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 (ATGCA GGCT) at −287/−279 and the FtrFeRE2 (AAGCGATTGCCA GAGCGC) 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 Fe3+ is reduced by FRE1/FRE2 to Fe2+ and is subsequently reoxidized to Fe3+ 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 Fe2+ transporter that also transports Cu+ and Zn2+ 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 gene when iron is abundant [25]. In barley, IDS1 and IDS2 induced specifically by iron-deficiency stress are gene when iron is abundant [25].

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 \(\mu\)g/mL arginine [41], and transformants were grown in either +Fe (18 \(\mu\)M Fe) or −Fe (0 \(\mu\)M Fe) TAP medium. Liquid cultures were grown under continuous light of 150 \(\mu\)mol \(m^{-2}\times s^{-1}\) at 25°C in shaking conditions of 250 rpm. Strains on TAP-agar plates were incubated at a light intensity of 100 \(\mu\)mol \(m^{-2}\times s^{-1}\) 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 *SalI/KpnI* sites of pJD54 or in *KpnI* 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 ampl efied 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 \(\times\) 10^5 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 \(\times\) 10^6 cells/mL. DNA (2 \(\mu\)g of construct and 10 \(\mu\)g pARG7.8 each) and cells (400 \(\mu\)L) were mixed with polyethylene glycol (100 \(\mu\)L 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.
### 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 ARS junction with a reverse primer ArsR(5′-TTCTGATGCTTCTCTCTGTC-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 of the culture supernatant was added to 500 μL of 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 × 10⁶ cell/mL.

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

| 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 |
| 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          | 5       | 3.49       | 0.55       | 6       |
| 291–194    | 133       | 26         | 5       | 3.49       | 0.55       | 6       |
| FtrM291F   | 186       | 29         | 6       | 5.41       | 0.81       | 7       |
| FtrM287F   | 186       | 29         | 6       | 5.41       | 0.81       | 7       |

Ars mRNA levels were assessed by real-time PCR in −Fe (0 μM) and +Fe (18 μM) conditions. Data were calculated by the 2ΔΔCt 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′Primer | The position |
|------------|-----------|----------|--------------|
| Ftr1179    | 1179F TCGTACCTTACTGCGCTTACTGCA | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −1179/+58 |
| Ftr917     | 917F TAAGTACCCAGAATAACCATGACGGC | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −917/+58 |
| Ftr718     | 718F TAAGTACCCCGAGTACCCGGCCTCG | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −718/+58 |
| Ftr511     | 511F CTGTGATCCGCCCGCAGCGTGAAT | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −511/+58 |
| Ftr355     | 355F ATTTGATCCGGACACCTTGTCCCA | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −355/+58 |
| Ftr291     | 291F GACGTAACCTCATTGACGGCTCTCCT | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −291/+58 |
| Ftr253     | 253F TAAGTACCAAGCTGCGAGAGGCAAGG | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −253/+58 |
| Ftr161     | 161F TAAGTACCAATGGGACCGATTAGG | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −161/+58 |
| Ftr59      | 59F TAAGTACCCAGGACGAGTGAACCG | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −59/+58 |
| Ftr24      | 24F ATCGTACCATGATACACTCTAAC | 58R GAAGTCGAC CGTCCTGATGAGAAAGG | −24/+58 |
| 355–162    | 355F GAACGTCGCCAGCACCATGCTCCACCG | 162R GAACGTCGCCAGCATGCTCCCTATGG | −355/-162 |
| 355–194    | 355F GAACGTCGCCAGCACCATGCTCCACCG | 194R GAACGTCGCCAGCATGCTCCCTATGG | −355/-194 |
| 334–194    | 334F GAACGTCGCCAGCACCATGCTCCACCG | 194R GAACGTCGCCAGCATGCTCCCTATGG | −334/-194 |
| 291–162    | 291F GAACGTCGCCAGCACCATGCTCCACCG | 162R GAACGTCGCCAGCATGCTCCCTATGG | −291/-162 |
| 291–194    | 291F GAACGTCGCCAGCACCATGCTCCACCG | 194R GAACGTCGCCAGCATGCTCCCTATGG | −291/-194 |
| 291–215    | 291F GAACGTCGCCAGCACCATGCTCCACCG | 215R GAACGTCGCCAGCATGCTCCCTATGG | −291/-215 |
| 291–236    | 291F GAACGTCGCCAGCACCATGCTCCACCG | 236R GAACGTCGCCAGCATGCTCCCTATGG | −291/-236 |
| 291–254    | 291F GAACGTCGCCAGCACCATGCTCCACCG | 254R GAACGTCGCCAGCATGCTCCCTATGG | −291/-254 |
| FtrD5(overlap extension PCR) | A,1179F AAGGTACCCGGAATCCCTAAAGTGGG | B,291R GAAATCGAGTGTCGTTACCTGATTG | −1179/+58 with removal |

Primers The position
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, 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 25μL with the following components: 0.2 pmoles of each primer, 1μL of cDNA, 12.5μL of SYBR Green Mix (Invitrogen SYBR Greenr QPCR), and water was used to adjust the volume to 25μL. 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, 18SrRNA (5’-TCAACTTTCGATGAGATGAG-3’) and 18SrRNAR (5’-CCGTGTCAGGATTGGTATTT-3’). Expression of this gene was measured and shown to be constitutive under all the conditions used in this work. Primers, ARSF1 (5’-ATGGGTGACGGATGAGATGAG-3’) and ARSR1 (5’-GTAGCGGATGACTTTGTGCAG-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−ΔΔCT) 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 fragment from −291 to +58 of FTR1. The cotransformation frequency was tested by PCR from 16 random selected constructs. 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). 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 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.

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 XSO4 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 ARSR1 (5′-ATGGGTGCCCTCGCAG-3′) was essential for the induction of expression of the arylsulfatase gene. The cotransformation frequency was tested by PCR from 16 random selected Arg independent transformants.
3.2. The FeREs of FTR1 Localize in the \(-291/\sim236\) Region and a Negative FeRE Localizes in the \(-319/\sim292\) Region. To verify that the \(-291/\sim254\) 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 \textit{Chlamydomonas} \(\beta2\)-tubulin gene \[42\]. Verified constructs were delivered into \textit{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 \(-291/\sim254\) 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 \(-291/\sim254\) region and the other in \(-253/\sim236\) 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 \(-319/\sim292\) 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 \(-291/\sim236\) and the \(-319/\sim292\) Regions of FTR1. After localizing the positive FeREs to the \(-291/\sim236\) region and the negative FeRE to the
−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, 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, FtrM259F, FtrM242R, and FtrM236R, 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 ATGACAGGCCT is critical for Fe-deficiency negative regulation, and this element was designated as Ftr-FeRE3.

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.

**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) displayed relatively lower arylsulfatase (Ars) activity than the control 291–236 (AAGCGATTGCCAGAGCGC) were determined by PCR with 16 Arg independent transformants tested in each line. 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.
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 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 ATGCAGGCT, which we designated as FtrFeRE1. The other FeRE localized in −253/−236 with the sequence AACGGATTGCAGGCG, 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 AGTACTGTTAAGCC (Figure 4).

Sequence analysis of FtrFeRE1 and FtrFeRE2 shows that the FtrFeRE1 contains a TGGCA-type-like FeRE with the sequence of ATGCAGGCT, belonging to TGGCA-type-like FeRE.

In the previous studies, two of the Fox1-like type FeREs have been found in the −789/−283 (CACATG) and the −261/−255 (CACCGG) 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 CACATG was not sufficient to FTR1 gene. Interestingly, CACCG, the consensus sequence of the FOX1 FeRE1 differs to CACATG 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 positive 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)(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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was detected by PCR with 16 Arg independent transformants tested in each line.

**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 determined by PCR with 16 Arg independent transformants tested in each line.

| Constructs | (−319/−292) | (−291/−294) | −Fe | +Fe |
|------------|-------------|-------------|-----|-----|
| 319/194    | TCACCTGGCCCGAGTAACTTAAAGCC | TCACA....ATGTC | 0.26 ± 0.10 | 0.38 ± 0.19 |
| FtrM319F   | GGCAGAAGCCGCGAGTAACTTAAAGCC | TCACA....ATGTC | 0.28 ± 0.15 | 0.34 ± 0.17 |
| FtrM33F    | TCACCTGGCCCGAGTAACTTAAAGCC | TCACA....ATGTC | 0.28 ± 0.08 | 0.46 ± 0.39 |
| FtrM36F    | TCACCTGGCCCGAGTAACTTAAAGCC | TCACA....ATGTC | 2.28 ± 1.96 | 0.43 ± 0.13 |
| FtrM299F   | TCACCTGGCCCGAGTAACTTAAAGCC | TCACA....ATGTC | 2.56 ± 1.83 | 0.57 ± 0.23 |
| 291/194    | TCACCTGGCCCGAGTAACTTAAAGCC | TCACA....ATGTC | 2.14 ± 0.83 | 0.43 ± 0.32 |

**Figure 5:** Sequence comparison of FtrFeRE1 and FtrFeRE2, FaeFeRE1 and FaeFeRE2, 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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