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An Fe Deficiency Responsive Element with a Core Sequence of TGGCA Regulates the Expression of *FEA1* in *Chlamydomonas reinharditii*

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Iron is essential to the unicellular green alga *Chlamydomonas*, but the molecular mechanism for response to iron deficiency remains largely unknown. In previous studies, we have identified FOX1 and ATX1 FEREs (Fe deficiency-responsive elements) as important regulation components of iron response in this organism. Here we present another iron regulated gene *FEA1*, which promoter was analysed by using a 5′-and 3′-end deletion and a scanning mutagenesis assay. The results reveal that the co-existence of −273/−188 and −118/−49 regions from transcriptional start site of *FEA1* were sufficient and necessary for Fe deficiency-induced expression. Further deletion analysis indicates both −273/−253 and −103/−85 regions are essential for inducible expression. The scanning mutagenesis analysis of these regions identifies two *cis*-acting elements: the FeaFeRE1 at −273/−259 (CTGCGGTGGCAAA GT) and FeaFeRE2 at −106/−85 (CCGCCGCNNNTGGCACCAGCCT). Sequence comparison of FeaFeRE1 and FeaFeRE2 reveals a core sequence of TGGCA, which had been found in our previously reported Fe-deficiency-inducible gene *ATX1*. Moreover, we show that the promoter region of several genes, including *FRE1*, *IRT1*, *ISCA*, *ZRT1*, *ZRT5*, *NRAMP2* and *COPT1*, also contains this core sequence, suggesting that at least two classes FeRE elements exist in *Clamydomonas*, one in FEA1 and ATX1 and others the second in *FOX1, FEA2, MTP4, NRAMP3* and *RBOL1*.

Key words: *Chlamydomonas reinharditii*, FEA1, FeREs, iron, promoter.

Abbreviations: FEA1, *Fe* assimilation 1; ATX1, antioxidant 1; FOX1, ferroxidase1; FER1, ferritin 1; Ars, arylsulfatase; FeREs, Fe deficiency-responsive elements.

Iron is an essential nutrient for virtually every organ on the earth, because iron participates as a cofactor in numerous essential enzymatic reactions involving transfer of electron. Symptoms of acquired or inherited iron deficiency have been reported in most organisms, but iron uptake and the regulation of iron metabolism are best elucidated at the molecular level in *Saccharomyces cerevisiae* (*1*, *2*). *Saccharomyces cerevisiae* has three known pathways for iron uptake, two for free iron and one for siderophore bound iron. Free iron can be acquired either by a high or by a low-affinity uptake system. Under iron deficient conditions, the high-affinity system is induced, which consists of an iron reductase (FRE1/FRE2) (*3*, *4*) and a transport complex consisiting of one of a multi-copper oxidase (FET3) and an iron transporter (FTR1) (*5*, *6*). Free Fe^{3+} is reduced by FRE1/FRE2 to Fe^{2+} and is subsequently re-oxidized to Fe^{3+} by FET3 at the site of FTR1, which transports the iron into the cell. Under iron sufficient conditions, iron uptake is facilitated by the low-affinity iron transporter FET4 (*7*). FET4 is a Fe^{2+} transporter that also transports Cu^{+} and Zn^{2+} into the cell (*8–10*). Siderophores are low molecular weight organic molecules excreted by some organisms to chelate iron specifically. *Saccharomyces cerevisiae* does not synthesize siderophores but has an uptake system for those made by other organisms. This system consists of the proteins transporting the siderophores to the cell wall (FIT1 to FIT3) and mediating the siderophore uptake in to the cell (ARN1 to ARN4) (*11*, *12*).

These three iron uptake systems are regulated by two transcription factors, Aft1p and Aft2p (*13–16*). These two transcription factors are paralogous (39% homology), they recognize a common DNA element (T/C) (G/A)CAC CC (*17*). Aft1p is localized in the cytoplasm under iron-replete conditions, but is relocated to the nucleus if the cell becomes iron deficient and thereby increases the expression of the iron regulation genes (*18*). The localization of Aft2p has not been determined. Although Aft1p and Aft2p bind to the same promoter motif, they do not control the same subset of genes (*19*). Some genes are regulated by both Aft1p and Aft2p (for example *fre1*, *ftr1* and *fet3*), but other genes are only regulated by one of them but not by the other (*20*, *21*).

In higher plants, two major strategies to acquire iron have evolved. Non-graminaceous plants use Strategy I,

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which is a reduction strategy. The solubility of Fe^{3+} is increased in the rhizosphere by an H^+-ATPase that extrudes protons. Soluble Fe^{3+} is then reduced to Fe^{2+} by an iron reductase, and taken up into the cell by an iron transporter. *Arabidopsis thaliana* is the best-studied Strategy I plant and several genes encoding proteins involved in iron uptake have been sequenced, e.g. *FRO2* encodes a reductase that catalyse Fe^{3+} to Fe^{2+} reduction (*22*), and *IRT1* and *IRT2* encode a Fe^{2+} transporter localized in external cell layers of the root subapical zone, which facilitates the Fe^{2+} uptake into the roots (*23–25*). Strategy II plants (graminaceous monocots) use a chelation strategy. Phytosiderophores are secreted into the rhizosphere where they form stable Fe^{3+} chelates, and these chelates are transported into the cells by specific transport systems.

So far some of the *cis*-acting elements of iron regulatory genes in higher plant have been identified. Among these are two types of *cis*-acting elements that function to derepress the expression of phytoferritin genes: the iron-dependent regulatory sequence (IDRS) in maize and the ferritin gene in *Arabidopsis*, the regulatory elements of which share a conserved sequence of CACGAG GCCGCCAC (*26*). The iron regulatory element (FRE) localized within an 86-bp iron response region in the soybean ferritin gene contains a symmetric sequence sufficient to confer derepression of ferritin gene in the presence of iron (*27*). In barley, two iron deficiency inducible genes *IDS1* and *IDS2* had been reported (*28*, *29*). Analysis of the promoter of *IDS2* had led to the identification of two iron deficiency inducible elements, the IDE1 and IDE2 (*30*). The tomato FER and its ortholog of FIT from *Arabidopsis* function as transcription factors in the iron-deficiency-signaling pathway (*31–33*). Both *FER* and *FIT* encode a basic helix–loop–helix (bHLH) transcription factor that is expressed in roots. Down regulation of the FIT mRNA leads to reduction in the mRNA levels of *FRO2* and *IRT1*. Recent reports show the iron deficiency regulation in Strategy I plants also needs BHLH38 and BHLH39 as the binding partner to control the expression of *FRO2* and *IRT1* in iron limitation (*34*, *35*). Presumably, FIT and BHLH38/39 act directly to induce expression of *FRO2* and *IRT1* because co-expression of *FIT* with either *BHLH38* or *BHLH39* in yeast cells leads to the activation of GUS expression driven from the *IRT1* and *FRO2* promoters (*35*). In Strategy II plants, a rice bHLH protein OsIRO2 was identified as an iron-deficiency-induced gene in microarray expression profiling (*36*). Although *IRO2* is well conserved in grasses, it is not closely related to *FIT* or *FER*. Overexpression of *OsIRO2* resulted in increased MAs secretion, and further analysis demonstrated that OsIRO2 positively regulated a large number of the genes related to strategy II response (*37*). Moreover, transcriptional regulatory elements binding to the IDE1 have been identified. The rice IDEF1 protein was identified by testing candidate genes in the ABI3/VP1 families for the ability to bind to the IDE1 sequence (*38*). The *IDEF2* gene, which binds to the IDE2 promoter element, was identified through a yeast one hybrid screen; this gene encodes a member of the NAC family of transcription factors (*39*).

The model photosynthetic eukaryote *Chlamydomonas reinhardtii* uses a high affinity system to acquire iron under iron deficient conditions (*40*), and the proteins involved in the uptake process exhibit a high degree of homology to their yeast counterparts (*41*). Several genes, including *ATX1*, *FOX1*, *FTR1*, *FER1* and *FEA1* (previously named *H43*) had been identified as iron deficiency expression (*41–44*). Other genes involved in iron or other metal ions transport are identified by genomic analysis. These include the CDF family (*MTP1* to *MTP5*), the ZIP family (*ZIP1* to *ZIP14*), the COPT family (*COPT1*), and the NRAMP family (*NRAMP1* to *NRAMP3*) (*45*, *46*).

In previous studies, we identified *FOX1*FeRE1 at −87/−82 (CACACG) and FeRE2 at −65/−61 (CACGCG), both of which are needed for induced *FOX1* expression under conditions of iron deficiency. Our scanning mutagenesis analysis identified a consensus sequence of the *FOX1* FeRE1 C(A/G)C(A/G)C(G/T) (*47*). We also identified *ATX1* AtxFeRE1 at −529/−515 (GTCGCACTGGCATGT) and AtxFeRE2 at −300/−286 (GCAGCGATGGCATTT), with a conserved sequence of GNNGCNNTGGCATNT (*48*), each contains a core sequence of TGGCA. FEA1, previously designated as H43, is a CO_2-inducible protein and its mRNA accumulates at high level in iron deficiency conditions (*44*, *46*). In this article, we use promoter deletion and scanning mutagenesis analyses to study these iron responsive elements. Moreover, by using real time PCR, we quantitated the mRNA level of the transfomant strains to confirm that the control of the expression is at transcriptional level. Our data indicate that two of the regions in its 5′-upstream region are sufficient and necessary for Fe deficiency expression, and that transcription of the gene is controlled by two of the *cis*-acting elements at transcriptional level.

MATERIALS AND METHODS

Strains and Culture Conditions—The recipient strain of all transformations, *C. reinhardtii* strain CC425 (cw15 arg2), was grown in TAP (Tris–acetate phosphate) liquid medium supplemented with 250 μg/ml arginine (*49*), and transformants were grown in either +Fe (18 μM Fe) or −Fe (0 μM Fe) TAP medium. Liquid cultures were grown under continuous light of 150 $\mu mol\, m^{-2}\, s^{-1}$ at 25°C and with an agitation of 250 rpm. Strains on TAP-agar plates were incubated at a light intensity 100 $\mu mol\, m^{-2}\, s^{-1}$at 22°C.

Deletion Constructs of FEA1—Primers using in constructing the deletion mutations of *FEA1* are listed in Table 1. PCR fragments generated using the 5′ and 3′ primers were inserted in *KpnI* site of pJD100 to make the deletion constructs.

Constructs Using in Scanning Mutagenesis Assay—Scanning mutations between –273 and –250 were generated by amplification of –273/–250 region using forward primer containing the target mutation sequence and reverse primer 84R. Mutations between –106 and –85 were generated by amplifying the –106/–85 region using forward primer 273F and reverse primer containing the target mutation sequence. Each of the mutated

Table 1. **Primers used in amplification of the fragments for making the deletion constructs.**

Constructs	5′ Primers	3′ Primers	The position
Fea507F	507F,GACGGTACCGCTTGCTTTCCCGCCGTGC	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–507/–49
Fea403F	403F,GACGGTACCCCTTTTAAGCGATGTGTGC	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–403/–49
Fea333F	333F,GACGGTACCAGTAGGGCAGGGCCGGCCG	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–333/–49
Fea273F	273F,GACGGTACCCTGCGGTGGCAAAGTTGGTG	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–273/–49
Fea187F	187F,GACGGTACCCTGCTTTGCCTTTCAGCGG	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–187/–49
Fea123F	123F,GACGGTACCGACACGCGCGCGCGACGCC	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–123/–49
Fea118R	507F,GACGGTACCGCTTGCTTTCCCGCCGTGC	118R,GACGGTACCCGTGTCGTGCGCGCTTTCC	–507/–119
Fea196R	507F,GACGGTACCGCTTGCTTTCCCGCCGTGC	196R,GACGGTACCAGACAAGAAAGTTAGAGTG	–507/–197
Fea267R	507F,GACGGTACCGCTTGCTTTCCCGCCGTGC	267R,GACGGTACCACCGCAGTCGTCCTGGCAG	–507/–268
Fea338R	507F,GACGGTACCGCTTGCTTTCCCGCCGTGC	338R,GACGGTACCCTAAACATACAAGAGTTATC	–507/–339
Fea252F	252F, GACGGTACCAGATTTTCGAGTTGGAGCTC	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–252/–49
Fea231F	231F, GACGGTACCTCTGTGTCTGTGTCAGACAC	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–231/–49
Fea209F	209F, GACGGTACCTAACTTTCTTGTCTCCTGTT	48R, GACGGTACCTTTATGCGCGGCGTGCAGC	–209/–49
Fea67R	273F,GACGGTACCCTGCGGTGGCAAAGTTGGTG	67R, ACGGTACCAGATTGGATCCAGCGCCAGG	–273/–68
Fea84R	273F,GACGGTACCCTGCGGTGGCAAAGTTGGTG	84R, GACGGTACCAGGCTGGTGCCAGCCGCGG	–273/–85
Fea103R	273F,GACGGTACCCTGCGGTGGCAAAGTTGGTG	103R, GACGGTACCCGGGCGTCGCGCGCGCGTG	–273/–104

fragments was cloned into *KpnI* site of pJD100. The integrity of all constructs (as well as every other construct mentioned in this article) were confirmed by DNA sequencing.

Transformation—The cells used for transformation [*C. reinhardtii* strain CC425 (cw15 arg2)] were at a cell density of $1–2 \times 10^6$ cells/ml, and constructs were introduced into the cells by the glass bead method (*50*) through co-transformation with the plasmid pARG7.8 containing the selectable marker arginosuccinyllyase (*51*). Cells were collected by centrifugation, washed twice and resuspended in TAP medium without arginine to a cell density of approximately 1×10^8 cells/ml. DNA (2–4 μg of construct and 4 μg pARG7.8 each) and cells (400 μl) were mixed with 100 μl 20% polyethylene glycol and 300 mg sterile glass beads, and vortexed for 15 s. Cells were washed from the glass-beads and plated on TAP agar without arginine. After 7 days colonies were transferred in duplicate to +/− Fe TAP agar plates without arginine.

Co-transformation Frequency detection—To determine the frequency of co-transformation, 16 of arginine independent transformants were transferred and maintained for 5 days on TAP agar medium. DNA purified from these transformants using the E-Z 96-well plant DNA kit (Omega Bio-tek) was used as templates for PCR to amplify the *FEA1*-Ars junction with a relative forward primer in *FEA1* promoter region and a reverse primer ArsR (5′-TTCTGAATGGCGTCCTGGTC-3′). The reverse primer locates at amino acids 36–42 of Ars coding sequence.

Arylsulfatase activity assay—Arylsulfatase activity was assayed as described by Davies *et al*. (*52*). Plates with −Fe TAP solid medium were added 300 μl 10 mM 5-bromo-4-chloro-3-indolyl sulfate (XSO4, Sigma Chemical Co.) and scribed before the clones were inculcated. After 1 day transformants expressing arylsulfatase activity were identified by a blue halo around the colonies. For quantitative analysis of arylsulfatase, cells were first collected by centrifugation. Hundred microliter of the supernatant were added to 500 μl of 0.1 M Glycine–NaOH pH 9.0, 10 mM Imidazole, 4.5 mM *p*-nitrophenyl sulfate and incubated in 27°C for 27 min. The reaction was stopped by the addition of 2 ml of 0.25 M NaOH and the absorbance at 410 nm was determined. The standard curve of *p*-nitrophenol (Sigma Chemical Co.) was done in 0.2 M NaOH (*53*).

Real-Time PCR—Transformants for real-time PCR analysis were cultured in −Fe (0 μm) and +Fe (18 μm) TAP liquid medium and reach the density of 2×10^6 to 5×10^6 cell/ml. RNA was extracted by using TRIzol Reagent (Shanghai Sangon Biological Engineering Technology Service Co.). Single-stranded cDNA was made by Bio-Rad Iscript selected cDNA synthesis kit using 100 ng RNA sample and random primer carrying out as 65°C 5 min, 25°C 5 min, 42°C 50 min. The Real time PCR was performed on BioRad iCycler iQ Real-TimePCR Detection System using SYBR Green as a fluorescent dye. Each reaction was made in a final volume of 25 μl with the following components: 0.2 pmol of each primer, 1 μl of cDNA, 12.5 μl of SYBR Green Mix (Invetrogen SYBR Greener QPCR) and water was used to adjust the volume to 25 μl. The iCycler run protocol was: denaturation at 95°C, 5 min; 40 · (denaturation at 95°C, 30 s; annealing at 54°C, 30 s; amplification at 72°C, 15 s). The specificity of the PCR amplification was checked by a melting curve program (55–100°C with a heating rate of 0.5°C/s). The 18S rRNA was used as control with the primers, 18SrRNAF (5′-TCAACTTTCGATGGTAGGATA GTG-3′) and 18SrRNAR (5′-CCGTGTCAGGATTGGGTA ATTT-3′). Expression of this gene was measured and shown to be constitutive under all the conditions used in this work. Primers, ARSF1 (5′-ATGGGTGCCCTC GC GGTGTTC-3′) and ARSR1 (5′-GTAGCGGATGTACTTGT GCAG-3′) were designed specifically for Ars cDNA. The amplification rate of each transcript (Ct) was calculated by the PCR Base Line Subtracted method performed in the iCycler software at a constant fluorescence level. Cts were determined over three repeats. Relative fold differences were calculated based on the relative quantification analytical method ($2^{-\Delta\Delta CT}$) using 18s rRNA amplification as internal standard (*54*).

RESULTS

FEA1 FeREs Localized in –273/–253 and –103/–85 Regions—To study the promoter region of FEA1, by using cDNA sequence information of FEA1 (GeneBank accession no AB042098) and sequence information from JGI *Chlamydomonas* genomic database (http://genome.jgi-psf.org), we first cloned a 459-bp fragment upstream of *FEA1* (from −507 to −49) into pBluescript II SK(+). The resulting plasmid was used to generate a series of 5′-end nested deletion constructs (Fig. 1, Fea507F to Fea123F) as well as 3′-end nested deletion constructs (Fig. 1, Fea118R to Fea338R). The 5′ deletion constructs possessed the 5′ upstream fragment from position −507, −403, −333, −273, −187 and −123 to −49 fused to the arylsulfatase gene in pJD100, which contains a minimal promoter element from the *Chlamydomonas* β2-tubulin gene (*52*); The 3′ deletion constructs possessed the fragment between −118, −196, −267, −338 and −507 were similarly cloned into pJD100. All of the deletion constructs from these manipulations were delivered into *C. reinhardtii* CC425 by co-transformation with pArg7.8 (*50*, *51*) and the resulting strains were used to analyse the response of these constructs to different iron concentrations.

Our results indicated that a region spanning nucleotide −273 to −188 was essential for Fe deficiency inducible expression (Fig. 1, Fea507F to Fea273F). On the other hand, deletion analysis from the 3′ end of −507/−49 region revealed that none of the tested constructs from −507 to −118 was sufficient to confer promoter activity under low Fe conditions (Fig. 1, Fea187F to Fea338R), suggesting that promoter activity requires both −273/−188 and −118/−49 regions, to precisely localize the FeREs within the −273/−188 and −118/−49 regions, we conducted another round of deletion analysis. Our results revealed that this region contains two FeRE elements; one locates within −273/−253 and the other in −103/−85 (Fig. 2).

Scanning Mutagenesis Analysis of the –273/–250 and –106/–85 of FEA1—After localizing the FeREs to regions of −273/−253 and −103/−85, we performed a scanning mutagenesis to further identify the core sequence of the regulatory elements. In the −273/−250 region, constructs FeaM273F, FeaM270F, FeaM267F, FeaM264F, FeaM262F and FeaM261F, showed relatively lower arylsulfatase (Ars) expression than the control Fea84R (construct with original nucleotide sequence from −273 to −85) in low Fe (0 μM) conditions (Fig. 3, 0.68, 1.38, 0.84, 0.97, 0.72 and 1.34 compared with 5.42 nmol *p*-nitrophenol × min^{-1} × 10^{-6} cells). The promoter activity of these mutants also did not significantly change of under low or high Fe (18 μM) conditions. However, in constructs FeaM258F, FeaM255F and FeaM258F, Ars was expressed 2–5 times higher in low-Fe than in high-Fe condition, and the activity was not

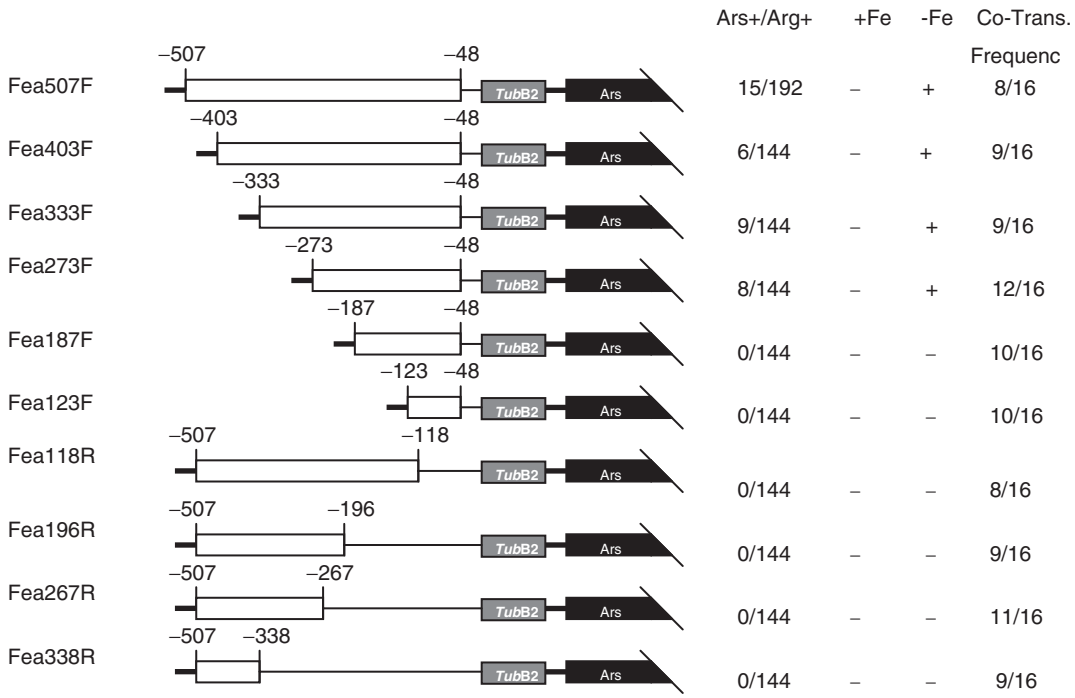


Fig. 1. **5′ and 3′ deletion analysis of the *FEA1* promoter region.** A series of 5′ and 3′ deletions from −507 to −49 of *FEA1* promoter region were amplified by PCR and fused to the Ars reporter gene in pJD100, and transformed into the arginine requiring *C. reinhardtii* strain CC425 by co-transformation with pArg7.8. Arginine independent colonies were transferred to +/− Fe TAP plates and sprayed with 10 mM x SO_4 to visualize arylsulfatase activity. These results identified regions −273/−188 and −118/−49 are sufficient and necessary for Fe-deficiency inducible expression. The fraction of arylsulfatase expressing colonies among the arginine independent colonies is indicated as Ars^+/Arg^+, and +Fe and −Fe indicate growth on +/− Fe TAP plates, and (+) and (−) indicate expression of the arylsulfatase gene. TubB2 stands for a minimal promoter element from the *Chlamydomonas* β2-tubulin gene (*52*). The co-transformation frequency was tested by PCR from 16 random selected Arg independent transformants.

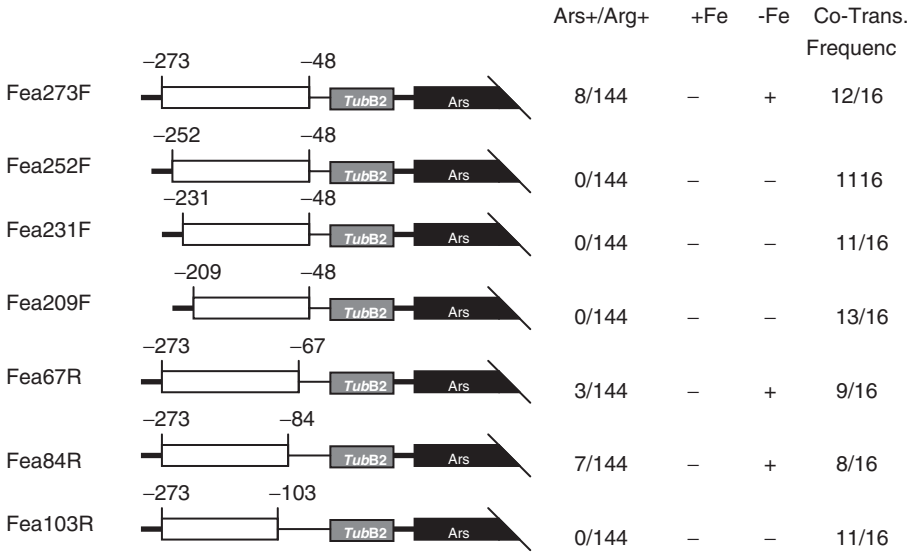
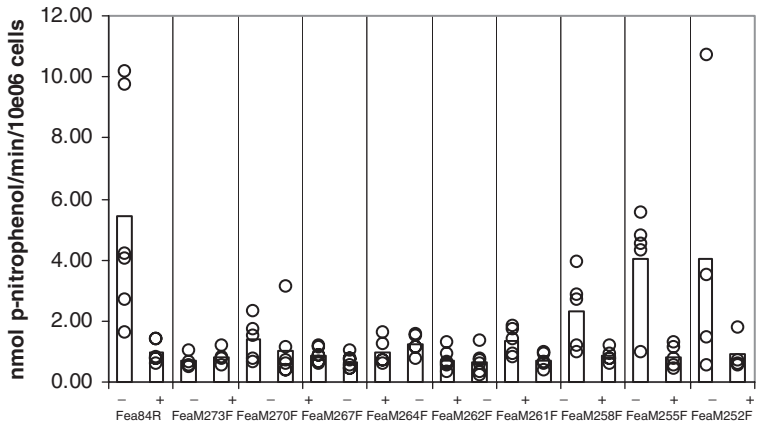
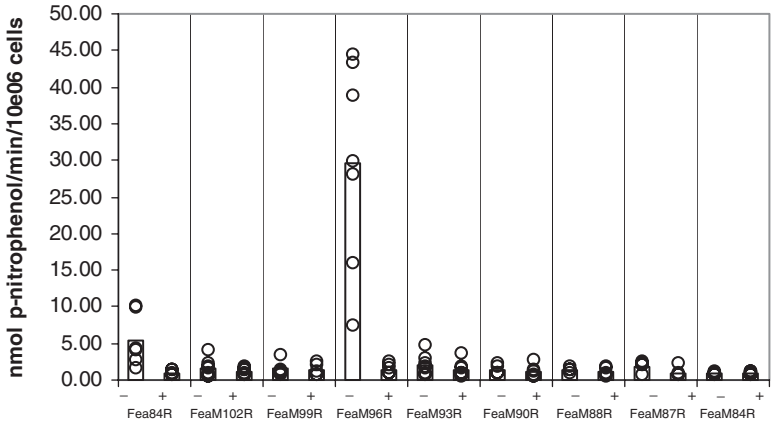


Fig. 2. **Deletion analysis of the *FEA1* promoter region.** Constructs Fea273F, Fea252F, Fea231F, Fea209F, Fea67R, Fea84R and Fea103R were generated by fusing the fragment of −273/−49, −252/−49, −231/−49, −209/−49, −273/−68, −273/−85 and −273/−104 with the Ars reporter gene in pJD100 respectively, and co-transformed into the arginine requiring *C. reinhardtii* strain CC425 with pArg7.8. Arginine independent colonies were transferred to +/− Fe TAP plates and sprayed with 10 mM × SO_4 to determine arylsulfatase activity. Results revealed the FeREs elements were in −273/−253 and −103/−85 region. The fraction of arylsulfatase expressing colonies among the arginine independent colonies is marked as Ars^+/Arg^+, and +Fe and −Fe indicate growth on +/− Fe TAP plates, and (+) and (−) indicate expression of the arylsulfatase gene. TubB2 stands for a minimal promoter element from the *Chlamydomonas* β2-tubulin gene (*52*). The co-transformation frequency was tested by PCR from 16 random selected Arg independent transformants.



Constructs	-273/-250	Ars activity -Fe	Ars activity +Fe	N	Fe respons.?	Co-Trans.Freq.
Fea84R	**CTGCGGTGGCAAAGT**TGGTGGAGA	5.42±5.96	0.95±0.86	6	Yes	
FeaM273F	AGTCGGTGGCAAAGTTGGTGGAGA	0.68±0.24	0.82±0.27	4	No	11/16
FeaM270F	CTGATTTGGCAAAGTTGGTGGAGA	1.38±0.69	1.04±1.06	5	No	9/16
FeaM267F	CTGCGGGTTCAAAGTTGGTGGAGA	0.84±0.24	0.66±0.21	7	No	12/16
FeaM264F	CTGCGGTGGATAAGTTGGTGGAGA	0.97±0.43	1.21±0.31	5	No	11/16
FeaM262F	CTGCGGTGGCACAGTTGGTGGAGA	0.72±0.35	0.65±0.39	6	No	9/16
FeaM261F	CTGCGGTGGCAACTGTGGTGGAGA	1.34±0.46	0.71±0.25	5	No	8/16
FeaM258F	CTGCGGTGGCAAAGTGTTTGGAGA	2.33±1.24	0.84±0.22	5	Yes	
FeaM255F	CTGCGGTGGCAAAGTTGGGTTAGA	4.03±1.78	0.83±0.37	5	Yes	
FeaM252F	CTGCGGTGGCAAAGTTGGTGGCTC	4.05±4.59	0.91±0.57	4	Yes	

Fig. 3. **Scanning mutagenesis analysis of the *FEA1*−273/−250 region.** A series of nucleotide substitutions were introduced into the −273/−250 region. Results revealed the FeaFeRE1 element in −273/−259 region with the sequence CTGCGGTG GCAAAGT. The substituted nucleotides are underlined and bold letters are the sequence of FeaFeRE1. The Ars activities (nmol *p*-nitrophenol × min^{-1} × 10^{-6} cells) expressed by the mutated constructs were measured under −Fe (0 μM) or +Fe (18 μM) conditions. Black and white circles represent the Ars activities in independent transformants under −Fe or +Fe conditions, respectively. Bars indicated the median values. The number of the transformants tested for the activity is represented by N. The co-transformation frequency of the constructs was determined by PCR with 16 Arg independent transformants tested in each line.



Constructs	-106/-85	Ars activity -Fe	Ars activity +Fe	N	Fe respons.?	Co-Trans.Freq.
Fea84R	**CCGCCGC**GGC**TGGCACCAGCCT**	5.42±5.96	0.95±0.86	6	Yes	
FeaM102R	AATACGCGGCTGGCACCAGCCT	1.54±1.22	1.13±0.42	8	No	9/16
FeaM99R	CCGCATAGGCTGGCACCAGCCT	1.52±1.09	1.42±0.79	5	No	8/16
FeaM96R	CCGCCGCTTATGGCACCAGCCT	29.7±14.0	1.38±0.66	7	Yes	
FeaM93R	CCGCCGCGGCGTTCACCAGCCT	1.94±0.36	1.33±1.00	8	No	12/16
FeaM90R	CCGCCGCGGCTGGACCCAGCCT	1.25±0.57	1.12±0.79	6	No	10/16
FeaM88R	CCGCCGCGGCTGGCAACAGCCT	1.34±0.35	1.09±0.59	5	No	9/16
FeaM87R	CCGCCGCGGCTGGCACACTCCT	1.90±0.68	1.10±0.75	5	No	8/16
FeaM84R	CCGCCGCGGCTGGCACCAGAAG	0.88±0.13	0.83±0.23	6	No	10/16

Fig. 4. **Scanning mutagenesis analysis of the FEA1−106/−85 region.** A series of nucleotide substitutions were introduced into the −106/−85 region. Results revealed the FeaFeRE2 element in −106/−85 region with the conservation sequence CCGC CGCNNNTGGCACCAGCCT. The changed nucleotides are underlined. The conservation sequence of FeaFeRE2 is highlighted in bold. The Ars activities (nmol *p*-nitrophenol × $min^{-1} \times 10^{-6}$ cells) expressed from constructs were measured under −Fe (0 μM) or +Fe (18 μM) conditions. Black and white circles represent the Ars activities in independent transformants under −Fe or +Fe conditions, respectively. Bars indicated the median values. N represents the number of the transformants tested for the activity. The co-transformation frequency of the constructs was detected by PCR with 16 Arg independent transformants tested in each line.

significantly different from the control (Fig. 3, 2.33, 4.03 and 4.05 compared with 5.42 nmol *p*-nitrophenol × $min^{-1} \times 10^{-6}$ cells). These results suggested that the element from −273 to −259 (CTGCGGTGGCAAAGT) was one of the core sequences of FeRE that allows the promoter to respond to Fe-deficient induction, and we designated this element as FeaFeRE1.

By analysing the −106/−85 region of constructs including FeaM102R, FeaM99R, FeaM93R, FeaM90R, FeaM88R, FeaM87R and FeaM84R, we observed lower promoter activities under Fe-deficient conditions compared with control (Fig. 4, 1.54, 1.52, 1.94, 1.25, 1.34, 1.90 and 0.88 compared with 5.42 nmol *p*-nitrophenol × $min^{-1}10^{-6}$ cells). Again these promoters did not respond to low Fe levels and exhibited similar activities in media containing low or high Fe levels (Fig. 4, FeaM102R, 1.54/1.13; FeaM99R, 1.52/1.42; FeaM93R, 1.94/1.33; FeaM90R, 1.25/1.12; FeaM88R, 1.34/1.09; FeaM87R, 1.90/1.10; FeaM84R, 0.88/0.83). However, in construct FeaM96R, Ars activity was 21-fold higher in low Fe than in high Fe conditions, and the activity was more than 5-fold higher than that of the control (Fig. 4). These results suggest that region spanning from −106 to −85 (CCGCCGCGGCTGGCACCAGCCT) is critical for Fe-deficiency induction, and this element was designated as FeaFeRE2 with the conservation sequence of C CGCCGNNNTGGCACCAGCCT.

Fe-regulated FEA1 Gene Expression Occurred at Transcriptional Level—To confirm that Fe-mediated gene regulation occurs at transcriptional level, we used real-time PCR to measure the mRNA levels under these conditions (Table 2). We selected several transformants containing the appropriate constructs for analysing mRNA levels as well as Ars activity. Our results indicated that a significant amount of Ars mRNA was accumulated in transformants containing Fea507F, Fea273F, Fea84R or FeaM96R, but not in those containing Fea252F, Fea187F, Fea118R or Fea103R. These results are in high degree agreement with the data obtained by Ars activity assay, indicating that the Ars activity corresponds to its transcriptional level in response to iron deficient induction and that the observed iron regulation of gene expression occurs at the transcription level.

DISCUSSION

In this study, we have analysed the Fe-deficiency-induction gene of *FEA1* using deletion and scanning mutagenesis methods. We found that FEA1, previously reported as a CO_2 responsive protein named H43, is co-induced with FRE1 in Fe deficiency cells. Its mRNA was remarkably higher in low Fe cells (540×) than in high Fe cells (*44*). Our results reveal two FeREs elements localized in −273/−253 and −103/−85 regions that controls gene expression in response to Fe deficiency (Figs 1 and 2). Further studies of these regions by scanning mutagenesis analyses identified one of the FeRE as CTGCGGTGGCAAAGT localized in −273/−259 region

Table 2. **The comparison of Ars mRNA levels and Ars activities of selective transformants.**

Strains transformed by constructs	Ars mRNA abundances			Ars activities (nmol *p*-nitrophenol × min^{-1} × 10^{-6} cells)		
	−Fe (0 μM)	+Fe (18 μM)	−Fe/+Fe	−Fe (0 μM)	+Fe (18 μM)	−Fe/+Fe
Fea507F	121	13	9	8.38	0.93	9
Fea273F	81	11	7	7.31	1.02	7
Fea252F	3	2	2	0.33	0.31	1
Fea187F	2	1	2	0.26	0.23	1
Fea118R	2	2	1	0.31	0.26	1
Fea103R	3	2	2	0.20	0.17	1
Fea84R	33	5	6	4.05	0.56	7
FeaM96R	1274	7	41	28.07	0.88	32

Ars mRNA levels were assessed by real-time PCR in −Fe (0 μM) and +Fe (18 μM) conditions. Data were calculated by the $2^{-\Delta\Delta CT}$method (*54*). The Ars activities of the selected transformants were detected as described by Davies *et al.* (*52*).

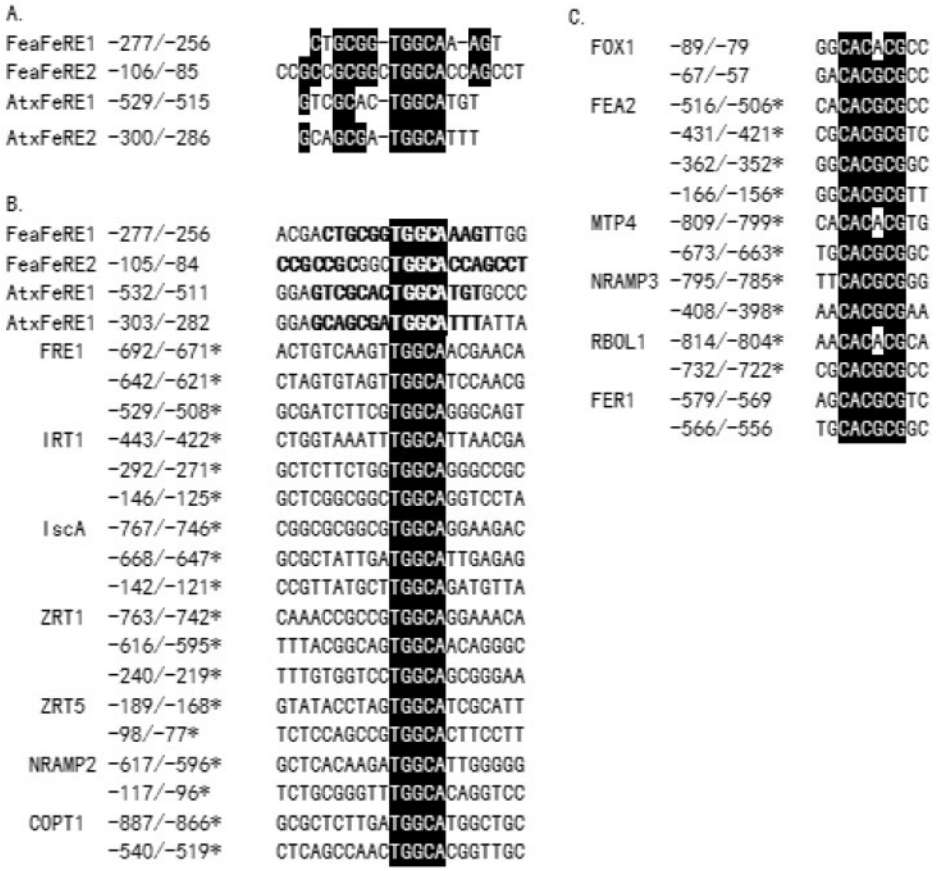


Fig. 5. **Sequence comparison of the FeaFeRE1 and FeaFeRE2, AtxFeRE1 and AtxFeRE2, and other promoters of Fe-regulated gene in *Chlamydomonas*.** (A) Sequence comparison of FeaFeRE1 and FeaFeRE2, AtxFeRE1 and AtxFeRE2, the core sequence of TGGCA was shaded. (B) TGGCA is present in promoters of other Fe-deficiency-inducible genes. (C) The presence of the conserved sequence of *FOX1* FeREs in promoters of other Fe-deficiency-inducible genes. White letters on black background indicate the conserved sequences. The distances from the transcriptional starting sites or translation starting sites are shown in front of the sequence (the distance from the translational starting sites are marked with asterisk).

and the other in −106/−85 with the conserved sequence of CCGCCGCNNNTGGCACCACGCCT (Figs 3 and 4).

The comparison of FeaFeRE1 and FeaFeRE2 led to the identification of a 12-bp conserved sequence, CNGCG GNTGGCANNAG (Fig. 5A). We compared this elements with several known FeRE elements, such the *cis*-acting elements of *FUR* (*55*, *56*), the iron-responsive element (IRE) of vertebrates (*57*), the IDRS of phytoferritin genes from maize, or *Arabidopsis* (*26*), the iron regulatory element (FRE) of ferritin gene from the soybean (*27*), and IDE1 and IDE2 in barley *IDS2* gene (*30*), and no similarity between the conserved sequence and those elements was detected. However, FeaFeRE1 and FeaFeRE2 shared a 5 bp sequence (TGGCA) with the core sequence of AtxFeRE1 and AtxFeRE2, previously identified by our group (Fig. 5A).

We examined the JGI *Chlamydomonas* database to determine whether the promoter region of the putative Fe regulated genes contains the TGGCA element. Our results show that the TGGCA element is present in some of the promoters. Among these, a *FRE1*(C_40004) gene related to NADPH oxidase and ferric chelate reductases that is co-induced with *FEA1* in *Chlorococcum littorale*, contains three regions with the core sequence in −692/−671, −642/−621 and −529/−508 from the translation start sites. Similarly, a *IRT1*(C_210127) gene, identified as a Fe^{2+}/Zn^{2+}-regulated transporter belonging to the ZIP family, also contains three such core sequence in −443/−422, −292/−271 and −146/−125 regions. Finally, an IscA- like protein (C_690044), related to Fe–S cluster biosynthesis conains three core sequences in −767/−746, −668/−647 and −142/−121 regions. Other genes including *ZRT1*(C_100025), *ZRT5*(C_980003), *NRAMP2* (C_570027) and *COPT1*(C_360043), all contain the core sequence in their promoter region (Fig. 5B).

We also searched the *FOX1* FeREs in the database. As we reported earlier (*47*), FoxFeRE1 in its −87/−82 of CA CACG and FoxFeRE2 in its −65/−60 of CACGCG and Fox FeRE1with the consensus sequence C(A/G)C (A/G)C(G/T)). We found that *FEA2* (C_70186, the homologue of *FEA1*), *MTP4* (C_110212, putative Mn transporter), *NRAMP3* (C_440074, Mn2+ and Fe2+ transporters, belonged to the NRAMP family) and *RBOL1* (C_30095, Ferric reductase-like transmembrane component) possess at least two of the conserved sequences in their promoter region (Fig. 5C). These results imply that there are at least two categories of FeRE elements exist in *Chlamydomonas*. One of them, harbored by *FOX1*, *FEA2*, *MTP4*, *NRAMP3*, *FER1* and *RBOL1*, carries the sequence of C(A/G)C(A/G)C(G/T) and CACGCG. The second, carried by genes such as *FEA1*, *FRE1*, *IRT1*, *ISCA*, *ZRT1*, *ZRT5*, *NRAMP2* and *COPT1* contains the TGGCA core sequence.

Moreover, we investigated the promoter region of *FEA1*, besides FeaFeRE1 and FeaFeRE2 a Fox-type FeRE with the sequence of CACGCG in −121/−115 region had been found. But only one Fox-type FeRE is not enough to drive the expression of *FEA1* under low Fe conditions (see Fig. 1, the transgenic result of Fea123). We also investigated the promoter region of another iron regulated gene *FTR1* (an iron permease gene). Two of the Fox-type like FeREs had been found in its −789/−283 (CACA**T**G) and −261/−255 (CACGCG) regions. We used overlap extension PCR to remove both of the regions from *FTR1* promoter. Results showed remain sequence could still drive the iron-deficiency- inducible expression. Although the sequence of CACA**T**G is only one base pair different from the *FOX1* FeRE1 (CACA**C**G) [C→T, The consensus sequence of the *FOX1* FeRE1 is C(A/G)C (A/G)**C**(G/T)], it can not be recognized by transcript factors that control the *FOX1* gene. The results indicate these two of Fox-type like FeREs are not critical to drive the expression in *FTR1*. After analysed *FTR1* promoter by 5′ and 3′ deletion and scanning mutagenesis assay, we identified the *FTR1* FeREs still contained the core sequence of TGGCA (unpublished data).

In *S. cerevisiae*, iron homoeostasis is maintained primarily through transcriptional control of gene expression. In response to variation of iron availability, the transcriptional regulator Aft1p/Aft2p directs the expression of a series of genes to control iron transport and sub-cellular compartmentalization. Promoters of these genes contain the consensus element CACCC recognized by the regulator (*19*). A few reports have studied transcriptional gene regulation in *Chlamydomonas*. Kucho *et al*. (*58*) identified two *cis*-acting enhancer elements, EE-1 and EE-2 that are responsible for gene regulation in response to low CO_2. These elements shared a conserved motif, GANTTNC. In another study, Quinn *et al*. (*59*) reported that the motif GTAC was absolutely essential for copper deficiency expression of the *CYC6* and the *CPX1* genes (encoding cytochrome*c6* and coprogen oxidase). In the high-affinity iron uptake pathway, a multicopper ferroxidase (*FOX1*), an iron permease (*FTR1*), a copper chaperone (*ATX1*) and ferritin (*FER1*) had been cloned based on sequence similarity between these genes and their yeast counterparts, suggesting that *Chlamydomonas* and *S. cerevisiae* share a similar iron assimilation pathway when Fe is scarce. That the *Chlamydomonas* genome does not harbor homologs of Aft1p/Aft2p suggests that although this organism uses a similar enzyme to regulate the affinity iron uptake under iron deficient conditions, the regulation mechanisms are different from that of the yeast. In our previous studies, we reported FoxFeRE1 in its −87/−82 with the sequence of CACACG and FoxFeRE2 in −65/−60 with the sequence of CACGCG (*47*). We also found that both AtxFeRE1 and AtxFeRE2 have a core sequence of TGGCA in the regulatory elements (*48*). Our current results indicate that FeaFeRE1 and FeaFeRE2 have the same core sequence of TGGCA as *ATX1* FeREs. Furthermore, our bioinfomatic analyses reveal at least two classes of iron responsive element in Chlamydomonas, suggesting the existence of at least two corresponding regulation mechanisms in response to Fe-deficiency in this organism.

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CONFLICT OF INTEREST

None declared.

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