). For example, *FEA1* expression is not significantly changed by copper- or zinc-deficiency. There is a small increase in the expression of the *FEA* genes in manganese-deficient cells but this is only a few percent of the change noted in iron-deficiency, and is in fact attributable to secondary iron-deficiency in manganese deficient cells (2).

***FEA1* and *FEA2* are differently regulated by high CO₂**

HCRI, a *Cc. littorale* homologue of the *Chlamydomonas* *FEA* genes had been identified initially as a gene induced by high CO₂, and a recent study showed that *FEA1* was also induced by high CO₂ (36, 76). Therefore, we tested the pattern of both *FEA1* and *FEA2* expression in response to CO₂ in wild-type cells and in a mutant, *cia5*, which is defective in the carbon concentrating mechanism (CCM) and hence unresponsive to CO₂ nutrition (31, 57). We found that the *FEA1* gene is down-regulated in low CO₂ in a pathway dependent on *CIA5* but *FEA2* is not (Table III). When we repeated the analysis with *lcr1*, another mutant of CCM (91), we

reached the same conclusion (data not shown).

***FEA1*, *FOX1* and *FTR1* are transcriptionally regulated by iron**

Regulation of iron assimilation occurs at the transcriptional level in many microorganisms, but at the post-transcriptional level in animal cells (reviewed in 73, 75). To distinguish the operation of an iron nutrition-responsive pathway in *Chlamydomonas*, we fused the 5' flanking DNA upstream of the putative 5' end of the mRNA (where +1 is defined by the sequence of the most 5' EST defined by accession BE237853) to a reporter gene, *ARS2*, driven by a minimal promoter from the *TUB2* gene to generate the test constructs p5'-*FOX1*-*ARS2*, p5'-*FTR1*-*ARS2* and p5'-*FEA1*-*ARS2* (see methods section, constructs available from the *Chlamydomonas* culture collection). The constructs were each introduced into *Chlamydomonas* strain CC425 by co-transformation with pArg7.8 and selection for growth in the absence of arginine. For each construct, colonies that expressed the reporter gene were analyzed to assess iron-responsive expression. In parallel, colonies resulting from co-transformation of pJD100 (containing no *FEA1* or *FOX1* or *FTR1* sequences) and pArg7.8 were analyzed. No colony appearing from the control transformation ever showed significant arylsulfatase expression (one representative is shown in Table IV). Quantitation of *ARS2* expression by enzyme assay (analysis of two representative colonies for for each construct is shown in Table IV) confirmed that the 5' flanking sequences from the *FEA1*, *FOX1* and *FTR1* genes confer iron responsiveness to a reporter gene. Furthermore, it appears that the magnitude of regulation of these genes is recapitulated with the reporter genes because the constructs carrying *FEA1* sequences appeared generally to show much greater reporter gene activity.

We conclude that components of the iron assimilation pathway in *Chlamydomonas* are transcriptionally regulated by iron availability in the medium. We suspect that regulation occurs at least in part by activation of gene expression in low Fe (as opposed to repression in high Fe) because the arylsulfatase expressing colonies carrying the 5'*FEA1*-*ARS2*, 5'*FTR1*-*ARS2*, or 5'*FOX1*-*ARS2* constructs always showed much higher expression than colonies carrying pJD100 (where *ARS2* is driven by a minimal promoter). Nevertheless, we can not rule out the presence of

repressing sequences, nor can we exclude post-transcriptional mechanisms overlaid on the primary transcriptional response because we did not test for these.

Function of FEA1 and FEA2

FEA1 and FEA2 are secreted to the medium in *Chlamydomonas* strain CC425 (which is defective with respect to cell wall structure), suggesting that the proteins are probably located in the extracellular space in a wild type cell (Figure 7). Therefore, we suggest that the proteins function as periplasmic substrate binding proteins to concentrate substrates in the vicinity of assimilatory transporters. Since FEA proteins were completely lost to the medium in strain CC425 (Figure 7), we were able to assess the function of cell associated FEA proteins in the context of iron nutrition. The rationale is as follows: a strain that loses the protein to the medium would be analogous to a loss of function *fea* mutant. Therefore, we analyzed the amount of cell-associated FEA proteins and iron nutrition-dependent growth of spores resulting from a cross of strain CC425 to strain CC124 (Figure 8). We analyzed 7 complete tetrads and noted that strains that had no cell-associated FEA1 and FEA2 were unable to grow in medium containing 0.1 - 0.2 μM iron ($p < 0.01$). We conclude that the FEA proteins are not essential for iron uptake but rather that they facilitate iron uptake by functioning as iron binding proteins in the extracellular space.

DISCUSSION

Studies of the impact of micronutrients on interpretation of the genome are generally facilitated in microorganisms because of the ease with which the growth medium can be manipulated, and *Chlamydomonas reinhardtii* has emerged as a powerful model for the study of the impact of nutrition on the photosynthetic apparatus (32, 55, 89). The photosynthetic apparatus, like the respiratory chain, is a major iron utilizing pathway in a photosynthetic cell. Recently, we showed that the organization and abundance of chlorophyll containing proteins is modified in a programmed pathway that responds sequentially and progressively to iron depletion (58). The mechanisms for sensing and responding to iron status are, however, unknown. In this work, we surveyed all candidate metal transporters in the genome and applied sub-proteomic analysis to expand our knowledge of components involved in iron assimilation in *Chlamydomonas*.

FEA1 and FEA2

Two homologous proteins, FEA1 and FEA2, are identified as extracellular proteins on the basis of their recovery from the growth medium after removal of cells. The recovery of both proteins is greater in strain CC425 (which lacks a cell wall and loses proteins to the medium) compared to wild-type cells, which confirms their extracellular location (Figure 7, and data not shown). Because their accumulation increases with progressively greater iron deficiency, we hypothesized that the molecules must function in iron assimilation. And indeed, we noted that cells containing FEA proteins appeared to have a greater facility for iron utilization (Figure 8). Cells that lose FEA proteins to the medium grow more poorly in iron-deficient medium.

FEA1 is several fold more abundant than FEA2, especially in iron-deficient cells (Figure 3). In wild-type cells (CC125), we note that *FEA1* is already maximally increased in expression when the iron content of the medium is reduced to 1 μM , while *FEA2* is maximally increased only in iron-limited (<0.2 μM) cells. It is possible that the two proteins might be functionally different with respect to iron-binding affinity. Interestingly, *Chlamydomonas FEA1* was identified independently as a “high CO_2 responsive” gene (36) and this is confirmed in our study

(Table III). For *Cc. littorale*, both *HCR1* and *HCR2*, homologues of *FEA1* and *FRE1*, are high CO₂-responsive (76). We do not understand the basis for regulation of *FEA1* by CO₂, but it might relate to modification of iron demand by carbon availability. Another possibility is that the response may be related to a role for (bi)carbonate in facilitating iron binding to FEA. We note that *FEA2* is not regulated by CO₂, and this may reflect specialization of function after gene duplication. The *Volvox* genome encodes 3 FEA proteins; perhaps this relates to its multicellularity and different metabolic demands of vegetative vs. reproductive cells.

Pathways of iron assimilation in the algae appear to be quite diverse. For instance, the *Ostreococcus* spp. genomes do not seem to encode homologues of the putative high affinity ferric transporter (FTR1) found in *Chlamydomonas*, *Volvox* and *Physcomitrella*, nor of the ferroxidase discovered in *Chlamydomonas* and *Dunaliella*, and it is suggested that they may synthesize siderophores (61). *D. salina* but not the *V. carteri* or *C. reinhardtii* (all within the *Chlamydomonadaceae*) uses an extracellular transferrin-like molecule for high affinity iron uptake (28). Our analysis of FEA function in *Chlamydomonas* suggests that periplasmic iron binding may be important for acquiring iron in a situation of limitation, but each organism may have devised / acquired different means for accomplishing this. An *FEA* homologue was among the ESTs from a dinoflagellate, indicating that the proteins are not restricted to the chlorophyte algae.

Regulation of iron assimilation pathway genes

Reporter gene analysis confirmed that *FEA1*, *FOX1* and *FTR1* are regulated largely if not entirely at the transcriptional level (Table IV). The magnitude of regulation of the *FEA1* and *FRE1* genes make their promoters useful targets for dissection of nutritional iron signaling in *Chlamydomonas*. It is likely that the signaling pathway responds primarily to iron status rather than to stress resulting from iron deficiency because the changes in gene expression seem to be highly selective for iron-deficiency (Figure 6). Zinc-deficient cells are also growth inhibited but the *FOX1*, *FTR1*, *FRE1* and *FEA* genes are not upregulated in this situation. Manganese-deficient cells do show increased expression of these genes, but this is attributed to secondary

iron-deficiency (2). The iron content of severely manganese-deficient cells is comparable to the iron content of cells grown in 1 μ M iron. The *FRE1* gene is much more responsive to manganese-deficiency than are the *FOX1*, *FTR1* and *FEA* genes, suggesting that it may respond also directly to manganese nutrition.

Copper independent iron uptake

In previous work, we had suggested that *Chlamydomonas* cells expressed a high affinity copper-independent uptake system that was expressed in copper-deficient medium (25, 47). We tested copper deficient cells for *FEA* gene expression and found the proteins unchanged in copper-deficiency and the mRNAs minimally changed (Figure 3 and 6) arguing against the function of the FEA proteins as substitutes for the FOX1/FTR1 pathway of *Chlamydomonas* (47).

We identified a number of gene models encoding candidate transporters and reductases and tested the pattern of expression with respect to transition metal nutrition to identify other components in iron assimilation. This approach did identify a putative reductase, *FRE1*, but none of the other genes showed changes in gene expression comparable to those noted for *FOX1* and *FTR1* (47). We looked also for iron responsive expression in combination with copper-deficiency in the hope of identifying the transport pathway that functioned in the copper-deficient *Chlamydomonas* cell, but again, no candidate was revealed by this approach. This does not rule out a function of these transporters in iron homeostasis because it is possible that there may be more than one nutritional iron signaling pathway and therefore changes in RNA abundance may play only a small role in the regulation of some assimilatory molecules. For instance, *FOX2* mRNA abundance is not affected by iron-deficiency in the experiments presented in this work, but the protein appears to increase in abundance when cells are maintained in iron-deficiency for prolonged periods (70).

Analysis of the *Chlamydomonas* genome indicates that some pathways, especially those related to chloroplast function, are homologous to those in plants, while other pathways are more similar to those in animals, or occasionally those in other protists (e.g. the pathways related to

mitochondrial function). The components of iron assimilation reflect this “mix and match” approach to pathway acquisition. The reductase is more similar to plant and other algal rather than animal or fungal homologues, the ferric transporter is related to the homologues in fungi, and the multicopper oxidase (although unique in terms of domain arrangement) shows better sequence relationship to the animal enzymes than the fungal ones.

Proposed mechanism of FEA1

In this work, we identify *FRE1* and the *FEA* genes as the most dramatically induced genes in iron-deficient *Chlamydomonas*. Although the ferroxidase had been noted previously to be one of the most abundant plasma membrane proteins in iron-deficient *Chlamydomonas* (43), we show here that the magnitude of regulation is much greater for *FRE1* and the *FEA* genes, and they respond most dramatically to iron deficiency than to other nutrient deficiencies. This is compatible with a site of action in an extracellular location as the first steps in a multi-step iron assimilation pathway.

How might FEA1 facilitate iron delivery given its periplasmic location in *Chlamydomonas*? In one model (Figure 9), FEA1 functions merely to concentrate iron in the periplasmic space in the vicinity of low affinity transporters, which makes them more effective. This model is analogous to the situation for inorganic carbon uptake where a secreted carbonic anhydrase provides the substrate for a specific transporter (26), and compatible with the finding that FEA1 can facilitate iron uptake in a heterologous system (74).

Many organisms secrete extracellular iron binding molecules like transferrins or siderophores when they are iron deprived. Although a transferrin like molecule has been identified in *Dunaliella spp.* (28), the *Chlamydomonas* genome does not appear to encode proteins related to the transferrins. FEA1 may be a functionally analogous equivalent (74). *S. cerevisiae* induces the cell wall mannoproteins to facilitate use of iron bound to siderophores synthesized by other organisms (66). The FEA proteins are not similar in sequence to the Fit proteins but they may have an analogous function.

A multiple alignment of the FEA proteins indicates a number of conserved residues that

could provide iron binding ligands. Among these are thiols, imidazoles, carboxylates and hydroxyl groups (Figure 4). As soft ligands, the thiols, which are important for function, would be more compatible with Fe(II) binding or of course they may be important for disulfide bond formation and protein stability in the extracellular space. In terms of a pathway for iron assimilation, it is attractive to place the FEA proteins either as Fe(II) carriers between the reductase and the ferroxidase (Figure 9) or as Fe(III) carriers that make substrate available to the reductase.

ACCEPTED

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FIGURE LEGENDS

Figure 1: **Transcripts for one of four candidate ferrireductases are increased in iron-deficient medium.** Candidate genes were analyzed by quantitative real time RT-PCR for expression during iron-deficiency growth. Strains 17D-, CC1021 and CC125 were tested on 0.2 (circles), 1 (triangles) or 20 (crosses) μM iron. Each 20 μl reaction contained cDNA equivalent to 100ng of input total mRNA. Fold induction was calculated according to the $2^{-\Delta\Delta C_T}$ method (50). Each data point is the average of technical triplicate and represents an individual experiment.

Figure 2: **Survey of candidate metal transporter genes for expression in iron-deficient medium.** Candidate genes for transporters were analyzed by real time RT-PCR for expression in strains CC125 and CC1021 grown in 0.1 (circles), 1 (triangles) or 20 (crosses) μM iron. Relative transcript abundance was calculated as described in the legend to Figure 1.

Figure 3: **FEA1 and FEA2 are secreted by iron-deficient cells.** Proteins secreted into the medium by *Chlamydomonas cw15* strain (CC400) were collected by ammonium sulfate/trichloroacetic acid precipitation and analyzed after electrophoretic separation on SDS-containing polyacrylamide (12% monomer) gels. Each lane contained extract corresponding to an equivalent number (7.3×10^7) of cells. The major proteins revealed by Coomassie blue staining were analyzed by MALDI-TOF mass spectrometry after in-gel trypsin digestion.

Figure 4: **Conserved residues in the FEA proteins.** FEA1 (BAA94959) and FEA2 (Protein ID 173281 in the version 3 draft genome) of *C. reinhardtii*, Hcr1 (BAA22844) of *Cc. littorale* and C_370140 (Fea1), C_520002 (Fea2), C_520026 (Fea3) of *V. carteri* (v 1.0 draft genome) were aligned using Multalin (<http://prodes.toulouse.inra.fr/multalin/multalin.html>, (11)). Signal peptides, indicated by lower case letters, and cleavage sites were predicted using TargetP

(<http://www.cbs.dtu.dk/services/TargetP/>, (24, 59)). All are predicted to be secreted proteins. Residues are indicated in white text on a black background if it is identical in all six sequences or on a grey background if it is identical in five of the six or by black text on a grey background if four of six are conserved. Potential metal binding ligands are indicated with a red background if they are identical or with a yellow background if the residues represent conservative replacements. Asterisks mark every 10th residue.

Figure 5: *FEA1* and *FEA2* are expressed coordinately with *FOX1* and *FTR1* genes under iron deficient conditions. A. Real-time PCR quantitation of gene expression in CC125. See legend to Figure 1. Each data point is the average of technical triplicate and represents an individual experiment. B. Blot hybridization. Wild-type (CC125) or wall-less cells (CC425) were grown in TAP medium with iron supplementation as indicated. RNA was isolated after 5 days growth corresponding to cell densities in the range of 5 to 7 x 10⁶ cell/ml. 5 µg of total RNA was loaded in each lane and analyzed by blot hybridization. The expression of *CβLP* is monitored as a loading control.

Figure 6: Metal selective response of *FEA* gene expression. Transcript abundance was assessed by real-time PCR (see legend to Figure 1). For copper and manganese deficiency, cells were adapted to deficiency by three sequential transfers (1:1,000 dilution at each transfer) into medium lacking the trace element while for zinc deficiency, cells were sampled after the second transfer into zinc-free medium. For iron-deficiency, the cells were washed once and transferred from iron replete (18 µM) to iron-deficient (1 µM) and sampled when they reached a density of 5 to 7 x 10⁶ cell / ml. The various RNAs collected from strains CC125, CC425 and CC1021 were tested for marker gene expression characteristic for each deficiency (*CYC6* for copper deficiency, *GPXI* for manganese deficiency, and *ZRT1* for zinc deficiency). For each experiment, the level of expression is normalized to the replete condition. Each data point is the average of technical triplicate and represents an individual experiment.

Figure 7: **Functional model for FEA proteins.** Top, FEA proteins (circles) associate within the periplasm in a cell wall containing *Chlamydomonas* strain. In a cell wall-less mutant, FEA proteins are lost to the medium and unable to function in iron assimilation. Bottom, Secreted and cell-associated fractions of CC125 (walled) and CC425 (wall-deficient) cells were isolated and separated by denaturing gel electrophoresis. FEA proteins were visualized by immunoblotting. Each lane corresponds to $\sim 2 \times 10^6$ cells.

Figure 8: **Loss of FEA proteins results in poor growth on low iron medium.** Spores from a tetrad of *cw15arg2mt+* x *CW15ARG2mt-* were analyzed for growth on iron limited ($0.1 \mu\text{M}$) TAP+arg medium (top) and cell-associated FEA proteins by immunoblot analysis (bottom) of washed iron-deficient cells (grown on TAP +arg with $1 \mu\text{M}$ Fe).

Figure 9: **Model for iron assimilation by *Chlamydomonas*.** The components of an iron assimilation pathway are shown. Ferric iron may be released from chelated forms by reduction to the ferrous form. The FEA proteins may bind the ferrous iron to keep it soluble and available as a substrate for the ferroxidase / ferric transporter complex. Alternatively, FEA might bind ferric ion, in which case they would function upstream of FRE1. FRE1 was found in a plasma membrane sub-proteome, which is consistent with the model proposed here (70).

Name	¹ Protein ID	² % Efficiency	Primer Pair
<i>ATX1</i>	163414	87	TCCTGGGAAAGCTGGATGGA CCACTCCTGGCAAAAGCACA
<i>CBR1</i>	163751	100	GCGCCGTGGACTTTGTCATC CTTCAGGATGGCGTTCAGCA
<i>CTP4</i>	195998	97	ACGGATGACGAACAGCAGCA CTCACACAGCCCCCTTCCACA
<i>FEA2</i>	173281	102	GCTGCCACCATCACCTTT AGCACCAGCTCGCGCATCAGGTC
<i>FOX1</i>	184156	92	GCTGGTGGCGGGGCCGACGGCAG CTCACATCGAAGTCGGCGCTCAG
<i>FOX2</i>	129541	97	GTGCAAATGGGCGAACACAA CCGGAGCCAGTAACGAACCA
<i>FRE1</i>	169091	96	CGCCAACGTAACCGAAATGG CGTGCGTGAGGATGGTCTTG
<i>FTR1</i>	137142	100	TCATCATCTACTGCGGCTTCTGGAG TAGCCTCACCCGCCTCAAGCGCCGC
<i>IRT1</i>	196755	97	CGACCCCAAGGACAAGGTCA TCACTCCACCGGCAACTTCA
<i>IRT2</i>	196635	92	GAACCTGACCACCGCCCTAA CCGAACAGGATGCCGAACAC
<i>RBOL1</i>	145439	112	GGCCAACCCAAAATGGTCAA TCGTCGTAGCCCAGCACACT
<i>RBOL2</i>	189216	87	CACAAAGGCCAACATCAGG AACACCTCTGCGTGACGTG
<i>UBQ2</i>	196845	96	GCGATTTCTCGTTGGGCAGT TGGCCCATCCACTTGTCTT
<i>ZIP2</i>	196644	104	GGGCTCTTCGCACGTCTTCA AGGGGACCAGGAAGCCAAAA
<i>ZIP4</i>	174212	97	GGGGCTACTGATGGCGGTGA GTAGATGGGTGCGGCGATGA

Table I: Primers used for real time PCR. The primer pairs for each gene model (¹corresponding to the Version 3.0 draft genome) are shown. The upper row of each set corresponds to the forward direction for the gene model and the lower row to the reverse direction. All primer sequences are written 5' to 3'. ²The percent efficiency for each primer pair is calculated based on the theoretical doubling of product at each cycle. For 100% efficient primers, the amount of product doubles at every cycle.

Band	Identity	Protein ID	Coverage	Peptides Identified
43 kDa	FEA1	129929	34%	105DTSSLDAAIR ₁₁₄ 122ATLAAGLIQVGTLK ₁₃₅ 136YHLHEVDEAYNK ₁₄₇ 210SYVNTAMINTVNEMLAAAR ₂₂₈ 229LSTLNIQAYDAAR ₂₄₁ 271RPAAEVEDAK ₂₈₀ 281TMIAVHWAYLEPMLK ₂₉₅ 301ASAVTELHHQLTASK ₃₁₅ 336SSELGAPQSAIIAANWK ₃₅₂
38 kDa	FEA2	173281	43%	84SFSGFASYATSGPELLHDSLAMGR ₁₀₇ 110TWLDVAIR ₁₁₇ 118TAFAEQNRPLVEGLILVAGVK ₁₃₈ 139YGLHEVDEGATK ₁₅₀ 231DNGTLSSAVYNASRVDLMR ₂₄₉ 250ELVLLGLQGVLHSSYK ₂₆₅ 274RPDADLEEAK ₂₈₃ 312TALTAARPNYMDVR ₃₂₅ 327AVVSVADAMGR ₃₃₇ 339MAEIGTPIHDR ₃₄₉ 353GWAGCSASR ₃₆₁

Table II. Identification of FEA proteins by mass spectrometry. Proteins were excised from an SDS gel, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry as described in the methods section. RMS error for identification was 66 ppm and 22 ppm for FEA1 and FEA2, respectively. Protein IDs correspond to the Chlamydomonas genome version 3.0. The position of the peptide in the protein model (accession AB042099 for H43 and derived from the gene model for FEA2) is indicated in subscripts. The identification was repeated on independent protein preparations.

Gene	Relative Transcript Abundance	
	Wild-type	<i>cia5</i>
<i>CA1</i>	4.1 x 10 ³	3.0
<i>FEA1</i>	-2.8 x 10 ²	-5.0
<i>FEA2</i>	-1.7	-2.0
<i>FTR1</i>	-2.5	-1.8
<i>FOX1</i>	-5.5	-1.1
<i>FOX2</i>	2.0	5.9
<i>CBR1</i>	-1.6	-2.2
<i>FRE1</i>	-2.6	1.4
<i>RBOL1</i>	2.4	1.2
<i>RBOL2</i>	-1.2	1.0

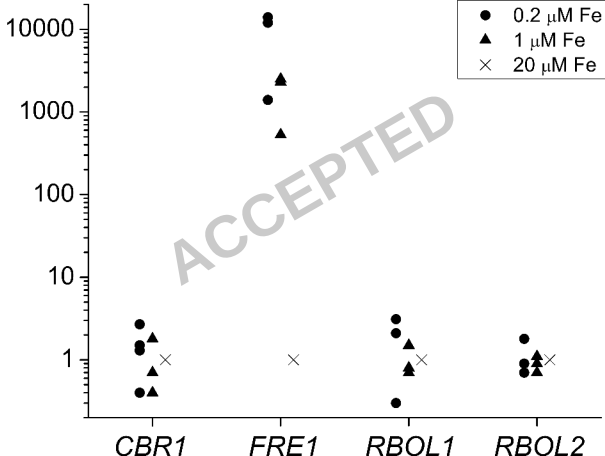
Table III. CCM1 mediated expression of iron responsive genes. Iron responsive genes were assessed by real-time PCR on low CO₂ grown wild-type and *cia5* mutants (described in 31). Expression was first normalized to *CβLP* expression and average C_T value was calculated from technical triplicates and expression was calculated relative to high CO₂ grown cells. Expression was calculated by the 2^{-ΔΔC_T} method (50). Negative numbers represent a suppression of gene expression compared to the high CO₂ grown cells.

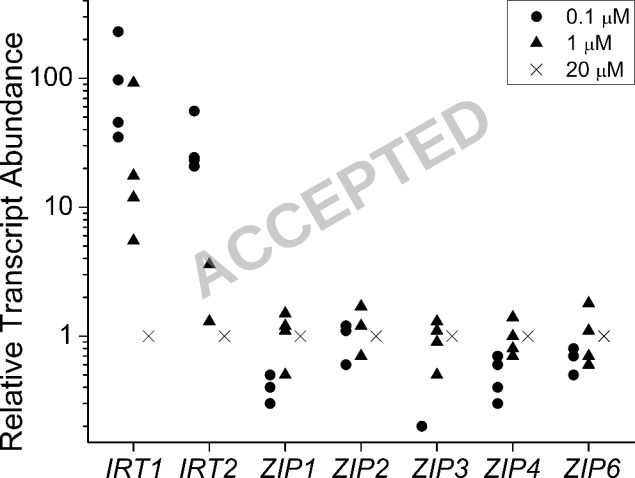
Reporter Construct	Arylsulfatase Activity	
	1 μ M Fe	200 μ M Fe
5'FOX1 #1	10 $\pm$ 1.2	3.3 $\pm$ 0.2
5'FOX1 #2	27 $\pm$ 1.5	3.0 $\pm$ 0.2
5'FTR1 #1	13 $\pm$ 1.1	3.0 $\pm$ 0.2
5'FTR1 #2	15 $\pm$ 1.6	0.3 $\pm$ 0.1
5'FEA1 #1	86 $\pm$ 1.9	0.2 $\pm$ 0.1
5'FEA1 #2	29 $\pm$ 5.0	1.6 $\pm$ 0.3
pJD100	0.1 $\pm$ 0.03	0.1 $\pm$ 0.02

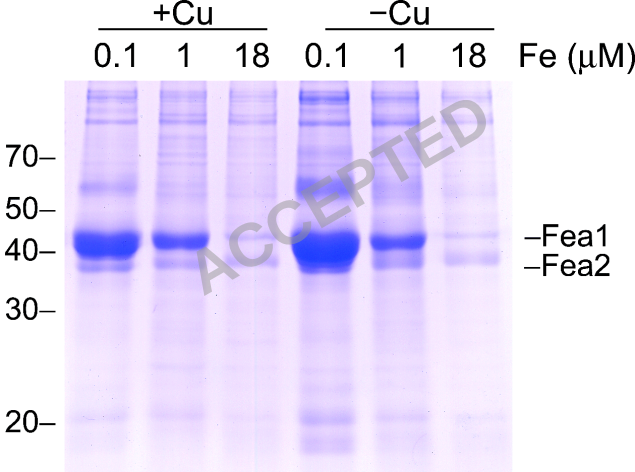
Table IV. Arylsulfatase activity in strains containing transgenic reporter genes.

Arylsulfatase activity of each strain was measured with *p*-nitrophenyl sulfate as the substrate (see Methods) and is expressed as pmol of *p*-nitrophenol / min / 10⁶ cells. Data represent average values from two independent experiments $\pm$ SD.

Relative Transcript Abundance







* 20 40 60 80
 Cr FEA1 : -----msvqgflvlalgalvvataQ-PTTLGTRFEFGSYAGNVIGYVNMMDYCDIKAAMAAAGNFTEALSITYSTGKNSFS : 73
 Cr FEA2 : -msrpsiaivlaaavallgvcalaQ-PSITGTRYKLSYSSNVGYVNMMDYCEIKAAALAGNWAEALTYLSSGKNSIS : 78
 Ccl HCR1 : mmnrkiaavsvllafvaagsvsgSVVFQDTLGLMLGPFFKSGNVAGYVNMMDYCDIQAAALYAGDTATALEYNFYFKNSER : 80
 Vc FEA1 : mfnrrmttaaitvsvallcaqQAQ-PTTLKTRFEGYTYASNVIIGYVNMMDYCDIQAAAITGNTFAALETYATGKNSYS : 79
 Vc FEA2 : mrsrvsvfvlpifaalfwttqQGO-NTTLGTRFDGYSYASNVIIGYVNMMDYCDIQAAITAGNFSAALGIYTTGKNSWN : 79
 Vc FEA3 : mrsrvsvfvlpifaalfwttqQGO-NTTLGTRFDGYSYASNVIIGYVNMMDYCDIQAAITAGNFSAALGIYTTGKNSWN : 79

* 100 120 140 160
 Cr FEA1 : GLARRTEFRFASYITANGSVEPLHDSILACSKDTSSLDAAATRAALADGKATLAAGLIQVGTLLKYHLHEVDEAYNKIKITYLA : 153
 Cr FEA2 : GLSRRSESGFASYATSGP--ELLHDSLAMCRNRTWLDVAIRTAFAEQNRPLVEGLILVAGVKYGLHEVDEGATKIVQYLA : 156
 Ccl HCR1 : GAAFRST---ALWAEANHTGQIFYDALGL-PSETYITDVFSGAAGDGDATMAMAVLDAIKIKYMFHEWDTAVSRTASGNP : 156
 Vc FEA1 : GLSRRTEYRFASFAPAAGVVEPLHDAALTACRNATWLDTLKDAISKRDGPLTLGLIQVAALKYFLHEVDEGEFNKIRTYFQ : 159
 Vc FEA2 : GLTRRTFYRFASFTFGAGVVEPLHDALAMCRNASWLDMSIKDAIAKRNGLAAGLIQVAVLYKYFFHEVDEGEFTNVATYLN : 159
 Vc FEA3 : GLTRRTFYRFASFTFGAGVVEPLHDALAMCRNASWLDMSIKDAIAKRNGLAAGLIQVAVLYKYFFHEVDEGEFTNVATYLN : 159

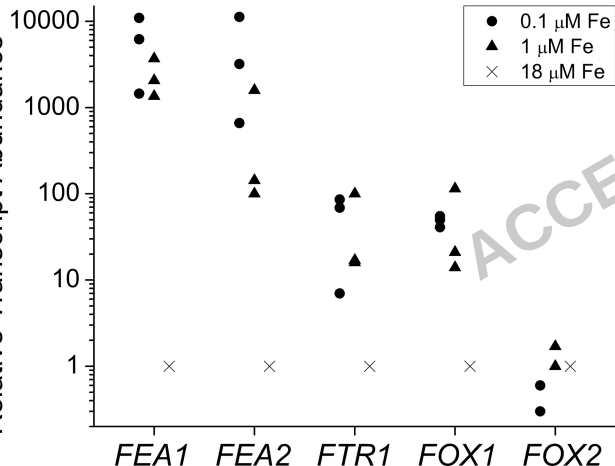
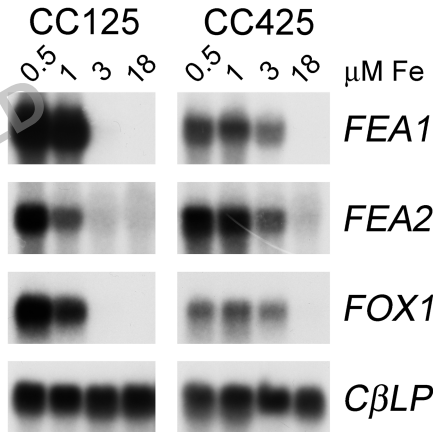
* 180 200 220 240
 Cr FEA1 : DGTGNLTNLVSDASGAPHNVDEAFALWAGGAANNCGTILSGWASSLCAAMGTEFLCKSYVNTAMINTVNM-LAAAEI--S : 230
 Cr FEA2 : D---NTLTNLVGDADGASHSVDEAFALWLTGGRDDHCGSAAAWAALGADMGTFLGCSYVNAATITFNET-LMSGKNDNG : 233
 Ccl HCR1 : D-----PSYGAPHNLDEAAAMYFAGTEGCGCGASVELHAM--NQAAMLKCMLYFGSSPSNTLMSTLMSFINAQDATL : 224
 Vc FEA1 : DPTNN-DILISDETGAPHNVDEAFALWAGGNPRACGTTISGWAARLGSDMDTELGKSFVNSAMTLALNEL-LASSET--A : 235
 Vc FEA2 : DTANN-GILISDATGAPHNVDEAFALWAGGSPSACTTILSSWAAQLGADLETTFVGRSYINSAMTLALNEL-QVAARK--G : 235
 Vc FEA3 : DTANN-GILISDATGAPHNVDEAFALWAGGSPSACTTILSSWAAQLGADLETTFVGRSYINSAMTLALNEL-QAAARK--G : 235

* 260 280 300 320
 Cr FEA1 : TLNIQAYDAARTNEVRLTLLGLQGVSVAAAYTADAFAACKRPAAEVEDAK-TMTAVHWAYLEPMILKLNFKASAVTELHH : 309
 Cr FEA2 : TLSSAVYNASRVLDLMEVLVLLGLQGVLSHYKAQAQAVACRRPDADLEAK-AYLSVHWTYLEPFLVARGVPADRITRLRT : 312
 Ccl HCR1 : TNNTAKIESAYATFLNQMVQLQRLRYTDAALLSYSCKCGNTTISSETILVSWMLTHQMLQMPQLTGMTVNHSTIETIDF : 304
 Vc FEA1 : --DLDIYNDTRAMVQRYITVMLGLQGVVRSLYRAEAFAVACKRSAQIAEAK-AMTIVHWTYLEPILFARNMRASIYQALGQ : 312
 Vc FEA2 : --NAVSYNDTRAMVQRYITLLGLQGVMSVYRAEFAVACKRPAQIAEAQ-AFTAVHLTYLEPMLLASNVPPALVQDLNR : 312
 Vc FEA3 : --NAVSYNDTRAMVQRYITLLGLQGVMSVYRAEFAVACKRPAQIAEAQ-AFTAVHLTYLEPMLLASNVRPALVQDLNR : 312

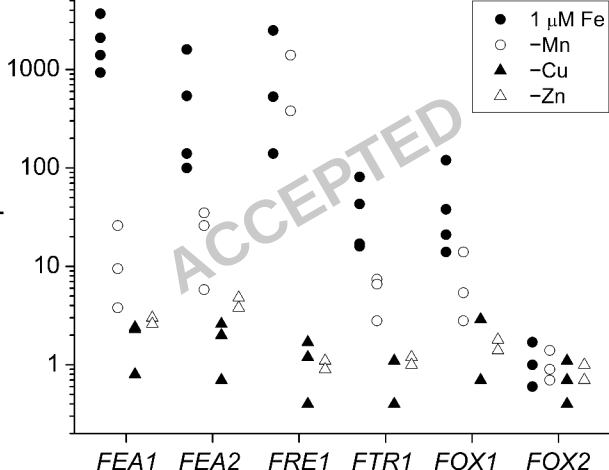
* 340 360 380
 Cr FEA1 : QLTASKLSYKK--VAAAVKGVLSAMGRRSSEL---GAFQSAIIAANW-KCSSKTLRSIA----- : 362
 Cr FEA2 : ALTAARPNYMD--VRRVAVSVADAMGRMAEI---GTETIHDRVTRGAGCSASRL----- : 363
 Ccl HCR1 : IIENTEECLMSQAIEIALKDVVMELGYEWADIFGNGTDYEIMDESMGLECGGETGWVLKGNRSN----- : 368
 Vc FEA1 : ALTNPSTPSYSR--ALPAIRTVSVMGRMSEI---GTEQFDRVITAGTTCNAAAVAPVAGHRQS----- : 371
 Vc FEA2 : ALTNPSTPSYSR--ALPAIRVVVSALGRMAEV---GEERFEVITAGTWNCKLNSPPAVGRRLLSREKRVWRAFTIS : 383
 Vc FEA3 : ALTNPSTPSYSR--ALPAIRVVVSALGRMAEV---GEERFEVITAGTWNCKLNSPPVVGRRLL----- : 370

A

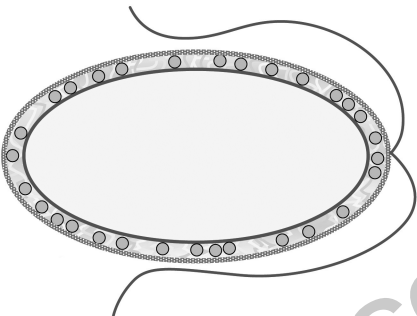
Relative Transcript Abundance

**B**

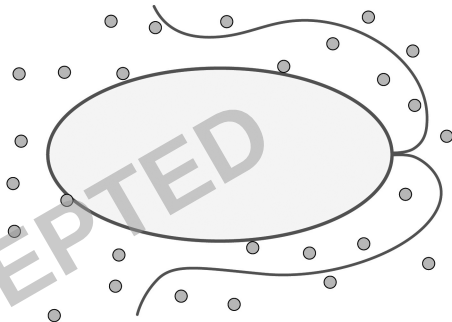
Relative Transcript Abundance



CC125



CC425

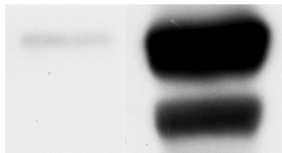
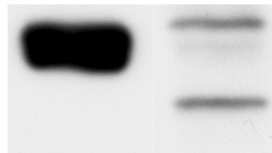


Secreted

Washed cells

Secreted

Washed cells



—FEA1

—FEA2

A

B

C

D

Growth in
 $0.1\mu\text{M Fe}$



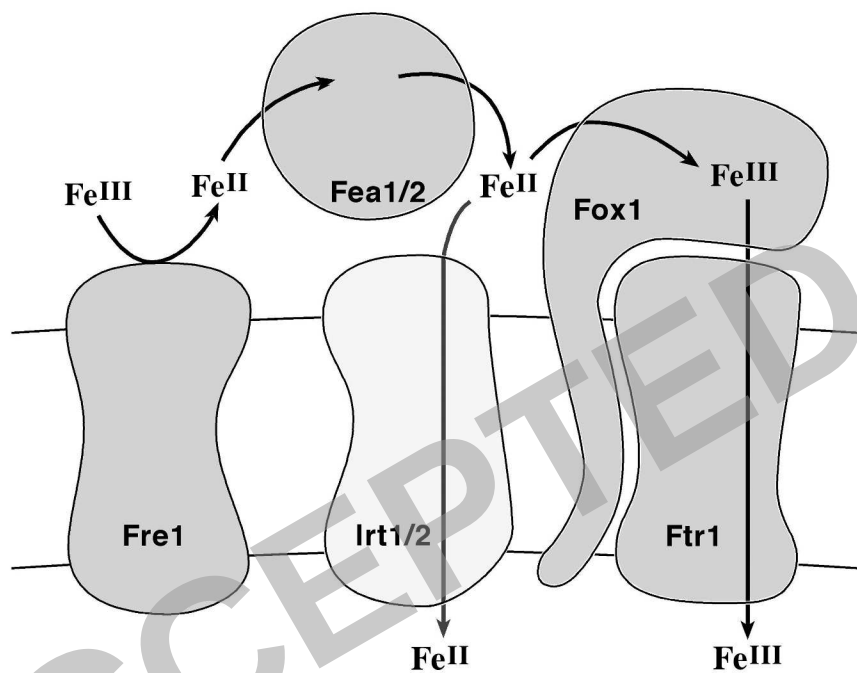
Washed Cells
Immunoblot



-FEA1

-FEA2

A



B

