Running head: GCN5 Regulates Iron Homeostasis Via FRD3

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GCN5-mediated Histone Acetylation of FRD3 Contributes to Iron Homeostasis in Arabidopsis thaliana

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One-sentence Summary:
Histone acetyltransferase plays important roles in regulating iron homeostasis in Arabidopsis
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Iron homeostasis is essential for plant growth and development. Here, we report that a mutation in GCN5 impaired iron translocation from the root to the shoot in Arabidopsis. Illumina high-throughput sequencing revealed 879 GCN5-regulated candidate genes potentially involved in iron homeostasis. ChIP assays indicated that five genes—At3G08040, At2G01530, At2G39380, At2G47160 and At4G05200—are direct targets of GCN5 in iron homeostasis regulation. Notably, GCN5-mediated H3K9/14ac acetylation of FRD3 determined the dynamic expression of FRD3. Consistent with the function of FRD3 as a citrate efflux protein, the iron retention defect in gcn5 was rescued and fertility was partly restored by overexpressing FRD3. Moreover, iron retention in gcn5 roots was significantly reduced by the exogenous application of citrate. Collectively, these data suggest that GCN5 plays a critical role in FRD3-mediated iron homeostasis. Our results provide novel insight into the chromatin-based regulation of iron homeostasis in Arabidopsis.
INTRODUCTION

Iron is an essential trace nutrition element for plants; its availability affects root morphogenesis, photosynthesis, nitrogen fixation, respiration, and flower color and fertility (Guerinot and Yi, 1994; Walker and Connolly, 2008). Iron deficiency impairs fundamental processes and causes a decrease in chlorophyll production, thus influencing crop productivity and quality (Schikora and Schmidt, 2001; Curie and Briat, 2003). Conversely, iron in excess is toxic to plants. Therefore, to ensure the effective acquisition of iron from the soil and to avoid excess iron in cells, iron uptake and distribution are strictly controlled in plants (Guerinot and Yi, 1994). Once taken up into the root, iron must be moved to the central vascular cylinder, where it can be loaded into the xylem and translocated to the aerial part of the plant (Rogers, 2006).

At the molecular level, several genes involved in iron uptake and mobilization have been characterized, including *Ferric Reductase Defective3* (*FRD3*), *Ferric Reductase Oxidase2* (*FRO2*), *ZIP family Iron Regulated Transporter1* (*IRT1*) and other genes (Curie and Briat, 2003; Walker and Connolly, 2008). In *Arabidopsis*, *FRO2* and *IRT1* are two important genes responsible for iron uptake. *IRT1* is the major transporter responsible for high-affinity iron uptake from the soil and is a key player in the regulation of plant iron homeostasis, as attested to by the severe chlorosis and lethality of the *irt1* mutant (Korshunova et al., 1999; Varotto et al., 2002; Vert et al., 2002). Encoding a low-iron-inducible ferric chelate reductase, *FRO2* is responsible for the reduction of iron at the root surface. When iron is loaded into the root xylem from the pericycle, iron distribution between the root and shoot is essential for tolerating fluctuations in iron availability (Long et al., 2010). Several studies revealed that *FRD3*, a multidrug and toxin efflux protein, facilitates Fe chelation to citrate and the subsequent transport of Fe-citrate from the root to the shoot (Green and Rogers, 2004; Grotz and Guerinot, 2006; Durrett et al., 2007).

Gene expression regulation is a crucial step for coping with iron deficiency or excess in plants. The tomato *FER* gene, encoding a basic helix-loop-helix (bHLH) transcription factor, was the first gene shown to be involved in iron deficiency responses and iron uptake (Ling et al., 2002). In *Arabidopsis*,
FIT (FER-like iron deficiency-induced transcription factor), a homolog of tomato FER, positively regulates the expression of the FRO2 and IRT1 genes (Colangelo and Guerinot, 2004; You Xi et al., 2005). In addition to FIT, the AtbHLH38, AtbHLH39, AtbHLH100 and AtbHLH101 proteins, which are classified into the Ib subgroup of basic helix-loop-helix (bHLH) transcription factors (Toledo-Ortiz et al., 2003), are required for the regulation of iron uptake and homeostasis in Arabidopsis (Wang et al., 2012).

With the discovery of the regulatory role of histone posttranslational modifications and their correlation with transcriptional states, studies have demonstrated that epigenetic mechanisms play important roles in iron homeostasis. For example, SKB1/AtPRMT5, which catalyzes the symmetric dimethylation of histone H4R3 (H4R3sme2), is involved in iron homeostasis in Arabidopsis. Histone H4R3 dimethylation negatively regulates iron homeostasis by affecting several Ib subgroup bHLH genes and iron uptake processes (Fan et al., 2013).

Histone acetylation and deacetylation are known to be important in the regulation of gene expression (Grunstein, 1997). Two enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC), are responsible for histone acetylation and deacetylation, respectively (Jenuwein and Allis, 2001). The Arabidopsis genome encodes at least 12 HAT and 18 HDAC genes (Pandey et al., 2002). Although the functions of HATs and HDACs in plant development and reproduction have been reported extensively, their roles in iron homeostasis are poorly understood. In this study, we find that a mutation in GCN5 (General control non-repressed protein 5) impairs iron transportation from the root to the shoot and affects iron homeostasis in Arabidopsis. We also confirmed that GCN5 bound to the chromatin at FRD3 under iron-sufficient conditions and that the enrichment of GCN5 was enhanced under iron-deficient conditions. Therefore, we conclude that GCN5 regulates iron distribution pathways by affecting the acetylation level of its direct target FRD3. In addition, we propose that HDA7 may negatively regulate FRD3 expression through FRD3 deacetylation.
RESULTS

The Mutation in GCN5 Caused Iron Retention in Roots and Affected Iron Homeostasis in Arabidopsis

Iron homeostasis comprises two successive steps: iron uptake at the root surface, and iron distribution in vivo (Curie and Briat, 2003). In this study, Perl’s stain method, a qualitative measure of the ferric iron content in living tissues, was employed to screen ten Arabidopsis T-DNA insertion mutants (gcn5, hda2c, hda5, hda7, hda9, hda13, hda14, hda15, hda18 and hda19). After 1 d of iron-deficiency treatment, iron accumulated to higher levels in the roots of three single mutants (gcn5, hda9 and hda19) than in the wild type (Figure 1A and Supplementary Figure 1). Moreover, after treatment with TSA, an inhibitor of HDACs, iron accumulation throughout the roots was not observed in the gcn5 mutant (Figure 1A). To better understand the underlying mechanism of action of GCN5 in iron homeostasis, we assessed the transcriptional response of GCN5 to iron deficiency in a wild-type background by using quantitative PCR (qPCR). Compared to the controls, the expression of GCN5 was higher after 1 d of iron-deficiency treatment, reaching a maximum at 3 d, then decreasing by 5 d (Figure 1B), indicating that GCN5 is an iron-deficiency-inducible gene. Collectively, these data indicate that GCN5 may play important positive regulatory roles in iron homeostasis.

Illumina High-throughput Sequencing Revealed 879 GCN5-regulated Candidate Genes Involved in Iron Homeostasis

To obtain detailed information about the expression profiles of GCN5-regulated genes involved in iron-deficiency responses, we used Illumina high-throughput sequencing to compare the expression profiles of four samples: designated Ws (wild type with sufficient iron), Ws-Fe (wild type with deficient iron), gcn5 (gcn5 mutant with sufficient iron), and gcn5-Fe (gcn5 mutant with deficient iron). The expression of GCN5 peaked at 3 d of iron-deficiency treatment, being upregulated by more than 5-fold over the control. Thus, seedlings with 3 d of iron-deficiency treatment were collected for RNAseq. All sequencing reads were aligned to the reference genome of Arabidopsis thaliana TAIR10 (http://www.arabidopsis.org/), using Bowtie2 software.
(Langmead and Salzberg, 2012), and only uniquely mapped reads were retained for the subsequent expression analysis by edgeR (Robinson et al., 2010). With two biological replicates for each sample, the correlation coefficients showed acceptable reproducibility, indicating the high reliability of our mRNA-seq data (Supplementary Figure 2). The accuracy of the transcriptome data was also validated by qPCR. Among the 16 randomly selected genes, the qPCR results of 15 genes were completely consistent with the RNAseq data (Supplementary Figure 3).

Under iron-deficient conditions, we found that 1917 genes were significantly induced in the Ws genotype compared with the gcn5 mutant, designated ‘Ws-Fe>gcn5-Fe’. In addition, 1278 genes displayed dramatic responses to iron-deficiency treatment in the wild-type background, designated ‘Ws-Fe>Ws’. Because GCN5 functioned as a positive regulator of transcription (Imoberdorf et al., 2006) and was upregulated by iron-deficiency treatment, the GCN5-regulated genes involved in iron homeostasis should be included in the overlapping 884 genes of ‘Ws-Fe>gcn5-Fe’ and ‘Ws-Fe>Ws’ (Figure 2A and Supplementary Table 2). Among the 884 overlapping genes, 5 genes belonged to the ‘gcn5-Fe>gcn5’ group, so these might not be regulated by GCN5. Therefore, 879 genes were considered to be GCN5-regulated candidate genes in iron homeostasis (Figure 2A and Supplementary Table 2).

Gene Ontology (GO) analysis of the 879 genes showed categories of significant enrichment. The most abundant category was metabolic processes (21%; including carbohydrate, glucuronoxylan and lipid metabolic processes), followed by the response to abiotic or biotic stimulus category (11%; including responses to ABA stimulus, auxin stimulus, high light intensity and hydrogen peroxide). A significant fraction (8%) represented genes implicated in responses to stress, such as salt, water deprivation, cold and heat stress. Up to 7% of genes were identified to be involved in transport, including nitrate, iron ion, lipid and calcium ion transports. The other categories involved developmental processes (7%), signal transduction (2%), cell organization and biogenesis (2%), DNA-dependent transcription (1%) and electron transport or energy pathways (1%). In addition, a large group of genes (40%) involved in other biological processes could not be simply classified and were
labeled as “others” (Figure 2B).

**Five Genes Are Direct Targets of GCN5 in Iron Homeostasis Regulation**

To identify the direct target genes in GCN5-regulated iron homeostasis, we focused on the candidate genes related to iron transport because the mutation in GCN5 resulted in iron retention in roots and the failure to transport this metal to the aerial parts of the plants. Through GO analysis of the 879 genes, we obtained all of the GO terms with P<0.01 (data not shown) and selected two iron binding or transportation-related GO terms (GO:0005506 and GO:0006826). Finally, we obtained 10 genes from the two GO terms based on their biological properties and described effects on iron transport (Green and Rogers, 2004; Feng et al., 2006) and their differential expression observed by RNAseq experiments (Table 1). Seven-day-old seedlings of Ws and gcn5 genotypes subjected to iron-deficiency conditions for 3 d were collected for ChIP and gene expression assays (Figure 3). ChIP-PCR analysis showed that GCN5 bound to the core promoter region of 5 genes in Ws to a significantly greater extent than in gcn5: FRD3 (At3G08040), MLP329 (At2G01530), EXO70H2 (At2G39380), BOR1 (At2G47160) and CRK25 (At4G05200) (Figure 3A). However, GCN5 was not enriched at the promoter of FRO6 (At5G49730), an important gene for iron homeostasis, indicating that this gene is an indirect target of GCN5 (Figure 3A).

Previous studies demonstrated that GCN5 is responsible for H3K14ac and for facilitating H3K9ac and H3K27ac, which is expected to be required for the expression of a large number of genes (Vlachonasios et al., 2003; Earley et al., 2007; Benhamed et al., 2008). To further confirm the direct regulation of these 5 potential target genes by GCN5, we performed ChIP-PCR assays using antibodies against H3K9ac and H3K14ac (Figure 3B). Consistent with the property of GCN5 as a histone acetyltransferase, the H3K9ac and/or H3K14ac levels of the 5 genes FRD3, EXO70H2, MLP329, BOR1 and CRK25 were significantly decreased due to the impairment of GCN5. Furthermore, the mRNA abundances of these 5 genes were significantly lower in gcn5 than in Ws under iron-deficiency conditions (Figure 3C). Based on these findings, we propose that GCN5 directly binds to the promoters of these five genes,
modulates their H3K9ac and/or H3K14ac levels, and in turn regulates their expression.

**GCN5-mediated Acetylation of FRD3 Determines the Dynamic Expression of FRD3**

Iron retention in the roots of the gcnc5 mutant was validated based on ICP-MS analysis. In addition, the gcnc5 mutants showed nearly two-fold higher levels of manganese and five- to six-fold higher levels of zinc in their roots than the wild type. Copper levels were unchanged in the mutant (Figure 4A). These phenotypes of gcnc5 are quite similar to those of frd3. Consistently, much less iron and manganese was detected in gcnc5 shoots than in the wild type. The zinc content was, somewhat lower in the gcnc5 mutants than in the wild type (Figure 4B). The micronutrient zinc plays an important role in the physiological and metabolic processes of plants. However, zinc can be toxic to the plant in excess concentrations, causing symptoms such as chlorosis (Marschner, 1995; Ramesh et al., 2004). According to the ICP-MS results, we believe that GCN5 also regulates zinc homeostasis in Arabidopsis. We propose that the misregulation of the distribution of other elements, such as zinc, also plays important roles in gcnc5 phenotypes.

Studies revealed that dynamic alteration in modifications of the histone H3 N-tail were correlated with gene activation in response to abiotic stress (Bruce et al., 2007). Here, FRD3 was used for further investigation because the frd3 and gcnc5 mutants showed a similar iron retention phenotype in roots (Green and Rogers, 2004). Seven-day-old seedlings of Ws were transferred to iron-deficient MS medium for 0, 1, 2, 3, 4, or 5 d treatments, and samples were collected for further ChIP-PCR and gene expression analysis. ChIP-PCR revealed that GCN5 association with the FRD3 promoter exhibited up to ~2-fold enrichment from 1 d to 4 d, compared to the control (0 d), but the enrichment of GCN5 at 5 d returned to similar levels to those of the control (Figure 5A). The quantity of GCN5 associated with the FRD3 promoter was correlated with the status of iron, suggesting that GCN5 regulates iron homeostasis when environmentally soluble iron is limited. Furthermore, we investigated whether the enhanced association of GCN5 to FRD3 leads to
dynamic changes in histone acetylation. We collected the seedlings at 5 time
points for ChIP-PCR assays specific to H3K14ac. Compared with the control,
the H3K14ac levels in the promoter region of \textit{FRD3} were clearly increased
after 2 d of iron-deficiency treatment, reached a maximum at 3 d, and then
were decreased at 4 d (Figure 5B). As shown in Figure 5C, \textit{FRD3} transcripts
were significantly upregulated after 3 d or 4 d iron-deficiency treatment
compared with the control.

qPCR analysis showed that the expression of \textit{FRD3} was approximately
3-fold lower in the \textit{gcn5} mutant than in the wild type (Supplementary Figure
4A). ChIP assays also revealed a significant greater association of GCN5 with
the \textit{FRD3} promoter in the wild type than in the \textit{gcn5} mutant (Supplementary
Figure 4B). To confirm that GCN5 regulates \textit{FRD3} gene expression by
modulating acetylation, we designed two pairs of primers for ChIP assays
against H3K9ac or H3K14ac, which spanned the core promoter and the
second exon, respectively. Consistent with the enrichment of GCN5, the
H3K9ac and H3K14ac levels in both the promoter and gene body regions of
\textit{FRD3} were significantly higher in the wild type than in the \textit{gcn5} mutant
(Supplementary Figure 4C). Collectively, these data provide further evidence
that the acetylation of specific histone Lys residues, regulated by GCN5, is
required for iron deficiency-induced \textit{FRD3} gene expression.

\textbf{GCN5 Plays a Major Role in FRD3-mediated Iron Homeostasis}

To investigate the function of \textit{FRD3} in GCN5-regulated iron homeostasis, we
generated \textit{FRD3} overexpression lines in the \textit{gcn5} mutant background. Owing
to the low pollen fertility of the \textit{gcn5} mutant, we first transformed the
\textit{35S::FRD3} construct into \textit{Ws}. Three independent transgenic plants
\textit{(35S::FRD3/Ws #1 to 3)} with significantly elevated \textit{FRD3} transcripts were
selected to be crossed with \textit{gcn5} mutants. Finally, we obtained three
homologous \textit{FRD3} overexpression lines in the \textit{gcn5} mutant background,
which exhibited significantly higher expression levels of \textit{FRD3} than the \textit{gcn5}
mutant, designated \textit{35S::FRD3/gcn5 #1 to 3} (Supplementary Figure 5).

\textit{FRD3} facilitates citrate release and the subsequent transport of Fe-
citrate from the root to the shoot (Green and Rogers, 2004; Grotz and
Iron retention was not detected in the three 35S::FRD3/gcn5 lines or the wild type, in contrast to the gcn5 mutant (Figure 6A). In addition, iron retention in the gcn5 mutant was successfully rescued by the exogenous addition of citrate to the MS plates (Figure 6A). Based on these data, we conclude that FRD3 is a critical direct target of GCN5 in iron distribution that regulates the citrate efflux in root tissues.

Iron in the leaves and chloroplasts is tightly correlated with the content of chlorophyll (Jacobson, 1945). As shown in Figure 6B, gcn5 plants exhibited less chlorophyll in the new leaves relative to Ws, and this defect was enhanced after a 3 d iron-deficiency treatment. Moreover, the observed defect in the gcn5 mutants was partially rescued by the exogenous application of citrate or overexpression of FRD3 (Figure 6B), suggesting that FRD3-mediated citrate efflux regulated by GCN5 plays an important role in chlorophyll synthesis.

Iron plays essential roles in pollen development (Lanquar et al., 2005; Roschztardtz et al., 2011). The gcn5 mutant exhibited clear reproductive defects compared to the wild type, with shorter siliques and far fewer seeds (Table 2). Electron microscopy analysis revealed that the 35S::FRD3/gcn5 or exogenous iron-treated gcn5 mutant showed enhanced pollen viability and compact stigma papillae compared to the gcn5 mutant (Figure 7 and Table 3). Based on these results, we propose that GCN5-controlled iron homeostasis through the regulation of FRD3 expression also affects anther and stigma development.

**Nutrition-deficiency Responses of the gcn5 Mutant Under Nutrition-replete Conditions**

Iron homeostasis consists of two main processes: iron uptake and translocation in vivo. After suggesting one manner in which GCN5 regulates iron translocation, we investigated whether there were simultaneous influences in the iron uptake processes. IRT1 and FRO2 are two important genes for iron uptake in Arabidopsis (Robinson et al., 1999; Vert et al., 2002). Consistent with other reports, these two genes were greatly induced by iron-starvation treatment in the wild-type background (Figure 8A). However, both...
genes are significantly induced in the gcn5 mutant, even under nutrition-replete conditions, which was consistent with our RNA-seq data. To clarify whether the induction of nutrition uptake-related genes was a common phenomenon in the gcn5 mutant plants, we analyzed the RNA-seq data of normally grown Ws and gcn5 mutant samples. Among all of the 233 genes that exhibited at least 2-fold higher expression levels in the gcn5 mutant than in Ws, 50 genes were involved in nutrition homeostasis, including iron transporters, nitrate transporters, phosphate-starvation-related genes, and other nutrition element-binding components (Table 4). In addition, the gcn5 mutant exhibited more and longer root hairs than Ws under normal conditions, which is a common morphology of malnourished plants (Figure 8B). However, given that 18 out of the 233 genes were related to 5 important phytohormones, we could not rule out the possibility that this phenotype might be partially caused by a change in phytohormones. Nevertheless, these results suggested that though grown under nutrition-replete conditions, gcn5 mutants exhibited nutrition-deficiency responses both in gene expression and morphogenesis phenotypes, which might be attributed to this mutant’s inefficient distribution of nutrition elements in vivo.

Histone Deacetylases Negatively Regulate FRD3 Expression

Histone acetylation and deacetylation often coordinate to precisely regulate gene expression (Shahbazian and Grunstein, 2007). We found that FRD3 transcripts were significantly upregulated (up to 3-fold) in 2 HDAC mutants (hda7 and hda14) (Figure 9A). Because HDA7 mainly catalyzes deacetylation in lys9 and lys14 sites of histone 3 in Arabidopsis (Cigliano et al., 2013), we designed 5 pairs of primers spanning the core promoter and gene body regions and examined the H3K9/14ac modifications at the FRD3 locus by performing ChIP-PCR experiments (Figure 9B). The H3K14ac levels at the FRD3 locus did not drastically change, whereas the H3K9ac levels were much higher in the hda7 mutant than that in the wild type, suggesting that HDA7 might negatively regulate FRD3 expression by reducing the H3K9ac levels of FRD3 in Arabidopsis.
DISCUSSION

Iron is essential for plant survival and growth. Recent studies have highlighted transcriptional responses to iron deficiency (Buckhout et al., 2009; Yang and Finnegan, 2010) and that the regulation of gene expression is accomplished by epigenetic mechanisms that modulate chromatin structure. Fan et al. (2013) reported that histone H4R3 dimethylation negatively regulated iron homeostasis by affecting several Irb subgroup bHLH genes and the iron uptake processes. Here, our data show that histone acetylation is also important for iron distribution.

**GCN5 Is Required for the Expression of Iron Deficiency-responsive Genes**

After iron is taken up by roots, its distribution is an important process for the mineral nutrition for plants (Curie and Briat, 2003). To date, several central genes responsible for iron distribution have been identified, but the underlying regulatory mechanism remains to be elucidated. In this study, we found that a gcn5 mutant exhibited iron retention in root tissues. Consistent with the upregulation of GCN5, global H3K14ac levels clearly increased more in Ws than that in the gcn5 mutants after iron deficiency treatment (Supplementary Figure 6). Moreover, the observed iron retention in the gcn5 mutants was rescued by TSA treatment. Thus, these data suggest that histone acetylation via GCN5 is an important mechanism for iron distribution in Arabidopsis.

GCN5 is required for the regulation of divergent sets of stress-response genes in yeast and Arabidopsis (Grant et al., 1997; Benhamed et al., 2008). To understand the mechanisms underlying the involvement of GCN5 in iron deficiency responses, we identified 1278 genes that were upregulated after iron deficiency treatment, of which 879 are dependent on GCN5. Gene Ontology (GO) analysis revealed a significant overrepresentation of genes involved in metabolic processes and responses to abiotic or biotic stimuli, including responses to ABA and auxin stimulus, salt, water deprivation and heat stresses. These data indicate that GCN5 is a general positive transcriptional regulator that regulates the expression of genes involved in a range of biological activities important for responding to iron-deficient
Our results show that iron retention in the gcn5 mutant was caused by inefficient iron transportation from the root to the shoot. Interestingly, we identified 5 iron transport-related targets of GCN5 using ChIP-PCR assays against GCN5 and H3K9/14ac, including FRD3, MLP329, EXO70H2, BOR1 and CRK25. It is likely that these genes are downstream components jointly responsible for GCN5-dependent iron homeostasis regulation. FRD3 has been well established as a citrate efflux protein, facilitating iron chelation to citrate and subsequent transport of iron-citrate (Green and Rogers, 2004; Rogers, 2006; Durrett et al., 2007). BOR1 was the first boron transporter reported in Arabidopsis (Takano et al., 2002) and is required for efficient xylem loading and the preferential translocation of boron into young portions of plants under boron-deficient conditions (Noguchi et al., 1997; Takano et al., 2001). The other three genes are less well characterized and were predicted to have iron ion transport activity, the roles of which in iron homeostasis require further investigation.

**FRD3 Is a Critical Target Gene in GCN5-dependent Iron Homeostasis**

Due to the poor solubility and high reactivity of iron, its translocation inside the plant body must be associated with suitable chelating molecules (Curie and Briat, 2003). Previous studies showed that FRD3 plays a dominant role in xylem iron transport. The iron retention phenotype, decreased expression and histone acetylation levels of FRD3 in gcn5 mutants suggested that FRD3 might be a critical gene in GCN5-regulated iron homeostasis. In addition, both the frd3 and gcn5 mutants show iron-deficiency responses under normal conditions. ChIP assays further confirmed that the enrichment of GCN5 on the FRD3 core promoter significantly increased after iron-deficiency treatment, which was consistent with the increased H3K9/14ac levels. Notably, the iron retention in gcn5 mutants could be successfully rescued by the overexpression of FRD3 or exogenous citrate application. These results provide an epigenetic mechanism of iron homeostasis in which GCN5 regulates FRD3 expression by modulating its H3K9/14ac levels.

Iron availability affects chlorophyll synthesis and plant fertility (Guerinot...
The \textit{frd3} mutant is chlorotic and almost completely sterile (Durrett et al., 2007; Roschztardtz et al., 2011). Our results showed that pollen viability was also severely impaired and that the chlorophyll content in new leaves was decreased in \textit{gcn5} mutants. Moreover, the reduced chlorophyll content and pollen sterility in \textit{gcn5} mutants was partially restored by the overexpression of \textit{FRD3}, which provides further evidence that \textit{FRD3} plays an important role in GCN5-regulated iron homeostasis. However, these defects of the \textit{gcn5} mutant were not fully rescued by \textit{FRD3} overexpression. Thus, to enable a comprehensive interpretation of the role of GCN5 in pollen development and chlorophyll synthesis, it is necessary to study the involvement of other target genes of GCN5 in these processes.

**Complex Mechanisms Underlie the Regulation of Iron Homeostasis by HAT and HDAC**

The antagonistic functions of the HAT/HDAC pair may operate on the acetylation of the same Lys residue (Jenuwein and Allis, 2001). In contrast to the decreased expression in \textit{gcn5} mutants, \textit{FRD3} expression increased in \textit{hda7} and \textit{hda14} mutants. It has been reported that GCN5 and HDA7 acted on the same sites of H3K9 and H3K14 but with opposite effects (Imoberdorf et al., 2006; Jin et al., 2010; Cigliano et al., 2013). Remarkably, the H3K9ac levels at the \textit{FRD3} locus were upregulated in \textit{hda7} mutants, indicating that HDA7 might antagonistically act with GCN5 to regulate \textit{FRD3} expression. It should be noted that \textit{hda9} and \textit{hda19} mutants also exhibited iron retention in root tissues (Supplementary Figure 1). Moreover, \textit{FRD3} expression was decreased in \textit{hda9} but did not change in \textit{hda19}. Generally, the expression of direct targets of HDA9 will be increased in \textit{hda9}. Thus, we hypothesize that HDA9 might regulate \textit{FRD3} expression and iron homeostasis indirectly. Collectively, these data indicate the complexity of the underlying regulation mechanisms for HAT and HDAC in iron homeostasis, which requires further investigation.

In conclusion, we revealed the roles of histone acetylation in the regulation of iron homeostasis and proposed a possible model for GCN5-regulated \textit{FRD3}-dependent iron homeostasis in \textit{Arabidopsis} (Figure 10). Briefly, the
impairment of GCN5 leads to iron retention in roots, impaired pollen viability and compromised chlorophyll content. Iron deficiency-induced GCN5 acted as an epigenetic modulator to positively control the expression of FRD3 via H3K9/14ac modifications. In the gcn5 mutant, iron deficiency signals from the shoot might induce the expression of IRT1 and FRO2 in roots even under normal conditions. The upregulation of IRT1 and FRO2, the downregulation of FRD3 and the increased production of root hairs jointly contributed to the accumulation of iron in roots, which might lead to iron toxicity in the root systems of the gcn5 mutant. In contrast, some HDACs, such as HDA7, may also participate in this process by negatively regulating FRD3 expression. This research into histone acetylation and iron homeostasis may help uncover prospects for improving iron nutrient utilization efficiency and crop yield.
METHODS

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* wild type Col and Ws, the mutant *gcn5-2* (Ws background), *hda7, had5, hda9, hda13, hda14, hda15, hda18, hda19*, and *hda2c* mutants (Col-0 background), were used in this study. Mutants were obtained in the homozygous state from ABRC or individual donor. Loss-of-function of these mutants was confirmed by PCR analysis. For seed germination, sterilized seeds were incubated at 4°C for 3 d; seeds were then sown on Murashige and Skoog (MS) plates containing 1% Sucrose and 0.6% agar. Seedlings were grown under a 16-h-light/8-h-dark condition at 22°C in a growth room.

Perls’ Stain for Ferric Iron

*Arabidopsis* roots were treated with Perls’ stain according to established histological methods for mammalian tissues. Briefly, equal amounts of solutions of 4% (v/v) HCl and 4% (w/v) potassium ferrocyanide were mixed immediately prior to use. The stain solution was vacuum infiltrated into 6- or 7-d-old *Arabidopsis* seedlings for approximately 15 min. Seedlings were rinsed in water and Perls’ staining was observed immediately in whole roots.

RNA Isolation, Library Preparation and Transcriptome Sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. RNA concentration was measured using a NanoDrop 2000 spectrophotometer (ND-2000, Thermo Fisher Scientific, Inc., USA). RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., CA, USA). Paired end (PE) sequencing libraries with average insert size of 200 bp were prepared with TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, USA) and sequenced on HiSeq2000 (Illumina, San Diego, USA) according to manufacturer’s standard protocols. Raw data obtained from Illumina sequencing were processed and filtered using Illumina pipeline (http://www.illumina.com) to generate FastQ files. Finally, approximately 16 G high quality 100-bp pair-end reads were generated from 8 libraries (Supplementary Table 3). The genes, with changes more than...
2 folds and false discovery rate (FDR) adjusted \( p < 0.01 \) (edgeR), were defined as differentially expressed genes. The groups of genes from RNAseq in this study are shown in Supplementary Table 2.

**Plasmid Constructions and Plant Transformations**

The cDNA clone of FRD3 gene containing the full-length open reading frame was amplified by PCR directed cloning based on annotation from The Arabidopsis Information Resource (TAIR), with the following primer pair: FRD3-ORF-L and FRD3-ORF-R, (see Supplemental Table 1). The sequence-confirmed clone containing the full-length cDNA of target genes were digested by KpnI and XbaI and cloned into the binary expression vector pCAMBIA1300 (driven by CaMV35S promoter). Binary vectors were transferred into Agrobacterium tumefaciens strain GV3101. Transgenic plants were generated by a floral dip method and screened on solid plates containing 25 mg/L hygromycin.

**Chromatin Immunoprecipitation**

ChIP assays were performed as described previously (Fiil et al., 2008). Seeds of Arabidopsis were sterilized, kept for 3 days at 4 °C, and grown in vitro under long-day conditions. At proper growing period or after Fe-deficiency treatments, seedlings were harvested and fixed in 1% formaldehyde for 15 min in a vacuum and then neutralized by 0.125 M glycine. After washing with sterilized water, the samples were ground in liquid nitrogen. Nuclei pellets were suspended in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 8, 10 mM MgCl2, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitors (one mini tablet per milliliter; Roche). The suspensions were transferred to microfuge tubes and centrifuged at 12 000× g for 10 min. The pellets were suspended in 1.7 M sucrose, 10 mM Tris-HCl, pH 8, 2 mM MgCl2, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF and protease inhibitors, and centrifuged through a layer of the same buffer in microfuge tubes. The nuclear pellets were lysed in a buffer containing 50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS and protease inhibitors. The lysed nuclei were sonicated four times for 15 s at 4 °C followed by centrifugation.
The supernatants containing chromatin fragments were diluted 10-fold with 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8 and 167 mM NaCl. Aliquots of the dilution were used for an immunoprecipitation assays. The antibody of GCN5 was provided by Prof. Zhou and described in (Benhamed et al., 2008). The H3K14ac and H3K9ac antibody were purchased from Upstate Biotechnology. CHS was amplified as an endogenous control. Immunoprecipitated DNA was analyzed by PCR using primer sets listed in Supplementary Table 1.

**Elemental Analysis**

Elemental levels were measured at the College of Resources and Environmental Sciences, CAU, in a VG AXIOM high resolution ion coupled plasma mass spectrometry (ICP-MS).

**Chlorophyll Measurement**

Ten-day-old seedlings of wild-type, gcn5 and 35S::FRD3/gcn5 grown on MS plates were transferred onto MS plates with 0 or 100 µM Fe-EDTA. After 4 days, the two newest leaves were collected and their fresh weight was determined. Chlorophyll was extracted in 1.0 ml of 80% acetone in the dark at room temperature (about 23 °C) for at least 12h until the leaves became blanched. The supernatant was then subjected to spectrophotometry at 470, 646 and 663 nm. The total chlorophyll content was calculated as described by (Hartmut, 1983).
ACKNOWLEDGMENTS

We thank Qixin Sun for providing constructive comments on the manuscript, Shunong Bai for providing seeds and Daoxiu Zhou for providing antibodies against GCN5.

AUTHOR CONTRIBUTIONS

J.X. and Z.N. conceived this project and designed all research. J.X. performed the experiments and analyzed the data with assistances from Z.L., Z.H., T.W., J.X., H.P., Y.Y., M. X., F. Y., D. Z. and J.X. and Z.N. wrote the article.
Figure 1. GCN5 Is Responsible for Iron Translocation and Responses to Iron Deficiency.

(A) Iron retention is observed in the roots of the gcn5 mutant. Perl’s staining was performed to detect the ferric iron content in the root. Seedlings (Ws and gcn5 mutants) were grown on normal MS plates for 7 d and then transferred to MS plates [(0 µM Fe-EDTA) or (0 µM Fe-EDTA, 0.5 µM TSA)] for 1 d. TSA, trichostatin A, an inhibitor of HDACs.

(B) GCN5 transcripts are induced in iron-deficient conditions. Seven-day-old seedlings of Ws on MS plates with iron were transferred onto MS plates without iron (0 µM Fe-EDTA) or with iron (100 µM Fe-EDTA) for 12 h, 1 d, 2 d, 3 d, 4 d or 5 d. Total RNA was isolated, and qRT-PCR showed the dynamic expression of the GCN5 transcript. The expression of β-Actin was used to normalize mRNA levels. Error bars represent SD values from at least three repetitions.

Figure 2. Identification of Potential GCN5 Targets Involved in Iron Homeostasis Using RNAseq.

(A) The overlap (879 genes, underscored by a black line) in the Venn diagram represents potential GCN5 targets that are also involved in iron homeostasis.

(B) Distribution of Gene Ontology categories (biological processes) for the 879 GCN5-regulated candidate genes.

Figure 3. Identification of the Direct Targets of GCN5 and Measurement of Their Acetylation States.

Seven-day-old seedlings of Ws and gcn5 mutants on MS plates with iron were separately transferred onto MS plates without iron (0 µM Fe-EDTA) for 3 d. Nuclei were extracted from the cross-linked seedlings, sonicated, and immunoprecipitated with antibodies specific to GCN5, H3K9ac or H3K14ac. Primers were designed at the core promoter region. Error bars represent SD values from at least 3 repetitions.

(A) ChIP assays (anti-GCN5) identified the direct target of GCN5.
(B) ChIP assays (anti-H3K9ac or -H3K14ac) were used to examine the H3K9ac or H3K14ac levels of the five GCN5 target genes. (C) qRT-PCR shows the dynamic expression of GCN5 target genes. Asterisks indicate significant differences (*P<0.05, **P<0.01; Student’s t test).

**Figure 4. gcn5 Mutants Accumulate More Fe, Mn and Zn in the Roots.**

Pooled samples of 2-week-old roots (A) and shoots (B) from plants grown on MS plates were subjected to elemental analysis using ICP-MS. Experiments were performed at least twice. Asterisks indicate significant differences (*P<0.05, **P<0.01; Student’s t test).

**Figure 5. The Regulation of FRD3 Expression by GCN5.**

Seedlings grown on normal MS plates for seven d were transferred to MS plates without iron for 1, 2, 3, 4 or 5 d, respectively. (A) Nuclei were extracted, and ChIP assays were performed with antibodies specific to GCN5. The gene AtCHS was used as a negative control. (B) Nuclei were extracted, and ChIP assays were performed with antibodies specific to H3K14ac. (C) qRT-PCR was performed to detect the FRD3 transcript levels. Asterisks indicate significant differences (*P<0.05, **P<0.01; Student’s t test).

**Figure 6. Iron Retention in Roots Caused by Impairment of GCN5 Affects Chlorophyll Content.**

(A) Perl’s staining to detect the ferric iron content in roots. Seedlings (Ws, gcn5 mutants and 35S::FRD3/gcn5 transgenic lines) were grown on normal MS plates for 7 d and then transferred to MS plates (0 µM Fe-EDTA) for 1 d. The gcn5 mutant was transferred to MS plates (0 µM Fe-EDTA, 3 mM sodium citrate) for 3 d. (B) Impairment of GCN5 lead to decreased total chlorophyll content in new leaves. Ten-day-old seedlings (Ws, gcn5 mutants and 35S::FRD3/gcn5 transgenic plants) were transferred to MS plates with 0 or 100 µm Fe for 3 d. In addition, gcn5 mutants were treated with 3 mM sodium citrate for 3 d. New leaves were collected and total chlorophyll was quantified (n=6, values are...
±SD). Asterisks denote values that are significantly different (*P < 0.05, **P<0.01; Student’s t test) compared with Ws.

**Figure 7. Reproductive Growth of gcn5 Is Impaired.**

Observation of the anther and stigma of the gcn5 mutant with water (A, B) or exogenous FeEDDHA (C, D) treatment and the normally grown 35S::FRD3/gcn5 plants (E, F). I2-KI staining to examine the pollen viability of gcn5 mutants (G) and 35S::FRD3/gcn5 plants (H).

**Figure 8. Nutrition-deficiency Responses of the gcn5 Mutant under the Nutrition-replete Condition.**

(A) Seven-day-old normally grown Ws seedlings were transferred to MS plates with or without iron for 3 d. qRT-PCR was performed to detect the expression of two iron uptake-related genes (IRT1 and FRO2).

(B) Phenotypes of root hair zones for normally grown Ws and the gcn5 mutant. The gcn5 mutant exhibited more and longer root hairs than Ws under normal conditions.

**Figure 9. HDA7 Negatively Regulates FRD3 Expression.**

(A) Normally grown ten-d-old seedlings of wild type (Col-0) and 9 HDAC mutants were collected. qRT-PCR was performed to detect the expression of FRD3 transcripts.

(B) Normally grown ten-d-old wild type (Col-0) and hda7 seedlings were collected and ChIP assays were performed using antibodies specific to H3K9ac or H3K14ac, respectively. The five primer sets (arrowheads) were numbered for FRD3.

**Figure 10. A Model for Acetylation Regulation of Iron Homeostasis.**

The transcription of GCN5 is induced by iron deficiency. GCN5 and some HDACs (especially HDA7) might antagonistically act on the expression of FRD3 by modulating its acetylation levels. M, mesophyll cell; p, pericycle; po, pollen grain; x, xylem.
Table 1. Potential GCN5-regulated, Iron Transport-related Genes.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G49730</td>
<td>FRO6</td>
<td>Ferric reduction oxidase 6</td>
</tr>
<tr>
<td>AT2G39380</td>
<td>EXO70H2</td>
<td>Exocyst subunit exo70 family protein H2</td>
</tr>
<tr>
<td>AT2G01530</td>
<td>MLP329</td>
<td>MLP-like protein 329</td>
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<tr>
<td>AT2G47160</td>
<td>BOR1</td>
<td>HCO3- transporter family</td>
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<tr>
<td>AT3G53420</td>
<td>PIP2;1</td>
<td>Plasma membrane intrinsic protein 2A</td>
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<tr>
<td>AT3G08040</td>
<td>FRD3</td>
<td>MATE efflux family protein</td>
</tr>
<tr>
<td>AT4G05200</td>
<td>CRK25</td>
<td>Cysteine-rich RLK (RECEPTOR-like protein kinase) 25</td>
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<tr>
<td>AT5G44610</td>
<td>PCAP2</td>
<td>Microtubule-associated protein 18</td>
</tr>
<tr>
<td>AT5G51160</td>
<td>-</td>
<td>Ankyrin repeat family protein</td>
</tr>
<tr>
<td>AT5G10580</td>
<td>-</td>
<td>Protein of unknown function, DUF599</td>
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</table>
Table 2. Seed Number and Silique Length in *gcn5* Mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Seed Number</th>
<th>Silique Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gcn5</em></td>
<td>2.2±1.4</td>
<td>4.3±1.16</td>
</tr>
<tr>
<td><em>gcn5</em>(+Fe)</td>
<td>4.1±1.1**</td>
<td>5.5±1.08*</td>
</tr>
</tbody>
</table>

Asterisks denote values that are significantly different from the control (*P<0.05, **P<0.01; Student’s *t* test).
Table 3. Pollen Viability in the *gcn5* Mutant and 35S::FRD3/*gcn5* Lines.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Fertile Pollen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gcn5</em></td>
<td>16.88±3.21</td>
</tr>
<tr>
<td>35S::FRD3/<em>gcn5</em> #1</td>
<td>30.27±3.18**</td>
</tr>
<tr>
<td>35S::FRD3/<em>gcn5</em> #2</td>
<td>24.37±4.45*</td>
</tr>
<tr>
<td>35S::FRD3/<em>gcn5</em> #3</td>
<td>25.8±3.14**</td>
</tr>
</tbody>
</table>

Asterisks denote values that are significantly different from the control (*P*<0.05, **P**<0.01; Student’s *t* test).
Table 4. GO Analysis of the 233 Genes with Higher Expression in the \textit{gcn5} Mutant than in Ws.

<table>
<thead>
<tr>
<th>GO ID</th>
<th>Gene Number</th>
<th>Annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutrition Homeostasis (Total 50)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0005506</td>
<td>9</td>
<td>Iron ion binding</td>
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<tr>
<td>GO:0046872</td>
<td>7</td>
<td>Metal ion binding</td>
</tr>
<tr>
<td>GO:0016036</td>
<td>5</td>
<td>Cellular response to phosphate starvation</td>
</tr>
<tr>
<td>GO:0000041</td>
<td>3</td>
<td>Transition metal ion transport</td>
</tr>
<tr>
<td>GO:0015706</td>
<td>4</td>
<td>Nitrate transport</td>
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<tr>
<td>GO:0046686</td>
<td>6</td>
<td>Response to cadmium ion</td>
</tr>
<tr>
<td>GO:0005507</td>
<td>2</td>
<td>Copper ion binding</td>
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<tr>
<td>GO:0008270</td>
<td>14</td>
<td>Zinc ion binding</td>
</tr>
<tr>
<td><strong>Hormone Response (Total 18)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO:0009830</td>
<td>4</td>
<td>Cell wall modification involved in abscission</td>
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<tr>
<td>GO:0009862</td>
<td>4</td>
<td>Salicylic acid-mediated signaling pathway</td>
</tr>
<tr>
<td>GO:0009751</td>
<td>4</td>
<td>Response to salicylic acid stimulus</td>
</tr>
<tr>
<td>GO:0009736</td>
<td>3</td>
<td>Cytokinin-mediated signaling pathway</td>
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<tr>
<td>GO:0009867</td>
<td>3</td>
<td>Jasmonic acid-mediated signaling pathway</td>
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</table>


