Supporting Online Material for

TATA box insertion provides a selected mechanism in apple for enhancing gene expression to adapt to Fe deficiency

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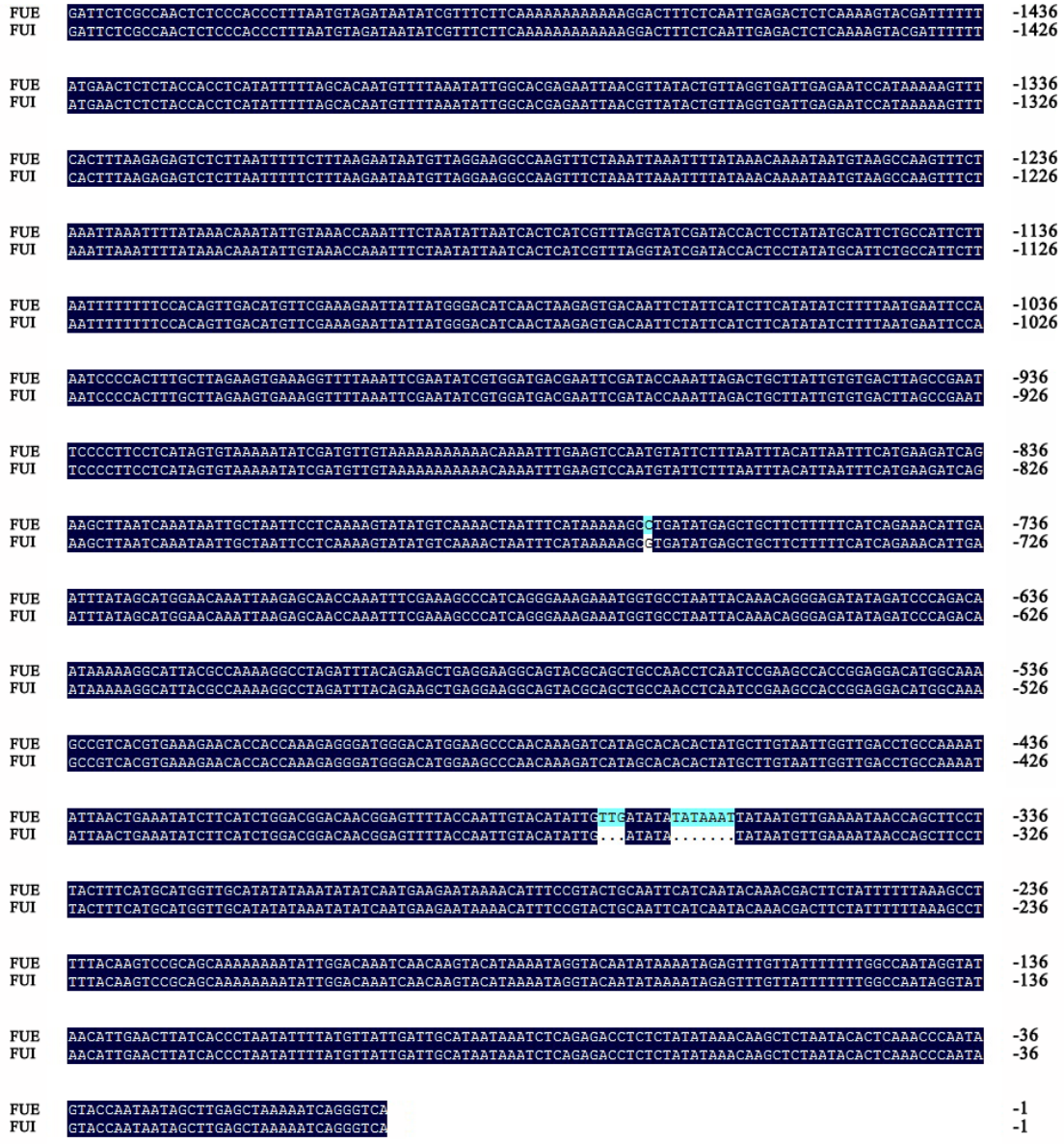
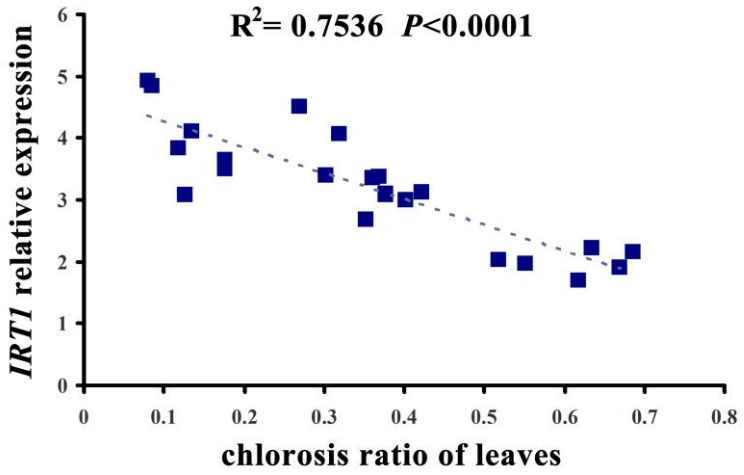
Supplemental Method S1. *A. thaliana* protoplast isolation and PEG-mediated gene transformation.

Supplemental Method S2. Modified yeast one-hybrid analysis protocol.

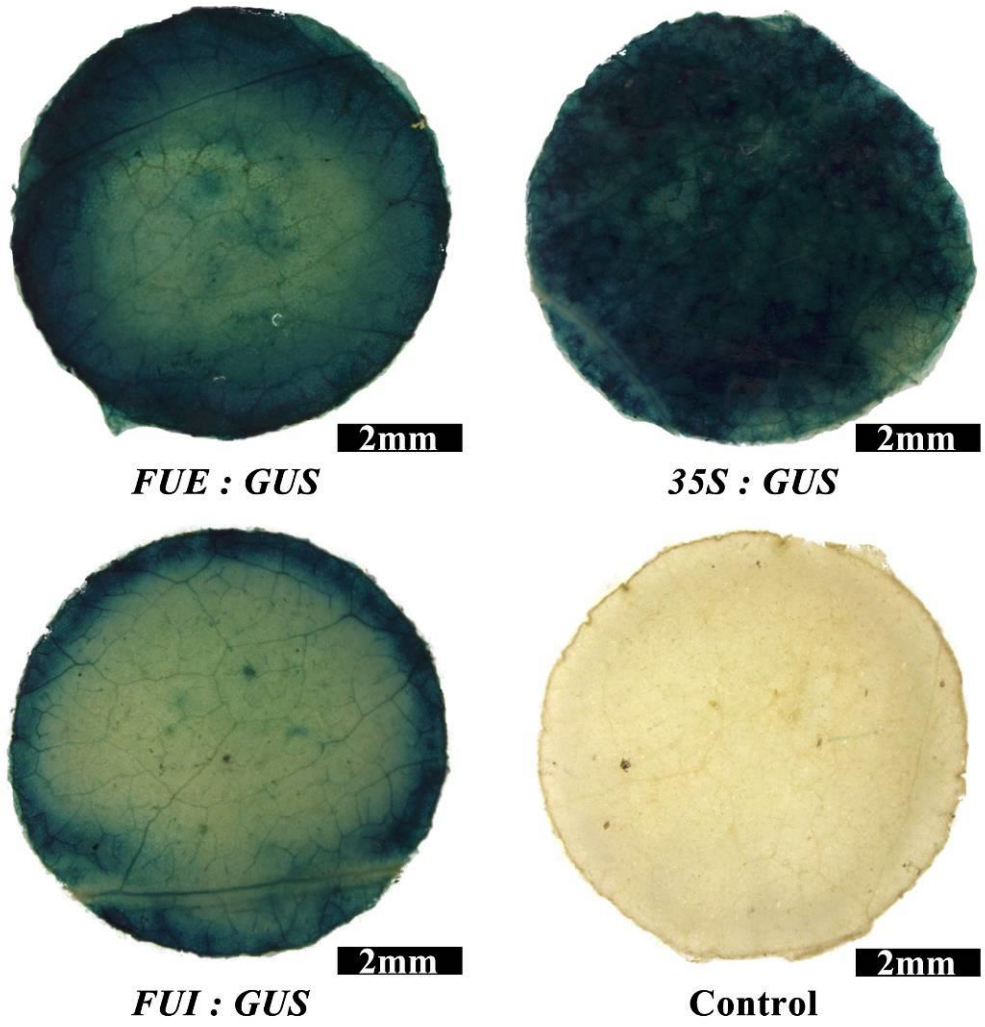
Supplemental Method S3. Functional complementation in yeast.



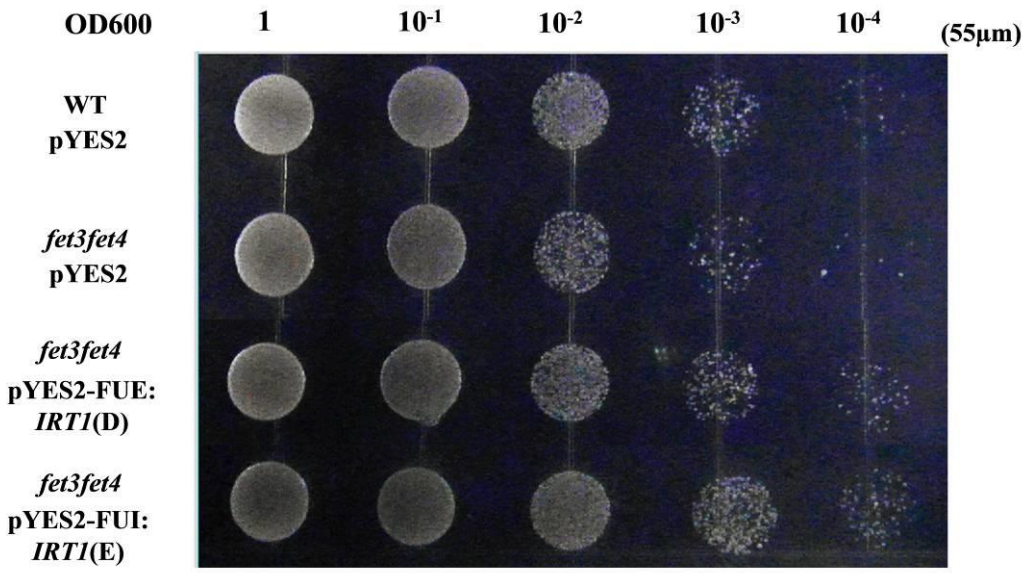
Supplemental Figure S1. Sequences of the 1500 bp FUE and FUI promoters.



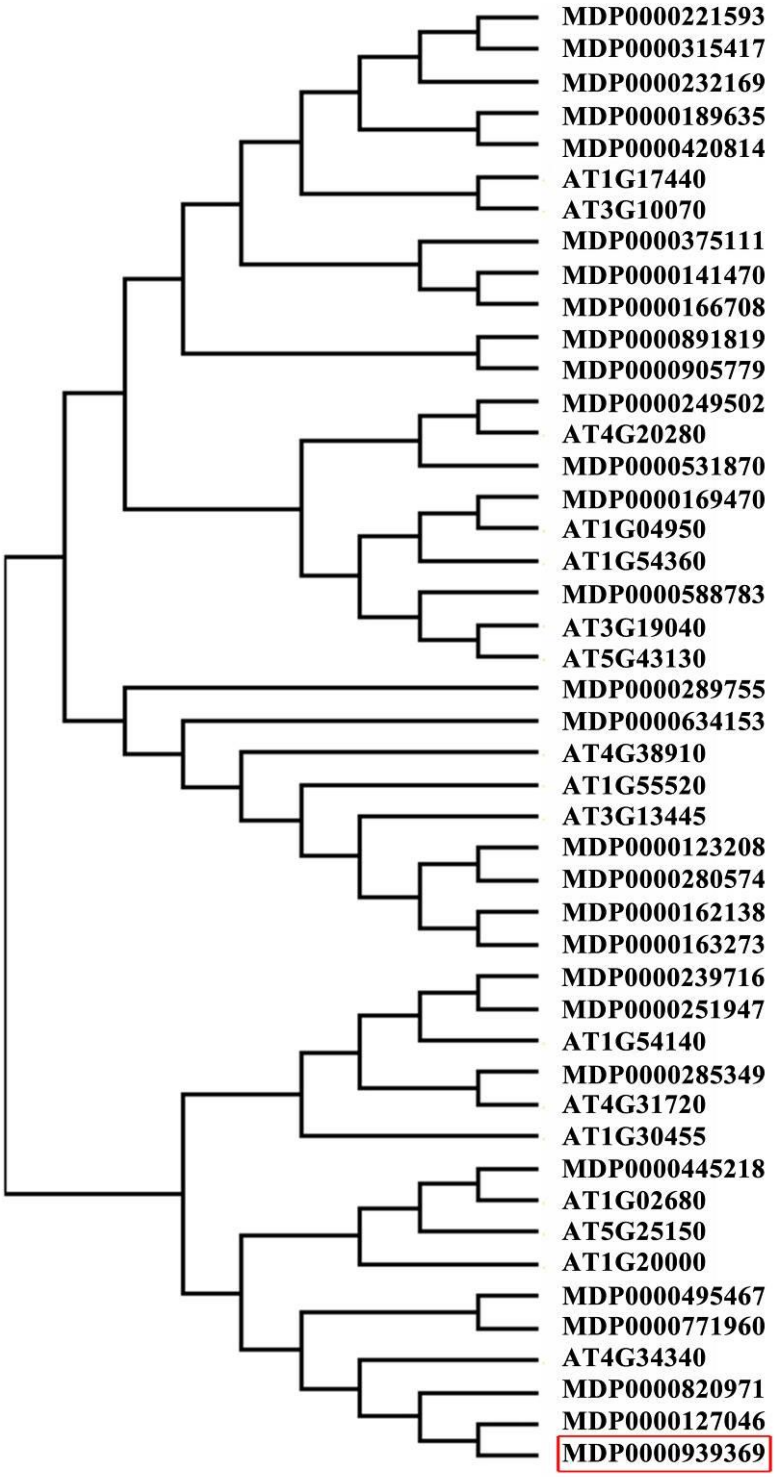
Supplemental Figure S2. Correlation analysis between chlorosis ratios and relative expression levels of *IRT1* in the progeny of *M. xiaojinensis* and *M. baccata* under Fe-deficiency treatment.



Supplemental Figure S3. Transient *GUS* expression under the control of the FUE and FUI promoters in wild type tobacco.



Supplemental Figure S4. Complementation test of *IRT1* Fe^{2+} uptake in the *fet3fet4* yeast mutant grown on Fe-deficient medium supplemented with 55 µM BPDS (bathophenanthrolinedisulfonic acid disodium salt), pH 5.8.



Supplemental Figure S5. Phylogenetic analysis of homologous transcription factors in the apple and *A. thaliana* genomes.

Supplemental Table S1. Primers for the transcription factor genes used in the yeast one-hybrid assay.

Gene		Primer sequences(5'-3')
MDP0000249502	Fwd	ATGAAGCAGCAATCCAAGGACCC
	Rev	CTACCGGAAGAGCCTAGACTGTGAT
MDP0000251947	Fwd	ATGGCAGGGGAAGATGAGGACTT
	Rev	CTACCTTGCGACATGCTTGG
MDP0000239716	Fwd	ATGGTTCATGCAGAATCGGC
	Rev	TCATCCTCCACCATTTCTTCCGTTA
MDP0000163273	Fwd	ATGGAGGCGCAAGGTTTCAA
	Rev	TTAAAGTTCATACCAATGCTGATTT
MDP0000162138	Fwd	ATGTTTGGGTCACGACGCGT
	Rev	TTAAAGTTCATACCAATGCTGATTT
MDP0000280574	Fwd	ATGGAAGAACAAGGTGGATCTGAAG
	Rev	TTACTGTTGCTGGACCTTCCTGAAC
MDP0000634153	Fwd	ATGACGTCAATCGGGCCGGT
	Rev	TYAAYYYTKMYCYGKYGCATCWTCT
MDP0000123208	Fwd	CGTTTTGCCGCGGTCATTAT
	Rev	TCATCCTTGAATGTCCTTGTTGCAC
MDP0000445218	Fwd	ATGGAAGACATCGTTGTGGAATATG
	Rev	TCAAAAGCCCTTCCTCAGCTT
MDP0000169470	Fwd	ATGAGCATTGTGCCCAAAGA
	Rev	TTACAAAAACAAACACATCTCGG
MDP0000166708	Fwd	ATGGCGGAAAACGGCCCATC
	Rev	TTAAAACCGAGTCATTTGCTGCATC
MDP0000141470	Fwd	ATGGCGGAAAACGGCCCATC
	Rev	TTAAAACCGAGTCATTTGCTGCAGC
MDP0000315417	Fwd	ATGGGCCGTGGCGGCGTCTC
	Rev	TTATGTAACTTCACGCATTTTAGAG
MDP0000221593	Fwd	ATGGATCAACAGAACTCCACC
	Rev	TTATGTAACTTCACGCATTTTAGAG
MDP0000232169	Fwd	ATGGATCAACAGAACTCCACCA
	Rev	CACAGCAAGACGCTCCTTGT
MDP0000375111	Fwd	ATGGGGGTGATGGGATCCAT
	Rev	TTAGATCTTCAATTGATCGTCATTA
MDP0000891819	Fwd	ATGATTGAYTTTTCATTATCCC
	Rev	TTATGTAACTTCACGCATTTTAGAG
MDP0000905779	Fwd	ATGATTGACTTTTCATTATCCCCAA
	Rev	TTAACAAGACCACCCTTTGCATTT
MDP0000531870	Fwd	ATGTCGGCTTTCTGGCTGTG
	Rev	TTAAAGCCCCACCTCAACATTTC
MDP0000588783	Fwd	ATGGCATCTTCTTCGGACTCAT
	Rev	TCMCCTTTTGCAAATTGAAGAAA

MDP0000430814	Fwd	ATGCAGGGAATGGGGATGAT
	Rev	TCAATTACTCACAGAATCAACAAAT
MDP0000189635	Fwd	ATGGACCAACAGAACTCCAC
	Rev	TCAATTACTCACAGAATCAACAAAT
MDP0000285349	Fwd	AATCAGGCTGGTAGCTGTCG
	Rev	TTACTCTTCCCTTGTTGCAGGAT
MDP0000939369	Fwd	ATGAGCCATGGAGATGCGGA
	Rev	CTACAACTGAGTGAGTTCCTGTGGG
MDP0000127046	Fwd	ATGAGCCATGGAGATGCGGA
	Rev	CTACAACTGAGTGAGTTCCTGTGGG
MDP0000820971	Fwd	ATGAGCCATGGAGATGCGGA
	Rev	CTACAACTGAGTGAGTTCCTGTGGG
MDP0000495467	Fwd	ATGACCAATGGGGKTGGGGA
	Rev	TTACAACTCCGCAAGCTCCTCTTG
MDP0000771960	Fwd	ATGACCGATGGGGGTGTGGA
	Rev	TTATAACTCAGCAAGCTCCTCTGGG
MDP0000289755	Fwd	ATGGGGGATCAAAGTGGATTAGAAG
	Rev	TCAAGCATGCTTGGGAAGTACTGGA

The primers in red were expressed in roots of *M. xiaojinensis* and selected for amplify the candidated transcription factors of yeast one-hybrid.

Supplemental Table S2. Probe sequences used for EMSA.

	Probes
FUE	TACCAATTGTACATATTGTTGATATATATAAATTATAATGTTGAAAATAACCAGCTTCCT
FUI	TACCAATTGTACATATTGATATATATAATGTTGAAAATAACCAGCTTCCT

TATA-box insertion in probes are shown in red.

Supplemental Data 1. Sequences of *IRT1* promoters with different numbers of the TATA-box insertion.

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1       AAGCCACCGG AGGACATGGC AAAGCCGTCA CGTGAAAGAA CACCACCAAA GAGGGATGGG
61      ACATGGAAGC CCAACAAAGA TCATAGCACA CACTATGCTT GTAATTGGTT GACCTGCCAA

P0121   AATATTAACT GAAATAT         CTTCA         TCTG GACGGACAAC GGAGTTTTAC
P1121   AATATTAACT GAAATAT         CTTCA         TCTG GACGGACAAC GGAGTTTTAC
P2121   AATATTAACT GAAATAT         CTTCAA TTATAATCTG GACGGACAAC GGAGTTTTAC
P3121   AATATTAACT GAAATATATT ATAACTTCAA TTATAATCTG GACGGACAAC GGAGTTTTAC


P0181   CAATTGTACA TATTGTTGAT ATATATAA         TGTTG AAAATAACCA GCTTCCTTAC
P1181   CAATTGTACA TATTGTTGAT ATATATAAAT TATAATGTTG AAAATAACCA GCTTCCTTAC
P2181   CAATTGTACA TATTGTTGAT ATATATAAAT TATAATGTTG AAAATAACCA GCTTCCTTAC
P3181   CAATTGTACA TATTGTTGAT ATATATAAAT TATAATGTTG AAAATAACCA GCTTCCTTAC

241   TTTCATGCAT GGTTGCATAT ATAAATATAT CAATGAAGAA TAAAACATTT CCGTACTGCA
301   ATTCATCAAT ACAAACGACT TCTATTTTTT AAAGCCTTTT ACAAGTCCGC AGCAAAAAAA
361   ATATTGGACA AATCAACAAG TATATAAAAT AGGTACAATA TAAAATAGAG TTTGTTATTT
421   TTTTGGCCAA TAGGTATAAC ATTGAACTTA TCACCCTAAT ATTTTATGTT ATTGATTGCA
481   TAATAAATCT CAGAGACCTC TCTATATAAA CAAGCTCTAA TACACTCAAA CCCAATAGTA
541   CCAATAATAA CTTGAGCTAA AAATCAGGGT CA
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Supplemental Method S1. *A. thaliana* protoplast isolation and PEG-mediated gene transformation.

Protoplasts were isolated using a modified version of a protocol developed for *Arabidopsis*. Grow *Arabidopsis* plant on nutrient soil in an environment-controlled chamber for 20–30days. Choose well-expanded and healthy leaves, then cut the leaves from 0.5 mm to 1mm with fresh sharp razor blade. We transfer leaf strips into the prepared enzyme solution [1.5% (wt/vol) cellulase R10, 0.4% (wt/vol) macerozyme R10, 0.4M mannitol, 20 mM MES (pH5.7), 20mM KCl. Warm the solution at 55 ℃ for 10 min and cool it to room temperature, then add 10mM $CaCl_2$, and 0.1% BSA]. Subsequently, Enzymatic hydrolysis reaction for 3.5 h with gentle shaking at 40 rpm and room temperature, in the dark. Check for the release of protoplasts in the solution under the microscope and dilute the enzyme solution with W5 solution [2mM MES (pH5.7), 154mM NaCl, 125mM $CaCl_2$, 5 mM KCl], then filtered through a 100-mesh stainless steel filter into 50 ml round-bottomed tube. Centrifuge the flow-through for 5 min at 700 rpm. Remove the supernatant and re-suspend the protoplast withW5 solution. Keep the protoplasts on ice for 30 min and remove the W5 solution. Then re-suspend the protoplast with MMG solution [4 mM MES (pH5.7), 0.4 M mannitol, 15mM $MgCl_2$]. We can use a hemocytometer to measure protoplast density.

We used a DNA-PEG–calcium transfection method for protoplast [40% (wt/vol) PEG4000 in ddH_2O was prepared with 0.2 M mannitol and 100 mM $CaCl_2$]. Add 10 ml target gene plasmid DNA (10–20 ug) and 10 ml reference gene plasmid DNA (10–20ug) to a 5-ml microfuge tube. Add 200ul protoplasts and well-mixed gently. Then add 220ul of a 40% PEG 4,000 solution quickly and well-mixed gently. Incubate the transfection mixture at room temperature for 15 min and stop the transfection with the addition of 2 ml W5 solution, mix well by gently rocking. Centrifuge at 700 rpm for 5 min at room temperature and remove the W5 solution. Add 1ml WI solution [4 mM MES (pH5.7), 0.5 M mannitol, 20 mM KCl] to incubate protoplasts at room temperature (23-25 ℃). After 16h, we analyzed for GFP expression. Harvest protoplasts by centrifuging at 700 rpm for 5 min and store at -80 ℃ for RNA extraction.

Supplemental Method S2. Modified yeast one-hybrid analysis protocol.

Integrating recombinant vectors into the yeast genome.

1. Yeast EGY48 streaked on the YPD plate, 28 ℃, 2-3d;

2. Select single colony into 25ml YPDA liquid medium, incubate at 28 ℃ for overnight with shaking at 200 rpm;

3. Centrifuge the culture at 3000rmp for 5min at room temperature;

4. Discard the supernatant and resuspend each cell pellet in 25 ml of sterile water;

5. Centrifuge the culture at 3000rmp for 5min at room temperature;

6. Discard the supernatant and resuspend each cell pellet in 25 ml of sterile water;

7. Equal to the 1.5ml centrifuge tubes;

8. Centrifuge at 3000rmp for 5mins at room temperature;

9. Resuspend each cell pellet in 100μl one-step-buffer (36μl 1M 10×LiAc, 240μl 50% PEG3350, 26μl salmon DNA, sufficient mixing);

10. Add 6μl salmon DNA, 2μl recombinant AD vector, 2μl recombinant BD vector;

11. Incubate at 45 ℃ for 30min, sufficient mixing every 10min;

12. Coated at corresponding plates, 28 ℃, 2-3d.

Chromogenic reaction

1. Select 5-10 single colonies into 3ml corresponding liquid medium, incubate at 28 ℃ for overnight with shaking at 200 rpm;

2. Get the 5μl bacterial liquid at the corresponding plates with x-gal;

3. 28 ℃, 2-3d, observe the color change.

Supplemental Method S3. Functional complementation in yeast.

The yeast strains used in the experiment included wild type (WT) strain DEY1457 and the yeast mutant *fet3fet4* DEY1453 (Eide et al., 1996; Dix et al., 1997), which were generously supplied by Dr. Yuanmei Zuo from China Agricultural University (Shen et al., 2014). The WT yeast cells transformed with pYES DEST52 were used as positive controls. The IRT1(+/-TATA box) cDNA was inserted into the yeast expression vector pYES DEST52 by using GATEWAY method (Invitrogen Biotechnology Co., Ltd., Beijing, China).

pYESDEST52-IRT1(+/-TATA box) were transformed into *fet3fet4* yeast mutant, respectively, for yeast functional complementation assays of IRT1. The transformed yeast cells were prepared according to a high-efficiency transformation protocol (Gietz & Woods, 2002) and selected on solid synthetic defined (SD)-Ura media plates. Yeast cells were grown on 1 % yeast extract, 2 % peptone, 40 mg l^{-1} adenine, 2 %

dextrose medium plates (pH 5.8) before transformation and on SD-Ura plates (pH 5.8) after transformation at 28 ℃ for 3 days. For iron starvation media, bathophenanthroline disulphonic acid disodium salt (BPDS) was added. The transformed cells were grown overnight and diluted to OD 1-10^{-4} at 600 nm. The diluted cells (10 μl) were spotted onto the tested medium and cultured at 28 ℃. Photographs of the yeast colonies were taken after 3 days growth. Three independent tests were performed.

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Shen H, Xiong H, Guo X, Wang P, Duan P, Zhang L, Zhang F, Zuo Y (2014) AhDMT1, a Fe^{2+} transporter, is involved in improving iron nutrition and N_2 fixation in nodules of peanut intercropped with maize in calcareous soils. Planta **239:** 1065-1077