***FER1* and *FER2* Encoding Two Ferritin Complexes in *Chlamydomonas reinhardtii* Chloroplasts Are Regulated by Iron**

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ABSTRACT

Two unlinked genes *FER1* and *FER2* encoding ferritin subunits were identified in the *Chlamydomonas* genome. An improved *FER2* gene model, built on the basis of manual sequencing and incorporation of unplaced reads, indicated 49% identity between the ferritin subunits. Both *FER1* and *FER2* transcripts are increased in abundance as iron nutrition is decreased but the pattern for each gene is distinct. Using subunit-specific antibodies, we monitored expression at the protein level. In response to low iron, ferritin1 subunits and the ferritin1 complex are increased in parallel to the increase in *FER1* mRNA. Nevertheless, the iron content of the ferritin1 complex is decreased. This suggests that increased expression results in increased capacity for iron binding in the chloroplast of iron-limited cells, which supports a role for ferritin1 as an iron buffer. On the other hand, ferritin2 abundance is decreased in iron-deprived cells, indicative of the operation of iron-nutrition-responsive regulation at the translational or post-translational level for *FER2*. Both ferritin subunits are plastid localized but ferritin1 is quantitatively recovered in soluble extracts of cells while ferritin2 is found in the particulate fraction. Partial purification of the ferritin1 complex indicates that the two ferritins are associated in distinct complexes and do not coassemble. The ratio of ferritin1 to ferritin2 is 70:1 in iron-replete cells, suggestive of a more dominant role of ferritin1 in iron homeostasis. The *Volvox* genome contains orthologs of each *FER* gene, indicating that the duplication of *FER* genes and potential diversification of function occurred prior to the divergence of species in the Volvocales.

ALTHOUGH iron is abundant on earth, its bioavailability is limited in the aerobic world because of the insolubility of ferric salts, and iron can be a limiting nutrient for most forms of life. A third of the agricultural land and the same fraction of the ocean are considered iron deficient (reviewed by BOYD *et al.* 2007). Therefore, iron nutrition is a key component of global productivity. Organisms have evolved multiple and varied pathways for assimilating iron in its various chemical forms and at a range of concentrations in the nutrient environment (STAIGER 2002; reviewed in CURIE and BRIAT 2003; HENTZE *et al.* 2004). Even though iron can be toxic to cells as a consequence of its propensity for participating in redox chemistry, cells do not generally excrete iron because it is a limiting nutrient (LIOCHEV and FRIDOVICH 1999). Instead, cells tend to store intracellular iron in a less reactive and hence nontoxic form. Because of the importance of iron for life, these pathways

of uptake and storage are subject to layers of homeostatic regulation.

We have developed *Chlamydomonas* as a model organism for understanding trace metal nutrition in plants, especially in the context of chloroplast function and photosynthesis (MERCHANT *et al.* 2006). As a microorganism, *Chlamydomonas* lends itself to nutritional studies because of the ease with which the aqueous growth medium can be manipulated. Furthermore, the absence of proteins or amino acid supplements that provide metal-binding ligands simplifies the provision of metal micronutrients. Previously, using genomic and proteomic approaches, we identified a number of proteins that might function in parallel pathways of iron assimilation (LA FONTAINE *et al.* 2002; ALLEN *et al.* 2007). We proposed that the *FRE1* gene encodes a ferrireductase that mobilizes iron by reduction of Fe³⁺ to Fe²⁺. Two uptake pathways appear to operate in *Chlamydomonas*: a high-affinity one involving a ferroxidase coupled to a ferric ion transporter, encoded by *FOX1* and *FTR1*, respectively, and one of likely lower affinity, involving inducible ZIP family transporters that we named IRT1 and IRT2 (ALLEN *et al.* 2007; CHEN *et al.* 2008). We also identified extracellular proteins FEA1 and FEA2 that appear to facilitate iron uptake when iron is present at low concentrations in the medium. The increased expression of *FOX1*, *FTR1*, and *FEA1* is attributed

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to transcriptional regulation (ALLEN *et al.* 2007; DENG and ERIKSSON 2007).

In most eukaryotic cells, ferritin is the major, and perhaps only, iron storage protein (THEIL 2004; KOORTS and VILJOEN 2007). Although iron can be “scavenged” from abundant iron-containing proteins (such as plant ferredoxins) in a situation of iron deficiency, this serves only as a last resort source of iron. Ferritin is a multi-meric complex, consisting of 24 ferritin subunit polypeptides that form a shell around a core that can hold up to 4.5×10^3 iron atoms as an insoluble iron-oxy-hydroxide mineral. In animals, there are two types of chains—the light (L) chain and the heavy (H) chain (THEIL 1987). The H chain carries ferroxidase active sites that oxidize ferrous to ferric iron, a prerequisite for mineralization in the core (reviewed by CHASTEEN 1998). The L chain, present only in vertebrates, does not have a ferroxidase site but is able to form a complex and bind iron at neutral but not acidic pH (LEVI *et al.* 1989). Therefore changes in the ratio of H/L subunits, *e.g.*, in response to iron nutrition or in particular cell types, can affect the iron storage capability of ferritin (reviewed by KOORTS and VILJOEN 2007). The importance of ferritin in biology is underscored by the lethal phenotype of the knockout in mouse (FERREIRA *et al.* 2000). In animal cells, ferritin is primarily a cytosolic protein, but a mitochondrial ferritin gene has also been identified (LEVI *et al.* 2001).

In vascular plants, ferritin is located in the plastids rather than in the cytosol and is the source of iron for *de novo* synthesis of the cytochromes and FeS proteins in the photosynthetic apparatus during chloroplast development (reviewed by LOBRÉAUX and BRIAT 1991; MERCHANT and DREYFUSS 1998). Accordingly, it is synthesized as an N-terminally extended precursor that is post-translationally targeted to the plastid. Although ferritin is encoded by a multigene family in plants (four *FER* genes in Arabidopsis; PETIT *et al.* 2001a), the subunits are all of one type with a ferroxidase active site and characteristics of both the H and the L chain (WADE *et al.* 1993). Plant ferritins also have a distinctive extension peptide (EP) not found in mammalian homologs. Although the function of this EP is not known, an involvement in protein stability has been proposed (VAN WUYTSWINKEL and BRIAT 1995). The iron mineral in plant ferritin also has a high P content, which affects iron release. Recently, ferritin was also found in plant mitochondria and it was suggested that this might be a consequence of dual targeting of one of the preproteins (ZANCANI *et al.* 2004).

In our previous study of iron assimilation components in *Chlamydomonas*, we described a cDNA sequence encoding preapoferritin and named the locus *FER1*. The abundance of *FER1* mRNAs increased in iron deficiency (LA FONTAINE *et al.* 2002)—which is counterintuitive for an iron storage protein—and we accordingly hypothesized a role for plastid ferritin in buffering iron

TABLE 1

List of primer sequences used in this study

Primer name	Sequence
FER1 F	GCTCATGGAGTACCAGAACC
FER1 R	CTTCTTCACAGCCTCGACCT
FER2 _{1–20}	AGTTCTGGCAGCAGAGAAG
FER2 _{295–314}	GCTCGTGAAGCAGGTAGTCC
FER2 _{374–357}	TCGGGCTGGTGTTCAGCC
FER2 _{439–420}	CCGTGGTGTATGCCGTTACA
FER2 _{613–595}	CGCTGGGTCTGGTAGTTCAT
FER2 _{657–676}	GACTCCAGGTCAGGCGAATA
FER2 _{821–802}	AGTTCTGGCAGCAGAGAAG
FER2 _{1398–1379}	TGAACCCGTGTCGCTCTTTT
C98 F	CTTGGGGTCCATGAGCTG
rFer1 F	TTGGATCCTGGCGCTCTGTGCTCG
rFer1 R	TTCTCGAGTTACGCGGCGGCACC
rFer2 F	TTGGATCCTGGGCGAGGTGCAGCCG
rFer2 R	TTCTCGAGGCCAGCCAGCGG

Primers were designed against the *Chlamydomonas* genome (version 3.0). Subscript numbers indicate the location of the primer with respect to the *FER2* cDNA. F, forward primer; R, reverse primer.

or holding it transiently as it was released from PSI by degradation (MOSELEY *et al.* 2002). The version 2.0 draft genome suggested the presence of a second *FER* gene although there was no EST evidence for its expression and the gene model was incomplete because of a gap in the sequence. By amplification and sequencing of genomic DNA and cDNAs, we have constructed a complete model of the *FER2* locus (incomplete also in version 3.0). Since *Chlamydomonas* cells contain only one plastid, the presence of a second gene opened the door to the possibility that the two gene products might be functionally distinct with respect to subcellular location or that they may coassemble in different proportions to generate biochemically distinct complexes. Monospecific antibodies raised against the products of the *FER1* and *FER2* genes, referred to as ferritin1 and ferritin2, have allowed us to characterize the proteins with respect to pattern of expression, subcellular location, and association in complexes.

MATERIALS AND METHODS

***FER2* cDNA assembly:** The *FER2* genomic model spans the ends of contigs 97 and 98 on Scaffold 2 in the current version of the *Chlamydomonas* genome (version 3.0). Standard molecular biology strategies were used to sequence the genomic DNA between contigs and assemble a gene model for *FER2* (supplemental Figure 1). The corresponding cDNA sequence has been submitted to GenBank under accession no. EU223296.

Expression of recombinant ferritins and partial purification of recombinant ferritin2: Plasmid constructs for expressing recombinant ferritin1 and ferritin2 were constructed by standard molecular biology methods using expression vector pET23d and gene-specific primer sets (Table 1). Plasmid constructs (pFer1_{1–249} and pFer2_{78–298}) were confirmed by sequencing and transformed into *Escherichia coli* BL21 (DE3) cells for expression. *E. coli* cultures transformed with pFer1_{1–249}

or pFer2_{78–298} were grown to an OD₆₀₀ of 0.8 and induced with 1 mM IPTG. After 4 hr cells were collected by centrifugation and resuspended in three-cell pellet volumes of buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA. The cells were broken by sonication (microtip, 30% intensity, 10 cycles of 30 sec) and the soluble and insoluble protein fractions separated by centrifugation (10,000 × g, 10 min). rFer1 was present in the insoluble pellet and rFer2 was in the soluble extract. To further purify rFer2 the soluble extract was heated at 70° for 10 min and cooled by incubation on ice for 10 min. After removing the resulting denatured proteins by centrifugation (10,000 × g, 10 min), rFer2 remained in the soluble fraction, confirming that the rFer2 polypeptide folds and assembles *in vitro* into a functional heat-resistant protein shell.

Growth conditions: *Chlamydomonas reinhardtii* strains CC1021 (referred to by its more common name 2137 in this article), 17D[−] (wild type in the 137c background), CC400, and CC425 were used in this study. Starter cultures were maintained in the standard Tris-acetate-phosphate (TAP) medium containing 18 μM Fe at 25° in 50 μmol m^{−2} sec^{−1} light with shaking at 175 rpm. For Fe-deficiency experiments, TAP medium was made in acid-washed glassware using trace elements without Fe, which was subsequently added to a final concentration of 0.2–200 μM from a solution of iron-EDTA (MOSELEY *et al.* 2000). To avoid carryover of iron, cells were collected by centrifugation (3000 × g, 5 min) and resuspended to a density of 10⁸ cells/ml in TAP containing 0.2 μM Fe. This cell suspension was used to inoculate medium to a final concentration of 10⁵ cells/ml. Cells were collected at midlog phase when the culture reached a density of 5 × 10⁶ cells/ml.

RNA isolation and quantitative real-time PCR: Total RNA was isolated from *Chlamydomonas* cells and quantitative real-time PCR was as described (ALLEN *et al.* 2007). Gene-specific primers are listed in Table 1.

Extraction of Chlamydomonas protein: *Chlamydomonas* proteins were extracted essentially as described previously (ALLEN *et al.* 2007). Cells were collected by centrifugation at 1000 × g for 5 min, washed in 10 mM sodium phosphate, pH 7.0, resuspended in the same buffer to a concentration equivalent to 4 × 10⁸ cells/ml, and stored at −80°. For denaturing gel electrophoresis, cells were lysed by freeze/thaw cycling as described (HOWE and MERCHANT 1992). For nondenaturing gels, cells were broken by sonication (microtip, 30% intensity, two cycles of 30 sec). Extracts were separated into soluble and insoluble protein fractions by centrifugation (10,000 × g, 10 min). The pellet was washed once and resuspended to the same volume as that of the soluble fraction. Protein concentration was determined with the BCA assay kit (Pierce, Rockford, IL) or the Lowry method against a bovine serum albumin standard.

Isolation of chloroplasts and mitochondria: Chloroplasts were isolated from a 4-liter culture of strain CC400. Cells were dark adapted for 2–3 hr prior to harvesting by centrifugation (4000 × g, 10 min). Cells were resuspended in 50 ml buffer A (0.3 M sorbitol, 50 mM Hepes-KOH, 2 mM EDTA, 5 mM MgCl₂, pH 7.8) supplemented with 0.1% BSA and broken in a Yeda press (4.5 bar, 30 sec). Crude lysate was centrifuged (1000 × g, 5 min). The pellet fraction was washed twice in buffer A, loaded onto a 45/75% Percoll step gradient in buffer A, and centrifuged (9300 × g, 20 min). Intact chloroplasts were collected at the 45/75% interface and were washed and pelleted twice in buffer A (1000 × g, 5 min). Isolated chloroplasts were lysed in buffer B (10 mM Tricine/NaOH, 2 mM MgCl₂, pH 7.8) and broken by two freeze/thaw cycles at −80°. Lysed chloroplasts were loaded onto a discontinuous sucrose gradient (0.4/1.0 M in buffer B) and centrifuged for 1 hr at 80,000 × g. The intact chloroplast fraction floating on top of the 0.4-M sucrose phase was collected. Mitochondria were isolated as

described (ERIKSSON *et al.* 1995) except that the cells were lysed in a Yeda press (4.5 bar, 30 sec).

Antibody production: Antibodies that were specific to ferritin1 *vs.* ferritin2 were supplied by Agrisera (Umeå, Sweden). The synthetic peptides used as immunogen (Figure 1) were designed by Environmental Proteomics (Sackville, NB, Canada).

Immunoblotting: Proteins were separated on SDS-containing polyacrylamide gels (15% monomer) or nondenaturing gels (6% monomer) and transferred (mini-Trans-Blot cell; Bio-Rad, Hercules, CA) onto PVDF (0.45 μm; Millipore, Bedford, MA) for 90 min under constant voltage (150 V) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol]. For immunoblot analysis, the rapid immunodetection method was used (MANSFIELD 1995). Primary antisera were diluted in antibody dilution buffer: 1% BSA in phosphate-buffered saline (10 mM Na-phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.2) with 0.05% Tween-20. In competition assays (Figure 3B), antisera were preincubated at room temperature for 1 hr at 1:500 in the same buffer containing 0.05 ng/μl of recombinant ferritin and diluted to 1:5000 before use. After a 1-hr incubation with primary antibody, the membranes were washed (phosphate-buffered saline with 0.1% Tween-20) until the membrane was completely wet (typically two to three washes of 15 min each), incubated for 30 min in a 1:5000 dilution of goat anti-rabbit horseradish peroxidase (Pierce Biotechnology) in the dilution buffer, and washed twice with that solution followed by a final wash in phosphate-buffered saline without Tween-20. Bound antibody was detected with the Supersignal West Pico or Supersignal West Femto (ferritin2 only) chemiluminescent substrate (Pierce Biotechnology).

For chloroplast keto-acid reductase isomerase (KARI) and COX2b immunoblot analysis, proteins were separated on an SDS-containing polyacrylamide gel (10% monomer for KARI or 15% monomer for COX2b) and transferred in a semidry blotter onto PVDF (0.45 μm, Millipore) for 1.5 hr under constant current (400 mA) in 25 mM Tris, 192 mM glycine, 0.01% SDS, 20% methanol. Membranes were blocked with 5% dry milk in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 0.05% (w/v) Tween-20. Primary antibodies were used at 1:10,000 (KARI) or 1:25,000 (COX2b) and a 1:5000 dilution of goat anti-rabbit horseradish peroxidase was used as the secondary antibody. Signals were detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

Iron staining: A total of 20 μg of soluble extracts from cells grown in TAP medium containing 0.2–200 μM iron-EDTA were separated on a nondenaturing polyacrylamide gel. Horse spleen ferritin (Sigma, St. Louis) was used as a control. Iron staining was essentially as described in LEONG *et al.* (1992).

Immunopurification of ferritin1: An affinity column was prepared as follows: 200 μl of polyclonal anti-ferritin1 (anti-FER1) serum (Agrisera) was coupled to 0.5 g of lyophilized CNBr-activated Sepharose 4B (GE Healthcare) in a total volume of 5 ml according to the manufacturer's instructions. Uncoupled antibody was washed out and the remaining active groups were blocked by incubation with 0.1 M Tris-HCl, pH 8, for 4 hr at room temperature. The material was packed in a column and washed with 10 column volumes binding buffer (50 mM sodium phosphate, 0.15 M NaCl, 5 mM EDTA, 1% Triton-X-100, pH 7.5), and nonspecific binding sites were blocked by washing with 0.2% BSA in binding buffer. Soluble cell extracts from *Chlamydomonas* strain 2137 were prepared as described above. One milliliter of the cell extract was diluted in 10 ml binding buffer and incubated for 2 hr with the column material by repeatedly loading on the column. The column was washed with 20 ml of binding buffer, and then the buffer was exchanged to 10 mM sodium phosphate, pH 6.8, and the bound protein was eluted with 100 mM glycine-Cl, pH 2.8. The eluate was collected in 0.5-ml fractions and immediately

neutralized using Na_2CO_3 solution. Fractions 2–5 contained the major amount of purified ferritin1 and were pooled and concentrated to ~1 ml. Thirty-microliter aliquots of the crude cell extract, the flow through from the binding step, the last wash step, and the pooled ferritin1 fractions were used for analysis.

Sequence analysis of ferritin polypeptides: The affinity-purified ferritin1 complex sample was analyzed by separation on SDS-containing gels. For mass spectrometry, the proteins were visualized by Coomassie staining. Two major bands in the region of 25 kDa were cut out separately, subjected to in-gel tryptic digest as described previously (NAUMANN *et al.* 2005), and analyzed by nanoLC-MS/MS as described (RAMACHANDRAN *et al.* 2006). The resulting data were searched against a Chlamydomonas protein database consisting of the version 3.1 protein models (<http://genome.jgi-psf.org/Chlre3/Chlre3.download.ftp.html>), using the Mascot database search engine (Matrix Science, London). In all searches, one missed tryptic cleavage was tolerated, and a mass tolerance of 0.3 Da was set for the precursor and product ions. In a more focused analysis, the mass spectrometry data were searched against a database consisting of only the two Chlamydomonas ferritins. In this case, the search did not specify a protease so that nontryptic peptides could be found. The results were examined manually to identify N-terminal nontryptic peptides only. For Edman sequencing, the proteins were transferred to a PVDF membrane and visualized by colloidal Coomassie staining. The doublet in the region of 25 kDa was cut out and subjected to Edman sequencing for five cycles by Jack Presley at the University of California (Davis, CA) Molecular Structure Facility (ABI 494-HT Procise; Applied Biosystems, Foster City, CA).

RESULTS

Two *FER* genes in Chlamydomonas: Previous work identified a ferritin, encoded by *FER1*, that was upregulated by Fe deficiency. In the version 2.0 draft, and subsequently in version 3.1, a second gene, named *FER2*, was identified on the basis of homology. The occurrence of multiple genes in Chlamydomonas is consistent with the situation in vascular plants as well as other organisms where ferritin is also encoded as multigene families. The gene model in version 2.0 was not supported by EST data and a considerable sequence gap prevented further analysis. The gap in the model was therefore closed by manual sequencing of cDNA and genomic DNA PCR products and assembly of sequence data from unplaced reads (see supporting supplemental online material). An EST was mapped to the *FER2* gene model in the version 3.1 genome sequence and the corresponding cDNA was also sequenced. The version 4.0 finished genome sequence confirms the one submitted under accession no. EU223296.

A multiple-sequence alignment of Chlamydomonas *FER* gene products with those of other algae suggests that both ferritins have N-terminal extensions and are therefore likely to be plastid localized (see below). The ferroxidase active site is highly conserved in all algal ferritins (Figure 1A), suggesting that they do indeed function as subunits of an iron-binding complex (see below). Sequence analysis of a partially purified ferritin1 complex (see below) by mass spectrometry and Edman

degradation provided excellent support for the derived sequence (see supplemental material). Analysis of the draft Volvox genome revealed the presence of two genes, annotated as *FEN1* and *FER2* (March 2008), orthologous to the Chlamydomonas *FER1* and *FER2*, respectively (Figure 1B). The *FER2* sequence has a small C-terminal extension that we refer to as a Gly-rich sequence, but since this is not conserved in Volvox, it may not be functionally significant.

Unique but overlapping pattern of expression of *FER* mRNAs: When we analyzed the pattern of expression of *FER* mRNAs as a function of iron nutrition, we noted that both *FER1* and *FER2* mRNAs were increased in abundance in iron-limited (0.2 μM) *vs.* iron-replete (20 μM) cells (Figure 2). Nevertheless, while *FER1* mRNA abundance increases in proportion to the degree of iron depletion, *FER2* mRNA is increased only when medium iron content drops below a threshold value. Since individual “wild-type” strains of Chlamydomonas respond slightly differently to external iron supply (see ALLEN *et al.* 2007, for example) we tested multiple strains and noted the same general pattern. Specifically, in iron-replete or iron-excess situations, *FER2* mRNA was in low abundance and increased dramatically only when the iron-deficiency status was severe enough to result in chlorosis (Figure 2). There is also some experiment-to-experiment variation and therefore the results from three separate experiments are shown. Since the ferritin subunits associate to assemble into complexes, this result allows the possibility of distinct ferritin complexes in cells experiencing different degrees of iron nutrition, assuming that both proteins are in the same compartment. By comparing cycle threshold values for *FER* mRNAs, we noted that *FER1* constitutes a larger fraction ($\sim 10^2$ - to 10^3 -fold relative to *FER2*) of the ferritin-encoding mRNAs in all strains tested (data not shown) and this is validated by Solexa tag sequencing of cDNAs derived from iron-replete cultures (M. CASTRUITA, D. CASERO, J. KROPAT, S. KARPOWICZ, M. PELLEGRINI and S. S. MERCHANT, unpublished results).

Accumulation of ferritin subunits: To distinguish between the gene products of *FER1* and *FER2*, antibodies were generated by Agrisera against antigenic peptides designed by Environmental Proteomics to correspond to unique regions of the predicted protein sequences (Figure 1). The antiserum preparations were tested against recombinant *FER1* and *FER2* produced in *E. coli* (see MATERIALS AND METHODS). Immunoblot analyses confirmed high selectivity of each antiserum to its respective antigen (Figure 3A).

When extracts of Chlamydomonas were tested for the presence of ferritin1 and ferritin2, we noted that each antibody recognized a distinct polypeptide. Anti-*FER1* recognized a protein with a mobility corresponding to 25 kDa while anti-*FER2* recognized a protein that migrated at ~60 kDa (Figure 3B). It is not unusual for ferritin and other iron storage proteins such as Dps to migrate as multimeric species during gel electrophoresis

FIGURE 1.—Comparison of algal ferritins. (A) Chlamydomonas FER1 (accession no. AAM27205) and FER2 (accession no. EU223296), Volvox FER2 (protein ID 58531) and FEN1 (protein ID 81290), *Ostreococcus lucimarinus* PFE1 (protein ID 27552) and PFE2 (protein ID 29953), and *O. tauri* FER1 (protein ID 5942) were aligned with Multalin (<http://prodes.toulouse.inra.fr/multalin/multalin.html>). Lowercase sequence indicates transit peptides for plastid targeting for Chlamydomonas FER1 based on the N terminus determined in this study. The sequences of peptides used to generate antibodies specific to Chlamydomonas FER1 and FER2 (anti-FER1 and anti-FER2, respectively) are highlighted in yellow. Peptides used to generate antibodies that recognize both ferritins (anti-FER_{core}, experimentally verified) are underlined and double underlined (Busch *et al.* 2008). Residues corresponding to the ferroxidase center are shown in red. (B) A phylogenetic tree was generated with MAFFT (<http://align.bmr.kyushu-u.ac.jp/mafft/online/server/phylogeny.html>).

FIGURE 2.—Transcripts encoding both ferritin subunits are increased in iron-limited cells. The abundance of *FER1* and *FER2* mRNAs was analyzed by quantitative real-time PCR. Strains 17D⁻ and 2137 were cultured in TAP medium supplemented with the indicated amounts of iron. cDNA corresponding to RNA isolated from *Chlamydomonas* strains (A) 2137 (top) or (B) 17D⁻ (bottom) was used as a template with primers specific to *FER1* or *FER2*. The fold change in abundance of *FER1* (left) or *FER2* (right) after normalization to *CBLP* and relative to the abundance in a 20- μM grown culture was calculated according to the $2^{-\Delta\Delta\text{CT}}$ method (LIVAK and SCHMITTGEN 2001). Each data point is the average of technical triplicates and represents a separate experiment.

even under denaturing conditions (CLEGG *et al.* 1980; GUPTA and CHATTERJI 2003). Because of the unexpected size of ferritin2, the identity of the signal was confirmed by competition with rFER1 or rFER2. Only rFER2, but not rFER1 competed with the 60-kDa species, suggesting that the 60-kDa species corresponds to an aggregate of ferritin 2 subunits, possibly a trimer. The ratio of monomer to trimer is modified by heating the samples prior to electrophoresis but only rarely is the conversion to monomer complete (data not shown). Quantitation of ferritin subunit abundance indicated a ratio of ferritin1: ferritin2 of $\sim 70:1$ (data not shown).

The availability of specific antibodies allowed us to test the localization of each protein by analyzing purified chloroplasts and mitochondria *vs.* total cell extract for ferritin content. Chloroplasts and mitochondria were purified from *Chlamydomonas* and analyzed for ferritin content. Both ferritins were detected exclusively in the chloroplast fraction (Figure 4).

Fractionation of cell extracts after sonication showed that ferritin1 is predominantly a soluble protein while ferritin2 is predominantly found in the particulate fraction, where it could be associated with membranes and this difference in localization is independent of iron nutrition (Figure 5A). The abundance of ferritin1 follows the pattern of *FER1* mRNA; *i.e.*, it increases with progressively increasing iron deficiency. However, the abundance of ferritin2 decreases as the iron content of the medium (and cells) is decreased, which is more typical of ferritin behavior in animal cells.

Fe content of the ferritin complex: When we analyzed *Chlamydomonas* extracts by native gel electrophoresis, we noted that both ferritin subunits were associated in high-molecular-weight complexes as is typical for ferritin (Figure 5B). Furthermore, the pattern of accumulation of the ferritin complexes followed the pattern of individual subunit accumulation; complexes containing ferritin1 increase in response to iron deficiency while those containing ferritin2 decrease. However, when we assessed the iron content of the complex using an iron stain procedure applied to the nondenaturing gels, we found that the iron content of the ferritin complex was decreased in iron-deficient and iron-limited cells (Figure 5C). Interestingly, we did not see an increase either in the abundance of ferritin subunits or in the iron content of the complex in cells grown in excess iron (compare 200 μM to 20 μM). Yet, we do know that cells grown in 200 μM iron do overaccumulate the metal (two to five times the amount found in iron-replete cultures grown at 20 μM iron). Taken together, this suggests that *Chlamydomonas* ferritin likely does not have a predominant function in iron storage.

When we used an antibody that recognized a core sequence of ferritin (Figure 1A), we invariably noted a protein of higher mobility in extracts from iron-limited cells (Figure 6). This higher-mobility form was not recognized by either of the subunit-specific antibodies (Figure 3), suggesting that this form results from a modification of ferritin that involves the antigen site for anti-FER1 and anti-FER2. Peptide sequencing and

FIGURE 3.—Specificity of anti-ferritin1 and anti-ferritin2. (A) Recombinant protein. Extracts from *E. coli* cells expressing recombinant ferritin 1, rFER1₁₋₂₄₉ (corresponding to residues 1–249 of the preprotein), or recombinant ferritin 2, rFER2₇₈₋₂₉₈ (corresponding to residues 78–298 of the preprotein), were separated by denaturing polyacrylamide gel electrophoresis (15% monomer). The lane on the left, containing 3 μg of extract, was stained with Coomassie Blue. The specificity of the antibodies was analyzed by immunoblotting after transfer to PVDF membranes. (B) Endogenous protein. Total protein extracts from *Chlamydomonas* strain CC400, corresponding to 35 μg protein were analyzed by immunoblotting as in A. Ferritin antibodies were incubated for 60 min with 0.01 μg of *E. coli* extract from cells expressing recombinant ferritin 1 (+rFER1), ferritin 2 (+rFER2), or no protein (none) prior to immunoblotting (see MATERIALS AND METHODS).

Edman degradation (see below and supplemental online material) indicate that the faster-migrating species represents an N-terminal cleavage product.

Two separate ferritin1 and ferritin2 complexes: In animals, various isoforms of ferritin consisting of different proportions of L and H chains are found (KOORTS and VILJOEN 2007). These molecules have different biochemical properties with respect to the kinetics of iron mineralization and release. Although plants have only one type of chain, sequence variants occur because the subunit is encoded by a multigene family. A recent study showed that recombinant soybean ferritin subunits can associate to form complexes consisting of a single subunit type or combinations of different subunits (MASUDA *et al.* 2007). Therefore, given that both *Chlamydomonas* ferritin polypeptides are localized to the chloroplast, we were prompted to test whether they might be found in the same or different complexes.

Two approaches were used. First, we tested fractions of *Chlamydomonas* cell extracts separated by anion exchange chromatography for the presence of ferritin1 and ferritin2. If the two proteins coassemble in a heteropolymer, we would expect them to cofractionate. However, the proteins eluted as different peaks (data not shown). In a second approach, we immunopurified ferritin1 on an anti-ferritin1 affinity column (Figure 7). Although both ferritin1 and -2 are present in the starting material (load lane), when we analyzed the complex bound to the column, we did not find any ferritin2 (eluate lane). Although these results do not rule out the possibility that a small proportion of ferritin1 and ferritin2 might coassemble, they do indicate that the proteins are assembled largely into separate complexes.

N-terminal degradation of ferritin1: The affinity-purified ferritin that eluted from the column was separated by denaturing gel electrophoresis to reveal two polypep-

tides ~25 and 23 kDa (supplemental Figure 2). Edman sequencing of the mixture (supplemental Table 1) indicated two different N-terminal sequences, one at position ⁴²GIVV and the other either at position ⁵⁹ATVDK or at position ³⁵ATVDK of preapoferritin1, respectively. On the basis of the size of the 23-kDa band and the fact that it did react with anti-FER_{core} but not with the subunit-specific anti-FER1 (see supplemental Figure 2), we suggest that the N terminus of ferritin1 corresponds to ⁵⁹ATVDK. The two forms were also analyzed by mass spectrometry after trypsin digestion (see supplemental Table 2). The identified peptides corresponded to ferritin1 and support the two proposed N termini (supplemental Table 3). Since peptides corresponding to the C terminus could be detected in both bands, we conclude that the mobility difference corresponds to N-terminal degradation that is stimulated in the iron-deficient ferritin complex. This is consistent with the structure of the subunit within the crystallized bullfrog ferritin complex (pdb no. 1MFR; HA *et al.* 1999): the N terminus is exposed on the surface of the complex and thus accessible to proteases, while the C terminus is buried inside the complex.

DISCUSSION

Two ferritins in *Chlamydomonas* are differently regulated and may have different functions: In this work, we have shown that both *FER* genes in *Chlamydomonas* are expressed (Figure 2). The corresponding proteins are both localized to the chloroplast (Figure 4) but it is likely that they assemble into individual complexes (Figure 7) and that they function in different subcompartments of the plastid (Figures 4 and 5A). The major difference between the two ferritins is a predicted longer extension peptide in ferritin2 and a C-terminal

FIGURE 4.—Ferritin1 and ferritin2 are located in the chloroplast. Proteins (20 μ g) from total cellular protein extracts of *Chlamydomonas* strain CC425 (T), isolated mitochondria (M), and chloroplasts (C) were separated on a denaturing polyacrylamide gel containing SDS and transferred to PVDF membranes for immunoblot analysis with anti-FER1, anti-FER2, anti-KARI (marker for chloroplast), and COX2b (marker for mitochondria).

gly-rich sequence. One or both of these domains might contribute to the membrane association of ferritin2 (Figure 5A) and to the stability of oligomers (dimers/trimers) of ferritin2 under denaturing conditions (Figure 3B). If the two proteins are compartmentalized within the chloroplast, this might explain why they do not coassemble in a single complex (Figure 7).

For ferritin1, the pattern of protein accumulation parallels the pattern of RNA increase while for ferritin2, the dramatic increase in RNA abundance is not recapitulated at the level of protein accumulation (Figures 2 and 5, A and B). This suggests that mechanisms that control translation or ferritin2 half-life may be in operation. For instance, the apoform of ferritin2 might be highly protease susceptible. In the case of ferritin1, we did note that N-terminal cleavage was significantly more pronounced in iron-limited cells (Figure 6) where the protein occurs in the apoform (Figure 5C) relative to iron-replete cells that contain iron in the ferritin complex. This may result from a structural difference between the apo- and the holoform of ferritin and hence increased protease susceptibility of the N terminus because of its accessibility in the ferritin shell or perhaps simply from increased expression of proteases in iron-deficient chloroplasts as part of an iron-scavenging program. The question of regulation of ferritin abundance in chloroplasts by mechanisms that act at the translational or post-translational level has not been addressed yet in plants.

Because of its abundance, ferritin1 is likely to be the dominant player in chloroplast iron homeostasis, but

given the occurrence of a ferritin2 ortholog in *Volvox*, it may well have a more specific function. One possibility is that ferritin2 may provide iron for membrane protein biogenesis. The different physical properties of the two proteins and the different pattern of expression as a function of iron nutrition (Figures 2 and 5) argue in favor of a distinct function for ferritin2. In other work, we noted that *FER1* and *FER2* also have a different pattern of expression in response to high light stress (J. C. LONG and S. S. MERCHANT, unpublished results). There may well be specific modifications of chloroplast iron homeostasis as part of the high light acclimation response.

The distinct pattern of expression of the homologs, the difference in their physical properties, and the relative abundance of each isoform underscore the importance of functional analysis of each member of a multigene family. The *FEA* genes in *Chlamydomonas*, also involved in iron homeostasis, also show distinct patterns of expression (ALLEN *et al.* 2007). Interestingly, like the *FER* genes, the *FEA* genes also respond differently to high light stress (J. C. LONG and S. S. MERCHANT, unpublished results), and they also respond differently to CO₂ (ALLEN *et al.* 2007). A relationship between carbon and iron metabolism is evident from measurements of iron content in *Chlamydomonas* cells grown under various types of carbon nutrition but the molecular basis of the relationship has not been determined (SEMIN *et al.* 2003).

Function of ferritin1 in the iron-deficiency response:

Surprisingly, and in contrast to the situation in animal cells, *FER* gene expression is increased in response to iron deficiency rather than iron excess. There is no significant difference in the abundance of *FER* RNA and *FER* protein in cells acclimated to 200 μ M vs. 20 μ M iron nor in the iron content of ferritin1 (Figures 2 and 5). Measurement of iron content of cells nevertheless indicates that these cells do have increased iron content, up to two to five times the iron maintained in an iron-replete culture grown with 20 μ M Fe-EDTA (J. C. LONG and S. S. MERCHANT, unpublished results). This raises the question of whether there is another, ferritin-independent, mechanism for sequestering iron in *Chlamydomonas*. In *Arabidopsis*, a homolog of *Saccharomyces cerevisiae* Ccc1p called VIT1 functions to transport iron to the vacuole (KIM *et al.* 2006). While a protein with some similarity to a *Schizosaccharomyces pombe* Ccc1 is found in the *Chlamydomonas* genome, whether it is a VIT1 homolog is not evident.

In cells acclimated to iron-poor growth conditions, the iron content of the ferritin complex is greatly reduced even though the abundance of the complex is increased (Figure 5, B and C). This is an important distinction. We conclude that the increase in ferritin1 in iron deficiency increases the capacity for iron binding in the chloroplast. This is consistent with a role for ferritin1 as a potential buffer for iron that is released

FIGURE 5.—Differential accumulation of ferritin1 as a function of iron nutrition. (A) *Chlamydomonas* strain 17D[−] was grown in TAP medium containing the indicated iron concentration to midlog cell density. Soluble (S) and insoluble (P) fractions were prepared from the cells (see MATERIALS AND METHODS). Five micrograms of protein from each growth condition were separated on a denaturing SDS-PAGE gel (15% monomer) and transferred to PVDF for immunoblot analysis with anti-FER1 and anti-FER2. Five micrograms of soluble protein (17D[−]) from each growth condition were separated on a nondenaturing gel (6% monomer) and analyzed with subunit-specific anti-FER1 and anti-FER2 (B) or stained for iron content with ferricyanide (C). Horse spleen ferritin (5 μg) was used as a marker (M).

from degradation of abundant iron-sulfur proteins in the chloroplast, like ferredoxins and photosystem I (MOSELEY *et al.* 2002). This iron may be then redistributed to other chloroplast iron proteins or to other organelles like the mitochondrion (NAUMANN *et al.* 2007). Interestingly, the iron content of ferritin1 is already decreased in cells grown in medium containing 3 μM iron-EDTA and is greatly reduced when the iron content of the medium is decreased to 1 μM, suggesting that it is mobilized, perhaps for use in maintenance of plastid iron proteins. This change is exactly paralleled by an increase in the expression of assimilatory components and precedes the appearance of chlorosis and the degradation of the photosynthetic apparatus, which confirms our description of cells grown in 1–3 μM iron-EDTA as being marginally iron deficient (MOSELEY *et al.* 2002; ALLEN *et al.* 2007).

In previous work, we compared the ferritin content of cells grown in 0.1 μM iron-EDTA *vs.* 1 μM iron-EDTA and concluded that the abundance of the protein did not change as a function of iron nutrition (LA FONTAINE *et al.* 2002). However, the fact that cells grown in 1 μM iron-EDTA were already iron deficient was not appreciated at that time, and indeed the abundance of ferritin1 in cells grown in 1 μM *vs.* 0.2 μM iron-EDTA is not significantly different (Figure 5).

In vascular plants, the iron-responsive element/iron-regulatory protein system is not present (ARNAUD *et al.* 2007). Instead, mechanisms that act at the transcriptional level regulate *FER* gene expression in the iron overload situation (WEI and THEIL 2000; PETIT *et al.* 2001b; TARANTINO *et al.* 2003). While a role in iron deficiency has not been described, it is possible that this might be a cell- or organ-specific phenomenon that is restricted to chloroplast-containing cells that are rich in iron proteins.

FIGURE 6.—Modification of ferritin1 in iron-limited cells. Protein (5 μg) from iron-replete (18 μM) *vs.* iron-limited (0.5 μM) cells was separated on a denaturing gel and analyzed for the abundance of ferritin by immunoblotting with anti-FER_{core}, which recognizes the internal segment of both ferritins on the basis of reactivity with the recombinant proteins (see Figure 1 legend).

FIGURE 7.—Ferritin2 is not associated with ferritin1. The ferritin1 complex was purified on an affinity column. Equivalent volumes of material from various steps in the immunopurification procedure were analyzed for the presence of ferritin1 (top) or ferritin2 (bottom) by immunodetection after separation on SDS-containing gels. Lane 1, total soluble cell extract; lane 2, flow through; lane 3, final wash; lane 4, eluate.

The upregulation of *FER* genes in senescent plants may be related to the iron-deficiency response described in this study (BUCHANAN-WOLLASTON and AINSWORTH 1997; HÖRTENSTEINER *et al.* 2000; MURGIA *et al.* 2007): specifically, that ferritin accumulation is increased to handle the iron that is released from degradation of the photosynthetic apparatus (HIMELBLAU and AMASINO 2000). Individual *FER* genes respond to various developmental cues, growth factors, pathogen attack, H₂O₂, and various stress situations that generate reactive oxygen species (LOBRÉAUX and BRIAT 1991; LOBREAUX *et al.* 1993; KIMATA and THEIL 1994; FOBIS-LOISY *et al.* 1995; SAVINO *et al.* 1997; PETIT *et al.* 2001a; DELLAGI *et al.* 2005; BOUGHAMMOURA *et al.* 2007). These studies suggest that cell-specific analyses of the abundance of RNA and protein for individual ferritin isoforms and the iron content of the ferritin complex may reveal the operation of mechanisms that act to buffer iron release from the photosynthetic apparatus as seems to be the situation in *Chlamydomonas*, degreening *Chlorella*, and senescent *Brassica*. *Arabidopsis* plants carrying a knockout in the *AtFER1* gene showed symptoms of accelerated aging in the light that was

attributed to an inability to handle iron toxicity in the presence of light-dependent reactive oxygen species, which emphasizes the importance of ferritin as an iron buffer during plastid development (MURGIA *et al.* 2007).

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