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Research Article

A Novel Negative Fe-Deficiency-Responsive Element and a TGGCA-Type-Like FeRE Control the Expression of *FTR1* in *Chlamydomonas reinhardtii*

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We have reported three Fe-deficiency-responsive elements (FEREs), *FOX1, ATX1,* and *FEA1*, all of which are positive regulatory elements in response to iron deficiency in *Chlamydomonas reinhardtii*. Here we describe *FTR1*, another iron regulated gene and mutational analysis of its promoter. Our results reveal that the FeREs of *FTR1* distinguish itself from other iron response elements by containing both *negative* and *positive* regulatory regions. In *FTR1*, the −291/−236 region from the transcriptional start site is necessary and sufficient for Fe-deficiency-inducible expression. This region contains two positive FeREs with a TGGCA-like core sequence: the FtrFeRE1 (ATGCAGGCT) at −287/−279 and the FtrFeRE2 (AAGCGATTGCCAGAGCGC) at −253/−236. Furthermore, we identified a novel FERE, FtrFeRE3 (AGTAACTGTTAAGCC) localized at −319/−292, which negatively influences the expression of *FTR1*.

1. Introduction

Iron is an essential nutrient for virtually every organism on the earth, because it participates as a cofactor in numerous essential enzymatic reactions involved in electron transfer and many other physiological processes. Symptoms of acquired or inherited iron-deficiency have been reported in many organisms, but iron uptake and the regulation of iron metabolism are best characterized at the molecular level in *Saccharomyces cerevisiae* [1, 2]. *S. 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 consisting of one of a multicopper oxidase (FET3) and an iron transporter (FTR1) [5, 6]. Free Fe^{3+} is reduced by FRE1/FRE2 to Fe^{2+} and is subsequently reoxidized 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 an Fe^{2+} transporter that also transports Cu^{+} and Zn^{2+} into the cell [8–10]. These three iron uptake systems are regulated by two transcription factors, Aft1p and Aft2p [11–14]. These two transcription factors are paralogous (39% homology); they recognize a common DNA element (T/C) (G/A)CACCC [15]. 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 [16]. 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 [17]. Some genes are regulated by both Aft1p and Aft2p (e.g., *fre1, ftr1,* and *fet3*), but other genes are only regulated by one of them but not by the other [18, 19].

In higher plants, two major strategies to acquire iron have evolved. Nongraminaceous plants use Strategy I, 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, for example, *FRO2* encodes a reductase that catalyze Fe^{3+} to Fe^{2+} reduction [20], and *IRT1* and *IRT2* encode an Fe^{2+} transporter localized in external cell layers of the root subapical zone, which facilitates the Fe^{2+} uptake into the roots [21–23]. 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.

Although studies on regulation of iron metabolism in photosynthetic eukaryotes are just getting started, recently more and more reports in this field have been published. Iron related elements of photoferritin gene have been identified as IDRS (iron-dependent regulatory sequence) in maize and *Arabidopsis* and FRE (iron regulatory sequence) in soybean. The IDRS harbors the conserved sequence CACGAGGCCGCCAC [24]; whereas the FRE contains a symmetric sequence sufficient to derepress the ferritin gene when iron is abundant [25]. In barley, IDS1 and IDS2 induced specifically by iron-deficiency stress are metallothionein-like genes [26, 27]. There are two iron-deficiency-inducible elements in *IDS2*, IDE1 (ATCAAGCATGCTTCTTGC) at −153/−136 and IDE2 (TTGAACGGCAAGTTTCACGCTGTCACT) at −262/−236 [28]. The tomato FER gene, together with its *Arabidopsis* ortholog FIT gene, functions as transcriptional factors in the iron-deficiency-signaling pathway [29–31]. Both *FER* and *FIT* encode a basic helix-loop-helix (bHLH) transcriptional factor that is expressed in roots. Down regulation of the *FIT* mRNA leads to reduction in the mRNA levels of *FRO2* and *IRT1*, whose expression also needs the binding partner BHLH38 and BHLH39 when iron is limited [32, 33]. Coexpression of *FIT* with either *BHLH38* or *BHLH39* in yeast cells activates the expression of GUS controlled by *IRT1* and *FRO2* promoters, indicating that FIT and BHLH38/39 act directly to induce expression of *FRO2* and *IRT1* [33].

In *Chlamydomonas reinhardtii*, the expression of several genes, including *FOX1*, *FTR1*, *FER1*, *ATX1*, and *FEA1* is induced by Fe-deficiency [34–37]. So far, two types of FeREs have been identified in *Chlamydomonas*. One is the FOX1-type with two regions that contain the core sequence C(A/G)C(A/G)C(G/T) [38], and the other is the TGGCA-Type that harbors the sequence TGGCA [39, 40]. The FTR1 gene codes an iron permease whose mRNA is accumulated 2 to high levels in Fe-deficiency cells [34]. However, th ecis-acting elements that govern such response are unknown. In this paper, we report the identification of three FeREs in *FTR1* gene, two as positive Fe-deficiency-responsive elements and one as a novel negative FeRE.

2. Materials and Methods

2.1. Strains and Culture Conditions. The recipient strain of all transformations, *Chlamydomonas reinhardtii* strain CC425 (cw15 arg2), was grown in TAP (tris-acetate phosphate) liquid medium supplemented with 250 μg/mL arginine [41], 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 μmol $\times$ $m^{-2}\times$ s^{-1} at 25°C in shaking conditions of 250 rpm. Strains on TAP-agar plates were incubated at a light intensity of 100 μmol $\times$ $m^{-2}\times$ s^{-1}at 22°C.

2.2. Deletion Constructs of FTR1. Primers used to make the deletion constructs of *FTR1* are list in Table 2.

Target DNA fragments were generated by PCR using the 5′ and 3′ primers, which were then inserted in *Sal*I/*Kpn*I sites of pJD54 or in *Kpn*I site of pJD100 to make the described deletion constructs. To make constructs FtrD5, an overlap extension PCR method was used. First, the DNA fragment between −1179 and −291 of *FTR1* was amplified with forward primer A and reverse primer B. Then the fragment between −253 and +58 was amplified by primer C and D. The PCR products of these two reactions were mixed and used as the template for a third reaction with primers A and D. This manipulation produced a fragment from position −1179 to +58 of *FTR1* but with the −291/−254 removed. The fragment was then cloned into the *KpnI* and *SalI* sites of pJD54.

2.3. Constructs Used in Scanning Mutagenesis Assay. Substitution mutations in *FTR1* promoter sequence were generated by amplifying the *FTR1* promoter region with one primer containing the target mutation and a second primer outside the FeREs region (−291 to −236 or −319 to −292) relative to the *FTR1* transcription start. Mutated fragments were cloned into the *KpnI* site of pJD100. All constructs (as well as every other construct mentioned in this paper) were confirmed by DNA sequencing.

2.4. Transformation. The cells used for transformation (*C. reinhardtii* strain CC425 (cw15 arg2)) were grown to a cell density of 1-2 $\times$ 10^6 cells/mL, and constructs were introduced into the cells by the glass bead method [43] through cotransformation with the plasmid pARG7.8 that contains the selectable marker arginosuccinyl lyase [44]. Briefly, cells were collected by centrifugation, washed twice and were resuspended in TAP medium without arginine to a cell density of approximately 1 $\times$ 10^8 cells/mL. DNA (2 μg of construct and 10 μg pARG7.8 each) and cells (400 μL) were mixed with polyethylene glycol (100 μL 20%) and 300 mg sterile glass beads. After being vortexed for 15 s on a bench-top mixer, cells were washed from the glass-beads and plated on TAP agar without arginine. After seven days colonies were transferred in duplicate to +/− Fe TAP agar plates without arginine.

Table 1: The comparison of Ars mRNA levels and Ars activities of selective transformants.

Constructs	Ars mRNA Abundances			Ars Activities (nmol p-nitrophenol $\times$ min$^{-1}\times 10^{-6}$ cells)		
	−Fe (0 μM)	+Fe (18 μM)	−Fe/+Fe	−Fe (0 μM)	+Fe (18 μM)	−Fe/+Fe
Ftr1179	115	22	5	2.97	0.58	5
Ftr291	372	36	10	6.86	0.76	9
Ftr253	2	2	1	0.31	0.32	1
319–194	1	1	1	0.29	0.26	1
291–194	133	26	5	3.49	0.55	6
FtrM291F	186	29	6	5.41	0.81	7
FtrM287F	3	2	2	0.33	0.29	1

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 [29]. The Ars activities of the selected transformants were detected as described by Davies et al. [42].

Table 2: Primers used in amplification of the fragments for making the deletion constructs.

Constructs	5′Primers	3′Primers	The position
Ftr1179	1179F TCGGTACCTTACTGGCTTACTGGCA	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−1179/+58
Ftr917	917F TAAGGTACCGAACCTAACAAATGGCA	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−917/+58
Ftr718	718F TAAGGTACCCCAAGATCACCCGTCTG	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−718/+58
Ftr511	511F CTTGGTACCTTCCAGCCCGACTGAAT	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−511/+58
Ftr355	355F ATTGGTACCCGACACACCTTGCTCCA	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−355/+58
Ftr291	291F GACGGTACCTCACATGCAGGCTCCTCCT	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−291/+58
Ftr253	253F TAAGGTACCAAGCGATTGCCAGAGCG	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−253/+58
Ftr161	161F TAAGGTACCATGCCACCGATTACGAG	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−161/+58
Ftr59	59F TAAGGTACCGGAGCGACGGTAAACGG	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−59/+58
Ftr24	24F ATCGGTACCATATAGCACTCTAAC	58R GAAGTCGAC CGTCCTGATGAGAAAGG	−24/+58
355−162	355F GACGGTACCCGACACACCTTGCTCCACCG	162R GACGGTACCGCATGCGTTCTCTTATTG	−355/−162
355−194	355F GACGGTACCCGACACACCTTGCTCCACCG	194R GACGGTACCGACATTATCAGCTAGCG	−355/−194
334−194	334F GACGGTACCGAGCGAGGCTACTGGTC	194R GACGGTACCGACATTATCAGCTAGCG	−334/−194
291−162	291F GACGGTACCTCACATGCAGGCTCCTCCT	162R GACGGTACCGCATGCGTTCTCTTATTG	−291/−162
291−194	291F GACGGTACCTCACATGCAGGCTCCTCCT	194R GACGGTACCGACATTATCAGCTAGCG	−291/−194
291−215	291F GACGGTACCTCACATGCAGGCTCCTCCT	215R GACGGTACCCAGTGCATG GCGACAGCC	−291/−215
291−236	291F GACGGTACCTCACATGCAGGCTCCTCCT	236R GACGGTACCGCGCTCTG GCAATCGCTTC	−291/−236
291−254	291F GACGGTACCTCACATGCAGGCTCCTCCT	254R GACGGTACCCGCGCGTGCCCGGTCGG	−291/−254
FtrD5(overlap extension PCR)	A,1179F AAGGTACCGAACCTAACAAATGGCA C,253F CTGTTAAGCCAAGCGATTGCCAGAG	B,291R GCAATCGCTTGGCTTAACAGTTACTG D,58R GAAGTCGACCGTCCTGATGAGAAAGG	−1179/+58 with −291/−254 removal

2.5. Cotransformation Frequency Detection. To determine the frequency of cotransformation, 16 out of arginine autotrophic transformants were transferred and maintained for 5 days on TAP agar medium. DNA from these transformants was isolated by using the E-Z 96 well plant DNA kit (Omega Bio-tek) and was used as templates for PCR amplification of the *FTR1-ARS* junction with a forward primer in the *FTR1* promoter region and a reverse primer ArsR(5′-TTCTGAATGGCGTCCTGGTC-3′), which is corresponding to amino acid 36 to 42 of the *ARS* coding sequence.

2.6. Arylsulfatase Activity Assay. Arylsulfatase activity was assayed as described by Davies et al. [42]. Before inoculation, 5-bromo-4-chloro-3-indolyl sulfate (XSO4) (Sigma Chemical Co.) was added to plates with –Fe TAP solid medium at a concentration of 10 mM. Transformants expressing arylsulfatase activity were identified by the appearance of a blue halo around the colonies one day after inoculation. For quantitative analysis of arylsulfatase, cells were first collected by centrifugation. 100 μL of the culture supernatant was added to 500 μL buffer that contains 0.1 M Glycine-NaOH pH 9.0, 10 mM Imidazole, 4.5 mM p-Nitrophenyl sulfate. The reaction was incubated at 27°C for 27 minutes. 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 established in 0.2 M NaOH [45].

2.7. Real-Time PCR. Transformants for real-time PCR analysis were cultured in −Fe(0 μM) and +Fe (18 μM) TAP liquid medium to a cell density of 2 to 5 $\times$ 10^6 cell/mL.

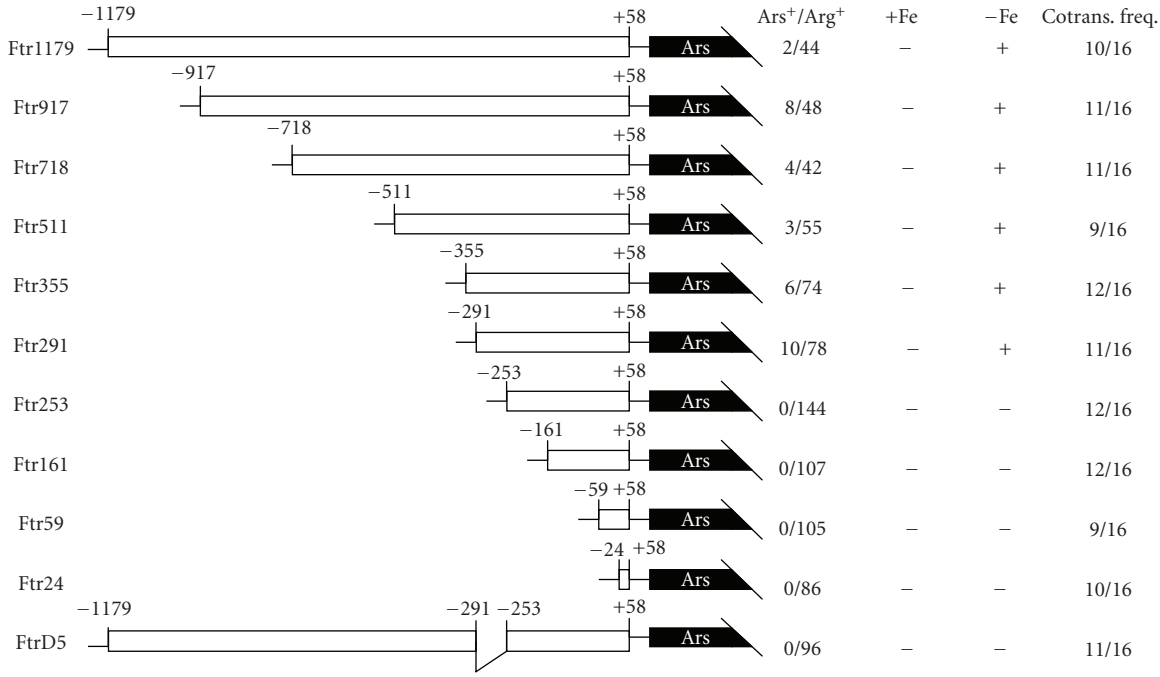


Figure 1: 5′ deletion analysis of the *FTR1* promoter region. A series of 5′ deletions from −1179 to +58 of *FTR1* promoter region were amplified by PCR and fused to the Ars reporter gene in pJD54, and transformed into the arginine requiring *C. reinhardtii* strain CC425 along with pArg7.8. Arginine independent colonies were transferred to +/− Fe TAP plates and sprayed with 10 mM XSO_4 to visualize arylsulfatase activity. FtrD5 was constructed by fusing the −1179/+58 fragment lacking the −291/−254 region to pJD54. The fraction of arylsulfatase expressing colonies among the arginine independent colonies is expressed as Ars^+/Arg^+; +Fe and −Fe indicate growth on +/− Fe TAP plates, and (+) and (−) indicate expression of the arylsulfatase gene. The cotransformation frequency was tested by PCR from 16 random selected Arg independent transformants.

RNA was extracted by using the TRIzol Reagent (Shanghai Sangon Biological Engineering Technology & Service Co.). Single strand cDNA was synthesized by Bio-Rad Iscript selected cDNA synthesis kit using 100 ng RNA and random primers performed at as 65°C 5 minutes, 25°C 5 minutes, 42°C 50 minutes. The real-time PCR was performed on a BioRad iCycler iQ real-timePCR Detection System using SYBR Green as a fluorescent dye. Each reaction was performed in a final volume of 25 μL with the following components: 0.2 pmoles 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: denaturing at 95°C, 5 min; 40 (denaturing at 95°C, 30 seconds; annealing at 54°C, 30 seconds; amplification at 72°C, 15 seconds). The specificity of the PCR amplification was examined by a melting curve program (55–100°C with a heating rate of 0.5°C/s). The 18S rRNA was used as controls with the primers, 18SrRNAF (5′-TCAACTTTCGATGGTAGGATAGTG-3′) and 18SrRNAR (5′-CCGTGTCAGGATTGGGTAATTT-3′). Expression of this gene was measured and shown to be constitutive under all the conditions used in this work. Primers, ARSF1 (5′-ATGGGTGCCCTCGCGGTGTTC-3′) and ARSR1 (5′-GTAGCGGATGTACTTGTGCAG-3′), were designed specifically for Ars cDNA. The amplification rate of each transcript (Ct) was calculated by the PCR Baseline 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 [46].

3. Results

3.1. The −291/−254 Region Is Essential for Fe-Deficiency-Mediated Induction of FTR1 Expression. To study the promoter region of *FTR1*, using cDNA information of *FTR1* (GeneBank accession number AF478411) and sequence information from the JGI *Chlamydomonas* genomic database (http://genome.jgi-psf.org/), we first cloned a 1237 bp (−1179 to +58) upstream fragment of *FTR1* into pBluescript II SK(+). The resulting plasmid was used to generate a series of 5′ end nested deletion constructs (Figure 1, Ftr1179 to Ftr24). Deletion constructs from this manipulation were delivered into *C. reinhardtii* CC425 by cotransformation with pArg7.8 [43, 44] and the response of these constructs to different iron concentrations was analyzed.

Our results showed that a region spanning nucleotide −291 to −254 was essential for the induction of *FTR1* under Fe-deficiency condition (Figure 1, Ftr1179 to Ftr24). Consistent with this notion, removal of the −291/−254 region abolished the low Fe-mediated induction of the reporter gene (Figure 1, FtrD5).

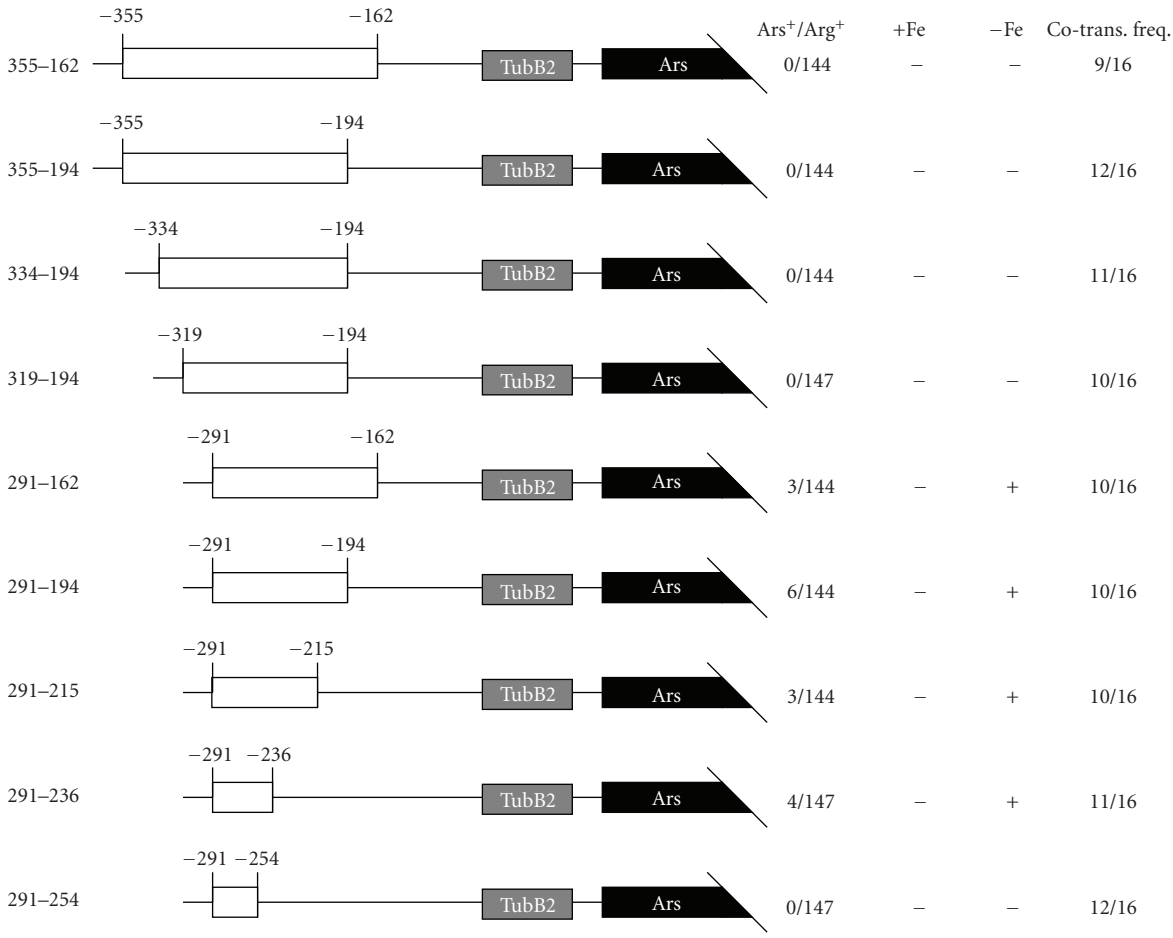
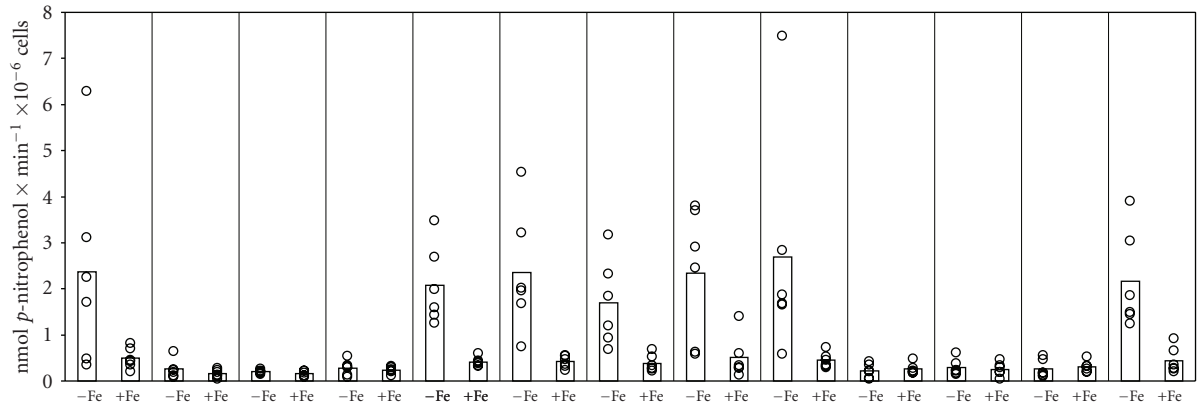


FIGURE 2: 5′ and 3′ deletion analysis of the *FTR1* −355/−162 promoter region. A series of 5′ and 3′ deletions from nucleotide −355 to −162 of the *FTR1* promoter region were amplified by PCR and fused to the Ars reporter gene in pJD100 and cotransformed into the arginine requiring *C. reinhardtii* strain CC425 with pArg7.8. Arginine independent colonies were transferred to +/− Fe TAP plates and sprayed with 10 mM XSO_4 to visualize arylsulfatase activity. 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* ß2-tubulin gene [42]. The cotransformation frequency was tested by PCR from 16 random selected Arg independent transformants.

3.2. The FeREs of FTR1 Localize in the −291/−236 Region and a Negative FeRE Localizes in the −319/−292 Region. To verify that the −291/−254 region is sufficient for iron responsive gene expression, a series of 3′ deletion constructs were generated as described in Figure 2 (355–162, 355–194, 334–194, 319–194, 291–162, 291–194, 291–215, 291–236, and 291–254). These constructs were fused to the arylsulfatase reporter gene harbored by pJD100, which is driven by a basal promoter element derived from the *Chlamydomonas* ß2-tubulin gene [42]. Verified constructs were delivered into *C. reinhardtii* CC425 by cotransformation with pArg7.8 and the response of the resulting strains to different iron concentrations was analyzed. The results indicated that the −291/−254 fragment was not sufficient to confer promoter activity under low Fe condition (Figure 2, 291–254, 0/147 of Ars^+/Arg^+). Instead, promoter activity requires a region that spans −291 to −236 (Figure 2, 291–236, 4/147). Furthermore, our analyses indicated that this region contains two FeREs, one in the −291/−254 region and the other in −253/−236 region.

Interestingly, comparing promoter activity of regions 291–194 and 319–194, 334–194 and 355–194 (Figure 2, 291–194, 319–194, 334–194, and 355–194) revealed a potential negative FeRE in the −319/−292 region. The existence of this element is also clear when promoter activity between regions 291–162 and 355–162 was compared (Figure 2, 291–162 and 355–162).

3.3. Scanning Mutagenesis Analysis of the −291/−236 and the −319/−292 Regions of FTR1. After localizing the positive FeREs to the −291/−236 region and the negative FeRE to the



Constructs	(−291/−236)	Ars Activities −Fe Average ± SD	Ars Activities +Fe Average ± SD	N	Fe Resp.	Co-Trans.Freq.
Ftr291/236	TCACATGCAGGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	2.17 ± 1.07	0.45 ± 0.28	6	Yes	11/16
FtrM291F	GACAATGCAGGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	2.37 ± 2.19	0.49 ± 0.23	6	Yes	
FtrM287F	TCACCGTCAGGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	0.26 ± 0.20	0.16 ± 0.09	5	No	10/16
FtrM284F	TCACATGACTGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	0.21 ± 0.04	0.16 ± 0.06	4	No	12/16
FtrM281F	TCACATGCAGTAGCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	0.28 ± 0.16	0.23 ± 0.07	4	No	11/16
FtrM278F	TCACATGCAGGCTAAGCCTGACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	2.07 ± 0.86	0.41 ± 0.10	6	Yes	
FtrM275F	TCACATGCAGGCTCCTAAGTACCGACCGGGCACGCGCGAAGCGATTGCCAGAGCGC	2.36 ± 1.33	0.42 ± 0.13	6	Yes	
FtrM271F	TCACATGCAGGCTCCTCCTGCAATCAAGGGCACGCGCGAAGCGATTGCCAGAGCGC	1.69 ± 0.94	0.38 ± 0.18	6	Yes	
FtrM264F	TCACATGCAGGCTCCTCCTGACCGACCTTTACCGCGCGAAGCGATTGCCAGAGCGC	2.34 ± 1.44	0.51 ± 0.46	6	Yes	
FtrM259F	TCACATGCAGGCTCCTCCTGACCGACCGGGCAATATATAAGCGATTGCCAGAGCGC	2.69 ± 2.46	0.45 ± 0.17	6	Yes	
FtrM248R	TCACATGCAGGCTCCTCCTGACCGACCGGGCACGCGCGCCTATCTTGCCAGAGCGC	0.22 ± 0.15	0.26 ± 0.12	6	No	10/16
FtrM242R	TCACATGCAGGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGAGGTAACGAGCGC	0.29 ± 0.17	0.25 ± 0.15	4	No	10/16
FtrM236R	TCACATGCAGGCTCCTCCTGACCGACCGGGCACGCGCGAAGCGATTGCCATCTATA	0.27 ± 0.19	0.31 ± 0.12	6	No	12/16

FIGURE 3: Scanning mutagenesis analysis of the *FTR1* −291/−236 region. A series of nucleotide substitutions were introduced into the −291/−236 region. The substituted nucleotides are underlined and red letters are the sequence of FtrFeRE1; and FtrFeRE2. 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 cotransformation frequency of the constructs was determined by PCR with 16 Arg independent transformants tested in each line.

−319/−292 region, we performed a scanning mutagenesis to further identify the core sequence of the regulatory elements.

In the −291/−236 region, constructs FtrM287F, FtrM284, FtrM281F, FtrM248R, FtrM242R and FtrM236R displayed relatively lower arylsulfatase (Ars) activity than the control 291–236 (construct with original nucleotide sequence from −291 to −236) under low Fe (0 μM) conditions (Figure 3, 0.26, 0.21, 0.28, 0.22, 0.29 and 0.27 compared with 2.17 nmol *p*-nitrophenol × min^{-1}× 10^{-6} cells). These mutations did not lead to significant changes in Ars activity under low or high Fe (18 μM) conditions. On the other hand, in the constructs FtrM291F, FtrM278F, FtrM275F, FtrM271F, FtrM264F and FtrM259F, low Fe induced Ars activity to 4 to 6 times higher than high Fe condition and such activity was indistinguishable from the control (Figure 3, 2.37, 2.07, 2.36, 1.69, 2.34 and 2.69 compared with 2.17 nmol *p*-nitrophenol × min^{-1}× 10^{-6} cells). These results suggest that the sequence from −287 to −279 of ATGCAGGCT and the sequence from −258 to −236 (AAGCGATTGCCAGAGCGC) were core sequences of FeREs that allowed the promoter to respond to Fe-deficient induction. We designated these elements as FtrFeRE1(ATGCAGGCT) and FtrFeRE2 (AAGCGATTGCCAGAGCGC), respectively.

In the −319/−292 region constructs FtrM319F and FtrM313F, we observed lower promoter activities under Fe-deficient conditions as control 319–194 (Figure 4, 0.28, 0.28, and 0.26 nmol *p*-nitrophenol × min^{-1}× 10^{-6} cells). Neither construct responded to low Fe levels, but both exhibited similar activities in media containing low or high Fe levels (Figure 4). However, constructs FtrM306F and FtrM299F remained highly responsive to low Fe conditions in a way similar to construct 291–194(Figure 4). These results suggest that sequence from −306 to −292 of AGTAACTGTTAAGCC is critical for Fe-deficiency negative regulation, and this element was designated as FtrFeRE3.

3.4. Fe-Regulated FTR1 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 1). We selected several transformants containing the appropriate constructs for analyzing mRNA levels as well as Ars activity. Our results indicated that a significant amount of Ars mRNA was accumulated in transformants containing Ftr1179F, Ftr291F, 291–194, or FtrM291F, but not in those containing Ftr253, 319-194, or FtrM287F. These results are in good agreement with the data obtained by Ars activity assay, indicating that the Ars activity is a true measurement of the transcriptional level in response to iron deficient induction

and that the observed iron regulation of gene expression occurs at the transcriptional level.

4. Discussion

In this study, we analyzed the Fe-deficiency-inducible gene *FTR1* using deletion and scanning mutagenesis methods. FTR1 originally was identified as an Fe transporter that forms a complex with FOX1 for transferring Ferric iron across the plasma membrane into the cytosol. The *FTR1* gene was believed to be involved in Fe-deficiency response in *Chlamydomonas* [34, 36]. For example, under low Fe condition, its mRNA accumulates remarkably higher. Our results indicate that the −291/−236 region is necessary and sufficient for Fe-deficiency-induced gene expression, and that the −319/−292 region contains a negative FeRE (Figures 1 and 2). Further studies of the −291/−236 region by scanning mutagenesis analysis revealed that one of the FeRE localized to −287/−279 with the sequence of ATGCAGGCT, which we designated as FtrFeRE1. The other FeRE localized in −253/−236 with the sequence AAGCGATTGCCAGAGCGC, which we designated as FtrFeRE2 (Figure 3). Similarly, analysis of the −319/−292 region identified a novel negative FeRE (FtrFeRE3) in −306/−292 with the sequence AGTAACTGTTAAGCC (Figure 4).

Sequence analysis of FtrFeRE1 and FtrFeRE2 shows that the FtrFeRE1 contains a TGGCA-type-like FeRE with the sequence of TG-CA and the reverse complement sequence of FtrFeRE2 contains the sequence TGGCA, suggesting that the positive FeRE in *FTR1* belongs to TGGCA-type-like FeRE (Figure 5).

In the previous studies, two of the Fox1-like type FeREs have been found in the −789/−283 (CACA**T**G) and the −261/−255 (CACGCG) regions by investigating the promoter of *FTR1* gene [38]. However, deletion of these regions from the promoter by overlapping extension PCR did not affect the iron-deficiency-inducible expression, suggesting that the sequence of CACA**T**G was not sufficient to *FTR1* gene. Interestingly, CACA**C**G, the consensus sequence of the FOX1 FeRE1 differs to CACA**T**G by only one nucleotide, implying that the nucleotide C is critical to the activities of the Fox1-type-like FeREs. In addition, the results in this study revealed that the TGGCA-type-like FeRE is a *cis*-acting element in iron-deficiency induction of the *FTR1* gene.

In *S. cerevisiae*, iron homeostasis is maintained primarily through transcriptional control of gene expression. In response to variations of iron availability, the transcriptional regulator Aft1p/Aft2p directs the expression of a series of genes to control iron transport and subcellular compartmentalization. Promoters of these genes contain the consensus element CACCC recognized by the regulatory protein [17]. In contrast, little information is available about the positive transcriptional gene regulation in *Chlamydomonas* [47, 48]. Similarly, only one report about a negative control region, the 358 bp silencer region in *CAH1* gene is in response to low CO2 induction has, been published [49]. In *Cyanobacterium, Synechococcus* sp. PCC 7942, a negative regulatory element has been shown in the promoter region of *cmpA*, which encodes a 42-kD low-CO2-inducible protein [50]. Although *Chlamydomonas* and *S. cerevisiae* share a similar iron assimilation pathway when Fe is scarce, *Chlamydomonas* genome does not harbor homologs of Aft1p/Aft2p. These observations suggest that despite these two organisms use of a similar enzyme to regulate the affinity iron uptake under iron deficient conditions, their regulation mechanisms of low Fe response may be different. In our previous studies, we reported two types of FeREs, one in *FOX1* gene with the core sequence C(A/G)C(A/G)**C**(G/T) in two of the promoter regions [38], the other in *FEA1* and *ATX1* with the core sequence TGGCA in two of the regions [39, 40]. These results suggest the existence of at least two potentially independent regulation mechanisms in response to Fe-deficiency in Chlamydomonas. The identification of a novel negative FeRE in this paper reveals the complex regulation mechanisms of Fe-deficiency-mediated gene expression in Chlamydomonas.

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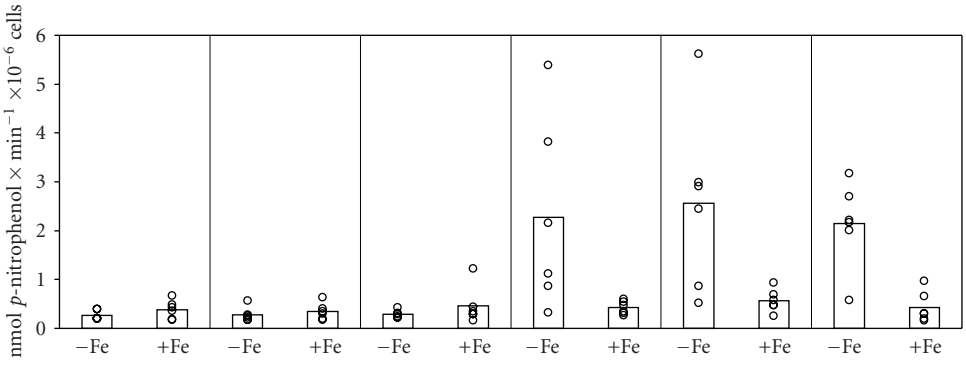

Constructs	(−319/−292)	(−291/−194)	Ars Activities −Fe Average ± SD	Ars Activities +Fe Average ± SD	N	Fe Resp.	Co-Trans.Freq.
319/194	TCACTCGCCCGGCAGTAACTGTTAAGCC	TCACA.....ATGTC	0.26 ± 0.10	0.38 ± 0.19	4	No	10/16
FtrM319F	GGCAGAGCCCGGCAGTAACTGTTAAGCC	TCACA.....ATGTC	0.28 ± 0.15	0.34 ± 0.17	5	No	9/16
FtrM313F	TCACTCTAAATTAAGTAACTGTTAAGCC	TCACA.....ATGTC	0.28 ± 0.08	0.46 ± 0.39	4	No	12/16
FtrM306F	TCACTCGCCCGGCCTGCCAGGTTAAGCC	TCACA.....ATGTC	2.28 ± 1.96	0.43 ± 0.13	6	Yes	
FtrM299F	TCACTCGCCCGGCAGTAACTTGGCCTAA	TCACA.....ATGTC	2.56 ± 1.83	0.57 ± 0.23	6	Yes	
291/194		TCACA.....ATGTC	2.14 ± 0.88	0.43 ± 0.32	6	Yes	10/16

Figure 4: Scanning mutagenesis analysis of the *FTR1* −319/−292 region. A series of nucleotide substitutions were introduced into the −319/−292 region. The changed nucleotides are underlined. The conservation sequence of FtrFeRE3 is highlighted in red. The Ars activities (nmol *p*-nitrophenol × min^{-1}× 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 indicate the median values. N represents the number of the transformants examined for the activity. The cotransformation frequency of the constructs was detected by PCR with 16 Arg independent transformants tested in each line.

```
 FtrFeRE1 -297/-276          TAAGCCCACATG-CAGGCTCCT
 FtrFeRE2 -254/-233  R.C     CACGGCGCTCTGGCAATCGCTT
 FeaFeRE1 -277/-256          ACGACTGCGGTGGCAAAGTTGG
FeaFeRE2 -105/-84          CCGCCGCGGCTGGCACCAGCCT
AtxFeRE1 -532/-511          GGAGTCGCACTGGCATGTGCCC
AtxFeRE2 -303/-282          GGAGCAGCGATGGCATTTATTA
```

Figure 5: Sequence comparison of FtrFeRE1 and FtrFeRE2, FeaFeRE1 and FeaFeRE2, AtxFeRE1 and AtxFeRE2. White letters on black background indicate the conserved sequences TGGCA. 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 *).

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