Supplemental data

Supplemental Materials and Methods

Hydroponic growth conditions

Seedlings at 8 days after germination (DAG) on agar medium were transferred to the control Hoagland solution (Ctrl, 50 μM Fe-EDTA, pH=5.3) or Hoagland solution supplemented with excess Fe (+Fe, 250 μM Fe-EDTA), and grown for an additional 3 d. The number of lateral root initiation events was determined in the proximal root and distal portions. Hoagland solution was modified accord with Xu et al. (2013) as the following nutrients: KNO_3, 0.5mM; $Ca(NO_3)_2$, 1.0mM; KH_2PO_4, 1.0mM; $MgSO_4$, 0.3mM; H_3BO_3, 13.3μM; $MnCl_2$, 3.0μM; $CuSO_4$, 0.5μM; $ZnSO_4$, 1.0μM; Na_2MoO_4, 0.1μM; NaCl, 2μM; $CoCl_2$, 0.01μM; $NiSO_4$, 0.1μM; and Fe-EDTA, 50 μM.

DR5:GUS-based auxin transport assay

The method, as described by Lewis and Muday (2009), was used to measure auxin transport. In brief, to measure basipetal auxin transport, plates with the control seedlings (5 DAG *DR5:GUS* plants) or IAA treated seedlings were incubated in the dark for 2 h. IAA treatment was conducted by placing a solidified agar block containing 0.1 μM IAA such that it overlapped with the root tip by ~0.5 mm. The entire seedling was then subjected to GUS staining for 16 h at 37 °C. Auxin transport was determined by comparing the distance of GUS staining from the site of IAA application of the treated seedlings with that of the controls.

Quantitative real-time reverse transcription PCR (qRT-PCR) analysis

qRT-PCR was carried out according to the method of Li et al. (2013). Total RNA was extracted from *Arabidopsis* roots. Gene sequences were available at the National Center for Biotechnology Information, and gene-specific primers for qRT-PCR were designed using Primer 5 software. CBP20 (nuclear-encoded cap-binding protein) was used as the housekeeping gene, and relative RNA abundance was normalized to the CBP20 internal control ($[mRNA]_{gene}/[mRNA]_{CBP20}$). The

primers used in this study as following: *AtACS5*: 5′-GTTTTAGCGGCTGGTTCGACATCT-3′ and 5′-CAACGCAGTGCCAAGTGGGTTA-3′; *AtACS7*: 5′-CCTGGGTTCCGTGAAAACGCATT-3′ and 5′-CGTCGTTAGGATCGGCGAGAATGA-3′; *CBP20*: 5′-ACCATCGGAAACGACAAAGAG -3′ and 5′-CTTCACCATCGTCATCGGAGT-3′.

Gravity stimulation

Five-day-old seedlings of similar size were transferred to new agar plates containing the appropriate treatments. Roots were placed vertically in rows, after recording the initial positions of the root tips, the plates were rotated by 90° and placed vertically for gravistimulation under control or excess Fe treatment in a cultivation chamber at time zero. Digital images of seedling growth were captured at regular, specified time points (as defined in the text) following gravistimulation with a Canon G7 (Canon Inc., Tokyo, Japan). The tip angles from the vertical were determined as described by Zou et al. (2012). The gravitropic angle refers to the angle of the root tip relative to the gravity vector.

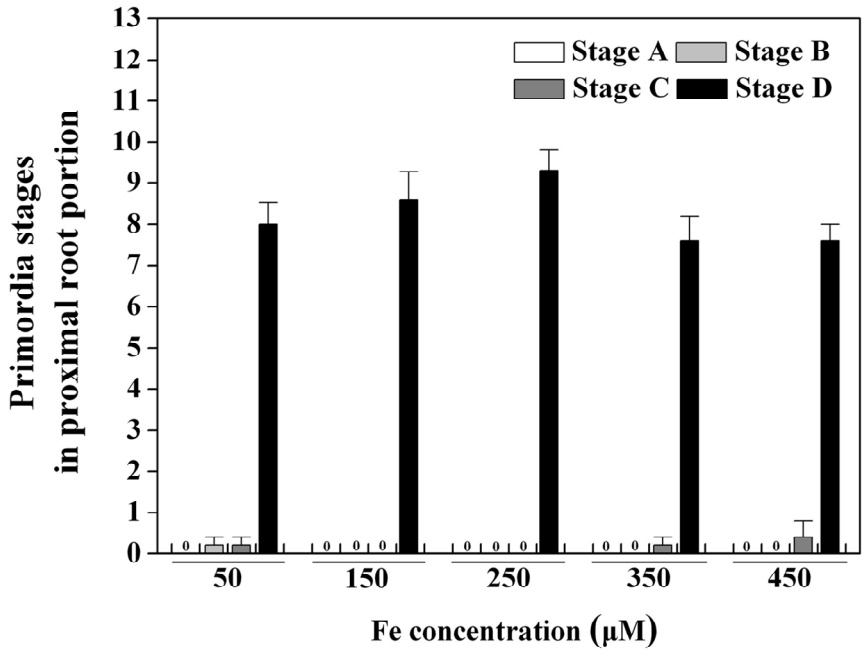
LITERATURE CITED

Lewis DR, Muday GK (2009) Measurement of auxin transport in *Arabidopsis thaliana*. Nature Protoc **4:** 437-451

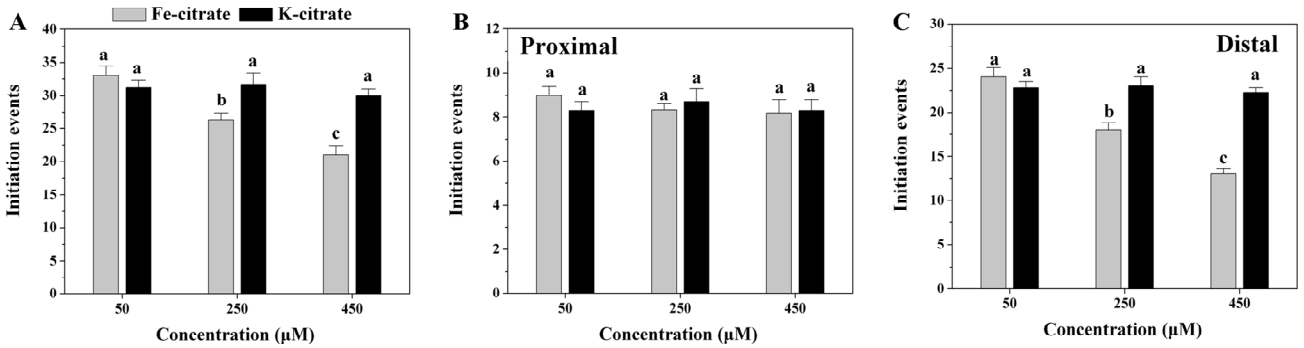
Li GJ, Li BH, Dong GQ, Feng XY, Kronzucker HJ, Shi WM (2013) Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*. J Exp Bot **64:** 1413-1425

Xu WF, Jia LG, Shi WM, Baluška F, Kronzucker HJ, Liang JS, Zhang JH (2013) The tomato 14-3-3 protein TFT4 modulates H^+ efflux, basipetal auxin transport, and the PKS5-J3 pathway in the root growth response to alkaline stress. Plant Physiol **163:** 1813-1828

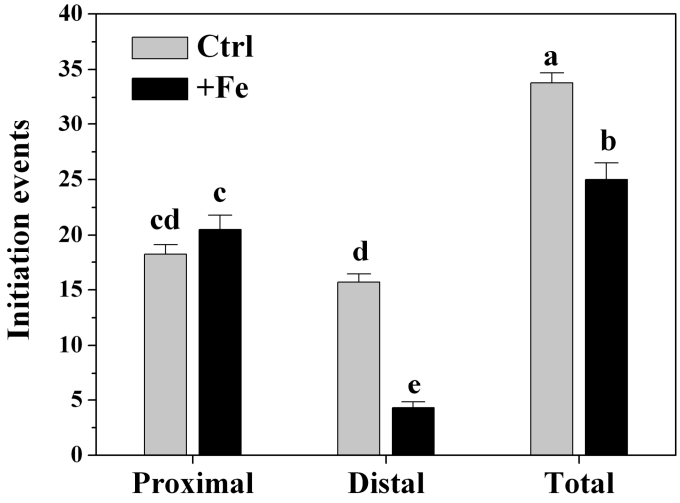
Zou N, Li BH, Dong GQ, Kronzucker HJ, Shi WM (2012) Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in *Arabidopsis*. J Exp Bot **63:** 3777-3788



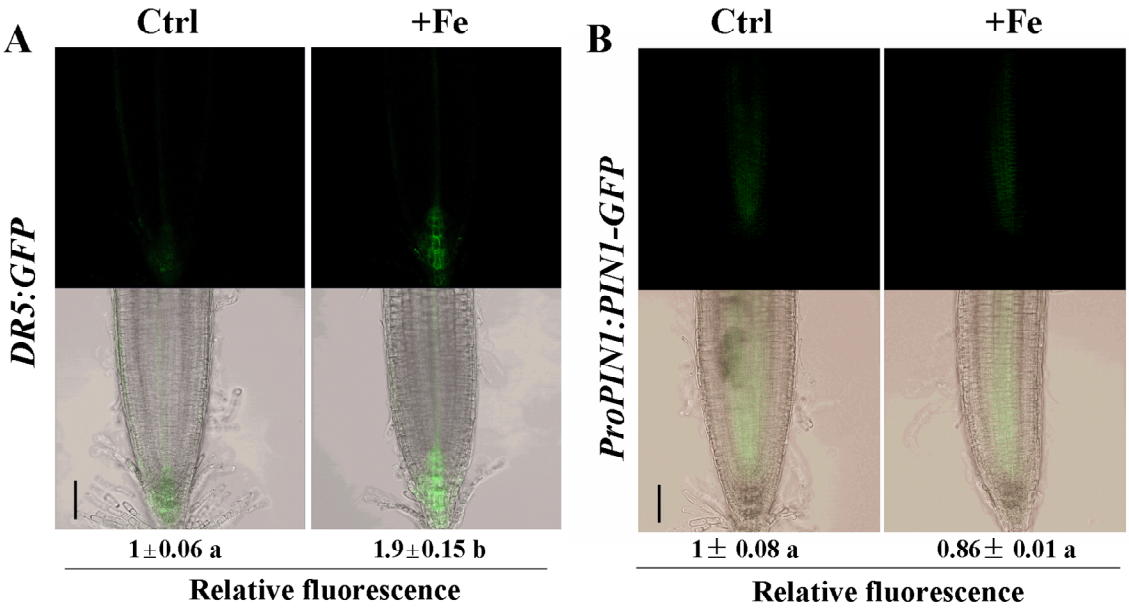
Supplemental Figure S1. Effects of excess Fe on the proportion of lateral root primordia in *Arabidopsis*. Seedlings at 5 days after germination (DAG) were transferred to varying concentrations of Fe (supplied as Fe-EDTA) and grown for an additional 5 d. The number of lateral roots initiated at each developmental stage was determined in the proximal root portion. Values are the means ± SE, n = 10.



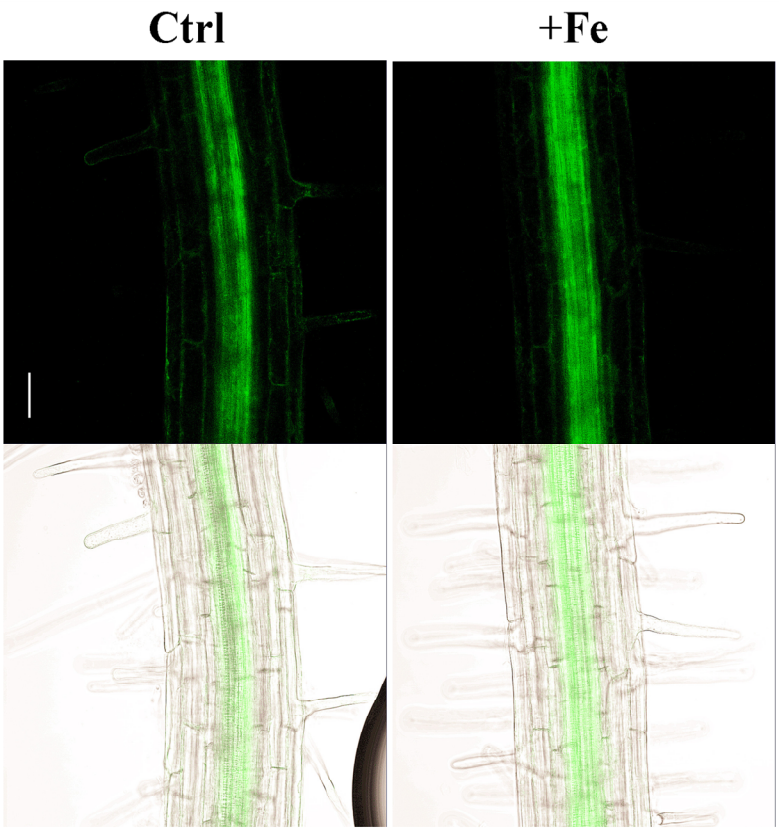
Supplemental Figure S2. Effects of Fe-citrate and K-citrate on lateral root initiation events in *Arabidopsis*. Seedlings at 5 days after germination (DAG) were transferred to varying concentrations of Fe and K (supplied as Fe-citrate and K-citrate) and grown for an additional 5 d. The number of lateral root initiation events was determined in the proximal root and distal portions. (A) Effect of total lateral root initiation events. (B) Effect of lateral root initiation events in proximal portion. (C) Effect of lateral root initiation events in distal portion. Means ± SE are shown (n = 6). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



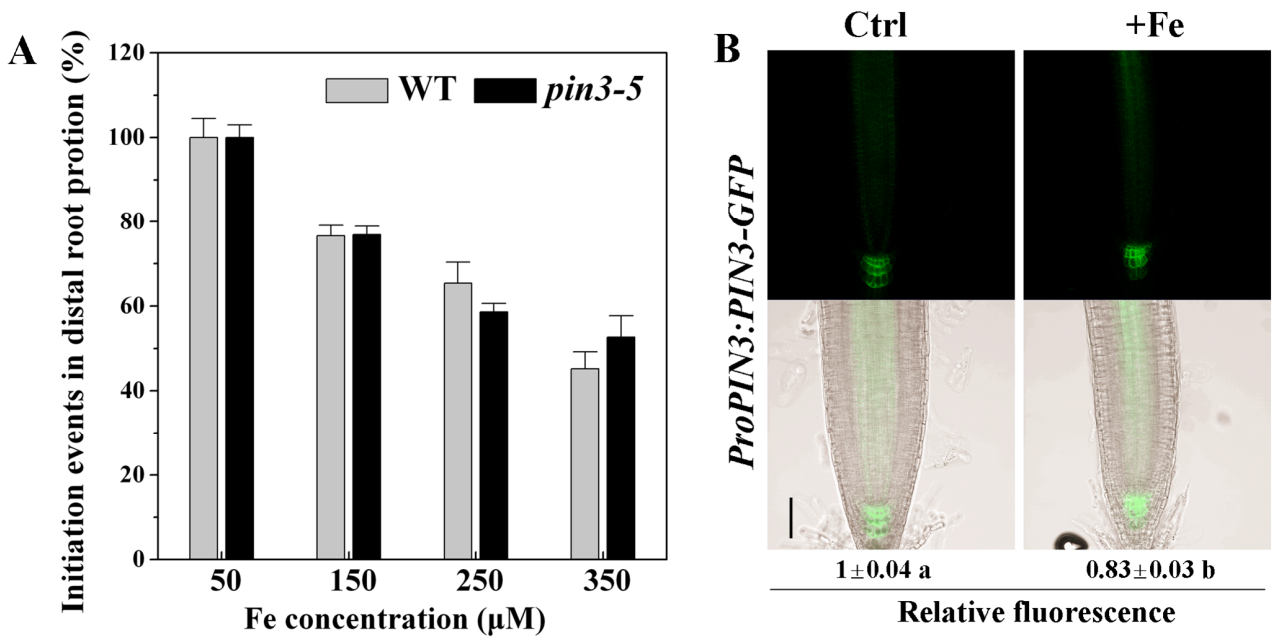
Supplemental Figure S3. Effects of Fe excess on *Arabidopsis* lateral root initiation events in hydroponic condition. Seedlings at 8 days after germination (DAG) on agar medium were transferred to the control Hoagland solution (Ctrl, 50μM Fe) or Hoagland solution supplemented with excess Fe (+Fe, 250μM Fe), and grown for an additional 3 d. The number of lateral root initiation events was determined in the proximal root and distal portions. Means ± SE are shown (n = 6). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



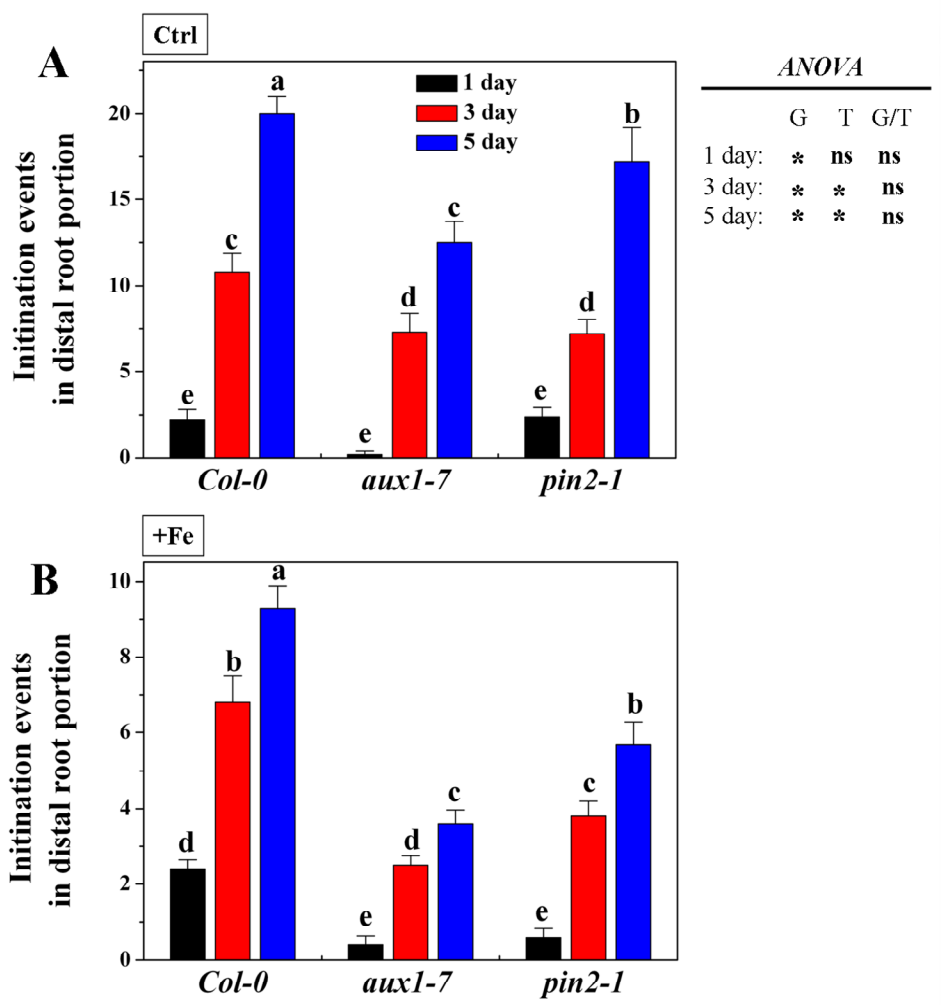
Supplemental Figure S4. Effects of excess Fe on the expression of *DR5:GFP* and *ProPIN1:PIN1-GFP* in *Arabidopsis*. Five-day-old WT seedlings, containing *DR5:GFP* or *ProPIN1:PIN1-GFP* constructs, were transferred onto control medium (Ctrl) or medium supplemented with excess Fe (+Fe, 250 μM). The fluorescence signal was detected with a Zeiss LSM710 confocal laser-scanning microscope at the specified time points. One representative image for each experiment is shown. Upper columns show the single GFP images; lower columns show the integration images of GFP and bright field. (**A**) Effects of excess Fe on the expression of *DR5:GFP* in roots. (**B**) Effects of excess Fe on the expression of *ProPIN1:PIN1-GFP* in roots. The images were captured using the same confocal setting and are representative of at least 10 roots obtained from at least three independent experiments. Scale of bars = 50 μm. Means ± SE are shown ($n \geq 5$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



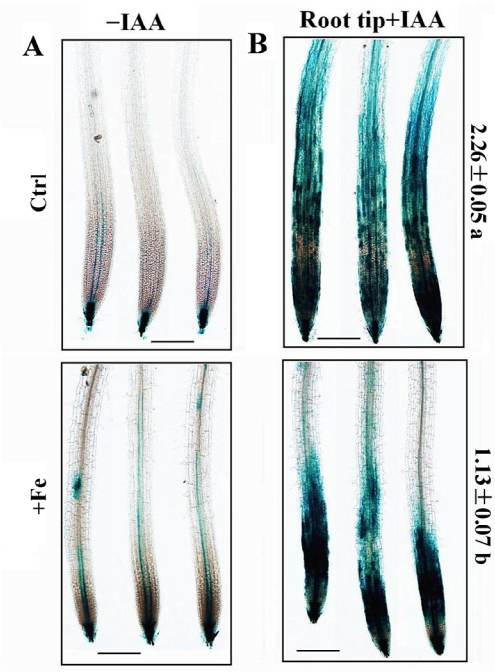
Supplemental Figure S5. Auxin activation of the *DR5* response in xylem-adjacent pericycle cells. Five-day-old WT seedlings, containing *DR5:GFP* construct, were transferred onto control medium (Ctrl) or medium supplemented with excess Fe (+Fe, 350 µM) for 3 d. Treatment with 1 µM IAA induces *DR5* response in pericycle cells adjacent to the xylem pole. The fluorescence signal was detected with a Zeiss LSM710 confocal laser-scanning microscope at the specified time points. One representative image for each experiment is shown. Scale of bars = 50 µm.



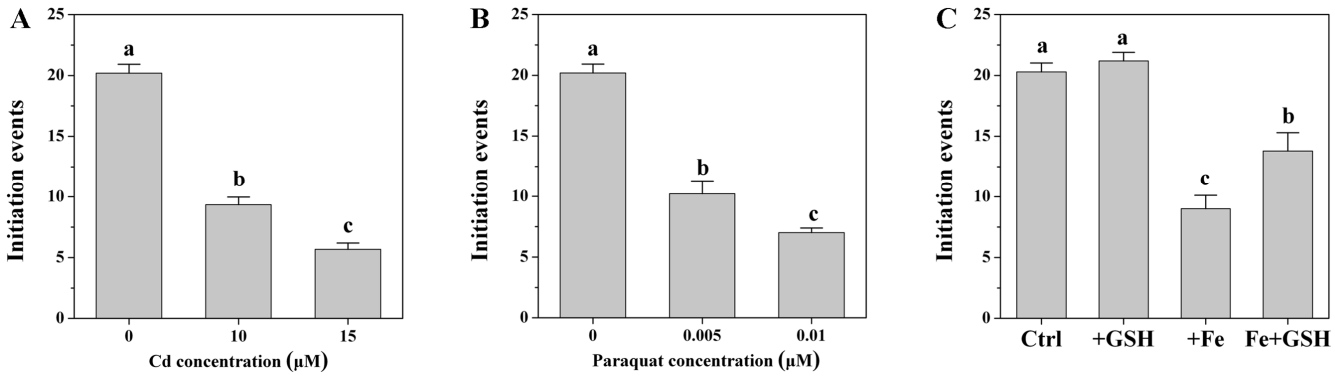
Supplemental Figure S6. Effects of excess Fe on the lateral root initiation of *pin3-5* and expression of *ProPIN3:PIN3-GFP* in *Arabidopsis*. (**A**) Effects of excess Fe on the lateral root initiation of *pin3-5*. Five-day-old WT and *pin3-5* seedlings were transferred to medium, and roots were supplemented with varying concentrations of Fe, for 5 d, after which LR initiation events were quantified. Total LR initiation events in WT and *pin3-5* in control were 21.4 ± 0.88 and 18.2 ± 0.48, respectively. Values are the means ± SE, n ≥ 5. (**B**) Effects of excess Fe on the expression of *ProPIN3:PIN3-GFP* in roots. Five-day-old WT seedlings, containing *ProPIN3:PIN3-GFP* constructs, were transferred onto control medium (Ctrl) or medium supplemented with excess Fe (+Fe, 250 μM). The fluorescence signal was detected with a Zeiss LSM710 confocal laser-scanning microscope at the specified time points. One representative image for each experiment is shown. Upper columns show the single GFP images; lower columns show the integration images of GFP and bright field. The images were captured using the same confocal setting and are representative of at least 10 roots obtained from at least three independent experiments. Scale of bars = 50 μm. Means ± SE are shown (n ≥ 5). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



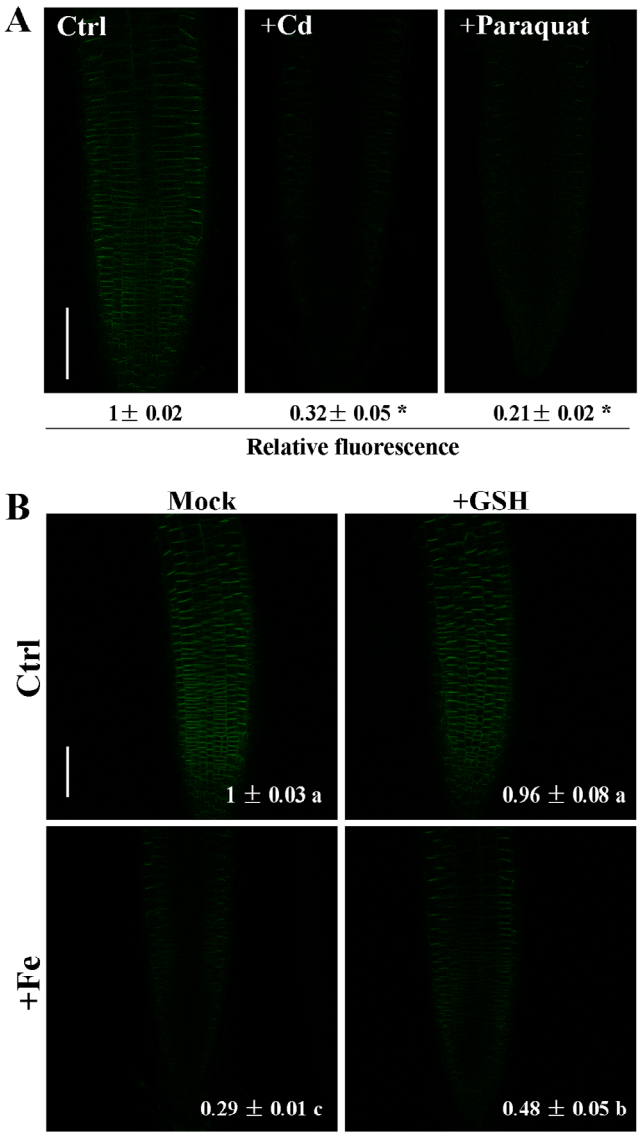
Supplemental Figure S7. Effects of excess Fe on LR initiation in *Arabidopsis* WT (*Col-0*), *pin2-1*, and *aux1-7*. Total lateral root initiation at one, three, and five days in control (Ctrl, 50 µM) and under excess Fe (+Fe, 350 µM) conditions. Values are the means ± SE, n ≥ 6. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test). A two-way analysis of variance (ANOVA) was used to detect the significance of interaction between genotype and environment. G: genotype (i.e. WT and *aux1-7*); T: treatment (i.e. Ctrl and +Fe). NS, *, indicate non-significant or significant differences at $P \leq 0.05$, respectively.



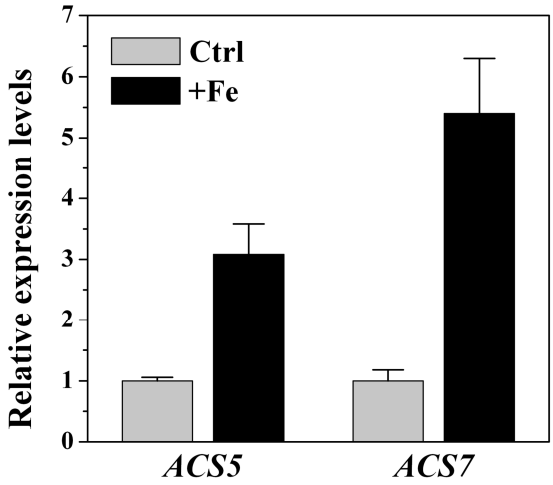
Supplemental Figure S8. Auxin induction of *DR5:GUS* expression in *Col-0*, with or without excess Fe. Basipetal auxin transport was determined by comparing the distance of GUS staining from the site of IAA application of the treated seedlings with that of the controls. Numbers to the right of microscopic images show the results of distance of GUS staining quantification (mm). At least six seedlings for each treatment were measured and the experiments were repeated twice independently. Values are the means ± SE. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test). (**A**) Expression of *DR5:GUS* in the primary apex of 5-DAG seedlings treated with or without 350 μM Fe (+Fe) for 3 d in the dark prior to staining for GUS expression. (**B**) Effects of the application of 0.1 μM indole acetic acid (IAA) to root tips on *DR5::GUS* expression in (**A**). Three representative plants from each treatment ($n \geq 6$ plants) are shown (A, B). Scale of bars = 0.3 mm.



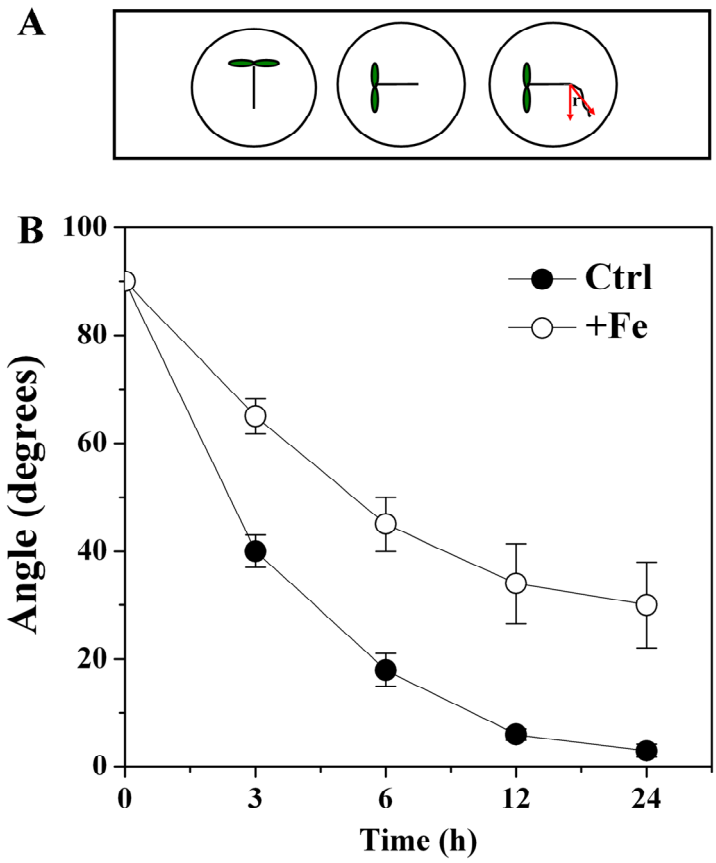
Supplemental Figure S9. Effects of cadmium, paraquat and glutathione on lateral root initiation in *Arabidopsis*. (**A**) Effects of cadmium on the number of lateral root initiation events in the distal root portion. Seedlings (*DR5:GUS* lines) at 5 days after germination (DAG) were transferred to varying concentrations of cadmium (Cd) treatment medium and grown for an additional 5 d. (**B**) Effects of paraquat on the number of lateral root initiation events in the distal root portion. Seedlings (*DR5:GUS* lines) at 5 days after germination (DAG) were transferred to varying concentrations of paraquat treatment medium and grown for an additional 5 d. (**C**) Effects of glutathione (GSH) on lateral root initiation events in the distal root portion. Seedlings (*DR5:GUS* lines) at 5 days after germination (DAG) were transferred to control (Ctrl, 50μM) and excess Fe (+Fe, 350μM) treatment medium with or without GSH (50μM) and grown for an additional 5 d. Values are the means ± SE, $n \geq 5$. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



Supplemental Figure S10. Effects of cadmium, paraquat and glutathione on the expression of *ProPIN2:PIN2-GFP* in *Arabidopsis*. Five-day-old WT seedlings, containing *ProPIN2:PIN2-GFP* constructs, were transferred onto control medium (Ctrl) or medium supplemented with cadmium (+Cd, 10μM), paraquat (+paraquat, 0.005μM) or excess Fe (+Fe, 250μM) with or without GSH (50μM) and grown for 48h. (**A**) Effects of cadmium and paraquat on the expression of *ProPIN2:PIN2-GFP* in roots. (**B**) Effects of GSH on the expression of *ProPIN2:PIN2-GFP* in roots. The images were captured using the same confocal setting and are representative of at least 10 roots obtained from at least three independent experiments. Scale of bars = 50 μm. Means ± SE are shown ($n \geq 3$). Asterisks indicate statistical differences between control and the treatments conditions (independent samples *t test*, $*P < 0.05$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



Supplemental Figure S11. Effects of excess Fe treatment on *AtACS5* and *AtACS7* transcript levels in *Arabidopsis*. Expression of root *AtACS5* and *AtACS7* were determined by quantitative real-time PCR after exposure of 5-d-old wild-type seedlings to 350 μM Fe for 6 h. Values are means ± SE of three replicates.



Supplemental Figure S12. Experimental system for examining agravitropic response in response to excess Fe. (**A**) Diagram shows an experimental design for the study of interaction between Fe stress and gravitropism. Seedlings at 5 days after germination (DAG) were transferred to control (Ctrl, 50 μM) and excess Fe (+Fe, 350 μM) medium in petri dishes vertically. The plates were then rotated 90^{o} and photographed at intervals following gravistimulation and excess Fe treatment. The angles (r) between gravity (g) and the roots were measured according to Zou et al. (2012). (**B**) The time course of root curvature of the seedlings during treatments. Values are means of 5 plants ± SE.

Supplemental Table S1. Comparison of the effects of IAA, ACC and AVG on total lateral root initiation events in *Arabidopsis.*

Background	LR initiation events
Ctrl	21.5 ± 0.39 c
+Fe	13.4 ± 0.67 d
+IAA	25.2 ± 0.73 b
Fe +IAA	25.5 ± 0.44 b
+ACC	20.1 ± 0.89 c
Fe +ACC	27.7 ± 0.31 a
+AVG	20.8 ± 1.38 c
Fe +AVG	5.33 ± 0.24 f

For growth conditions and treatments, see Table 1. The means ± SE are reported, with $n \geq 5$ ($P \leq 0.05$).

Supplemental Table S2. Comparison of the effects of excess Fe on total lateral root initiation events in *Arabidopsis* wild-type (*Col-0*) and mutants.

Background	LR initiation events
Col-0	
Ctrl	21.8 ± 1.1 a
+Fe	12.2 ± 0.37 d
aux1-7	
Ctrl	14.2 ± 0.37 c
+Fe	4.2 ± 0.39 f
pin2-1	
Ctrl	17.0 ± 0.31 b
+Fe	8.4 ± 0.74 e
eto1-1	
Ctrl	14.4 ± 0.81 c
+Fe	15.0 ± 0.54 c

For growth conditions and treatments (+Fe, 250 μM Fe), see Table 2. The means ± SE are reported, with $n \geq 5$ ($P \leq 0.05$).