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A putative protein kinase Femu9p contributes to the iron deficiency-inducible expression of *FOX1* gene in *Chlamydomonas reinhardtii*

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Abstract

FOX1 gene encoding a multicopper ferroxidase is vital in iron metabolism in the single-celled green algae *Chlamydomonas reinhardtii*. Molecular characteristics of this gene have been extensively studied. However, little information is available about its regulatory factors in *C. reinhardtii*. In this study, we isolated a regulatory mutant of *FOX1* gene by using an arylsulfatase gene driven by the *FOX1* promoter. The corresponding gene was also identified via thermal asymmetric interlaced polymerase chain reaction and named *Femu9p*. Gene structure analysis shows that the *Femu9p* gene encodes a protein of 757 amino acids which was predicted to contain a protein kinase domain. We demonstrate that *Femu9p* is essential for the regulation of *FOX1* gene expression because *Femu9p* RNAi silencing leads to a significant decrease in *FOX1* promoter activity. Moreover, our analysis indicates that *Femu9p* positively affects total chlorophyll (Ch a+b) content and the regulation of other iron-uptake-related endogenous gene expressions such as *FTR1*, *FEA1*, *ATX,* and *NRAMP2* in *C. reinhardtii*.

Keywords: *Chlamydomonas reinhardtii*, *FOX1* gene, protein kinase, regulatory mutant, RNAi silencing.
Abbreviations: ARS-arylsulfatase, FOX1-multicopper ferroxidase, XSO_4- 5-bromo-4-chloro-3-indolyl sulfate, TAIL PCR-thermal asymmetric interlaced polymerase chain reaction.

Introduction

Iron is an essential micronutrient because it acts as an enzyme cofactor that catalyzes redox reactions in fundamental metabolic processes such as respiration and photosynthesis (Moseley et al., 2002). Iron is abundant in the environment. However, it is mostly present in the insoluble ferric (Fe^{3+}) state, resulting in the survival of numerous organisms under iron-deficient conditions in vast regions. In order to meet cellular metabolic requirements, multiple pathways are employed for iron uptake, distribution, and utilization in plants and microorganisms (Eckhardt and Buckhout, 1998; Curie et al., 2001). In higher plants, two major strategies to acquire iron have been described. Dicotyledons and nongraminaceous monocotyledons use strategy I, Fe^{3+} is solubilized in the rhizosphere by the iron-deficiency induced H^+-ATPase, and then it is reduced to Fe^{2+} by an iron reductase. Subsequently, Fe^{2+} is absorbed and is transported by a series of cellular iron transport proteins (Yi et al., 1996; Fox et al., 1998; Robinson et al., 1999). Grasses (graminaceous monocots) use another strategy for iron uptake, in which phytosiderophores are secreted into the rhizosphere and chelate Fe^{3+}, the resulting complexes are transported into the root cells by specific transport systems (Takagi et al., 1984; Römheld and Marschner, 1986). On the molecular level, a high-affinity iron uptake system is best characterized in *Saccharomyces cerevisiae*. Under iron deficiency conditions, the yeast metalloreductases FRE1/FRE2 reduce Fe^{3+} to Fe^{2+}, which is subsequently reoxidized by the plasma membrane multicopper oxidase (FET3). The produced Fe^{3+} is transported into the cell by the iron permease FTR1, which contains seven membrane-spanning helices (Askwith et al., 1994; Martins et al., 1998; Severance et al., 2004).

Evidence has shown that E185 and D409 of FET3 and a DASE motif in the extracellular loop 6 of FTR1 are required for Fe^{3+} trafficking from FET3 to FTR1 in *S. cerevisiae* (Severance et al., 2004; Kwok et al., 2006). The model photosynthetic eukaryote *Chlamydomonas reinhardtii* acquires iron in a similar manner as that of *S. cerevisiae* (Terzulli and Kosman, 2010). Several components involved in high-affinity iron assimilation pathway were identified, including a multicopper ferroxidase (FOX1), an iron permease (FTR1), a copper chaperone (ATX1), a copper-transporting ATPase, a ferritin (FER1), and FEA1 (previously named H43) (Fontaine et al., 2002; Rubinelli et al., 2002). Other genes involved in iron transport were also identified by using genomic analysis, including the cation diffusion facilitator family (*MTP1* to *MTP5*), the ZIP family (*ZIP1* to *ZIP14*), and the natural resistance-associated macrophage protein (NRAMP) family (*NRAMP1* to *NRAMP3*) (Hanikenne et al., 2005; Allen et al., 2007). The promoter elements that control expression of *FOX1*, *ATX1*, *FEA1*, and *FTR1* in *C. reinhardtii* were studied in detail (Deng and Eriksson, 2007; Fei and Deng, 2007; Fei et al., 2009; Fei et al., 2010). However, the regulatory mechanisms of these genes remain unclear in *C. reinhardtii*.

FOX1 is anchored in the plasma membrane by a single amino-terminal membrane-spanning helix. It constitutes a high-affinity iron import complex with FTR1 together in *C. reinhardtii* (Terzulli and Kosman, 2010). Two iron-responsive elements, the FeRE1 at the -87/-82 regions (CACACG) and FeRE2 at the -65/-60 regions (CACGCG) from the transcription start site, were also identified in *FOX1* gene (Deng and Eriksson, 2007). In this study, plasmid insertional mutagenesis of *C. reinhardtii* was performed to isolate regulatory factors that control *FOX1* gene expression. The *C. reinhardtii* strain 2A38 harboring an arylsulfatase (ARS) reporter gene driven by the *FOX1* promoter was transformed with plasmid pSP124S, which confers zeocin resistance. Based on loss of the *FOX1* promoter activity, we isolated a regulatory mutant *mu9* and identified a putative protein kinase gene *Femu9p* that contributes to the iron deficiency-inducible expression of *FOX1* gene. *C. reinhardtii* is proposed to use an iron uptake system similar to that of strategy I plants (Eckhardt and Buckhout, 1998). Therefore, our studies provide new insight regarding the regulation of iron uptake-related gene in higher plants.

Results

Isolation of regulatory mutants of FOX1

In our previous study, we generated a transgenic strain *C. reinhardtii 2A38*, in which the promoter activity of *FOX1* gene is monitored by ARS enzyme activity (Deng and Eriksson, 2007). In the present study, *C. reinhardtii 2A38* was treated as the host strain, and transformed with KpnI-linearized plasmid pSP124S, which contains the *ble* gene that confers resistance to the antibiotic zeocin (bleomycin). Finally, we screened up to 50,000 plasmid insertional mutants and observed that 68 colonies exhibited little or no ARS activity under iron-deficient conditions (Fig 1), suggesting that plasmid insertion affected genes involved in controlling *FOX1* gene expression at the transcriptional level. Only the mutant *mu9* is described hereafter.

Isolation and sequencing of flanking DNA

The flanking DNA was amplified from the *mu9* strain by using Tail-PCR analysis as described by Liu et al. (1995). Our result showed that the plasmid was inserted from the site of 982, 186 on chromosome 7 (Fig 2). In order to identify the DNA sequence length that was disrupted by mutagenesis, six genes around the *ble* insertion site including *182468*, *205870*, *187498*, *296161*, *391725*, and *142766* [protein ID in *C. reinhardtii* database of DOE Joint Genome Institute (JGI)], were amplified by using DNA of the mutant strain and *C. reinhardtii* 2A38 as template. The results showed that target bands of *182468*, *296161*, *391725*, and *142766* could be amplified in both *C. reinhardtii* 2A38 and the mutant strain. However, genes *205870* and *187498* could only be amplified in *C. reinhardtii* 2A38 (Fig 2), suggesting that the plasmid insertion deleted two genes *205870* and *187498*. In the subsequent experiment, RNAi silencing vectors of *205870* and *187498* were constructed and then transformed into *C. reinhardtii* 2A38. However, no significant differences in ARS enzyme activity were observed in *205870* RNAi silencing lines (data not shown), suggesting that *205870* was not a candidate gene to activate the *FOX1* promoter. Thus, only gene *187498*, which is designed as *Femu9p*, is considered hereafter.

Femu9p bioinformatics analysis

Data from the JGI *C. reinhardtii* database showed that *Femu9p* cDNA comprises a 2274 bp open reading frame and a 414 bp 3'-untranslated region. The 5'-upstream region was not identified. Gene structure analysis showed that the cDNA was predicted to encode a protein of 757 amino acid residues with a molecular mass of 80.6 kD and a pI value of 6.72. Our analysis also indicated that *Femu9p* was predicted to contain a conserved protein kinase domain on the region of 401 to 656 amino acid residue by using PROSITE program (Fig 3A). A similarity search on the GenBank database using the predicted amino acid sequence revealed that except for the protein kinase domain, the other *Femu9p* region shows no sequence similarity with any other proteins (Fig 3B). The conserved domain exhibited higher levels of similarity with that of green algae such as *Volvox carteri* and *Chlorella variabilis* (54% and 39% identity, respectively) (Fig 3B). By contrast, other animal, plant, and bacterium kinase proteins exhibited lower degree of similarities.

Loss of FOX1 promoter activity due to RNAi-mediated Femu9p silencing under iron-deficient conditions in C. reinhardtii 2A38

To confirm that *Femu9p* is vital for regulating *FOX1* gene expression, the RNAi plasmid vector pMaa7IR/*Femu9p*IR was constructed and transformed into *C. reinhardtii* 2A38. A total of 125 *Femu9p* RNAi lines were generated. Five of those exhibited little ARS enzyme activities were selected for further analysis including quantitative analysis of ARS enzyme activity and examination of *Femu9p* mRNA levels. Wild-type *C. reinhardtii* CC425 served as a negative control. *C. reinhardtii* 2A38 and those transformed with the empty vector Maa7/XIR (Maa7-), were used as positive controls. In positive-control experiments, the cultures became dark blue after the addition of 5-bromo-4-chloro-3-indolyl sulfate (XSO_4) into the media without Fe (Fig 4A), indicating a normal induction of the ARS enzyme activity under iron-deficient conditions in *C. reinhardtii*. In contrast, cultures from the *Femu9p* RNAi lines became light blue, similar to those of the *mu9* strain (Fig 4A). Quantitative analysis of ARS enzyme activity also showed that *Femu9p* silencing led to a significant decrease by 76% (IR-2), 76% (IR-4), 89% (IR-6), 85% (IR-7), and 62% (IR-11), respectively (Fig 4B). These results demonstrated that impaired ARS enzyme activation occurred after *Femu9p* silencing in *C. renhardtii* 2A38. On the other hand, RNAi lines showed a consistent 46% to 65% reduction in *Femu9p* transcripts that were measured under iron-deficient conditions (Fig 5), revealing high-efficiency *Femu9p* silencing in *C. reinhardtii* 2A38. These data also suggested that *Femu9p* gene silencing resulted in loss of the *FOX1* promoter activation because the expression of *ARS* gene is driven by the *FOX1* promoter in *C. reinhardtii* 2A38.

Femu9p affects chlorophyll (Chl a+b) concentration in C. reinhardtii

To determine whether *Femu9p* expression affected chlorophyll concentration, total chlorophyll (Ch a+b) content was measured in *Femu9p* RNAi lines under iron-deficient conditions. As shown in Fig 6, compared with the control, the chlorophyll content showed a 3.5-fold decrease in the *mu9* strain, and 1.3- to 2.9-fold decrease in five RNAi lines (Fig 6). The results suggested that the *Femu9p* gene expression is associated with chlorophyll concentration in *C. reinhardtii*.

Femu9p is essential in endogenous FOX1, FTR1, FEA1, ATX1, and NRAMP2 expression in C. reinhardtii

To determine whether *Femu9p* gene expression affected other

Table 1. Primers used for real-time RT-PCR and amplifying the genes around the *ble* insertion site.

Gene name	Primer sequence
18S gene	Forward primer: 5'-TCAACTTTCGATGGTAGGATAGTG-3';
	Reverse primer: 5'-CCGTGTCAGGATTGGGTAATTT-3'
Femu9p	Forward primer: 5'-ACCGAGACCTGAAACCCG-3';
	Reverse primer: 5'- ATAAGGACGCCCAACGAG-3'
ARS	Forward primer: 5'-ATGGGTGCCCTCGCGGTGTTC-3';
	Reverse primer: 5'-GTAGCGGATGTACTTGTGCAG-3'
FOX1	Forward primer: 5'-GACGTGGAGGCCCAGAAG-3';
	Reverse primer: 5'-CGCGACGAAGTAGGTGTTG-3'
FTR1	Forward primer: 5'-TCTTTCGGGAGACCATTGAG-3';
	Reverse primer: 5'-GAAGCATAGCAAAGCCAAGG-3'
FEA1	Forward primer: 5'-CTCAAGTACCACCTGCACGA-3';
	Reverse primer: 5'-ACATAGCTCTTGCCGAGGAA-3'
ATX1	Forward primer: 5'-AGCTCGTGTCCTCGTAAAGC-3';
	Reverse primer: 5'-CTGCAACAGGTTCCGTGTAA-3'
NRAMP2	Forward primer: 5'-CTGTCGCAGGTGATCCTGT-3';
	Reverse primer: 5'-TTTGCACCACCAGGTTAATG-3'
205870	Forward primer: 5'-GCCATGAGCCGCATCTAC-3';
	Reverse primer: 5'-GCCACTCCTCCATCCACA-3'
187498	Forward primer: 5'-ACCGAGACCTGAAACCCG-3';
	Reverse primer: 5'-ATAAGGACGCCCAACGAG-3'
182468	Forward primer: 5'-GGCGGAAGCCGACATT-3';
	Reverse primer: 5'-CCCAACTCGCAGTCCAAAT-3'
296161	Forward primer: 5'-TGAGACCATCACGGACATCG-3';
	Reverse primer: 5'-CCTCAGTGCTTATGGCGTAGC-3'
391725	Forward primer: 5'-TAACCACCGAGACCCTTTCC-3';
	Reverse primer: 5'-AGCCCGTCCAGCACCTT-3'
142766	Forward primer: 5'-ATGCGAGCCTCCAACAA-3';
	Reverse primer: 5'-TGCTGCGGACCTTGATT-3'

endogenous genes involved in high-affinity iron uptake system in *C. reinhardtii*, mRNA levels of *FOX1*, *FTR1*, *FEA1*, *ATX1*, and *NRAMP2* were measured in the *mu9* strain and the *Femu9p* RNAi line (IR-6). Maa7- was used as control. Compared with the control, mRNA levels of endogenous *FOX1* gene were decreased by 45% and 78% in *Femu9* and IR-6, respectively (Fig 7), suggesting that *Femu9p* is associated with the activation of endogenous *FOX1* expression under iron-deficient conditions. Likewise, transcript levels of other iron-absorbed related genes *FTR1*, *FEA1*, *ATX1*, and *NRAMP2* were significantly decreased after *Femu9p* gene expression inhibition (Fig 7), indicating that the *Femu9p* gene is important in iron metabolism in *C. reinhardtii*.

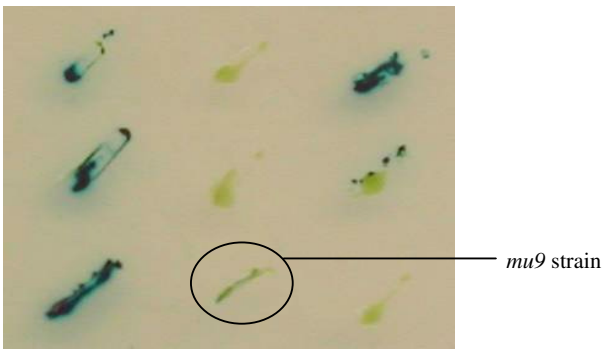


Fig 1. Generation and screening of mutants that exhibit no or little *FOX1* promoter activity.
The algae strain *C. reinhardtii 2A38* harboring ARS reporter gene driven by *FOX1* promoter was transformed with plasmid pSP124S DNA by using the glass bead method (Kindle, 1990) and the transgenic cells were plated onto the TAP agar plates with 10 μg/mL of zeocin. Seven days later, the transformants were inoculated into XSO_4-TAP agar medium without Fe. After 24 h, the mutants were selected based on the absence of a blue halo around the colonies. The black circle indicates the mutant *mu9* strain analyzed in the present study.

Discussion

C. reinhardtii expresses FOX1 homologous to a ferroxidase which is essential in iron metabolism in bacteria, fungi, algae and higher plants (Kosman, 2002; Petrak and Vvoral, 2005; Terzulli and Kosman; 2010). Deng and Eriksson (2007) have reported that two separate iron-responsive elements are necessary for iron deficiency-inducible *FOX1* gene expression. However, little information is available regarding the regulatory factors of *FOX1* gene at the transcriptional level in *C. reinhardtii*. In the present study, a promoter/reporter screening system was used for isolating the regulatory mutants of *FOX1* gene in *C. reinhardtii*. Using *ARS* gene as a reporter to examine promoter activity is effective because the gene can be easily visualized (Davies et al, 1992). The product of ARS action on XSO_4 is a blue compound (blue colonies). Based on the absence of a blue halo around the colonies, we isolated 68 regulatory mutants of *FOX1* gene under iron-deficient conditions. Among which only the mutant *mu9* was analyzed.
The TAIL-PCR result indicated that the *Femu9p* gene was likely responsible for regulating *FOX1* promoter activity (Fig 2). This finding has been confirmed by the results of *Femu9p* silencing in *C. reinhardtii* 2A38. mRNA level of *Femu9p* was reduced by approximately 65% in the best RNAi line (IR-6), in which a significant decrease by 89% in ARS enzyme activity was occurred (Fig 4 and Fig 5). The results demonstrated that *Femu9p* was crucial for the activation of the *FOX1* promoter. Considering that *Femu9p* contains a conserved protein kinase

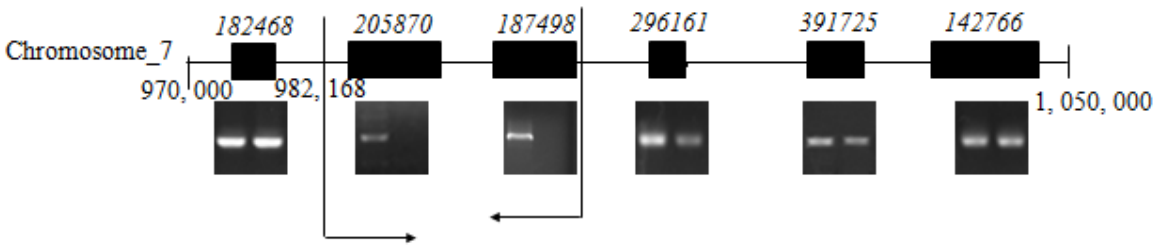
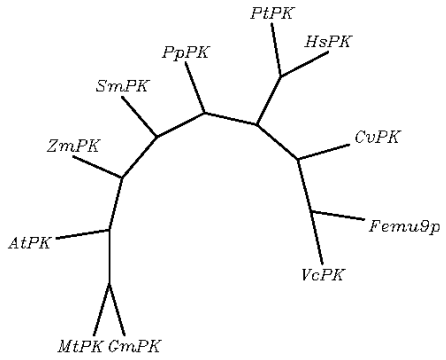


Fig 2. Schematic of the genome site that plasmid pSP124 inserted in the mutant *mu9* strain. Plasmid pSP124S was inserted at the site of 982, 186 on chromosome 7, and deleted two genes 205870 and 187498. The left-hand lane of the gel electrophoresis image is a PCR product from *C. reinhardtii* 2A38, whereas the right-hand lane is from the mutant *mu9*. The arrows show the location of deletion of genes.

MRLKGASAGLLGWTLWLCCALVRGQQAARGTVDCKNGQDIVRALQDPEVHTAYLMYNMVLSESDWAGVSTPYTLTRNFTVAKHPTLLDMPILDLSFATGHVQ
LMQAVVLSFVDLVLTRFRGDTTVAQAPGFDLLTTSQPGLVPPPVLSIVSGGLVMRACMPVAFAAMATVTMPRPPQYGGNHSVDWRLPQPACVNDSVQAPAPPLA
RCWPARGMYRDLKTYGANIDAQGSTVELSGLAEQRSTEMLMTDECAQQLGPLGCWLFMFGRASTPSPPPSASHPAGIGSAVDGTGIDPTKQPSASPADSSSGGDG
TDGSGPSSDTVAAIVGGVVGGEVVLHVDGAGGAGGMAVARLTLALLVKSRWCVYGAADEAAAPLVVVTPFTPPRADFQELDDTQAAQELRLVPVTLGKGTYGR
VMAGEYRGRRVAVKLLTDPEGMPPDQLEPFLETFAREAEVLGRCEHANVVKLLAANLRPPRVCLPGVNLLPLSTMLQISIQVARGLEYLHPTIVHRDLKPGNVLV
NGADTPERLVAKLTDFGLSRLRNTVLVTKDPEAGTPAYTAPECFDPTNYEISHQADMYSLGVLMWEMIVGERPFDGLQMVAIAYRVTVQGKRPPWPAGLERSRCP
KRLRALIEECWDPVPRRRPAAAEVVKRLMMILQSVQSKIDTSSHENSDSEHRSCDGASAAPGAGGGFGGGGARGSKRQMLPADLDGKPLPPRVMHGVDLARISA
ELAKSEQVARGGSGIADLFSRKSHRRPSN

(A)



(B)

Fig 3. Predicted amino acid sequence of *Femu9p* and sequence comparison with protein kinase domains. (A) Primary structure of *Femu9p*. The highly conserved protein kinase domain is highlighted. (B) Phylogenic relationship of protein kinase domains. The sequences were aligned by employing the ClustalW program, which uses the neighbor-joining method. VcPK (NCBI accession number XP_002955538) from *Volvox carteri f. nagariensis*; CvPK (EFN58216) from *Chlorella variabilis*; HsPK (Q02779) from *Homo sapiens*; PtPK (XP_003316399) from *Pan troglodytes*; PpPK (EFA76341) from *Polysphondylium pallidum* PN500; SmPK (XP_002970062) from *Selaginella moellendorffii*; ZmPK (NP_001147952) from *Zea mays*; AtPK (NP_568893) from *Arabidopsis thaliana*; MtPK (XP_003593589) from *Medicago truncatula*; GmPK (XP_003529330) from *Glycine max*.

domain (Fig 3), it may function as a signal transducer that affects *FOX1* promoter activity. The mitogen-activated protein kinase and c-jun n-terminal kinase-mediated signal transduction pathways have been reported to be involved in iron metabolism in *C. reinhardtii* (Yu and Richardson, 2011). In *S. cerevisiae*, the iron deficiency-inducible expression of FET3 and FTR1 were also controlled by the Snf1 protein kinase through the Aft1p transcription factor (Haurie et al., 2003). Since protein kinase usually plays a role via phosphorylation, we propose that Femu9p may interact with other proteins such as transcription factor to regulate *FOX1* gene expression. Iron scarcity could cause chlorosis (chlorophyll deficiency) in photosynthetic organisms (Moseley et al., 2002). In this study, chlorophyll content was significantly decreased after *Femu9p* silencing in *C. reinhardtii*, a model organism to study photosynthesis (Fig 6), suggesting that *Femu9p* silencing caused an impaired high-affinity iron uptake system, resulting in chlorosis in *C. reihardtii*. In addition, Compared to the control, the transcriptional levels of several iron uptake-related genes including *FOX1*, *FTR1*, *FEA1*, *ATX1*, and *NRAMP2* were reduced in the mutant *mu9* and the *Femu9p* RNAi line (IR-6) (Fig 7). *Femu9p* may act upstream signal pathways or become a potential node for crosstalk between signal pathways. The characterization of other regulatory mutants of *FOX1* gene and identification of the Femu9p targets by using one-hybrid or two-hybrid screening methods will enable us to further understand the molecular mechanisms of *FOX1* gene regulation and signal transduction under iron-deficient conditions in *C. reinhardtii*.

Materials and methods

Strains and culture conditions

In our previous study, *C. reinhardtii* CC425 (cw15 arg2) cells were cotransformed with the plasmids pARG7.8 and pJF103

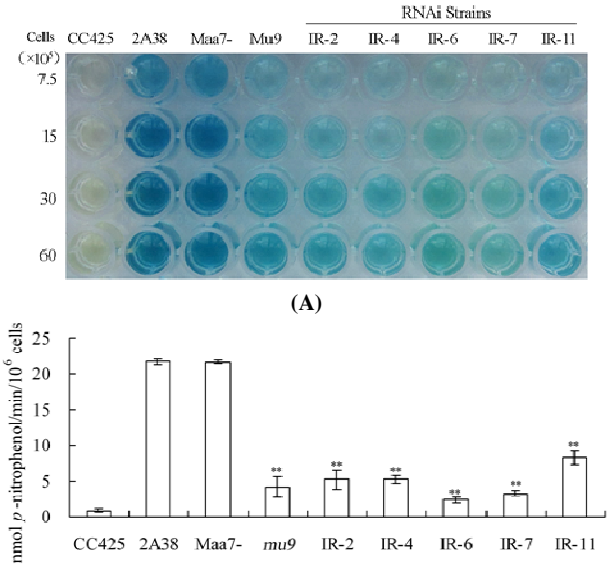


Fig 4. Qualitative and quantitative analysis of ARS enzyme activity in the *mu9* strain and *Femu9p*-RNAi lines.
(A) Qualitative analysis of ARS enzyme activity in controls and *Femu9p*-RNAi lines. The algal cells were adjusted to different concentration including 7.5×10^5, 15×10^5, 30×10^5 and 60×10^5 using TAP medium without Fe, and then incubated with XSO_4 for 30 min, respectively. The image was captured with a canon IXUS100IS camera. Maa7- is *C. reinhardtii* 2A38 transformed with the empty vector Maa7/XIR.
(B) Quantitative analysis of ARS enzyme activity in controls and *Femu9p*-RNAi lines. The data shown are means (±SD, n=3). Significance is indicated as **p < 0.01. The algal cells were incubated with Glycine-NaOH, Imidazole and p-Nitrophenyl sulfate at 27 °C for 27 min. The reaction was stopped by NaOH, and the absorbance at 410 nm was determined.

that contained the *FOX1* promoter-driven *ARS* reporter gene (Deng and Eriksson, 2007). A transformant was selected in tris-acetate-phosphate (TAP) medium without arginine and was labeled *C. reinhardtii* 2A38 (Deng and Eriksson, 2007). In the present study, *C. reinhardtii* 2A38 was used as the host strain for transformation. The transformants were cultured in TAP medium either with 18 μM Fe (+Fe) or free Fe (-Fe). The liquid cultures were maintained at 25 °C in a 250-rpm incubator shaker and were exposed to continuous light of 150 μmol m^{-2} s^{-1}. Strains grown on TAP agar plates were incubated at a light intensity of 100 μmol m^{-2} s^{-1} at 22 °C.

Generation and screening of mutants

C. reinhardtii 2A38 was transformed with 1 μg of KpnI-linearized pSP124S DNA harboring the *ble* gene (Lumbreras et al., 1998) by using the glass bead method as described previously (Kindle, 1990). The transgenic cells were allowed to recover in 10 mL of TAP overnight in the dark at 25 °C with 150 rpm shaking and then collected by centrifugation (3000 g, 3 min), resuspended in 300 μL of TAP, and plated onto the TAP agar plates with 10 μg/mL of zeocin (Invitrogen, USA). After seven days, the transformants were transferred in duplicate to the +/-Fe TAP agar plates. For mutant screening, the transformants were inoculated into the -Fe TAP agar medium with 300 μL of 10 mM XSO_4 (Sigma Chemical Co.). After 24 h, the mutants were selected for further analysis based on the absence of a blue halo around the colonies.

Genomic DNA preparation

Genomic DNA extraction was performed following a protocol described by Newman et al. (1990) with certain modifications. A total of 1.5 mL of cells in mid-log phase were collected and centrifuged for 30 s at 12000 rpm, and the supernatants were aspirated. The cells were resuspended in 150 μL of H_2O on ice, and 300 μL of SDS-Buffer (2% SDS, 400 mM Na_2EDTA, 100 mM Tris-HCl, pH 8.0) and 5 μL of RNase (20 μg/ μL) were added. The mixture was vortexed and maintained at room temperature for 15 min. After a series of phenol–chloroform extractions, DNA was precipitated with two volumes of absolute ethanol and then washed with 70% ethanol. The air-dried pellet was dissolved in 40 μL of H_2O. DNA concentration was determined via spectrophotometry, and integrity was checked via agarose gel electrophoresis.

TAIL-PCR, and amplified fragment sequencing

Genomic clones containing the flanking regions of the plasmid DNA insertion was isolated via TAIL-PCR (Liu et al., 1995). The forward primers for primary, secondary, and tertiary reactions were ChlamyTail1 5'-CCGAGGAGCAGGACTA-ACCG-3', ChlamyTail2 5'-GATCCCCGCTCCGTGTAAATG-3', and ChlamyTail3 5'-ACGGCGGTGGATGGAAGATA-3', respectively. The six arbitrary degenerate primers were AD1 5'-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3', AD2 5'-NGT-CGA(G/C)(A/T)GANA(A/T)GANA(A/T)GAA-3', AD3 5'-(A/T)GTGNAG(A/T)ANCANAGA-3', AD4 5'-AG(A/T) GNAG(A/T)ANCA(A/T)AGG-3', AD5 5'-NTCGWGWTSCN-AGC-3', and AD6 5'-WGNTCWGNCANGCG-3'. The PCR parameters are listed in Supplementary Data 1. TAIL-PCR reactions were carried out in a 25 μL system containing 2.5 μL of PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$), 0.5 μL of 10 mM dNTPs, 10 pmol of primers, 50 pmol of RMD227, and 2.5 U of Taq polymerase. One μL of 40-fold diluted primary PCR reaction product served as the template for secondary PCR reaction, and 1 μL of 20-fold diluted secondary PCR reaction product served as the template for tertiary PCR reaction. The products from the tertiary PCR reaction were purified by using an agarose gel DNA purification kit (Takara, Japan) and were cloned into pMD18-T vector (Takara, Japan) following the manufacturer's instructions. Sequencing was performed at Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). A series of primers around the *ble* insertion site was designed to identify the DNA sequence length that was disrupted by the mutagenesis. The primers are shown in Table 1.

Identification and analysis of gene structures

Database searches were conducted in the JGI *C. reinhardtii* v4.0 (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) portal and the NCBI database (http://www.ncbi.nlm.nih.gov/). Molecular weights and pI values were calculated by using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/), whereas the motifs were annotated by using PROSITE program (http://prosite.expasy.org/). Localization and classification of proteins were predicted by using Euk-mPLoc 2.0 (http:// www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/) and NLStradamus

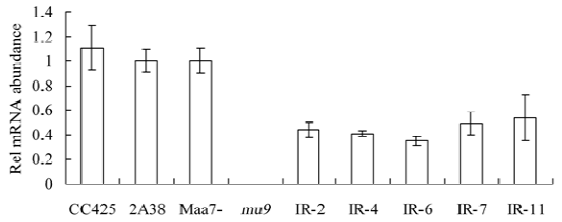


Fig 5. Quantitative real-time PCR analysis of *Femu9p* mRNA levels in control lines and in five *Femu9p*-RNAi lines. Maa7- is used as control. Three replicates were averaged, with standard deviation shown.

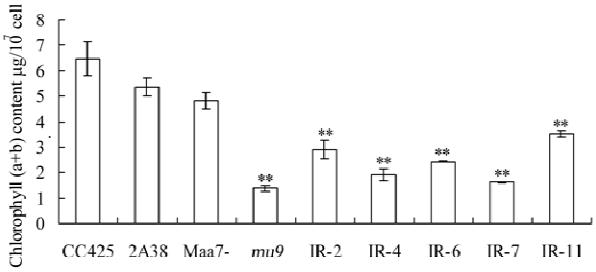


Fig 6. Total chlorophyll (Chl a+b) content in *Femu9p* RNAi lines. *C. reinhardtii* 2A38 transformed with the empty vector Maa7/XIR (Maa7-) was used as control. The experiment was performed thrice. Significance is indicated as **p < 0.01.

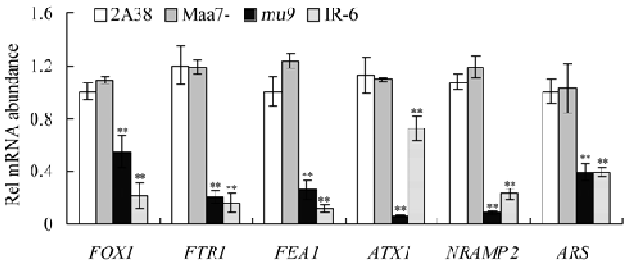


Fig 7. Quantitative real-time PCR analysis of *ARS* gene and several other genes involved in the high-affinity iron uptake system of *C. reinhardtii*. The data shown are means (±SD, n = 3). Significance is indicated as **p < 0.01.

(http://www.moseslab.csb.utoronto.ca/NLStradamus/). In silico identification of other homologous genes were performed by employing the Blastp programs from the NCBI database with the use of *Femu9p* sequence. Multiple sequence alignments and the phylogenenic tree were generated by using the ClustalW program (http://clustalw.genome.ad.jp/).

RNA extraction, RNAi vector construction and C. reinhardtii transformation

Total RNA was extracted as described by Deng et al. (2011). To generate a *Femu9p* RNAi construct, a fragment of *C. reinhardtii* 18S gene was amplified with primers 5'-CGAACTTCTGCGAAAGCAT-3' and 5'- TCAGCCTTGC-GACCATACT-3' and then inserted into the pMD18-T vector (Takara, Japan) to give a pMD18T-18S vector. Primers 5'-CGGGGTACCGCCGTTCCTGGAGACATT-3' and 5'- CGCG-GATCCATAAGGACGCCCAACGAG-3' were used to amplify a sense amplicon of *Femu9p* from the *C. reinhardtii* cDNA, whereas primers 5'-CCCAAGCTTGCCGTTCCTGGAGACA-TT-3' and 5'CGCGTCGACATAAGGACGCCCAACGAG-3' were used to amplify an antisense amplicon. The sense and antisense amplicons were double-digested with KpnI/BamHI and HindIII/SalI, respectively, and then ligated into pMD18T-18S vector. The resulting hairpin insert was excised by KpnI/HindIII digestion. After end blunting, the fragment was ligated into the pMaa7/XIR that was digested with EcoRI. The resulting plasmid was designated as pMaa7IR/*Femu9p*IR. The *C. reinhardtii* strain 2A38 was transformed by using the glass bead procedure (Kindle, 1990) and was allowed to recover for 2 d. The pMaa7IR/XIR and pMaa7IR/*Femu9p*IR transgenic strains were isolated on the TAP medium that was supplemented with 1.5 mM of L-tryptophan, 5 mg/mL of paromomycin, and 5 mM of 5-FI. Plates were incubated under dim light (approximately 50 mmol m^{-2} s^{-1} of photosynthetically active radiation). Isolated transgenic strains were maintained under a constant selective pressure to prevent the loss of integrated inverted repeat (IR) transgenes (Rohr et al., 2004).

ARS activity assay

ARS activity was assayed as described by Davies et al. (1992). For mutant screening, 300 µL of XSO_4 were added into TAP agar plates without Fe before clone inoculation. For qualitative analysis of enzyme activity in *mu9* strain and *Femu9p* RNAi lines, the algal cells were inoculated into 50 mL of TAP media without Fe and cultivated for six days. Each culture was then adjusted to different concentrations including 7.5×10^5, 15×10^5, 30×10^5 and 60×10^5, 200 µL of cultures were pippeted out and incubated with 6 µL XSO_4 for 30 min, respectively. The image was captured with a Canon IXUS100IS camera. For quantitative analysis, 100 µL of the sample was added to 500 µL of 0.1 M Glycine-NaOH at pH 9.0, 10 mM Imidazole, and 4.5 mM p-Nitrophenyl sulfate. The mixture was incubated at 27 °C for 27 min. The reaction was stopped by the addition of 2 ml of 0.25 mM NaOH, and the absorbance at 410 nm was determined. Statistical analysis was performed by using SPSS statistical software.

Real-time PCR analysis

Total RNA was prepared as described above. All RNA samples were digested with RQ1 RNase-free DNase (Promega, USA). First-strand cDNA was synthesized by using PrimeScript® Reverse Transcriptase (Takara, Japan) and was primed with a random primer in a volume of 20 µL following manufacturer's instructions. The quantitative PCR (qPCR) was performed on the Mx3005P™ real-time PCR system (Stratagene, USA) with a SYBR Green I fluorescent dye. The qPCR protocol followed the manufacturer's instructions for the SYBR® Premix Ex Taq™ kit (Takara, Japan). The PCR amplification specificity was checked by a melting curve program (55 °C to 100 °C at a heating rate of 0.5 °C/s). The *18S* rRNA gene was used as the internal control for each qPCR. The primers and the gene-specific primers are listed in Table 1. The amplification rate of each transcript (Ct) was determined over three repeats. Relative fold differences were calculated based on the relative quantification analytical method ($2^{-\Delta\Delta CT}$) via *18S* rRNA gene amplification as internal standard. Statistical analysis was performed by using SPSS statistical software.

Chlorophyll content measurement in Femu9p RNAi lines under iron-deficient conditions

To determine chlorophyll a, b content in the *Femu9p* RNAi lines, algae strains grown in TAP agar plates for 7 d were

inoculated into 50 mL of TAP medium without Fe, maintained in a 230 rpm incubator shaker at 24 °C, and exposed to continuous light of 150 μmol m^{-2} s^{-1} for 6 d. Thereafter, 1 mL of algae culture was transferred into a new 10 mL tube, with 4 mL of acetone added. The mixture was vortexed for 30 s, kept at 4 °C for 1 h, and centrifuged at 5000 g for 5 min at 4 °C. The supernatant was pippeted out, and the absorbance was read at 645 and 663 nm by using a spectrophotometer (Biorad, USA). Total chlorophyll a and b content was calculated as described by Yoshida et al. (1976). The formula is given below:

$$\text{Chlorophyll (a+b)}\left(\mu g/\text{cell}\right)=\frac{[20.2\times \text{OD}_{645}+8.02\times \text{OD}_{663}]\times V}{1000\times \text{cell density}}$$

V = Volume of acetone in the culture (mL)

The cell density was calculated by using a blood cell counting plate. *C. reinhardtii* 2A38 transformed with the empty vector Maa7/XIR (Maa7-) was used as control. Statistical analysis was performed by using SPSS statistical software. Analysis of variance (with LSD multiple-range test) was conducted. The experiment was performed thrice.

Conclusion

We isolated a regulatory mutant of *FOX1* gene that plays a significant role in iron metabolism in *C. reinhardtii*. The mutant *mu9* showed loss of *FOX1* promoter activity under iron-deficient conditions. We identified a candidate gene *Femu9p* from the mutant. Quantitative analysis of ARS enzyme activity revealed that RNAi silencing of *Femu9p* resulted in a significant reduction (-77.6%) of ARS enzyme activity compared with wild-type cells, indicating that *Femu9p* gene is essential to activate the *FOX1* promoter that controls *ARS* gene expression under iron-deficient conditions in *C. reinhardtii* 2A38. Our result also showed that mRNA level of *Femu9p* has a positive correlation with chlorophyll concentration in *C. reinhardtii*, implying that *Femu9p* is likely to participate in photosynthesis. In addition, mRNA abundance dramatically decreased in other iron uptake-related endogenous genes *FTR1*, *FEA1*, *ATX1*, and *NRAMP2* after *Femu9p* gene silencing. These data suggest that *Femu9p* gene is important in iron metabolism in *C. reinhardtii*. In future studies, analysis of molecular details of *Femu9p* gene would help us to better understand the occurrence of iron metabolism in higher eukaryotes.

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