

# MIR164b represses iron uptake by regulating the NAC domain transcription factor5-Nuclear Factor Y, Subunit A8 module in Arabidopsis

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## Abstract

Recent findings have revealed the important roles of microRNAs (miRNAs) in the secondary responses to oxidative damage caused by iron (Fe) excess. However, the functional importance of miRNAs in plant responses to Fe deficiency remains to be explored. Here, we show that the expression level of miR164 in Arabidopsis (*Arabidopsis thaliana*) roots was repressed by Fe deficiency. Primary root length, lateral root number, ferric reductase activity, and mRNA abundance of *IRON-REGULATED TRANSPORTER1 (IRT1)* and *FERRIC REDUCTION OXIDASE2 (FRO2)* were higher in the *mir164b* mutant than in the wild-type (WT) under Fe-deficient conditions. Analysis of the Fe concentrations and ferric reductase activities in the roots of miR164 knockdown transgenic plants showed that members of the miR164 family had different functions in Fe-deficiency responses. Promoter::GUS analysis showed that *NAM/ATAF/CUC (NAC) domain transcription factor5 (NAC5)* is regulated at both transcriptional and posttranscriptional levels under Fe-deficient conditions. Transgenic Arabidopsis plants overexpressing *NAC5* were more tolerant of Fe deficiency than the WT. *NAC5* has transactivation activity and directly transactivates the expression of *Nuclear Factor Y, Subunit A8 (NFYA8)*, as demonstrated by chromatin immunoprecipitation followed by quantitative polymerase chain reaction, electrophoretic mobility shift assay (EMSA), and dual-luciferase reporter assay. Like overexpression of *NAC5*, overexpression of *NFYA8* increases primary root length, lateral root number, ferric reductase activity, and mRNA abundance of *IRT1* and *FRO2* under Fe-deficient conditions. Thus, *MIR164b* is important for Fe-deficiency responses by its regulation of the *NAC5*–*NFYA8* module.

## Introduction

As the cofactor for many important enzymatic reactions, iron (Fe) is essential for all organisms. The fixation of insoluble Fe in the field, however, reduces the availability of Fe to plants, especially in calcareous soils with high pH and high bicarbonate content. Fe deficiency has become a major

factor limiting crop yield worldwide (Briat et al., 2015). Because limited Fe availability in soil also reduces the Fe content of plant-derived food, Fe deficiency-induced anemia is a common nutritional disorder for humans in developing countries (Grillet et al., 2018; Narayanan et al., 2019). An

improved understanding of the genetic mechanisms of Fe homeostasis in plants could help increase the Fe content in crops.

To cope with Fe-deficient environments, plants have evolved two responsive strategies. Gramineaceous species use a chelation-based Fe acquisition mechanism (Strategy II) in that they secrete phytosiderophores and uptake Fe(III)-phytosiderophores via the Yellow Stripe Like transporters (Römheld and Marschner, 1986; Curie et al., 2001). With the exception of gramineaceous species, most plants, including *Arabidopsis* (*Arabidopsis thaliana*), employ a reduction-based Fe-acquisition mechanism (Strategy I). They first acidify the rhizosphere by excreting protons and phenolic compounds to increase the solubility of Fe (Kobayashi and Nishizawa, 2012). The Fe(III)-chelates is then reduced to Fe<sup>2+</sup> by the ferric-chelate reductase FERRIC REDUCTION OXIDASE2 (FRO2), which is the rate-limiting step for mobilizing Fe from soil (Robinson et al., 1999; Satbhai et al., 2017). Finally, the reduced Fe<sup>2+</sup> is transported across the root plasma membrane by the metal transporter IRON-REGULATED TRANSPORTER1 (IRT1) (Eide et al., 1996; Vert et al., 2002).

Although recent research has demonstrated the importance of posttranslational modifications in plant Fe-nutritional responses (Shin et al., 2013; Dubeaux et al., 2018; Gratz et al., 2019), the transcriptional regulation of gene expression plays a crucial role in Fe deficiency responses in plants (Riaz and Guerinot, 2021; Sun et al., 2021). The basic helix–loop–helix (bHLH) transcription factors FERLIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) and POPEYE (PYE) are the key regulators of the Fe-deficiency response in *Arabidopsis* (Colangelo and Guerinot, 2004; Yuan et al., 2005; Long et al., 2010). The nonoverlapping locations of the target genes of FIT and PYE indicate that the two transcription factors are involved in separate gene regulons. FIT physically interacts with four subgroup 1b bHLH proteins, bHLH038, bHLH039, bHLH100, and bHLH101, to upregulate its target genes (Yuan et al., 2008; Wang et al., 2013; Cai et al., 2021). PYE acts as a transcriptional repressor and is involved in the internal mobilization of Fe in the root and its transport to the shoot (Long et al., 2010). To date, 17 of 133 members in the bHLH family has been determined to be involved in Fe homeostasis in *Arabidopsis* (Gao et al., 2015; Cui et al., 2018; Lei et al., 2020), indicating the importance of the bHLH family in plant adaptation to Fe deficiency.

NAM, ATAF1/2, and CUC1/2 (NAC) proteins constitute a large plant-specific family of transcription factors, with over 100 members in *Arabidopsis* (Olsen et al., 2005). Several reports indicate that the NAC family could be associated with Fe homeostasis in Strategy II plants. In wheat (*Triticum aestivum* L.), a NAC transcription factor, *NAM-B1*, accelerated senescence and increased grain protein, zinc, and Fe content (Uauy et al., 2006). In rice (*Oryza sativa*), IDE-binding factor 2 (*IDEF2*), which belongs to an uncharacterized branch of the NAC transcription factor family, regulates the

genes involved in Fe homeostasis by recognizing Fe deficiency-responsive element 2 in their promoters (Ogo et al., 2008). However, an understanding of NAC in Fe-deficiency responses in Strategy I plants is lacking.

A subset of NAC transcription factors, including *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, *NAC domain transcription factor1* (*NAC1*), *NAC4*, *NAC5* (At5g61430), and *ORE1* are posttranscriptionally regulated by miRNA164 (Laufs et al., 2004; Mallory et al., 2004; Guo et al., 2005; Kim et al., 2009). Plant miRNAs are involved in various developmental processes. Recent findings have revealed the important roles of miRNAs in plant adaptation to nutrient deficiency or excess, such as copper deficiency (Yamasaki et al., 2009; Pilon, 2017), phosphorus deficiency (Kuo and Chiou 2011), and copper/Fe excess (Sunkar et al., 2006). The expression and phenotypic analyses of transgenic plants over-expressing Fe and other heavy metal-related miRNAs have suggested that the miRNAs mainly function in the secondary responses to oxidative damage caused by heavy metal stress. The functional importance of miRNAs in Fe-deficiency responses in plants remains to be explored.

Here, we report that the expression of miR164 in *Arabidopsis* roots is repressed by Fe deficiency. Analysis of *mir164b* knockout plants, miR164 knock-down transgenic plants, and *NAC5*-overexpressing lines showed that the post-transcriptional regulation of *NAC5* by miR164 is important for Fe uptake in *Arabidopsis*. We also demonstrated that *NAC5* could directly activate the expression level of *Nuclear Factor Y, Subunit A8* (*NFYA8*). *NFYA8*-overexpressing *Arabidopsis* was tolerant of Fe deficiency. Taken together, our results reveal the importance of the *MIR164b*–*NAC5*–*NFYA8* module in Fe homeostasis in *Arabidopsis*.

## Results

### miR164 expression is repressed by Fe deficiency in *Arabidopsis* roots

To identify miRNAs and other endogenous small RNAs that respond to Fe deficiency, we used Solexa high-throughput sequencing technology to generate and sequence two small RNA libraries from Fe-sufficient and -deficient *Arabidopsis* roots. The Fe-deficient treatment was effective because the expression of the marker gene, *AtIRT1* (At4G19690), was significantly induced by Fe deficiency as reported previously (Eide et al., 1996; Vert et al., 2002; Supplemental Figure S1). The two small RNA libraries yielded a total of >23 million raw reads, and after 5' and 3' adaptor trimming, >87% of the remaining raw reads ranged in size from 18 to 26 nt (Supplemental Table S1). Of these, >16 million reads corresponding to 3.1 million unique sequences could be perfectly mapped to *Arabidopsis* genome (The *Arabidopsis* Information Resource 9). Sequences that could not be mapped to *Arabidopsis* genome were discarded and only those perfectly mapped were analyzed further. The genome-matched sequences were assigned to several RNA groups, including known miRNAs, rRNAs, tRNAs, repeats, snRNAs, snoRNAs, and others (Supplemental Table S2). The known

miRNAs accounted for 8.34% and 7.89% of all sequence reads for Fe-sufficient and -deficient small RNA libraries, respectively. Based on the difference in numbers of normalized sequence reads between Fe-deficient and -sufficient roots, 38 conserved miRNAs with > 1.5-fold relative change were identified (Supplemental Table S3).

miR164 attracted our attention because of its high expression levels in roots (Mallory et al., 2004; Guo et al., 2005; Supplemental Table S3). Fe deficiency significantly reduced the expression levels of *MIR164a* and *MIR164b* in Arabidopsis roots (Supplemental Table S3). To confirm the results obtained with Solexa high-throughput sequencing technology, we conducted small RNA northern blot analysis. Consistent with the Solexa high-throughput sequencing results, the expression level of miR164 in roots reduced by ~60% (relative to unstressed plants) when plants were subjected to Fe deficiency for 3 d (Figure 1A). Interestingly, the expression level of miR164 in shoots was upregulated by Fe deficiency (Figure 1A). We further collected xylem sap of Arabidopsis and found that miR164 existed in the xylem sap under both Fe-sufficient and -deficient conditions (Supplemental Figure S2), which indicated that miR164 could be moved from root-to-shoot. In addition, the expression level of miR164 was enhanced by Fe excess in both shoots and roots of Arabidopsis (Supplemental Figure S3).

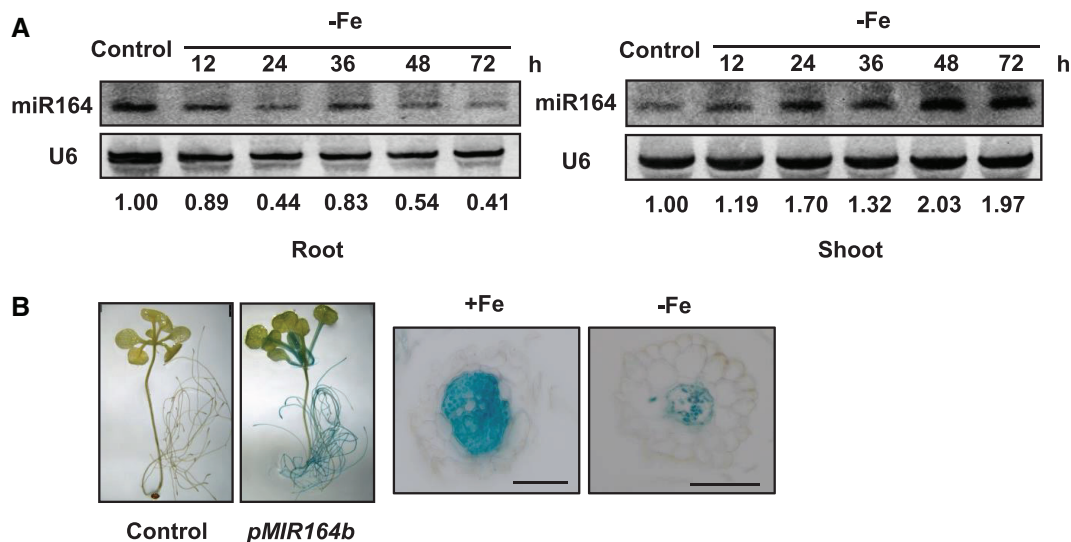
The miR164 family in Arabidopsis has three members, among which miR164b is the most abundant (Raman et al., 2008). We therefore selected miR164b for further study. We first used the promoter::GUS transgenic lines to examine the expression pattern of *MIR164b*. Glucuronidase (GUS) staining patterns in several transgenic lines showed that the promoter was active mainly in petiole and root vascular systems of 14-d-old Arabidopsis under Fe-sufficient conditions (Figure 1B). In

agreement with the small RNA northern blot analysis, Fe deficiency caused a substantial decrease in the GUS activity in Arabidopsis roots (Figure 1B).

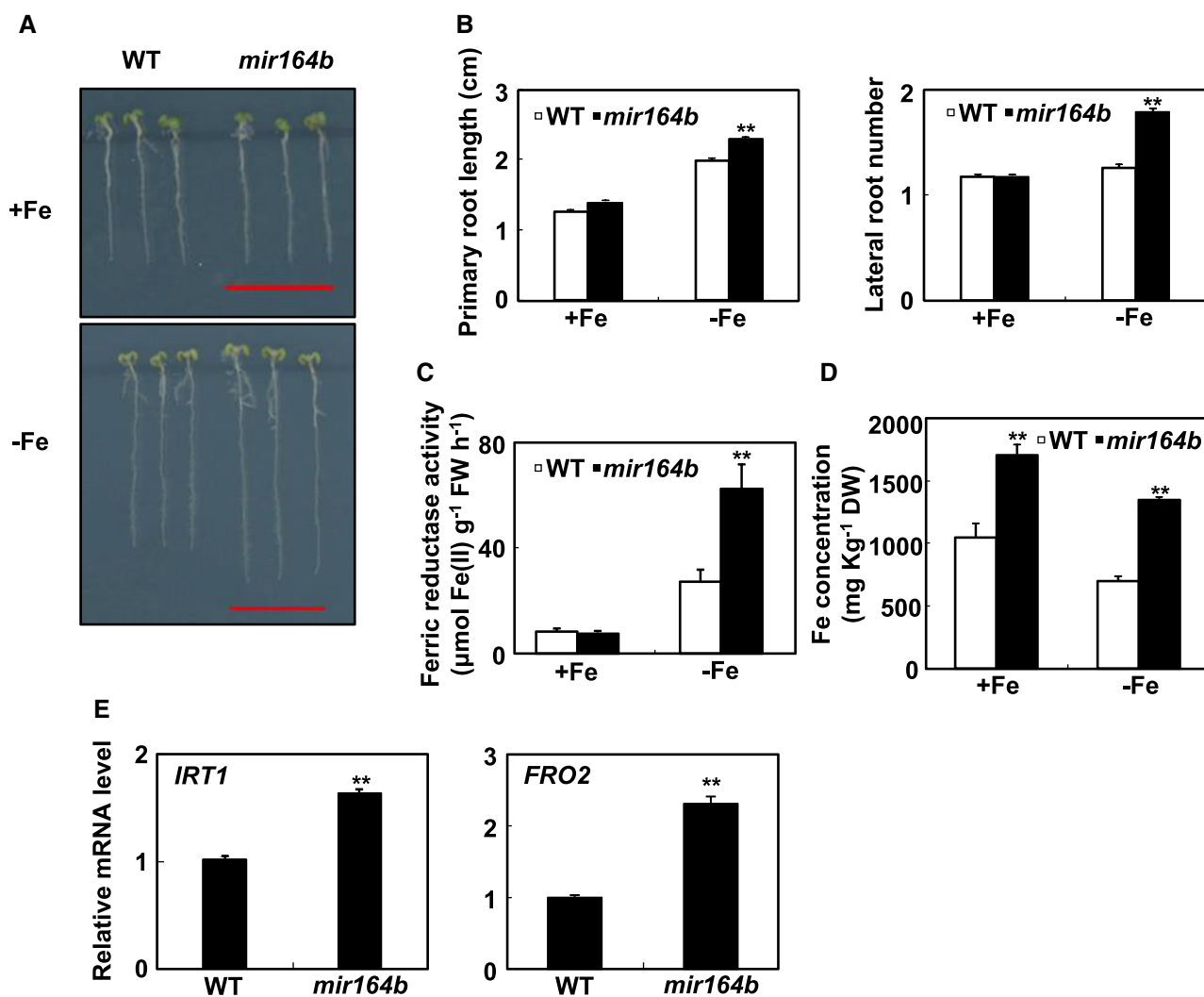
### The *mir164b* mutant is tolerant of Fe-deficient stress

The downregulation of miR164 by Fe deficiency and its strong expression in roots prompted us to analyze its potential role in Arabidopsis responses to Fe deficiency. To investigate the function of miR164b, we searched the publicly available T-DNA collections and obtained a T-DNA insertion mutant (SALK\_136105C in the Columbia background) from the Arabidopsis Biological Resource Center. Plants homozygous for the T-DNA insertion were identified by PCR and sequencing of the T-DNA flanking region confirmed the insertion site in loop of the predicted hairpin precursor of *MIR164b* (Supplemental Figure S4A). Reverse transcription PCR (RT-PCR) showed that *MIR164b* transcripts were absent in the T-DNA line designated as *mir164b* (Supplemental Figure S4B). The hybridization signal from the miR164 probe was much weaker in the *mir164b* lines than in wild-type (WT) plants (Supplemental Figure S4C), indicating that *mir164b* represented a null allele as described by Mallory et al. (2004).

In a previous study, the *mir164b* mutant promoted lateral root emergence when grown on Murashige and Skoog (MS) medium (Guo et al., 2005). When we grew the *mir164b* mutant on 0.5 MS medium, we observed no difference in root morphology between the *mir164b* mutant and WT Arabidopsis (Figure 2, A and B). When the *mir164b* loss-of-function mutant was germinated and grown on Fe-free 0.5 MS medium for 7 d, however, primary root length and lateral root number were significantly higher for the *mir164b* mutant than for the WT (Figure 2, A and B). We also generated transgenic Arabidopsis plants overexpressing *MIR164b*,



**Figure 1** Regulation of miR164 by Fe deficiency. A, Assay of miR164 abundance in Arabidopsis roots and shoots by small RNA northern blot. U6 was used as the loading control. Numbers below each lane indicate the expression level of miR164 relative to U6. B, *MIR164b*<sub>pro</sub>::GUS expression pattern in transgenic Arabidopsis roots. Scale bars = 50  $\mu$ m.



**Figure 2** *mir164b* mutants have increased tolerance to Fe deficiency. A, Representative images of *mir164b* mutant seedlings grown on Fe-deficient medium for 7 d. Scale bars = 1 cm. B, The primary root length and lateral root number of *mir164b* mutant seedlings grown on Fe-deficient medium for 7 d. C, The ferric reductase activity in the roots of *mir164b* mutant seedlings grown on Fe-deficient medium for 7 d. D, The Fe concentration in the roots of 18-d-old hydroponically grown Arabidopsis, either supplied with Fe or transferred to a Fe-free solution for 36 h. E, The effects of Fe deficiency on the expression levels of *IRT1* and *FRO2* in 18-d-old hydroponically grown WT and *mir164b* mutant plants. RT-qPCR quantifications were normalized to the expression of *TUB2*. Error bars represent standard errors of three independent experiments, with 20–30 plants per experiment (B–D). Error bars represent standard errors of three biological replicates (E). Asterisks indicate significant differences between Fe-sufficient and Fe-deficient treatments at  $P < 0.01$  according to  $t$  tests.

and found that primary root length was much shorter for *35S::MIR164b* transgenic plants than for the WT under Fe-deficient conditions (Supplemental Figure S5). In parallel, the rate of leaf discoloration was higher for *35S::MIR164b* transgenic plants than for the WT (Supplemental Figure S5). Interestingly, the ferric reductase activity was 2.3 times greater for *mir164b* mutant plants than for WT plants under Fe-deficient conditions (Figure 2C). These results indicated that the root system is more developed for *mir164b* mutant plants than for WT plants and that Fe acquisition might be more efficient in *mir164b* mutant plants than in the WT plants under Fe-deficient conditions.

To test the latter hypothesis, we used inductively coupled plasma mass spectrometer (ICP-MS) to measure the Fe concentrations in the roots of the *mir164b* mutant and the WT. Given the small root biomass of Arabidopsis seedlings, we used hydroponically grown 18-d-old Arabidopsis plants. Under both Fe-sufficient and -deficient conditions, the Fe concentrations in the roots were much higher in the *mir164b* mutant than in the WT (Figure 2D). After the plants were subjected to Fe deficiency for 36 h, the Fe concentration in the roots of the *mir164b* mutant was  $1,352 \text{ mg kg}^{-1} \text{ DW}$ , which was  $\sim$ two times greater than that in WT plants (Figure 2D). Consistent with these results, the

mRNA abundance of *IRT1* and *FRO2* in the roots under Fe-deficient conditions was much higher for the *mir164b* mutant than for the WT (Figure 2E). These results supported our hypothesis that *mir164b* mutant has increased tolerance to Fe deficiency.

### Functional specialization in Fe-deficiency responses of miR164 members

Analysis of *mir164abc* triple-mutant plants revealed that miR164a, miR164b, and miR164c control flower development in a redundant manner (Sieber et al., 2007). However, different expression patterns of the individual miR164 genes suggested that these genes may be redundant for some functions but not for others (Sieber et al., 2007). *mir164a-4* had deepened serrations of the leaf margins (Nikovics et al., 2006), while the phenotype of *mir164b-1* was the same as that of the WT (Mallory et al., 2004). These results suggested that miR164 family members have different functions during plant development. To check whether functional redundancy existed in Fe-deficient response among the members in miR164 family, we designed the short tandem target mimic transgenic lines targeting *MIR164a*, *MIR164b*, and *MIR164c* as described by Yan et al. (2012). The destruction of *MIR164a*, *MIR164b*, and *MIR164c* was verified by small RNA northern blot, and two independent knock-down lines (*TM-3* and *TM-5*) were chosen for analysis of their expression levels (Figure 3A). Under both Fe-sufficient and -deficient conditions, *TM* transgenic seedlings had longer primary roots and more lateral roots than WT seedlings (Figure 3, B and C). Consistent with the observation in the *mir164b* mutant, the ferric reductase activities in roots were higher for *TM* transgenic seedlings than for WT seedlings under Fe-deficient conditions (Figure 3D).

*TM* transgenic plants were similar to WT plants during vegetative development, except that the leaf margins of *TM* transgenic plants developed deepened serrations (Supplemental Figure S6). After the plants were subjected to Fe deficiency for 36 h, the Fe concentrations in the roots of 18-d-old hydroponically plants were higher for *TM* transgenic plants than for WT plants (Figure 3E). However, the increased ratios of Fe concentrations and ferric reductase activities in the roots of *TM* transgenic plants versus the WT were comparable to those of *mir164b* mutants versus the WT. These results suggested that miR164 family members have at least partial functional specialization in their Fe-deficiency responses.

### *NAC5* is regulated at both transcriptional and posttranscriptional levels under Fe-deficient conditions

miR164 was previously verified to posttranscriptionally regulate a subset of NAC-domain transcription factors, including *CUC1*, *CUC2*, *NAC1*, *NAC4*, *NAC5* (At5g61430), and *ORE1* (Laufs et al., 2004; Mallory et al., 2004; Guo et al., 2005; Kim et al., 2009). Of the six targets directly cleaved by miR164, only *NAC1* and *NAC5* were highly expressed in roots

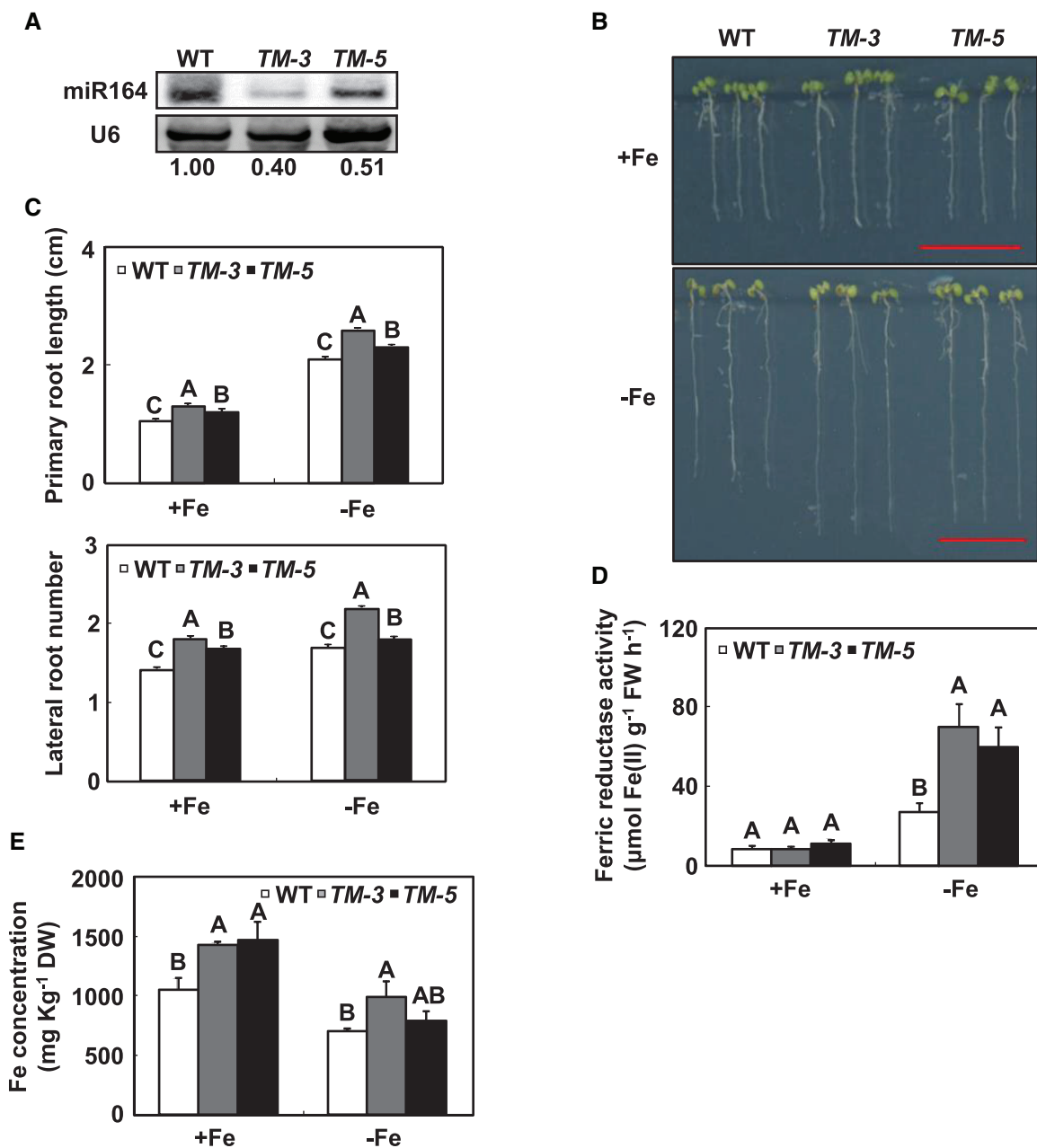
(Mallory et al., 2004). *NAC1* was mainly observed in root tips and lateral root initiation sites (Xie et al., 2000), which was inconsistent with the expression patterns of *MIR164b*. In addition, Fe deficiency did not alter the mRNA abundance of *NAC1* in Arabidopsis roots (Supplemental Figure S7). We, therefore, investigated the function of *NAC5*.

The mRNA abundance of *NAC5* was higher in the *mir164b* mutant than in the WT (Supplemental Figure S8), further demonstrating the posttranscriptional regulation of *NAC5* by *MIR164b*. In contrast to the downregulation of miR164 expression by Fe deficiency, *NAC5* was strongly induced by Fe deficiency in roots (Figure 4A). To determine whether the induction of *NAC5* in response to Fe deficiency was caused by increased promoter activity or altered RNA stability, we constructed *NAC5* promoter::GUS transgenic lines. In agreement with the expression patterns of *MIR164b*, *NAC5* expression was high in the vascular tissues of petioles and roots during vegetative development under Fe-sufficient conditions (Figure 4B). Fe deficiency enhanced the GUS activity of *NAC5* (Figure 4B). Unlike the expression pattern of *MIR164b* under Fe-deficient conditions, *NAC5* was expressed in both root vascular tissue and root cortical tissue under Fe-deficient conditions (Figure 4B), indicating that Fe deficiency also induced *NAC5* mRNA abundance at transcriptional level.

### 35S::NAC5 transgenic plants, like the *mir164b* mutant, are tolerant of Fe deficiency

To characterize the potential roles of *NAC5* in Fe-deficiency tolerance, we generated transgenic Arabidopsis plants overexpressing the full-length coding sequence (CDS) of *NAC5*. Two transgenic lines (#3 and #4) were chosen for further analysis based on their high level of *NAC5* (Figure 5A). Under both Fe-sufficient and -deficient conditions, the primary root length and lateral root number of 35S::NAC5 transgenic Arabidopsis seedlings, and the Fe concentrations in the roots of 18-d-old hydroponically grown 35S::NAC5 transgenic Arabidopsis plants, were significantly higher than those of WT plants (Figure 5, B–D). Correspondingly, the mRNA abundances of *IRT1* and *FRO2* in roots were much higher in 35S::NAC5 transgenic plants than in WT plants under Fe-deficient conditions (Figure 5E).

To further characterize the function of *NAC5* in Fe-deficiency responses, we identified a *NAC5* loss-of-function mutant and designated as *nac5* (Supplemental Figure S9). In contrast with WT plants, *nac5* loss-of-function mutant had shorter primary root and higher rates of leaf discoloration under Fe-deficient conditions (Supplemental Figure S9). We also detected the mRNA level of *FIT* and *PYE* in 35S::NAC5 transgenic Arabidopsis plants and *nac5* loss-of-function mutant under Fe-deficient conditions. Transcriptional levels of *NAC5* did not affect *FIT* and *PYE* mRNA abundance (Supplemental Figure S10). In addition, knock-out of *FIT* and *PYE* did not affect expression level of *NAC5* (Colangelo and Guerinot, 2004; Long et al., 2010). These results suggested



**Figure 3** The responses of miR164 knock-known transgenic plants (TM) to Fe deficiency. A, miR164 levels in TM transgenic plants as indicated by small RNA northern blot. U6 was used as the loading control. Numbers below each lane indicate the expression level of miR164 relative to U6. B, Representative images of TM transgenic seedlings grown on Fe-deficient medium for 7 d. Scale bars = 1 cm. C, The primary root length and lateral root number of TM transgenic seedlings grown on Fe-deficient medium for 7 d. D, The ferric reductase activity in the roots of TM transgenic seedlings grown on Fe-deficient medium for 7 d. E, The Fe concentration in the roots of 18-d-old hydroponically grown Arabidopsis, either supplied with Fe or transferred to a Fe-free solution for 36 h. Error bars represent standard errors of three independent experiments, with 20–30 plants per experiment (C–E). Means with the same letter are not significantly different at  $P < 0.01$  according to one-way ANOVA followed by Tukey's multiple comparison test.

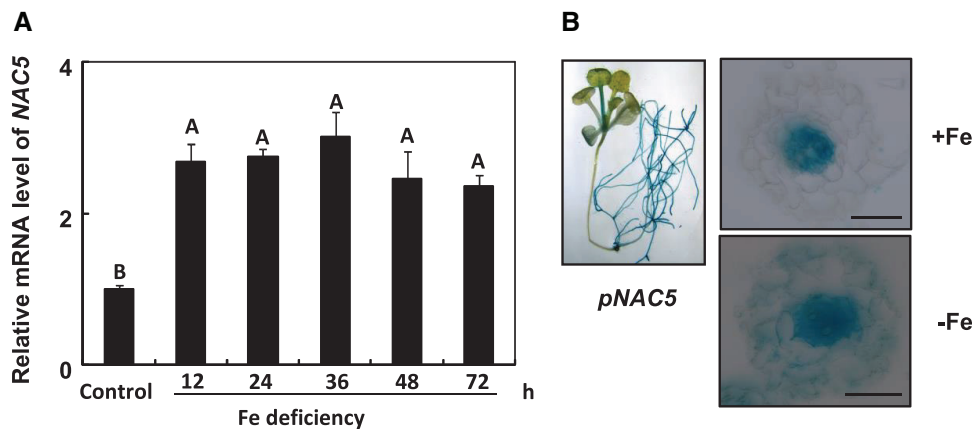
that NAC5, FIT, and PYE were involved in separate gene regions of Fe-deficient responses in Arabidopsis.

### NAC5 has transactivation activity

The UniProt program predicted the nuclear localization of NAC5 protein (<https://www.uniprot.org/locations>). To confirm the subcellular localization of NAC5 protein, we generated NAC5-GFP and YFP-NAC5 constructs, and separately

introduced the constructs into Arabidopsis leaf protoplasts. NAC5-GFP and YFP-NAC5 signals were detected only in the nucleus (Figure 6A). The localizations were confirmed by colocalization with the nucleus marker gene, NFYA4 (Figure 6A; Zhou et al., 2015).

NAC5 contains an NAC domain, indicating that NAC5 should have transcription activity. To test this hypothesis, NAC5 CDS encoding different fragments of NAC5 were fused



**Figure 4** Regulation of *NAC5* by Fe deficiency. A, Relative quantification of *NAC5* mRNA abundance in Arabidopsis roots in response to Fe deficiency by RT-qPCR. The expression levels were normalized to that of *TUB2*. Error bars represent standard errors of three biological replicates. Means with the same letter are not significantly different at  $P < 0.01$  according to one-way ANOVA followed by Tukey's multiple comparison test. B, *NAC5<sub>pro</sub>::GUS* expression pattern in transgenic Arabidopsis roots. Scale bars = 50  $\mu$ m.

to the GAL4 activation domain on the pGBKT7 vector in order to generate pBD-*NAC5* fusion plasmids. The pBD-*NAC5* fusion plasmids were co-transformed with the pGADT7 AD vector into the yeast strain Y2Hgold. The full-length *NAC5* protein and the C-terminal 202–335 amino-acid fusion protein had transactivation activities, leading to the  $\alpha$ -galactosidase activity and survival in a medium without Trp/His/Ade/Leu (Figure 6B). Deletion of the C-terminal domain showed that amino acids 1–150, 1–201, and 151–335 abolished the activation of the expression of the *LacZ* reporter gene (Figure 6B). These results indicated that the C-terminal is essential for the transactivation activities of *NAC5* protein and that at least two NAC repression domains (amino acids 1–150 and 151–201) exist in the N-terminal of the *NAC5* protein.

### *NAC5* directly transactivates the expression of *NFYA8*

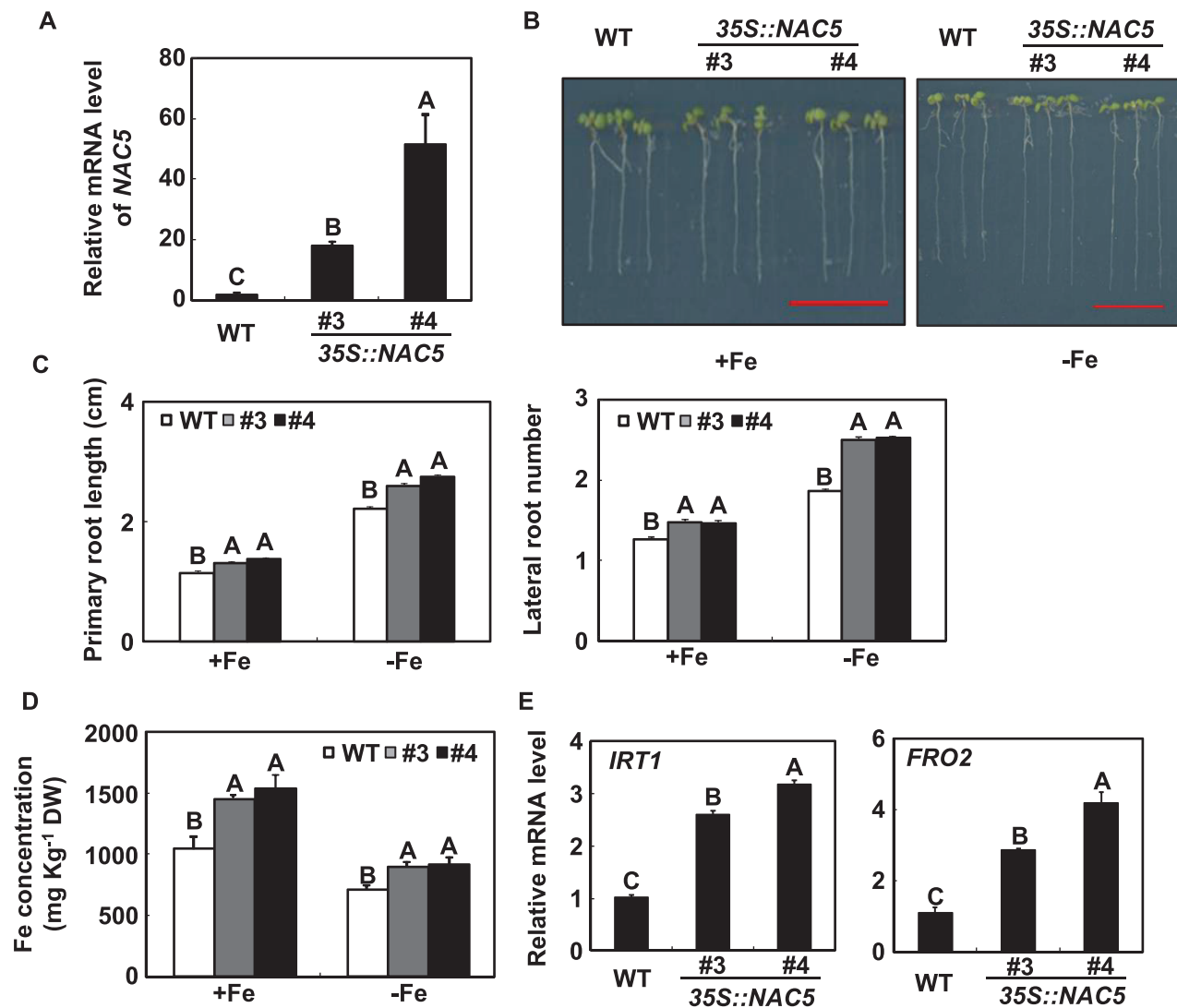
To further characterize the function of *NAC5* in Fe homeostasis, we identified *NAC5* targets by determining its genome-wide DNA-binding profile. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) was carried out using 35S::*myc-NAC5* transgenic plants (Supplemental Figure S11). As a control, the same experiments were performed in parallel with the WT. A total of 7,217 peaks were unique to 35S::*myc-NAC5* transgenic plants. Among these peaks, 1,101 were located in the core promoter regions (–1 kb to +100 bp of the transcription start site) of 1,007 genes. *NFYA8*, a member of the CCAAT-binding transcription factor (CBF-B/*NFYA*) family attracted our attention because the core promoter regions (1,000 bp) of *IRT1* and *FRO2* contain two and four CCAAT boxes, respectively (Figure 7A; Supplemental Figure S12), and because *NFYA8* expression was consistent with the location of *NAC5* (Supplemental Figure S13). To verify the ChIP-seq data, we performed ChIP-qPCR and found that the ChIP-seq detected peak of

the *NFYA8* promoter was enriched by up to 14-fold in 35S::*myc-NAC5* transgenic plants (Figure 7B). In contrast, promoter fragments of *NFYA6*, the negative control, were not enriched in 35S::*myc-NAC5* transgenic plants (Supplemental Figure S14).

We also utilize the dual-luciferase (LUC) reporter assay system to confirm the direct regulation of the *NFYA8* promoter by *NAC5*. *NAC5* significantly induced the expression of LUC driven by the *NFYA8* promoter with the *NAC5*-binding site (–650 bp), but not by the *NFYA8* promoters without the *NAC5*-binding site (–390 bp) (Figure 7C). To further confirm the direct binding of *NAC5* to the *NFYA8* promoter, we performed electrophoretic mobility shift assays (EMSA). The results of the EMSAs showed that *NAC5* was bound to the *NFYA8* promoter. When unlabeled *NAC5* probe was added to the system as a competitor, the signal was suppressed (Figure 7D), further demonstrating the direct binding of the *NFYA8* promoter by *NAC5*. Furthermore, the mRNA abundance of *NFYA8* significantly increased in 35S::*myc-NAC5* transgenic plants (Figure 7E). These results strongly suggested that *NAC5* regulates the expression of *NFYA8* in vivo.

### 35S::*NFYA8* transgenic plants are also tolerant of Fe deficiency

The direct binding of the *NFYA8* promoter by *NAC5* suggested that *NFYA8* was a directly downstream target of *NAC5*. To test the hypothesis, we overexpressed *NFYA8* in *nac5* mutant (Supplemental Figure S15). *NFYA8* overexpression almost completely rescued the retarded root development and leaf discoloration of *nac5* mutant under Fe-deficient conditions (Figure 8A; Supplemental Figure S16). Then, we constructed *NFYA8* overexpression lines and tested the responses of the 35S::*NFYA8* transgenic plants to Fe deficiency. There are four splicing variants of *NFYA8* transcripts (Figure 7A). Fortunately, these splicing variants share



**Figure 5** The responses of 35S::NAC5 transgenic plants to Fe deficiency. A, Detection of NAC5 mRNA in 35S::NAC5 transgenic Arabidopsis. RT-qPCR quantifications were normalized to the expression of *TUB2*. Error bars represent standard errors of three biological replicates. B, Representative images of 35S::NAC5 transgenic Arabidopsis seedlings grown on Fe-deficient medium for 7 d. Scale bars = 1 cm. C, The primary root length and lateral root number of 35S::NAC5 transgenic Arabidopsis seedlings grown on Fe-deficient medium for 7 d. D, The Fe concentration in the roots of 18-d-old hydroponically grown Arabidopsis, either supplied with Fe or transferred to a Fe-free solution for 36 h. Error bars represent standard errors of three independent experiments, with 20–30 plants per experiment (C and D). E, The effects of Fe deficiency on the expression levels of *IRT1* and *FRO2* in 18-d-old hydroponically grown WT and 35S::NAC5 transgenic plants. Error bars represent standard errors of three biological replicates. Means with the same letter are not significantly different at  $P < 0.01$  according to one-way ANOVA followed by Tukey's multiple comparison test.

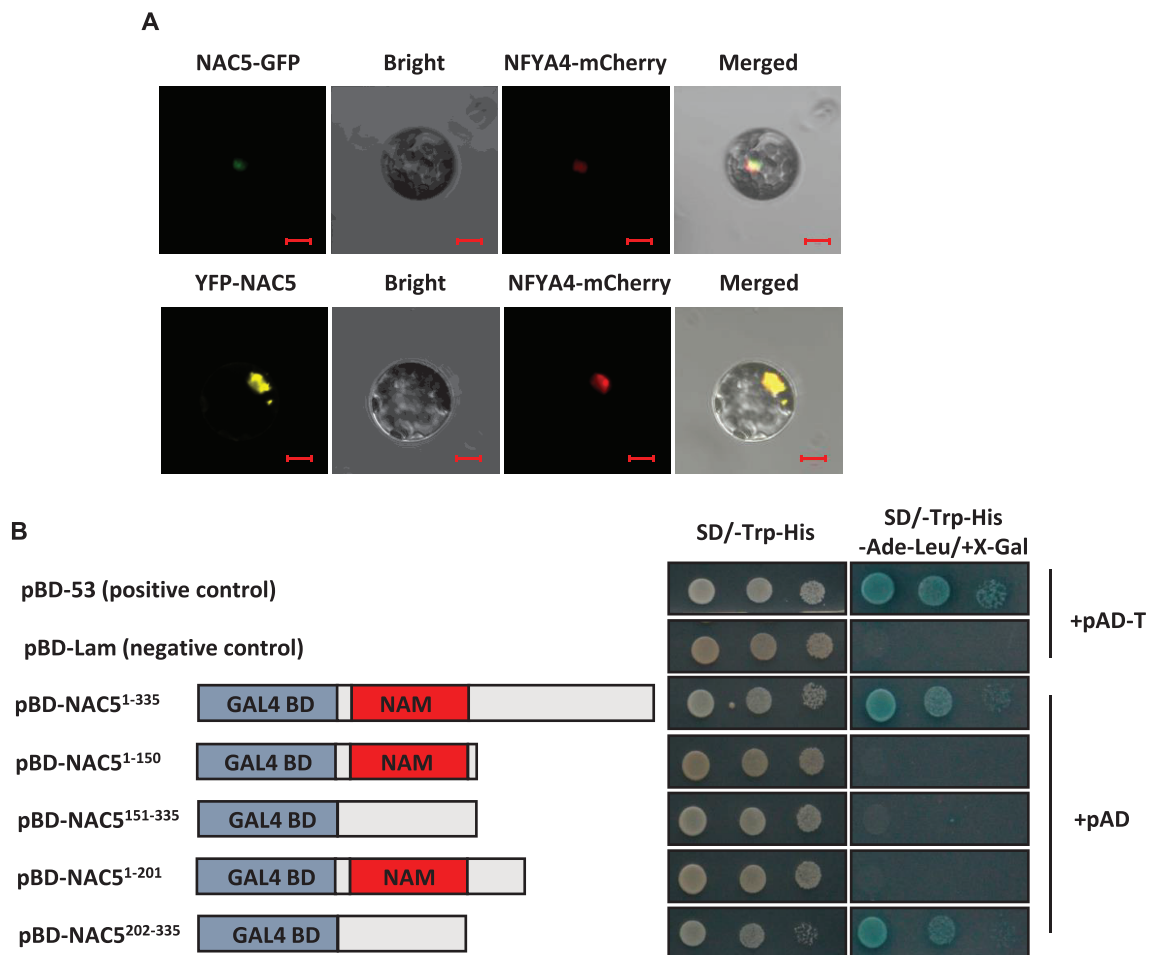
a common coding region, and the full-length CDS region of *NFYA8* was therefore used for the construct (Supplemental Figure S17). Consistent with *mir164b* mutant and 35S::NAC5 transgenic plants, the primary root length, lateral root number, ferric reductase activity, Fe content, and mRNA abundance of *IRT1* and *FRO2* were higher in 35S::NAC5 transgenic plants than in WT plants under Fe-deficient conditions (Figure 8, B–F). We also used the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Protein 9 (Cas9) system to generate *NFYA8* loss-of-function mutants (Supplemental Figure S18A). Cas9-free transgenic plants were identified for the phenotypic analysis. Compared with the WT, *NFYA8* loss-of-function mutants

were more sensitive to Fe deficiency (Supplemental Figure S18, B and C). These results indicated that *NFYA8* plays important role in plant tolerance to Fe deficiency.

## Discussion

Recent research has clarified the transcriptional responses, especially of bHLH family members, to Fe deficiency in plants (Colangelo and Guerinot, 2004; Yuan et al., 2005, 2008; Long et al., 2010; Wang et al., 2013; Zhang et al., 2015; Li et al., 2016; Liang et al., 2017; Cui et al., 2018). In this study, we identified a transcription factor from the NAC family, *NAC5*, that is important for Fe homeostasis in





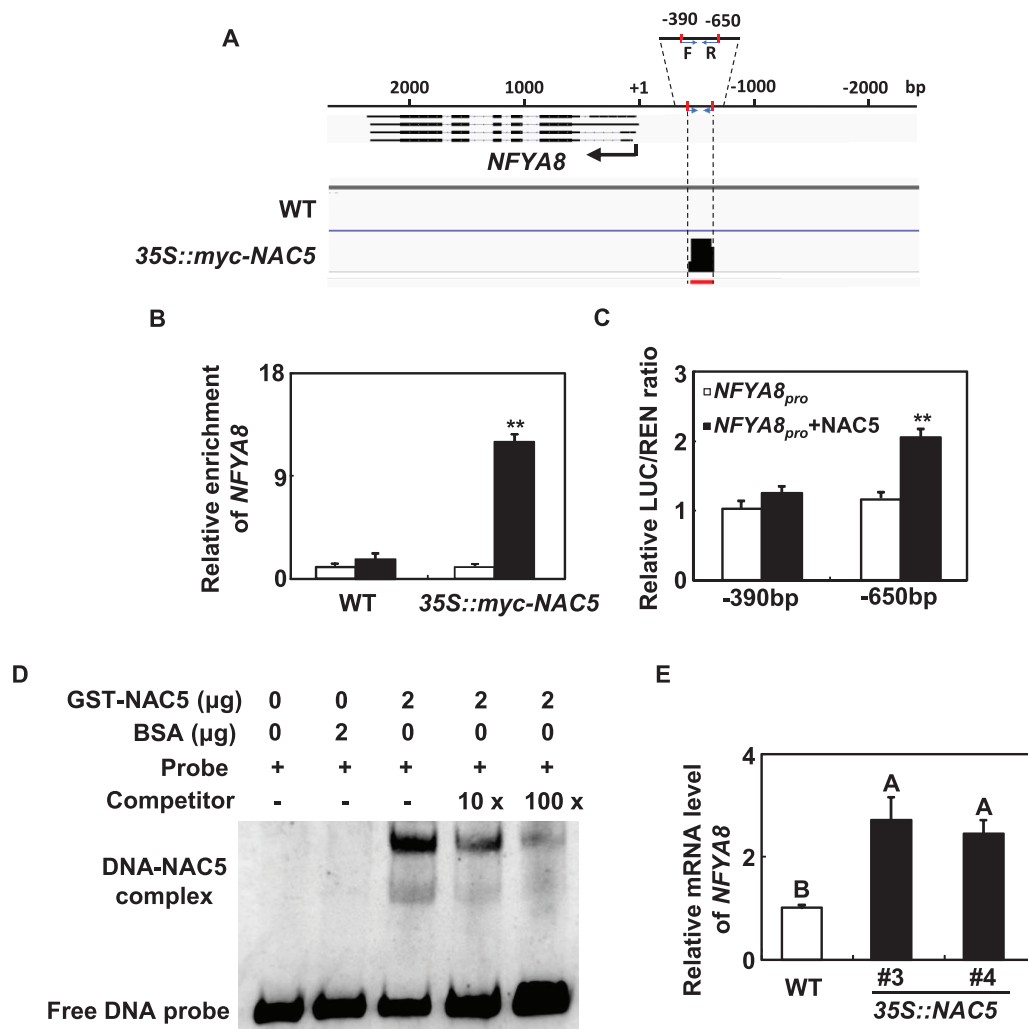
**Figure 6** Subcellular localization and transcriptional activation assay of the NAC5 protein. A, NAC5-GFP and YFP-NAC5 constructs were transiently coexpressed with the nuclear marker NFYA4-mCherry in Arabidopsis leaf protoplasts. GFP, YFP, and mCherry signals were imaged by confocal microscopy. Scale bars = 10  $\mu$ m. B, Transcriptional activation assay of NAC5 protein in yeast. NAC5 cDNAs encoding different fragments of NAC5 were fused to the GAL4 activation domain on the pGBKT7 vector to generate pBD-NAC5 fusion plasmids. pBD-53 and pBD-Lam were used as a positive control and a negative control, respectively.

Arabidopsis. Although the NAC family was previously determined to be associated with Fe homeostasis in Strategy II plants, the current study demonstrates that NAC protein could be involved in Fe-deficiency responses in Strategy I plants.

NAC5 is a functional transcription factor because (1) both NAC5-GFP and YFP-NAC5 are localized in the nucleus; (2) NAC5 contains a conserved NAC DNA-binding domain at the N terminus (amino acids 17–141); and (3) NAC5 has transcriptional activation activity as demonstrated by autoactivation testing in yeast cells and dual-LUC transient expression assay in Arabidopsis mesophyll protoplasts. In Arabidopsis, *FIT* and *PYE* regulons are the two key networks that regulate Fe homeostasis. NAC5 might define another Fe-deficiency-responsive transcriptional cascade. The NAC5 transcriptional cascade is important for Fe-deficiency responses because *mir164b* knockout plants, miR164 knock-down transgenic plants, and NAC5-overexpressing lines are

tolerant of Fe deficiency. Compared with the WT, these mutants and transgenic plants produce longer primary roots and more lateral roots under Fe-deficient conditions. Another related NAC transcription factor, NAC1 increases lateral root emergence by affecting auxin signals (Xie et al., 2000; Guo et al., 2005; D'haeseleer et al., 2011). NAC1 and NAC5 might be involved in separate gene regulons for root development, as indicated by the following observations: (1) under normal conditions, 35S::NAC5 transgenic plants but not 35S::NAC1 transgenic plants had longer primary roots than the WT (Guo et al., 2005); (2) the mRNA abundance of NAC5, but not of NAC1, accumulated under Fe-deficient conditions (this study); and (3) the lateral root number of 35S::NAC5 transgenic plants increased only under Fe-deficient conditions (this study).

By determining the genome-wide DNA-binding profile of NAC5, we identified 1,007 cis-acting targets. Gene ontology (GO; <http://systemsbiology.cau.edu.cn/agriGOv2/>) analysis

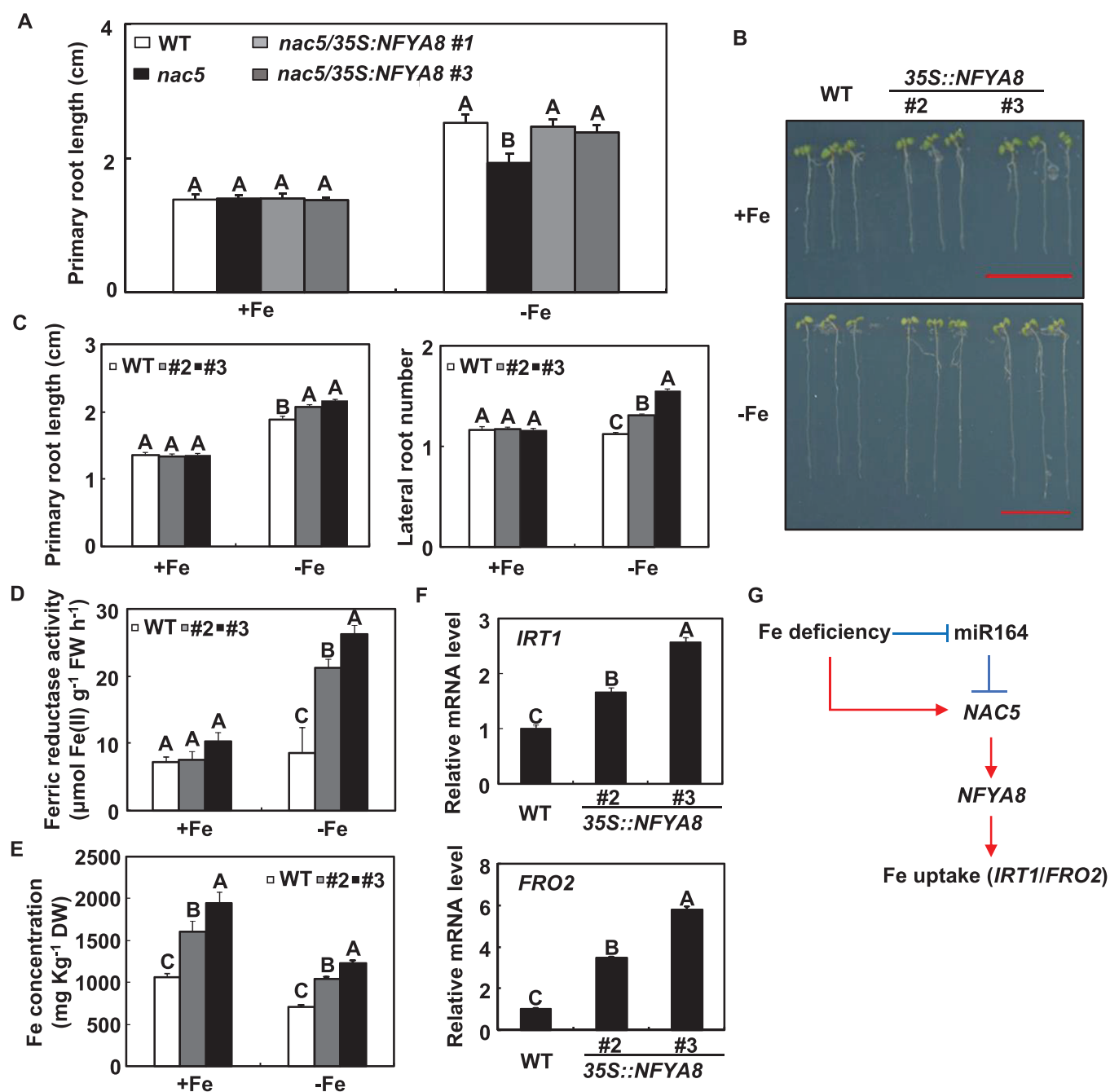


**Figure 7** NFYA8 is the direct target of NAC5. A, NAC5 binding peak in the promoter region of NFYA8 detected by ChIP-seq. F and R indicate the positions of forward and reverse primers used in ChIP-qPCR. B, ChIP-qPCR assays verified the in vivo binding activity of NAC5 to the promoter of NFYA8. TUB2 was used as an internal control. Error bars represent standard errors of three biological replicates. Asterisks indicate significant differences ( $P < 0.01$ ) between the amount of NFYA8 fragment in the immunoprecipitate and the amount in the input according to  $t$  tests. C, Transient transactivation assay of NAC5 protein with the promoter of NFYA8 in Arabidopsis mesophyll protoplasts. Relative REN activity was used as an internal control and the relative LUC/REN ratios are shown. Error bars represent standard errors of three biological replicates. Asterisks indicate significant differences based on  $t$  tests ( $P < 0.01$ ). D, EMSA with NAC5 protein using the probes derived from the NFYA8 promoter. Competition for the labeled sequences was tested by adding an excess of unlabeled probes as indicated. The free probes and DNA-NAC5 are marked. E, Detection of NFYA8 transcripts in 35S::NAC5 transgenic plants by RT-qPCR. Quantifications were normalized to the expression of TUB2. Error bars represent standard errors of three biological replicates. Means with the same letter are not significantly different at  $P < 0.01$  according to one-way ANOVA followed by Tukey's multiple comparison test.

indicated that these genes were related to various metabolic processes, including cytochrome-c oxidase activity (GO:0004129,  $P = 0.00019$ ), oxidoreductase activity, acting on a heme group of donors, oxygen as acceptor (GO:0016676,  $P = 0.00019$ ), NADH dehydrogenase activity (GO:0003954,  $P = 0.00027$ ), and mitochondrion (GO:0005739,  $P = 2.3e^{-24}$ ). These results suggest that NAC5 is involved in oxidative stress.

The peak in the core promoter region of NFYA8 (202 bp) contains eight NAC-binding sites (<http://jaspar.genereg.net/>). The binding of NFYA8 was verified by

ChIP-qPCR, dual-LUC reporter assay, EMSAs, and the corresponding mRNA abundance in 35S::myc-NAC5 transgenic plants. NF-Y is a universal transcription factor with high affinity and sequence specificity for the CCAAT box in ~25% of eukaryotic gene promoters. There are two and four CCAAT boxes located in core promoter regions of IRT1 and FRO2, respectively, and 35S::NFYA8 transgenic plants are as tolerant as 35S::NAC5 plants to Fe deficiency, indicating that NAC5 affected Fe-deficient responses in Arabidopsis by regulation of the expression of NFYA8. The 202-bp sequence



**Figure 8** Responses of *35S::NFYA8* transgenic plants to Fe deficiency. A, Rescue of the *nac5* phenotype by *NFYA8* overexpression. B, Representative images of *35S::NFYA8* transgenic *Arabidopsis* seedlings grown on Fe-deficient medium for 7 d. Scale bars = 1 cm. C, The primary root length and lateral root number of *35S::NFYA8* transgenic *Arabidopsis* seedlings grown on Fe-deficient medium for 7 d. D, The ferric reductase activity in the roots of *35S::NFYA8* transgenic *Arabidopsis* seedlings grown on Fe-deficient medium for 7 d. E, The Fe concentration in the roots of 18-d-old hydroponically grown *Arabidopsis*, either supplied with Fe or transferred to a Fe-free solution for 36 h. F, The effects of Fe deficiency on the expression levels of *IRT1* and *FRO2* in 18-d-old hydroponically grown WT and *35S::NFYA8* transgenic plants. Error bars represent standard errors of three independent experiments, with 20–30 plants per experiment (A, C–E). Error bars represent standard errors of three biological replicates (F). Means with the same letter are not significantly different at  $P < 0.01$  according to one-way ANOVA followed by Tukey's multiple comparison test. G, A model for the co-function among *MIR164b*, *NAC5*, and *NFYA8* in *Arabidopsis* under Fe-deficient conditions. The solid arrows and solid lines with perpendicular lines represent the upregulation and downregulation of corresponding mRNA abundance, respectively.

was not conserved among other members of the *NFYA* family, and promoter fragments of *NFYA6*, another member of the *NFYA* family, were not enriched in *35S::myc-NAC5* transgenic plants, which suggests that

the *NAC5*-*NFYA8* route is specific for Fe-deficiency responses in *Arabidopsis*.

Transcriptional induction may explain part of the *NAC5* transcript accumulation under Fe deficiency. Our results

suggest that the posttranscriptional regulation of *NAC5* transcripts by miR164 is also important for Fe homeostasis in Arabidopsis. Under Fe-sufficient conditions, long primary roots were produced by *MIR164a/b/c* TM transgenic plants, but not by *mir164b* mutant plants. Given the variations of Fe concentrations and ferric reductase activities in the roots of TM transgenic plants and *mir164b* mutants, we inferred that *MIR164b* is the member of the miR164 family that is mainly responsible for Fe-deficiency responses in Arabidopsis. GUS staining patterns showed that *MIR164b* expression was consistent with the location of *NAC5*, and *mir164b* plants were as tolerant of Fe deficiency as 35S::*NAC5* and 35S::*NFYA8* transgenic plants. We, therefore, propose a model for the co-function among *MIR164b*, *NAC5*, and *NFYA8* in Arabidopsis under Fe-deficient conditions (Figure 8G). Fe deficiency reduces *MIR164b* abundance and thereby, relieves miR164-guided repression of *NAC5* in Arabidopsis roots. *NAC5* directly transactivates the expression of *NFYA8*, which induces the mRNA accumulation of the Fe transporters *IRT1* and of the ferric-chelate reductase *FRO2*. In addition, the expression levels of miR164 were induced by Fe excess in Arabidopsis. This increases the tolerance of Arabidopsis to Fe stress.

## Materials and methods

### Plant materials and growth conditions

Arabidopsis (*A. thaliana*) Col-0 ecotype plants were grown hydroponically in a growth chamber with a light intensity of  $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 60%–70% relative humidity, and a 22°C/18°C day/night temperature regime. The hydroponic culture was performed as previously described (Zhao et al., 2011). After they had grown for 18 d, the plants were supplied with Fe free or 500  $\mu\text{M}$  Fe nutrient solutions to simulate Fe-deficient and excess conditions. Nutrient solutions were renewed daily to ensure pH stability. Shoots and roots were separately harvested, flash frozen, and stored at  $-80^\circ\text{C}$ . Arabidopsis seedlings in 0.5 MS (Murashige and Skoog, 1962) nutrient agar medium were grown under continuous light ( $70 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) at  $23^\circ\text{C} \pm 1^\circ\text{C}$ .

### Constructs and generation of transgenic plants

The full-length CDS of *NAC5* (AT5G61430) and *NFYA8* (AT1G17590) was amplified by PCR to generate 35S::*NAC5*, 35S::*NFYA8* transgenic plants. A fragment surrounding the *MIR164b* (AT5G01747) sequence including the fold-back structure was amplified from genomic DNA. The amplified fragments were introduced into the pENTR/D-TOPO vector (Invitrogen, Waltham, MA, USA) and cloned into pMDC32 (Curtis and Grossniklaus, 2003) by LR reactions (Invitrogen).

To generate the miR164 knock-down mutant, the short tandem target mimic transgenic lines targeting *MIR164a*, *MIR164b*, and *MIR164c* were designed as described by Yan et al. (2012). To generate 35S::*myc-NAC5* transgenic plants, the full-length CDS of *NAC5* was amplified. Thereafter, the fragments were cloned into the pCPB vector using the *Bam*H I restriction site by In-Fusion reaction.

The CRISPR/Cas9-mediated *NFYA8* editing was performed as described by Xing et al. (2014) with some modifications. In brief, two gRNAs that direct target sequences located at the first and fifth exon of *NFYA8* were produced. The fragments were cloned into the pHEC401 vector.

For *MIR164b*, *NAC5*, and *NFYA8* promoter::GUS constructs, the 906-bp, 831-bp, and 1,955-bp fragments upstream from the initiation codon were amplified and separately cloned into the pMDC164 vector following Gateway recombination.

The fusions of yellow fluorescent protein (YFP) to the C-terminal end and green fluorescent protein (GFP) to the N-terminal end of *NAC5* were generated and introduced separately into pAN580 and pCPB vectors using the *Bam*HI restriction site by In-Fusion reaction. The plasmids were transiently expressed in Arabidopsis mesophyll protoplast. YFP/GFP images were collected with a Leica SP2 confocal microscope. YFP and GFP were detected at 488 nm laser excitation, their collection bandwidth was at 515–535 nm and 510–550 nm, respectively (intensity at 3%, Gain < 20%).

The plasmids were electroporated into *Agrobacterium tumefaciens* GV3101, and Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with the use of 35  $\mu\text{g}$  mL<sup>-1</sup> hygromycin or 45  $\mu\text{M}$  glufosinate ammonium. T<sub>3</sub> or T<sub>4</sub> homozygous lines were used for all experiments. The sequences of the primer pairs used in the experiments are listed in Supplemental Table S4.

### Small RNA isolation, library construction, and sequencing

Total RNA was extracted with Trizol reagent (Invitrogen) from roots subjected to Fe deficiency for 2 d. Small RNA isolation, library construction, and data analysis were performed as previously described (Gao et al., 2015). Solexa sequencing was performed at BGI (Shenzhen, China). The unique sequencing data were aligned to the Arabidopsis genome (The Arabidopsis Information Resource 9). Only perfectly matching sequences were considered for further analysis. The count information was used to determine normalized miRNA expression levels as reads of exon model per million mapped reads (RPM).

### Gene expression analysis

For quantitative reverse transcription PCR (RT-qPCR), 1  $\mu\text{g}$  of total RNA isolated with the RNeasy plant mini kit was used for the first-strand cDNA synthesis with the SuperScript III first-strand synthesis supermix (Invitrogen). Specific primers were used to detect the expression levels of *NAC5*, *NFYA8*, *IRT1* (AT4G19690), and *FRO2* (AT1G01580). Primer efficiencies were determined (Ramakers et al., 2003). For stem-loop RT-qPCR, 0.5  $\mu\text{g}$  of total RNA was used for the cDNA synthesis with miR164 stem-loop RT primer according to the protocol of Varkonyi-Gasic et al. (2007). RT-qPCR was carried out in an ABI 7500 system using the SYBR Premix Ex Taq (perfect real time) kit (TaKaRa Biomedicals, San Jose, CA, USA) as previously described

(Zhao et al., 2011). Each experiment was replicated three times. The comparative Ct method was applied. The sequences of the specific primers are listed in [Supplemental Table S4](#).

### Transactivation assay in yeast cells

The yeast strain Y2Hgold (*MATa trp1-901 leu2-3 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1UAS-Gal1TATA-His3 GAL2<sub>UAS</sub>-Gal2<sub>TATA</sub>-Ade2 URA3::MEL1<sub>UAS</sub>-Mel1<sub>TATA</sub> AUR1-C MEL1*), which contains *HIS3* and *lacZ*, was used in the assay. Different fragments of *NAC5* were obtained by PCR and cloned into the pGBKT7 vector to generate pBD-*NAC5* fusion plasmids. The fusion plasmids were transformed into Y2Hgold according to the manufacturer's instructions (Clontech). The transformants were selected on synthetic medium lacking Leu and His. The positive clones were plated on SD/-Leu-Trp-His-Ade selective dropout medium. The transactivation was further confirmed by chloroform overlay  $\alpha$ -galactosidase plate assay without Leu, Trp, His, and Ade.

### ChIP assay

Two-weeks-old 35S::*myc*-*NAC5* transgenic plants grown on 0.5 MS medium were used for ChIP assays as described by [Huysmans et al. \(2018\)](#). A 3-g quantity of ground plant tissue was cross-linked in 1% (v/v) formaldehyde. The chromatin complexes were isolated and sonicated with a Bioruptor to obtain 200- to 800-bp DNA fragments. Protein–DNA complexes were immunoprecipitated using an anti-Myc Tag antibody (Millipore, Burlington, MA, USA). The purified DNA fragments were quantified by ChIP-qPCR with the primers listed in [Supplemental Table S4](#). The enrichment was calculated based on the amount of fragment in the immunoprecipitate relative to the amount in the input.

### The dual-LUC transient expression assay

*NFYA8* promoters with and without *NAC5* binding site were amplified by PCR. The amplified fragments were cloned into the pGreen0800-LUC double reporter vector using the *Bam*H I restriction site to generate reporters. The coding sequence of *NAC5* was amplified and introduced into the pRI101-AN vector to generate effectors. The constructs with various combinations were infiltrated into *Arabidopsis* mesophyll protoplasts as previously described ([Yoo et al., 2007](#)). The activities of Firefly LUC and Renilla LUC (REN) were measured using the Dual-LUC Reporter Assay System (Promega, Madison, WI, USA). The relative reporter ratio (LUC/REN) was normalized with respect to the average values of the *NFYA8* promoter.

### EMSA

EMSA were performed as previously described ([Liu et al., 2014](#)). Affinity-purified GST-*NAC5* recombinant protein was used for EMSAs. For the generation of GST-*NAC5* recombinant protein, full length coding region of *NAC5* was amplified and cloned into pGEX-4T vector using the *Bam*H I restriction site by In-Fusion reaction. GST-*NAC5* construct was expressed in *Escherichia coli* BL21 and purified by

glutathione-Sepharose 4B (GE Healthcare, Stockholm, Sweden). The DNA probe from *NFYA8* promoter was amplified by PCR and labeled DIG-ddUTP using DIG Gel Shift Kit (Roche, Basel, Switzerland). The same unlabeled fragment was used as competitor probe and the bovine serum albumin (BSA) alone was used as the negative control.

### Determination of Fe concentration

After heating to 120°C for 30 min, the roots of hydroponic *Arabidopsis* plants were dried at 65°C for 72 h and milled to a fine powder. The samples were weighed into PTFE digestion tubes and digested in HNO<sub>3</sub> using a microwave digester (CEM Mars6 Xpress). The Fe concentration in the samples was determined by ICP-MS (Agilent 7700x; Agilent Technologies, Santa Clara, CA, USA).

### Assay of Fe(III)-chelate reductase activity

The activity of Fe(III)-chelate reductase was assayed according to the procedure of [Connolly et al. \(2003\)](#) and [Satbhai et al. \(2017\)](#). Eight 7-d-old *Arabidopsis* plants were pooled together and whole roots of the plants were submerged in a medium containing 0.1 mM Fe(III)-ethylenediaminetetraacetic acid (EDTA) and 0.3 mM ferrozine. After incubation for 30 min in darkness at 25°C, the transmittance of Fe(II)-ferrozine at 562 nm was determined.

### GO analysis

GO enrichment was performed using AgriGO version 2.0 (<http://systemsbiology.cau.edu.cn/agriGOv2/>) with default parameters. GO terms were considered significantly enriched at false discovery rates < 0.05.

### Statistical analysis

In this study, data were presented as means  $\pm$  standard errors. Number of replicates can be found in the corresponding figure legends. The Student's *t* test was used to determine statistical significance between two groups, asterisk indicate significant difference at  $P < 0.01$ . For multiple group comparisons, the data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and means with the same letter are not significantly different at  $P < 0.01$ .

### Accession numbers

*Arabidopsis* Genome Initiative numbers for the genes discussed in this article are as follows: *MIR164b*, AT5G01747; *NAC1*, AT1G56010; *NAC5*, AT5G61430; *NFYA8*, AT1G17590; *IRT1*, AT4G19690; *FRO2*, AT1G01580; *NFYA6*, AT3G14020; *NFYA4*, AT2G34720; *PYE*, AT3G47640; *FIT1*, AT2G28160; *TUB2*, AT5G62690.

### Supplemental data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Marker gene expression is significantly induced by Fe deficiency in *Arabidopsis*.

**Supplemental Figure S2.** Detection of mature miR164 expression by stem–loop RT-qPCR in the xylem sap of *Arabidopsis* under Fe-sufficient and -deficient conditions.

**Supplemental Figure S3.** Regulation of miR164 by Fe excess.

**Supplemental Figure S4.** Diagram of the T-DNA insertion site in the *MIR164b* locus and detection of *MIR164b* abundance.

**Supplemental Figure S5.** The responses of 35S::*MIR164b* transgenic plants to Fe deficiency.

**Supplemental Figure S6.** Leaf phenotype of WT and *mir164b* mutant and *TM* transgenic plants.

**Supplemental Figure S7.** Relative quantification of *NAC1* mRNA abundance in *Arabidopsis* roots in response to Fe deficiency by RT-qPCR.

**Supplemental Figure S8.** The mRNA abundance of *NAC5* in *mir164b*.

**Supplemental Figure S9.** The responses of *nac5* to Fe deficiency.

**Supplemental Figure S10.** The relative mRNA level of *FIT1* and *PYE* in WT, 35S::*NAC5* transgenic plants, and *nac5* mutants.

**Supplemental Figure S11.** *NAC5* protein abundance in 35S::*myc-NAC5* transgenic plants.

**Supplemental Figure S12.** Schematic diagram of CCAAT box distribution in the core promoter regions of *IRT1* and *FRO2* genes.

**Supplemental Figure S13.** *NAC5<sub>pro</sub>::GUS* and *NFYA8<sub>pro</sub>::GUS* expression pattern in transgenic *Arabidopsis* roots.

**Supplemental Figure S14.** *NAC5* did not bind to the promoter of *NFYA6*.

**Supplemental Figure S15.** Detection of *NFYA8* mRNA in *nac5/35S::NFYA8* transgenic *Arabidopsis*.

**Supplemental Figure S16.** The effects of Fe deficiency on leaf color of WT, *nac5* mutant, and *nac5/35S::NFYA8* transgenic *Arabidopsis*.

**Supplemental Figure S17.** Detection of *NFYA8* mRNA in 35S::*NFYA8* transgenic *Arabidopsis*.

**Supplemental Figure S18.** The responses of *nfya8* to Fe deficiency.

**Supplemental Table S1.** Statistics of small RNA sequences from *Arabidopsis* roots with or without Fe.

**Supplemental Table S2.** Different categories of small RNAs in two small RNA libraries.

**Supplemental Table S3.** Expression profiles of conserved miRNAs in response to Fe deficiency in *Arabidopsis* roots.

**Supplemental Table S4.** Oligos used as primers in the experiment.

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*Conflict of interest statement.* We declare that we have no conflict of interest.

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