

Auxin Resistant1 and PIN-FORMED2 Protect Lateral Root Formation in Arabidopsis under Iron Stress¹[OPEN]

Guangjie Li, Haiyan Song, Baohai Li, Herbert J. Kronzucker, and Weiming Shi*

State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China (G.L., H.S., B.L., W.S.); and Department of Biological Sciences, University of Toronto, Toronto, Ontario, Canada M1C 1A4 (H.J.K.)

ORCID IDs: 0000-0003-4603-6722 (G.L.); 0000-0002-9905-5890 (B.L.).

A stunted root system is a significant symptom of iron (Fe) toxicity, yet little is known about the effects of excess Fe on lateral root (LR) development. In this work, we show that excess Fe has different effects on LR development in different portions of the Arabidopsis (*Arabidopsis thaliana*) root system and that inhibitory effects on the LR initiation are only seen in roots newly formed during excess Fe exposure. We show that root tip contact with Fe is both necessary and sufficient for LR inhibition and that the auxin, but not abscisic acid, pathway is engaged centrally in the initial stages of excess Fe exposure. Furthermore, Fe stress significantly reduced PIN-FORMED2 (PIN2)-green fluorescent protein (GFP) expression in root tips, and *pin2-1* mutants exhibited significantly fewer LR initiation events under excess Fe than the wild type. Exogenous application of both Fe and glutathione together increased *PIN2-GFP* expression and the number of LR initiation events compared with Fe treatment alone. The ethylene inhibitor aminoethoxyvinyl-glycine intensified Fe-dependent inhibition of LR formation in the wild type, and this inhibition was significantly reduced in the ethylene overproduction mutant *ethylene overproducer1-1*. We show that Auxin Resistant1 (AUX1) is a critical component in the mediation of endogenous ethylene effects on LR formation under excess Fe stress. Our findings demonstrate the relationship between excess Fe-dependent PIN2 expression and LR formation and the potential role of AUX1 in ethylene-mediated LR tolerance and suggest that AUX1 and PIN2 protect LR formation in Arabidopsis during the early stages of Fe stress.

Iron (Fe) is an essential trace element for plants (Pilon et al., 2009), and species differ greatly in how much Fe they require for optimal growth (Wheeler and Power, 1995; Batty and Younger, 2003). As Fe is frequently limiting, Fe deficiency is more commonly studied than toxicity arising from excess Fe exposure (Lei et al., 2014; Bashir et al., 2015; Briat et al., 2015). Fe is also a major focus for efforts in biofortification by targeting Fe transporters (Zhai et al., 2014; Pinto and Ferreira, 2015). However, the excessive presence of Fe in soils is equally common, in particular in soils characterized by low pH and hypoxic or anoxic conditions (Connolly and Guerinot, 2002). Toxicity arising from excess Fe exposure is recognized as one of the major plant diseases attributable to abiotic factors that impact the development and yield potential in the world's leading

cereal crops, rice (*Oryza sativa*) and wheat (*Triticum aestivum*; Becker and Asch, 2005; Khabaz-Saberi et al., 2012). Understanding the mechanisms underlying excess Fe toxicity is therefore essential.

Plastic responses in the plant's root system architecture are known to constitute a major mechanism by which plants cope with fluctuating environments. Lateral roots (LRs), which typically comprise the majority of the root system, contribute pivotally to nutrient acquisition from soil, and modulating LR development is a very important avoidance strategy for plants when confronted with unfavorable edaphic conditions, such as high salinity or heavy metals (Ivanov et al., 2003). In the case of excess exposure to Fe, stunting of the root system is among the chief symptoms of toxicity (Becker and Asch, 2005). However, while some information has been emerging on the primary root axis (Li et al., 2015), the specific role of the plant's LR apparatus remains poorly studied. Yamauchi and Peng (1995) reported retardation of root growth and a reduction in LR length and number under excess Fe conditions. Recently, Reyt et al. (2015) showed that excess Fe had no significant effect on LR initiation in the LR branching zone and that ferritins play an important role in LR emergence under excess Fe in this portion of the root, although the authors had not investigated LR development in the root portions near the growing tip of the primary root. Because LR initiation is restricted to specific pericycle cell files adjacent to a xylem pole in the basal region of the meristem (De Smet et al., 2007; Fukaki and Tasaka, 2009), and LR formation

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* Address correspondence to wmshi@issas.ac.cn.

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in this new growing root portion may be more susceptible to stress stimuli, such as observed with exposure to high NH_4^+ and salt (Duan et al., 2013; Li et al., 2013), it is reasonable to suggest that modulation of LR formation near the growing tip of the primary root is critical to the response to excess Fe stress.

In *Arabidopsis thaliana*, the development of LRs proceeds through the following stages: lateral root primordia (LRP) initiation, establishment, emergence, activation into mature LRs, and final maintenance of LR elongation (Fukaki and Tasaka, 2009; Péret et al., 2009). The hormones abscisic acid (ABA) and auxin are important internal negative and positive regulators during LR development, respectively (Fukaki and Tasaka, 2009). ABA has been implicated in LRP emergence and meristem activation independent of auxin (De Smet et al., 2003). Auxin is an important internal positive regulator during LR development (Fukaki and Tasaka, 2009), and auxin transport is critical (Blilou et al., 2005). Mutants in auxin efflux carriers such as PIN-FORMED (PIN) and P-Glycoprotein show significant defects in LR formation (Fukaki and Tasaka, 2009; Péret et al., 2009). For example, LR initiation frequency was significantly reduced in *pin2* and *pin3* mutants (Dubrovsky et al., 2009), and PIN2 was also shown to be involved in exogenous and endogenous signal-mediated LR development (by brassinosteroid, jasmonate, and fungal challenge; Li et al., 2005; Felten et al., 2009; Sun et al., 2009). Similarly, Auxin Resistant1 (AUX1), an auxin influx carrier, also regulates LRP positioning and initiation (De Smet et al., 2007). While both AUX1 and PIN2 are required specifically for the basipetal transport of auxin through the outer root cell layers (Fukaki and Tasaka, 2009), PIN1 localized at the basal end of vascular cells is responsible for direct acropetal auxin flow in the root stele (Blilou et al., 2005). Recently, the roles of ethylene on LR development have also been highlighted, and the ethylene-mediated LR formation is dependent on the auxin pathway (Ivanchenko et al., 2008; Lewis et al., 2011). Ethylene treatment could mediate fluorescence of AUX1 and PIN2 fluorescent protein fusions at the root tip (Růzicka et al., 2007; Lewis et al., 2011). Although ABA, auxin, and ethylene signals have been implicated as important for LR development, it is not known whether and how the three hormones are involved in the response of LR formation to Fe stress.

The previously described phenotypes and physiological processes related to Fe toxicity do not clarify the effect of excess Fe on LR formation. In this study, we employed the *Arabidopsis* wild type and ABA-, auxin-, and ethylene-related mutants to explore the LR formation response to Fe toxicity and to elucidate the roles of ABA, auxin, and ethylene. Potential mechanisms involved in the early stress response to Fe stress are discussed.

RESULTS

Effect of Excess Fe on LR Development

To investigate the effects of excess Fe toxicity on LR development, *Arabidopsis* seedlings were grown on

standard growth medium for 5 d and then transferred either to standard medium or medium supplemented with varying concentrations of Fe-EDTA for periods of up to 5 d. The total LR initiation events (including LRPs and emerged LRs) were significantly reduced by exposure to excess Fe stress, and the reduction of LR initiation events correlated positively with Fe concentrations (Fig. 1A). To further explore the effect of excess Fe on LR formation, two root portions were analyzed separately: the proximal root portion, representing the root that formed prior to the onset of the treatment with excess Fe, and the distal root portion, representing the root that formed following the transfer to media containing excess Fe, (Fig. 1B; following the definition by Dubrovsky and Forde [2012]). Following treatment of *Arabidopsis* seedlings over a wide range of Fe concentrations, we observed no significant changes in the total LR initiation events in the proximal portion (Fig. 1C), similar to the results of Reyt et al. (2015), who found no effect of excess Fe on LR initiation in the LR branching zone. Furthermore, we did not observe any strong effects on the distribution of the developmental stages for preemergent LR primordia (Supplemental Fig. S1). However, the total LR initiation events in the distal portion displayed dose-dependent inhibition (Fig. 1C). Although the number of LRP at the four developmental stages also decreased with increasing exogenous Fe concentrations, the proportion in the four stages was not significantly modified in the distal portion (Fig. 1D). To ascertain that the observed LR phenotypes were specifically due to high Fe concentrations and not to the organic chelate, similar experiments were performed using Fe-citrate instead of Fe-EDTA (Supplemental Fig. S2). The LR development parameters were affected similarly by Fe regardless of the chelation ligands used (Supplemental Fig. S2). Furthermore, the response of LR development to excess Fe was also observed under hypoxic hydroponic conditions (Supplemental Fig. S3). Thus, excess Fe affected LR initiation strictly in the distal primary root portion that elongated during the treatment. Based on these and previous results (Reyt et al., 2015), we focused on LR development in the distal portion in further experiments. To analyze LR initiation in the distal portion under excess Fe in more detail, LR initiation events were also quantified at 1, 3, and 5 d after transfer. There were no significant changes in total LR initiation events between control and excess Fe treatments at 1 d, but a clear suppression was found in excess Fe treatments growing on days 3 to 5 (Fig. 1E).

Our results show that cortical cell length was reduced by excess Fe in the distal root portion (Fig. 1F). To better understand the effect, we normalized for cell length. For this, a parameter called the LR initiation frequency, which defines how many LR primordia are formed along a parent root portion corresponding to 100 cortical cells in a file, was used. The LR initiation frequency reveals how LR initiation is affected in a genotype or by a treatment (Ivanchenko et al., 2008, 2010; Dubrovsky and Forde, 2012). Under Fe treatment, cell length and overall root length significantly decreased, and, therefore, LRP density increased compared with untreated

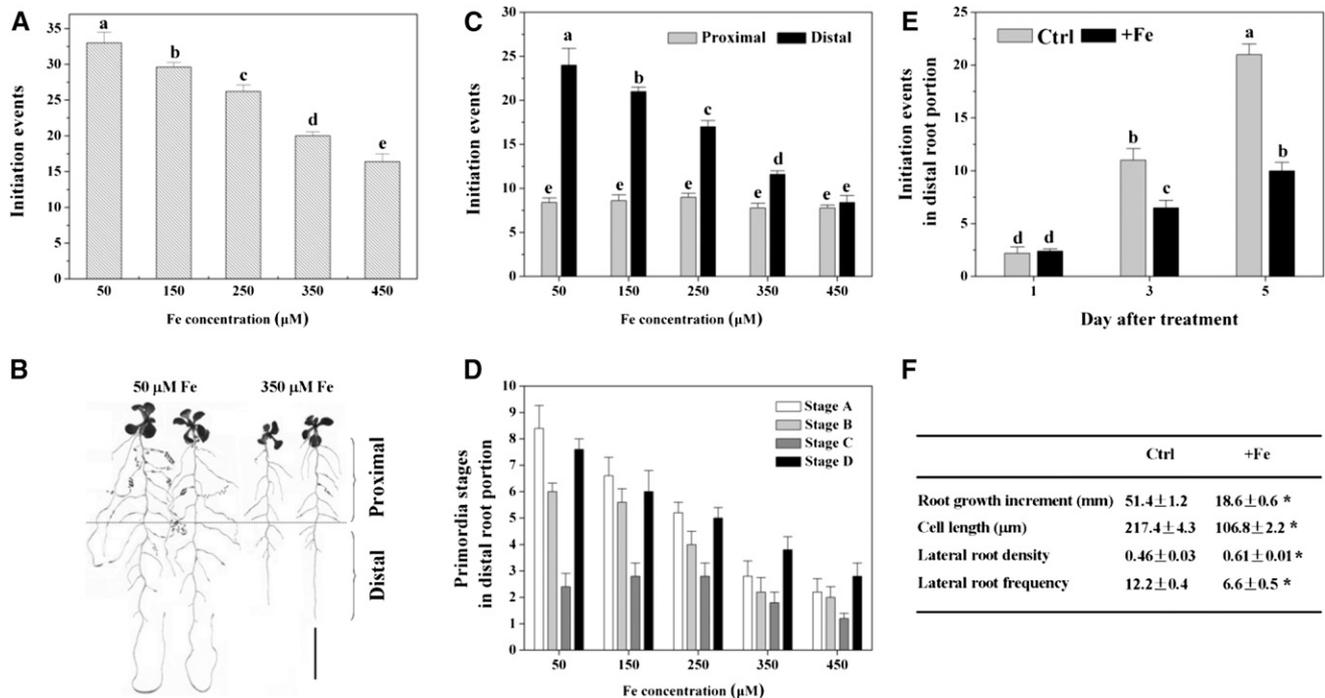


Figure 1. Effect of excess Fe on LR formation in *Arabidopsis* (Col-0). Seedlings (*DR5:GUS* lines) at 5 d after germination were transferred to varying concentrations of Fe (supplied as Fe-EDTA) treatment medium and grown for an additional 5 d. A, Inhibition of LR initiation events by varying concentrations of Fe. B, Photograph of representative seedlings after 5 d of vertical growth in medium lacking or supplemented with, 350 μM Fe. C, The number of LR initiation events in the proximal and distal root portions. D, The number of LR initiation events at three developmental stages, as indicated, was determined in the distal root portions. E, Effects of excess Fe on LR initiation in the wild type. Total LR initiation at 1, 3, and 5 d in control (Ctrl, 50 μM) and under excess Fe (+Fe, 350 μM) conditions. F, Effect of excess Fe (+Fe, 350 μM) on primary root growth, cortical cell length, and LR initiation. LR density refers to number of initiation events per mm, and LR frequency refers to number of initiation events per 100 cortical cells. Values are means of 10 plants \pm SE. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). Asterisks indicate statistical differences between control and excess Fe treatment (+Fe) conditions (independent samples, Student's *t* test, * P < 0.05). Bars = 1 cm.

roots (Fig. 1F). However, estimating the LRP frequency per 100 cells, we found that excess Fe can modify the LR initiation frequency. After excess Fe treatment (at 350 μM), the LR frequency decreased approximately 50% compared with control roots (Fig. 1F).

Given the strong impact of excess Fe exposure on LR initiation, we proceeded to determine if this was a localized or systemic response. We first tested the growth response of roots to localized Fe supply (Fig. 2A) and found that the inhibitory effect was identical whether Fe was applied to the whole root or only the tip (Fig. 2B). In a further experiment, no growth inhibition occurred when the shoots and root body were in contact with Fe, but the root tip did not touch the Fe-containing medium (Fig. 2B). These observations demonstrate a highly localized response to excess Fe in the inhibition of LR initiation phenotype.

Inhibitory Action of Excess Fe on LR Initiation Does Not Involve the ABA-Mediated Pathway

ABA is a negative regulator of LR development (De Smet et al., 2003), and has been suggested to mediate inhibitory effects of several stresses (Deak and Malamy, 2005; Xiong et al., 2006), and, thus, was examined here.

Specifically, the involvement of ABA in the regulation of LR development under excess Fe has not been investigated. For this purpose, we first treated roots with varying concentrations of ABA to determine if this hormone treatment had an effect similar to excess Fe. As previously shown (De Smet et al., 2003), ABA treatment arrested meristem activation of LRP without affecting LR initiation in the distal portion of the root (Fig. 3, A and B), different from the inhibitory effect of excess Fe on LR development (Fig. 1, C and D). Further, ABA-insensitive mutant *abi1-1* and ABA-deficient mutants *aba3-1* and *aba2-1* were used to test whether inhibition of LR initiation under Fe stress involves ABA mediation. The mutants and wild-type seedlings were exposed to varying concentrations of Fe continuously for 5 d, and the LR initiation events were analyzed (Fig. 3C). Fe had a similar impact on inhibition of LR initiation events in *abi1-1*, *aba3-1*, *aba2-1*, and wild-type plants.

Involvement of Auxin in the Response of LR Formation to Excess Fe

Given the established role of auxin in LR formation (Casimiro et al., 2003; Péret et al., 2009), we examined

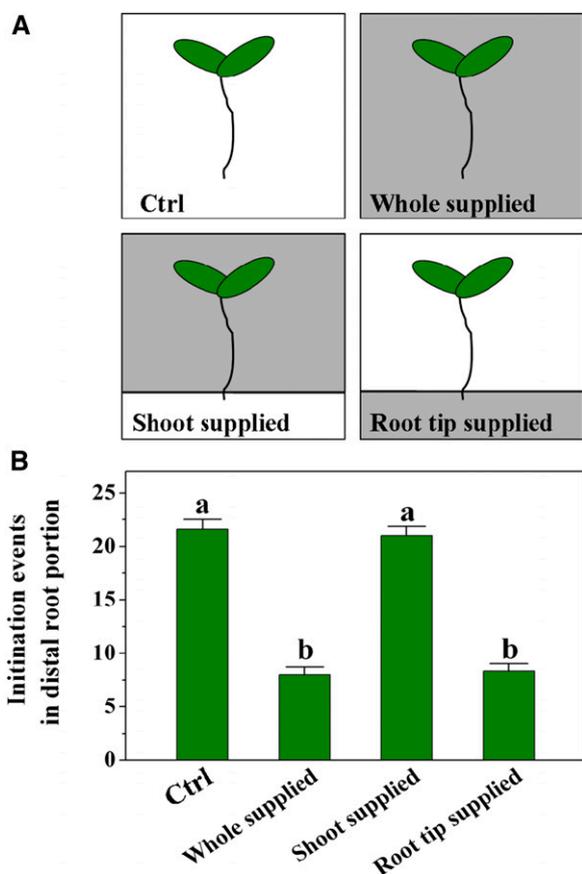


Figure 2. Effect of local supply of Fe on LR initiation in Arabidopsis. A, Schematic diagram of the experimental setup for applying excess Fe to the whole root versus the root tip and the shoot-mature root continuum. White sections indicate the basal growth medium with control medium, and gray sections indicate the Fe-enriched medium ($350 \mu\text{M}$). B, The effect of locally supplied Fe on LR initiation at 5 d. Values are means of 10 plants \pm SE. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). Ctrl, Control.

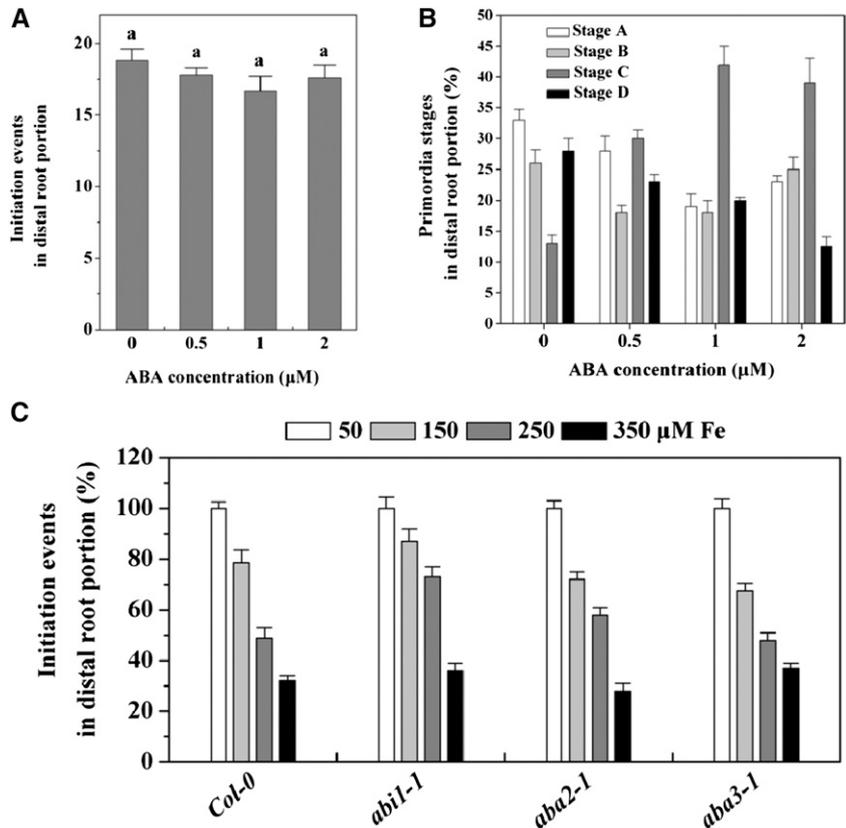
whether auxin is involved in excess Fe inhibition of LR development. As shown in Figure 4A, application of naphthylacetic acid (NAA) markedly promoted LR initiation events in both proximal and distal portions and significantly alleviated the excess Fe-mediated inhibition of LR formation in the distal portion. Furthermore, applications of indole-3-acetic acid (IAA) significantly promoted LR initiation frequency in the distal root portion under Fe stress (Table I; Supplemental Table S1). We first examined the spatial expression of the *Direct Repeat5 (DR5):GUS* reporter gene at several stages of LR development to determine whether excess Fe affects the auxin response in LR primordia. Compared with controls, *DR5:GUS* expression in LRP was not significantly modified in seedlings treated with excess Fe (Fig. 4B), and this might account for the above-described LRP proportion phenotype (Fig. 1D). However, the levels of *DR5:GUS* in the primary root apex were significantly enhanced under excess Fe stress

compared with control (Fig. 4C). Furthermore, the fluorescence intensity in Fe-treated *DR5:GFP* roots was also significantly higher than that in untreated roots (Supplemental Fig. S4A). Similarly, the expression of *ProIAA2:GUS* was higher in the primary root apex under excess-Fe treatment than under control condition (Fig. 4D). In this study, we observed uniform activation of *DR5:GFP* expression in xylem-adjacent pericycle cells after treatment with IAA in both controls and excess Fe treatments, suggesting that the sensitivity of root cells to auxin may be not altered by Fe stress (Supplemental Fig. S5). It was previously shown that transport inhibitor response1 (TIR1) acts as an auxin receptor (Dharmasiri et al., 2005) and that the *tir1-1* mutant shows reduced sensitivity to auxin as well as reduced LR formation (Ruegger et al., 1998). Here, we found that the response of LR initiation events to Fe stress in the *tir1-1* mutant was similar to the wild type (although slight sensitivity was observed at $350 \mu\text{M}$; Fig. 4E), suggesting that excess Fe may not affect the sensitivity of root cells to auxin.

To further evaluate the role of auxin in LR formation under excess Fe conditions, we examined the response of *pin1-1*, *pin2-1*, *pin3-5*, and *aux1-7* mutants defective in auxin transport to excess Fe. Total LR initiation events were decreased in the *pin1-1* and *pin3-5* mutants to a similar extent as in wild-type plants. However, *pin2-1* and *aux1-7* plants were found to be significantly more sensitive to excess Fe than the wild type under all tested elevated Fe concentrations (Fig. 4E; Supplemental Fig. S6A; Supplemental Table S2). Furthermore, the wild type and AUX1-complemented *aux1* and PIN2-complemented *pin2* mutants showed similar LR initiation sensitivity to excess Fe as controls (Fig. 4E). To analyze LR formation in the *aux1-7* and *pin2-1* mutants in more detail, LR initiation events in the distal portion were counted at 1, 3, and 5 d after transfer. Under control conditions, *aux1-7* and *pin2-1* mutants showed a reduction in total LRP formation compared with the wild type (Supplemental Fig. S7). Although excess Fe conditions decreased the LR initiation events in the distal portion in both *pin2-1* and *aux1-7* when compared with control, *pin2-1* and, in particular, *aux1-7* always showed lower LR initiation events than the wild type at the indicated treatment times of growth under excess Fe conditions (Supplemental Fig. S7). Furthermore, the LR frequency in the mutants slightly decreased under our growth conditions, compared with the wild type, and was approximately 83% of the wild type in *pin2-1* and approximately 73% in *aux1-7* (Table II). When the mutants were grown on media supplemented with excess Fe, the LR frequency in *pin2-1* and *aux1-7* plants decreased more significantly than the wild type, with LR frequency being reduced by approximately 35%, 58%, and 81% in wild-type, *pin2-1*, and *aux1-7* plants, respectively (Table II).

The appropriate expression and location of the AUX1 and PIN2 proteins in root tip epidermal cells are required for normal LR formation (Péret et al., 2009). Thus, we first examined whether AUX1 protein expression was altered when plants were treated with excess Fe, using a *ProAUX1:AUX1-Yellow Fluorescent Protein (YFP)* line.

Figure 3. ABA-mediated signaling is not required for the inhibitory action of Fe on LR initiation in Arabidopsis. Five-day-old wild-type, *abi1-1*, *aba3-1*, and *aba2-1* seedlings were transferred to medium, and roots were supplemented with varying concentrations of Fe (supplied as Fe-EDTA) or in combination with varying concentrations of ABA for 5 d, after which LR initiation events were quantified. **A**, Effect of ABA on LR initiation in *DR5:GUS* lines in seedlings supplied with varying concentrations of ABA for 5 d. **B**, The number of LR initiation events at each indicated developmental stage was determined in distal root portions. Values are means of 10 plants \pm SE. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). **C**, Effect of varying concentrations of Fe on LR initiation in wild-type and ABA mutant seedlings. Total LR initiation events in the wild type, *abi1-1*, *aba2-1*, and *aba3-1* in control were 19.7 ± 0.5 , 18.2 ± 0.8 , 19.2 ± 0.6 , and 22.8 ± 0.9 , respectively. Values are the means \pm SE ($n \geq 5$).



Intriguingly, the expression and localization patterns of *ProAUX1:AUX1-YFP* in roots was not affected by varying concentrations of Fe compared with those in untreated *ProAUX1:AUX1-YFP* roots at different treatment times, although an overall reduction in root length was observed (Figs. 5 and 6). Furthermore, Fe treatment did not change the *PIN1-GFP* expression profile in *ProPIN1:PIN1-GFP* plants, although a slight decrease was observed in the expression of *ProPIN3:PIN3-GFP* in roots (Supplemental Figs. S4B and S6B). By contrast, the fluorescence intensity of *ProPIN2:PIN2-GFP* in root apices decreased gradually with concentration and time compared with that in untreated roots, indicating the involvement of PIN2 during excess-Fe-modulated LR formation (Figs. 5 and 6). Using transgenic lines containing the *ProPIN2:GUS* reporter, we observed that the expression of the PIN2 gene was also influenced at the transcriptional level (Fig. 6B). However, *ProPIN2:GUS* expression was reduced relative slowly; typically, 48 h of exposure were required until significant inhibitory effects were observed (Fig. 6B).

To establish whether PIN2 expression in root tips is regulated by local Fe, we monitored PIN2 expression in root tips by employing *ProPIN2:PIN2-GFP* lines. Excess Fe localized to the root tip markedly decreased *PIN2-GFP* in roots (Fig. 7A). Quantification of PIN2-derived fluorescence confined to the root revealed that the localized provision of excess Fe significantly decreased the fluorescence intensity, similar to provision to the whole root (Fig. 7B). However, provision of excess Fe to the

shoot-mature root continuum had no impact on the fluorescence intensity of *PIN2-GFP* compared with control (Fig. 7B).

Further considering the involvement of PIN2-dependent basipetal auxin transport in the root tip (from the root tip toward the base of the root) in LR initiation (Luschnig et al., 1998; Ivanchenko et al., 2010), we assayed basipetal auxin transport by examining auxin induction of *DR5:GUS* expression (Lewis and Muday, 2009). Consistently, auxin-induced *DR5:GUS* expression was shown within a much shorter region of the root under excess Fe treatment (Supplemental Fig. S8).

Iron excess has been documented to be associated with enhanced reactive oxygen species (ROS) production and oxidative stress in general. The possible role of oxidative stress in Fe inhibition of PIN2 expression and LR development was investigated. We first examined the response of the LR phenotype to paraquat (*N,N'*-dimethyl-4,4'-bipyridinium dichloride), which is often used to stimulate ROS production in plant cells (Chu et al., 2010). Paraquat significantly inhibited both *PIN2-GFP* expression and the number of LR initiation events in the distal portion of the root (Supplemental Figs. S9B and S10A). The effects of glutathione, a powerful antioxidant (Chen et al., 2012), on *PIN2-GFP* expression and LR initiation events in the presence of excess Fe were also investigated. Our results show that exogenous application of both Fe and glutathione together could increase *PIN2-GFP* expression in Fe-treated seedlings (Supplemental Fig. S10B) and resulted

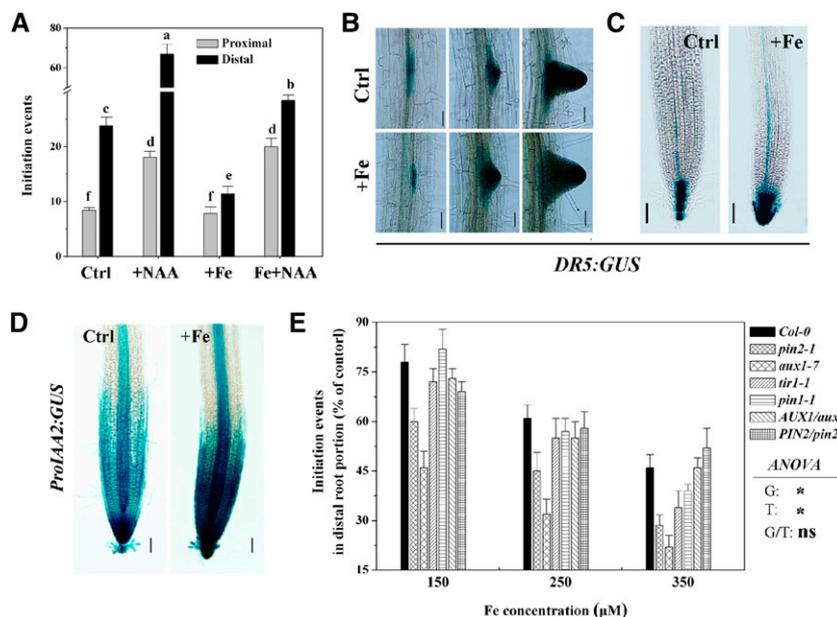


Figure 4. Effect of excess Fe on auxin response (*DR5::GUS* and *ProIAA2::GUS*) and LR initiation in the Arabidopsis wild type and auxin mutants. **A**, The effect of NAA ($0.05 \mu\text{M}$) on the number of LR initiation events in the proximal and distal root portions under control (Ctrl, $50 \mu\text{M}$) and excess Fe conditions (+Fe, $350 \mu\text{M}$) after 5 d. Values are the means \pm SE, $n \geq 5$. Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). **B** and **C**, The expression of *DR5::GUS* in LRP (**B**) and the primary root tip (**C**) with and without excess Fe ($350 \mu\text{M}$) for 3 d. **D**, The expression of *ProIAA2::GUS* with and without excess Fe ($350 \mu\text{M}$) for 3 d. **E**, Effect of varying concentrations of Fe, applied for 5 d, on LR initiation in auxin response and auxin transport mutants. Total LR initiation events in the wild type, *pin2-1*, *aux1-7*, *tir1-1*, *pin1-1*, *AUX1/aux1*, and *PIN2/pin2* in control were 21 ± 1.04 , 18.8 ± 1.1 , 12.5 ± 1.2 , 16.4 ± 0.45 , 23.4 ± 1.6 , 21.8 ± 0.8 , and 21.2 ± 1.2 , respectively. Values are the means \pm SE ($n \geq 5$). A two-way ANOVA was used to detect the significance of interaction between genotype and environment. NS and asterisk indicate nonsignificant or significant differences at $P \leq 0.05$, respectively. G, Genotype (i.e., the wild type and *aux1-7*); T, treatment (i.e., control and +Fe). Bars = $50 \mu\text{m}$.

in less inhibition of LR initiation events compared with excess Fe treatment alone (Supplemental Fig. S9C).

Involvement of Ethylene in the Response of LR Formation to Excess Fe

Ethylene has been shown to be involved in LR development (Ivanchenko et al., 2008; Lewis et al., 2011)

and has been suggested to alleviate Fe toxicity in some cases (Becker and Asch, 2005; Li et al., 2015). To elucidate the relationship between ethylene accumulation and Fe-mediated LR development, we first measured ethylene production under Fe stress by use of gas chromatography. Excess Fe significantly increased ethylene levels in roots (Fig. 8A). The effect of excess Fe on ethylene production was further investigated by monitoring the *AtACS5::GUS* and *AtACS7::GUS* activity

Table 1. Comparison of the effects of IAA, ACC, and AVG on primary root growth, cortex cell length, and LR initiation in Arabidopsis

Seedlings were treated with $250 \mu\text{M}$ Fe (+Fe) with or without $0.01 \mu\text{M}$ IAA, $0.05 \mu\text{M}$ ACC, and $1 \mu\text{M}$ AVG for 5 d. The means \pm SE are reported with $n \geq 5$ ($P \leq 0.05$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan's post hoc test).

| Background | Root Growth Increment | Cell Length | LR Density ^a | LR Frequency ^b |
|------------|-----------------------|--------------------|-------------------------|---------------------------|
| | mm | μm | | |
| Control | 51.8 ± 0.67 b | 226.3 ± 5.8 ab | 0.41 ± 0.01 e | 12.8 ± 0.73 c |
| +Fe | 21.1 ± 0.44 e | 156.8 ± 3.8 d | 0.64 ± 0.03 c | 9.02 ± 0.79 d |
| +IAA | 51.2 ± 0.66 b | 212.3 ± 3.7 bc | 0.49 ± 0.02 d | 14.8 ± 0.37 b |
| Fe + IAA | 20.8 ± 0.22 e | 154.4 ± 3.8 d | 1.26 ± 0.02 a | 17.7 ± 0.39 a |
| +ACC | 35.8 ± 0.37 c | 196.2 ± 4.8 c | 0.56 ± 0.03 d | 11.4 ± 1.15 c |
| Fe + ACC | 26.8 ± 0.28 d | 163.7 ± 2.1 d | 1.03 ± 0.01 b | 16.2 ± 0.85 a |
| +AVG | 54.8 ± 0.37 a | 233.5 ± 12.1 a | 0.38 ± 0.03 e | 12.2 ± 0.37 c |
| Fe + AVG | 10.0 ± 0.34 f | 103.4 ± 2.2 e | 0.56 ± 0.02 d | 3.50 ± 0.24 e |

^aNo. of initiation events per millimeter.

^bNo. of initiation events per 100 cortical cells.

Table II. Comparison of the effects of excess Fe on primary root growth, cortex cell length, and LR initiation in the wild type (*Col-0*) and mutants in *Arabidopsis*

For growth conditions and treatments (+Fe, 250 μM Fe), see Fig. 8. The means \pm SE are reported with $n \geq 5$ ($P \leq 0.05$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan's post hoc test).

| Background | Root Growth Increment | Cell Length | LR Density | LR Frequency |
|---------------|-----------------------|---------------------------------|--------------------|-------------------|
| | <i>mm</i> | <i>μm</i> | | |
| <i>Col-0</i> | | | | |
| Control | 50.4 \pm 0.24 a | 219.1 \pm 6.1 a | 0.43 \pm 0.02 cd | 13.4 \pm 0.59 a |
| +Fe | 23.0 \pm 1.1 d | 144.2 \pm 3.9 d | 0.53 \pm 0.02 b | 8.6 \pm 0.24 cd |
| <i>aux1-7</i> | | | | |
| Control | 45.2 \pm 0.58 b | 189.1 \pm 4.1 b | 0.31 \pm 0.01 e | 9.8 \pm 0.48 bc |
| +Fe | 12.1 \pm 0.77 g | 97.6 \pm 2.7 g | 0.35 \pm 0.03 de | 1.8 \pm 0.37 f |
| <i>pin2-1</i> | | | | |
| Control | 44.6 \pm 1.2 b | 190.8 \pm 5.6 b | 0.38 \pm 0.01 de | 11.1 \pm 0.31 b |
| +Fe | 16.0 \pm 0.31 f | 116.1 \pm 2.3 f | 0.53 \pm 0.04 b | 4.6 \pm 0.39 e |
| <i>eto1-1</i> | | | | |
| Control | 28.2 \pm 0.91 c | 157.9 \pm 2.2 c | 0.51 \pm 0.02 bc | 7.2 \pm 1.3 d |
| +Fe | 19.2 \pm 0.58 e | 128.5 \pm 2.9 e | 0.78 \pm 0.02 a | 11.3 \pm 0.44 b |

(two genes encoding 1-aminocyclopropane-1-carboxylic acid [ACC] synthase, the pivotal enzyme in the ethylene biosynthetic pathway of plants), in which the GUS reporter gene is driven by the *AtACS5* and *AtACS7* promoter. Excess Fe induced a marked increase of *AtACS5:GUS* and *AtACS7:GUS* expression in the root tip compared with control (Fig. 8B). The stimulatory effect of excess Fe on the expression of the *AtACS5* and *AtACS7* was also further confirmed by using quantitative reverse

transcription-PCR (Supplemental Fig. S11), with results consistent with a previous report from our laboratories (Li et al., 2015).

To investigate the possible role of ethylene in Fe-induced inhibition of LR formation, a genetic approach was adopted, using the ethylene overproduction mutant *ethylene overproducer1-1* (*eto1-1*). Exposure of the *eto1-1* mutant to varying Fe concentrations led to less inhibition of LR initiation than in the wild type, with LR

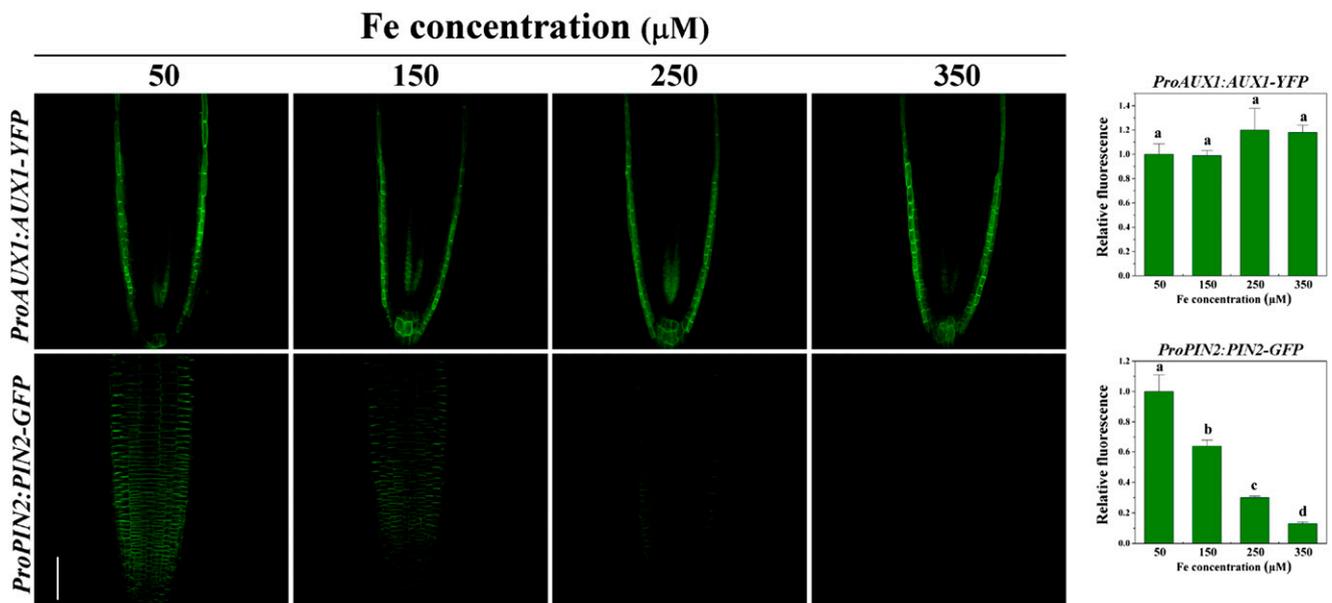


Figure 5. Effects of varying concentrations of Fe on expression of AUX1 and PIN2 in *Arabidopsis*. Five-day-old wild-type seedlings, containing *ProAUX1:AUX1-YFP* or *ProPIN2:PIN2-GFP* constructs, were transferred onto medium supplemented with varying concentrations of Fe-EDTA for 2 d. The fluorescence signal was detected using a Zeiss LSM710 confocal laser-scanning microscope at the specified time points. One representative image for each experiment is shown. The images were captured using the same confocal setting and are representative of at least 10 roots obtained from at least three independent experiments. Fluorescence is expressed relative to that of the control. Error bars represent SE ($n \geq 5$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). Bars = 50 μm .

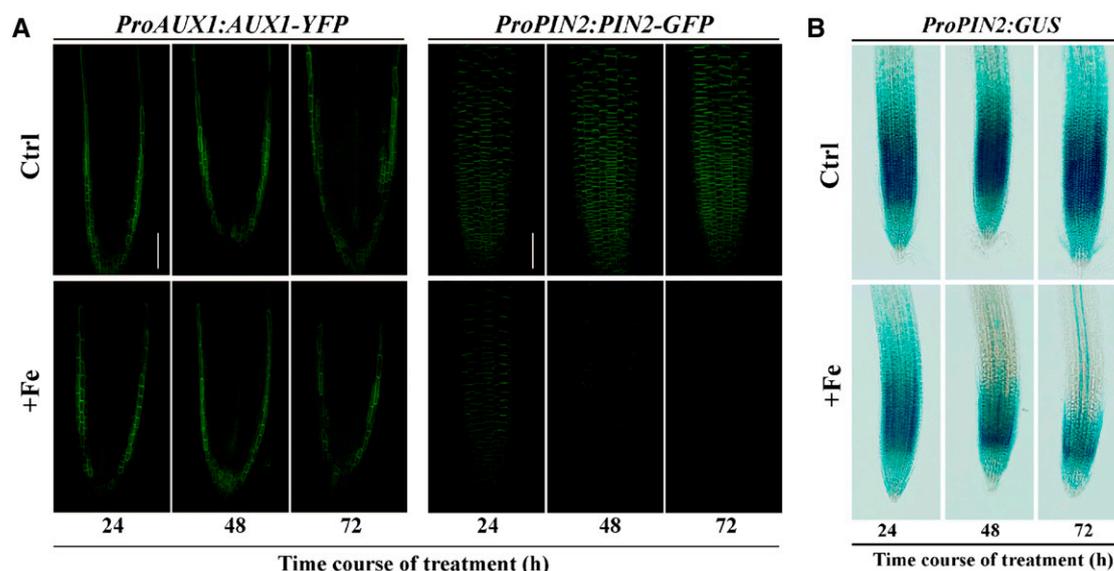


Figure 6. Effects of excess Fe on expression of AUX1 and PIN2 in Arabidopsis. Five-day-old wild-type seedlings, containing *ProAUX1:AUX1-YFP*, *ProPIN2:PIN2-GFP*, or *ProPIN2:GUS* constructs, were transferred onto control medium (Ctrl) or medium supplemented with 250 μM Fe (+Fe) at varying treatment times. The fluorescence signal was detected using a Zeiss LSM710 confocal laser-scanning microscope at the specified time points. One representative image for each experiment is shown. A, Effects of excess Fe on the expression of *ProAUX1:AUX1-YFP* and *ProPIN2:PIN2-GFP* in roots. The images were captured using the same confocal setting and are representative of at least 20 roots obtained from at least three independent experiments. B, The expression of *ProPIN2:GUS* with and without excess Fe for the indicated time periods. Bars = 50 μm (A) and 100 μm (B).

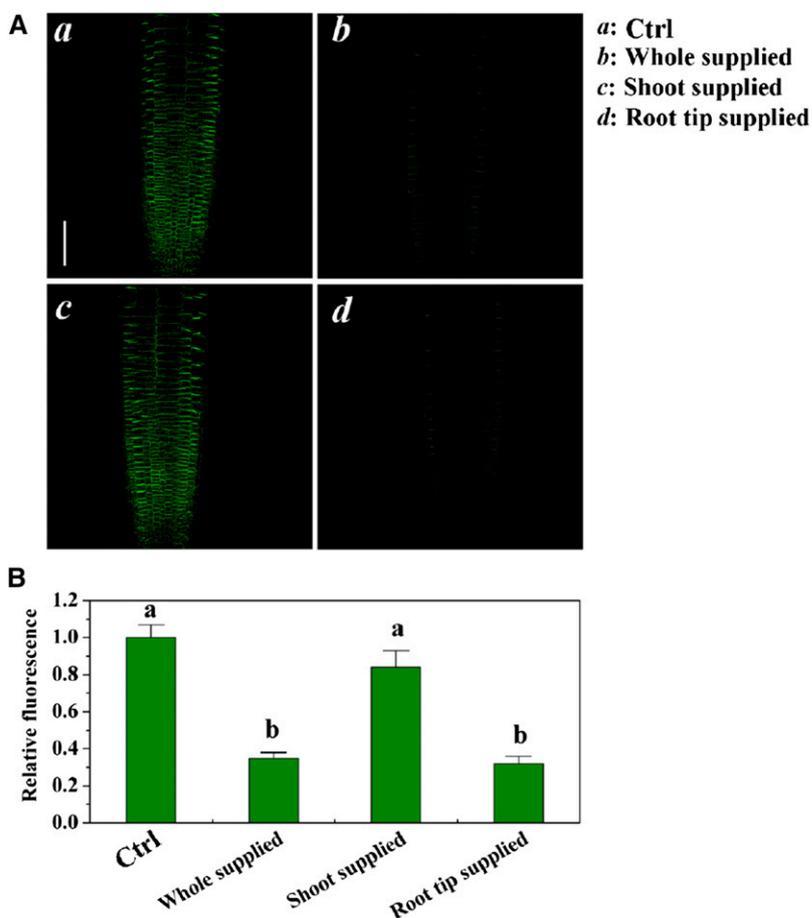
initiation events being reduced by approximately 66% and 24% in wild-type and *eto1-1* plants, respectively, upon exposure to 350 μM Fe (Fig. 8C). We further investigated the effects of aminoethoxyvinyl-glycine (AVG; an inhibitor of ethylene biosynthesis) on LR formation under varying Fe concentrations. Supplementation with AVG significantly enhanced the Fe-induced inhibition of the total LR initiation event in wild-type plants (Fig. 8D). We next determined if application of the ethylene biosynthesis inhibitor AVG would suppress the LR formation tolerance phenotype in the *eto1-1* mutant. We found that supplementation with AVG prevented LR formation tolerance in association with elevated ethylene levels in *eto1-1* seedlings exposed to excess Fe (Fig. 8E). Furthermore, supplementation with ACC (an ethylene precursor) effectively reversed the decreased rate of total LR initiation events and the reduced LR frequency seen under excess Fe, compared with the mock condition (Table I; Supplemental Table S1). Consistently, exposure of the *eto1-1* mutant to excess Fe stress also led to increased LR frequency compared with the wild type (Table II; Supplemental Table S2). However, AVG enhanced the Fe-induced reduction of LR frequency (Table I).

Involvement of Auxin in Ethylene-Regulated LR Formation during Fe Stress

The data above indicate that excess Fe represses PIN2 expression and up-regulates ethylene production,

while increasing the tolerance of the LR formation. To investigate whether increased ethylene could influence the Fe-modulated expression of PIN2, PIN2 expression was analyzed in the roots of *ProPIN2:PIN2-GFP* seedlings treated with excess Fe plus AVG or with Fe alone. Similar inhibition of PIN2-GFP abundance in the root apex was found in Fe-treated seedlings with or without AVG (Fig. 9, A and B). However, the localization of the PIN2 protein appeared to be altered only slightly (Figs. 7A and 9A). After extending the master gain of the GFP channel from 679 to 1,017, it was found that PIN2-GFP was diffusely distributed in the root tips in seedlings treated with excess Fe plus AVG (Fig. 7A, b; Fig. 9A, b). Surprisingly, excess Fe plus AVG markedly decreased AUX1-dependent fluorescence at the root apex compared with Fe alone (Fig. 9C). Quantification of AUX1-derived fluorescence confined to the root apex revealed that provision of excess Fe plus AVG decreased the AUX1 signal intensity by approximately 40% relative to excess Fe alone (Fig. 9D). In addition, the localization of the AUX1 protein appears altered in the root tip of seedlings grown on excess Fe plus AVG and becomes dispersed near the root tip (Fig. 9C, a–d). To further explore the role of AUX1 in the ethylene-induced LR formation tolerance phenotype, the LR initiation events of the distal portion were analyzed in the wild type, the *eto1-1* mutant, the *aux1-7* mutant, and the double mutant (*eto1/aux1*) under excess Fe (Fig. 9E). Our results show that *aux1-7* significantly weakens the LR formation tolerance characteristic of *eto1-1*.

Figure 7. Effect of locally supplied Fe on *PIN2-GFP* expression in the Arabidopsis root tip. A, Locally supplied Fe (250 μM) on *PIN2-GFP* expression in root tips at 2 d. B, The fluorescence of *PIN2-GFP* in A. Fluorescence is expressed relative to that of the control. Error bars represent SE ($n \geq 5$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). Bars = 50 μm .



DISCUSSION

Excess Fe Regulates LR Initiation Acting at the Tip of the Growing Primary Root

Varying initiation of LRP, frequency of LR emergence, and growth rate of both primary root and LR provide significant combinatorial potential for modifying root system architecture to respond to changing nutrient availability (Liao et al., 2006; Fujiwara and Matoh, 2009; Gruber et al., 2013). Fe is both an essential micronutrient and is highly toxic to root growth when in excess (Becker and Asch, 2005; Kobayashi and Nishizawa, 2012), but the toxicity mechanisms in terms of root development remain largely unknown. Recently, Reyt et al. (2015) found that root in Arabidopsis, rather than LR initiation within the LR branching zone, represented the principal target of inhibition. Whether, and how, LR formation near the growing tip of the primary root responds has not been examined to date. We conclude from the evidence presented here that excess Fe treatment in Arabidopsis not only directly impairs primary root growth, but also strongly arrests LR initiation by acting at the tip of the growing primary root. We show that excess Fe inhibits total LR initiation but not subsequent LR development (Fig. 1A; Supplemental Fig. S1B) and that these effects are only

seen in newly grown roots that elongated during exposure to excess Fe (Fig. 1, A and B). Similar results were found in the arrest of salt-mediated LR initiation events, which was, as well, confined to newly grown roots (Duan et al., 2013). Decrease in overall LR number with increasing Fe concentrations could simply reflect the shorter length of the primary root. Excess Fe significantly inhibited both primary root growth and the total LR initiation events, consistent with this simple explanation. However, IAA in our study did not affect primary root growth compared with mock conditions but significantly increased the number of LR initiation events in both control and excess-Fe conditions (Table I; Supplemental Table S1), consistent with results in a previous report (Ivanchenko et al., 2010). Furthermore, ACC, at the concentration applied in our study, significantly reduced primary root growth but had no effect on LR initiation events (Table I; Supplemental Table S1), similar to the results of Ivanchenko et al. (2008) and results obtained with the *eto1-1* mutant under excess Fe stress (Table II; Supplemental Table S2). Thus, both our present and previous results suggest a more complex interconnection between root length and LR initiation frequency under excess Fe conditions than a simple correlation between length and number. LR development is initiated by asymmetric divisions in pairs of

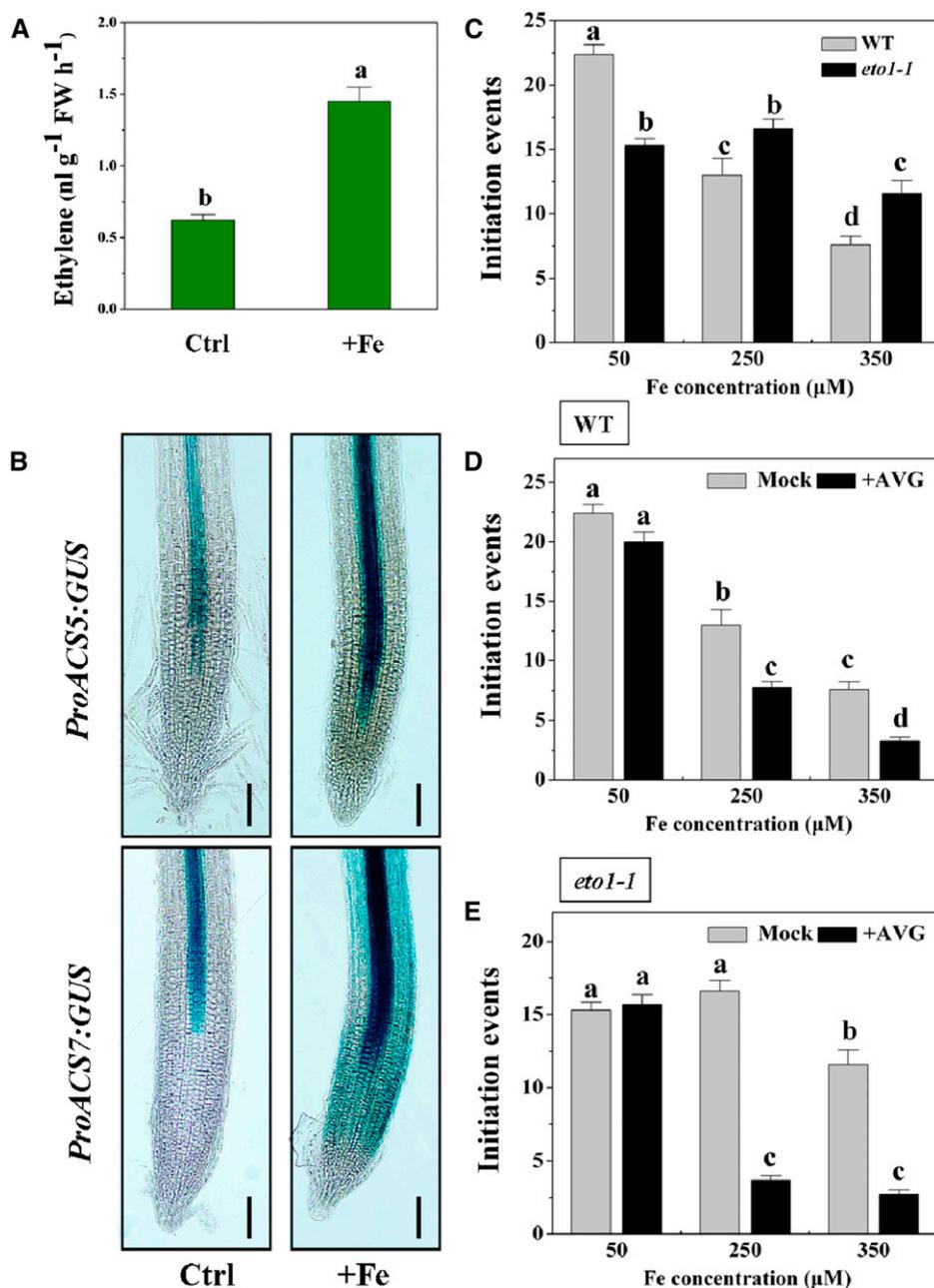


Figure 8. Effect of ethylene on the LR formation under excess Fe stress in Arabidopsis. **A**, Ethylene evolution in the root. Seedlings at 4 d after germination were exposed to 350 μM Fe in the roots for 4 d, and then ethylene evolution was determined. Values are the means ± SE of three replicates. **B**, Activity of *AtACS5:GUS* and *AtACS7:GUS* in the root tip. Seedlings at 5 d after germination were exposed to 350 μM Fe in the roots for 2 d, and then GUS activity was determined. One representative sample from each treatment (10 plants) is shown. **C**, Effect of excess Fe on LR initiation in the wild type and ethylene-overproducing mutant *eto1-1*. Data are from one of three experiments. Seedlings at 5 d after germination were exposed to varying concentrations of Fe for 5 d. Values are the means ± SE ($n \geq 7$). **D**, Effect of the ethylene inhibitor AVG on LR initiation of wild-type seedlings grown in varying concentrations of Fe. Values are the means ± SE ($n \geq 7$). **E**, Effect of the ethylene inhibitor AVG on LR initiation of *eto1-1* seedlings grown in varying concentrations of Fe. Values are the means ± SE ($n \geq 7$). Different letters represent means statistically different at the 0.05 level (one-way ANOVA with Duncan post hoc test). Ctrl, Control; WT, wild type; FW, fresh weight. Bars = 50 μm.

founder cells within xylem pole pericycle cells close to the root tip (De Smet et al., 2007; Overvoorde et al., 2010). Excess Fe may inhibit xylem-adjacent pericycle cell activation during primordium morphogenesis, because the frequency of LR initiation, estimated as the number of initiation events per 100 cortical cells, is decreased under Fe toxicity (Fig. 1C). This also provides an explanation for the absence of an arrest of initiation events in previously formed roots (proximal portion), because LR patterning in the proximal portion occurred before treatment of roots with excess Fe. No information is as yet available to suggest how Fe stress may inhibit the commitment, or activation, of xylem-adjacent

pericycle cells. Our data show that LR initiation frequency is associated with auxin (Table I). However, the sensitivity of root cells to auxin may not be altered by excess Fe (Fig. 4E; Supplemental Fig. S5). Furthermore, *aux1-7* and *pin2-1* mutants exhibited a higher degree of sensitivity in LR frequency to excess Fe (Table II), suggesting that the effect may, at least partly, be mediated by AUX1 and/or PIN2. However, the issue of how AUX1 and/or PIN2 are involved in Fe-mediated modulation of LR frequency still needs to be explored.

Moreover, we show that the impact of Fe toxicity on LR formation in Arabidopsis is not a systemic response. Split-plate results reported here provide strong demonstration

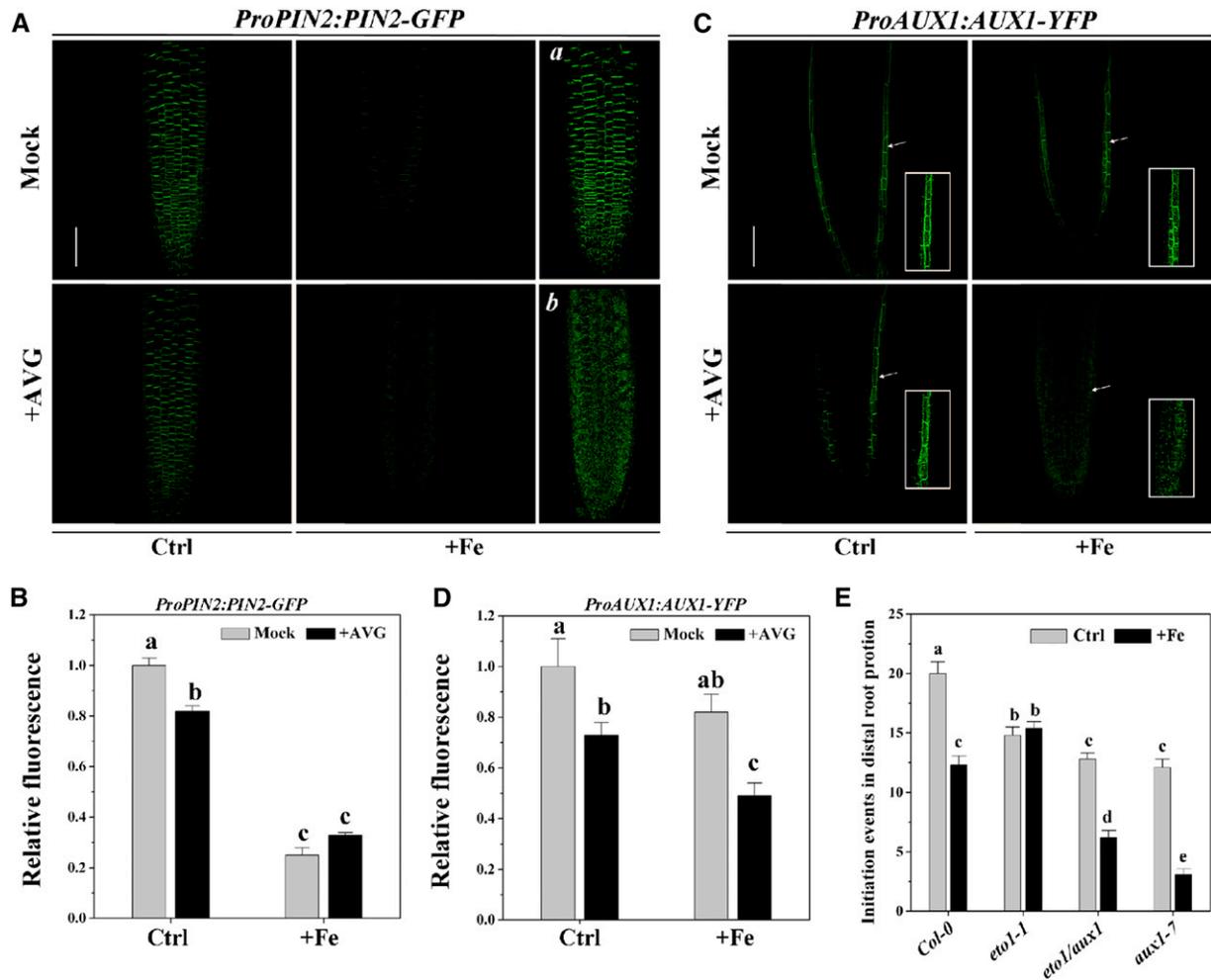


Figure 9. Effects of AVG on the expression of AUX1 and PIN2 in Arabidopsis. Five-day-old wild-type seedlings, containing *ProAUX1:AUX1-YFP* or *ProPIN2:PIN2-GFP* constructs, were transferred onto control medium (Ctrl) or medium supplemented with 250 μM Fe (+Fe) with or without 1 μM AVG for 48 h. A, The expression of *ProPIN2:PIN2-GFP* with and without excess Fe plus AVG or Fe alone treatment. The treatments were maintained under the same confocal setting, and the inserts (a and b) show roots under Fe treatment, extending the master gain of the GFP channel from 679 to 1,017. B, The fluorescence of PIN2-GFP in A. Fluorescence is expressed relative to that of the control without AVG. Error bars represent SE ($n \geq 5$). C, The expression of *ProAUX1:AUX1-YFP* with and without excess Fe plus AVG or Fe alone treatment. The treatments were maintained under the same confocal setting, and the inserts (a–d) show images of partial enlargement, as indicated by white arrows. D, The fluorescence of AUX1-YFP in C. Fluorescence is expressed relative to that of the control without AVG. Bars represent SE ($n \geq 5$). E, Effect of excess Fe (250 μM) on LR initiation events in wild-type (Col-0), *eto1-1*, *aux1-7*, and *eto1/aux1* seedlings after 5 d. Values are the means $\pm \text{SE}$ ($n \geq 7$). Bars = 50 μm .

that physical contact of the primary root tip with excess Fe is necessary, and sufficient, for LR formation inhibition in newly grown roots (Fig. 2). Previously, we found that the root tip was also the primary sensing site for the root elongation response to excess Fe stress (Li et al., 2015). In a previous study on NH_4^+ toxicity in Arabidopsis (Li et al., 2010), it was similarly observed that contact with the root tip was both essential and sufficient to explain the development of the NH_4^+ toxicity syndrome and, more specifically, the inhibition of primary and LR growth. Several studies have indicated that nutrients, such as N and P, with heterogeneous distribution in the soil and rhizosphere (Hodge, 2004; Britto and Kronzucker, 2013;

Hufnagel et al., 2014), are sensed by root-localized mechanisms (Forde and Lorenzo, 2001; Forde and Walch-Liu, 2009). Iron toxicity as a syndrome in plants is typically associated with an excessive amount of the ferrous form (the Fe^{2+} ion) in the soil (Vigani, 2012). The presence of the Fe^{2+} ion is increased by low pH and hypoxic or anoxic conditions, and there is an increasing presence in vertically lower strata (Ratering and Schnell, 2000; Becker and Asch, 2005). Thus, we propose that, when the primary root tip reaches the excess Fe zone, plants sense the stress stimulus and trigger a developmental switch in both the primary root and the LR apparatus from indeterminate to determinate growth, the

reduction of cell elongation and the LR initiation acting at the tip of the growing primary root. Such a change may adjust root system architecture to restrict excessive Fe absorption, which also occurs predominantly in the Fe^{2+} form (Vigani, 2012; Li et al., 2015), and prevent serious Fe toxicity. In addition, relatively stable LR number and length in the branching zone, or the proximal root portions, helped by Fe sequestration in the vacuole and by components such as ferritins (Vigani, 2012; Reyt et al., 2015), may play a role in maintaining the absorption of other nutrients in the less stressed areas of the root system.

The Role of AUX1 and PIN2 in LR Formation under Excess Fe Conditions

The inhibitory effects of excess Fe on LR initiation and xylem differentiation resemble the phenotypes of seedlings defective in auxin transport or perception (Casimiro et al., 2003; Fukaki and Tasaka, 2009; Péret et al., 2009). Analysis of the effect of excess Fe on auxin response and auxin transport in roots suggests that an important part of inhibition on LR formation is mediated by the auxin pathway (Fig. 4; Table I). However, we found that *DR5:GUS* expression in LRP was not significantly modified in seedlings treated with excess Fe (Fig. 4B). This, together with an account of ferritin gene expression reported previously (Reyt et al., 2015), might account for the above-described LRP proportion phenotype (Fig. 1D). Further screening of auxin-related mutants for excess Fe-responsive LR formation showed that LR initiation was inhibited by excess Fe to a greater extent in *aux1-7* and *pin2-1* mutants than in wild-type plants, at varying concentrations of Fe and with varying treatment time (Fig. 4E; Supplemental Fig. S7), indicating that disruptions in either AUX1 or PIN2 enhance the sensitivity of LR formation under Fe stress. By contrast, wild-type and *pin1-1*, *pin3-5*, and *tir1-1* mutants showed similar LR initiation sensitivity to excess Fe as controls (Fig. 4E; Supplemental Fig. S6A). Although the expression and localization of AUX1 were unaffected, PIN2 transcript and protein were decreased gradually under early Fe stress (Figs. 5 and 6). The alterations in PIN2 gene expression and PIN2 abundance may be expected to interfere with the distribution of auxin in root tips and with basipetal auxin transport (Abas et al., 2006; Wisniewska et al., 2006). This is supported by the observed reduction in basipetal auxin transport and the increase in auxin accumulation in the root apex under excess Fe stress (Fig. 4, C and D; Supplemental Fig. S8). This suggests a tight relationship for PIN2 in the excess-Fe-induced inhibition of LR formation. Moreover, our analysis of locally supplied Fe (Fig. 7) indicates that Fe supply to only the root tip, but not the shoot-mature root continuum, decreased *PIN2:GFP* expression in the root tip, consistent with the split-plate results (Fig. 2), and highlights the correlation between Fe-dependent PIN2 expression and LR initiation. The LR development of the *pin2-1* mutant was also

reported to be more sensitive to NH_4^+ toxicity (Li et al., 2011). Furthermore, auxin distribution within the root under Fe stress responded to alterations in PIN2 gene expression and PIN2 abundance, and this may modify the direction of root growth away from the stress stimulus (Supplemental Fig. S12; Wisniewska et al., 2006). In addition to participating in AUX1-mediated basipetal auxin transport, PIN2 is seen as a general stress target on account of its pronounced sensitivity to environmental stresses, such as cold, dark, salt, and aluminum and cadmium toxicity, and underlies root avoidance of stress (Supplemental Fig. S10A; Baluska et al., 2010). Iron excess has been linked to oxidative stress, and ROS homeostasis is emerging as a parameter controlling root system architecture more generally (Manzano et al., 2014). However, although antioxidant partially increased *PIN2-GFP* expression and LR initiation events, it could not rescue Fe inhibition of LR formation completely (Supplemental Figs. S9 and S10). Cadmium, which also induces oxidative stress via Fenton reactions, could inhibit root system development (Supplemental Fig. S9A; Jonak et al., 2004), with various hormones (nitric oxide, jasmonic acid, and salicylic acid) reported to be involved in tolerance (Rodríguez-Serrano et al., 2006; Lin et al., 2012). Our present data suggest that Fe-induced oxidative stress is partly involved in the suppression of *PIN2-GFP* expression and of LR formation in the distal portion of the root.

Using both pharmacological and genetic approaches, we further demonstrate that ethylene plays an important role in LR formation under excess Fe exposure. Ethylene evolution was enhanced in the root upon exposure of seedlings to excess Fe (Fig. 8A), similar to

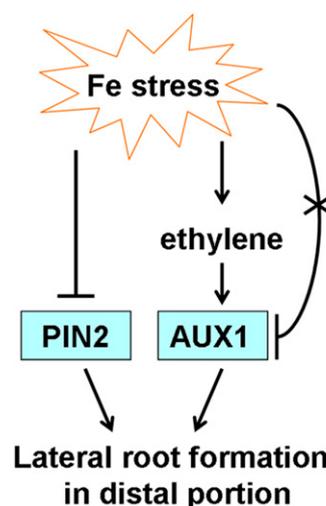


Figure 10. A proposed model for LR formation in Arabidopsis under excess Fe stress in the distal root portion. The expression of PIN2 protein in root tips is dramatically reduced and involved in arresting LR initiation near the growing tip of the primary root in the early response to excess Fe stress. Fe-induced endogenous ethylene enhances the tolerance of LR formation to Fe stress, and AUX1 plays a role in ethylene-mediated LR formation.

previous reports (Majerus et al., 2007; Li et al., 2015). Here, we identify *ACS5* and *ACS7* as two key genes responsible for the observed Fe-induced ethylene production in root tips (Fig. 8B; Supplemental Fig. S11). Remarkably, the excess-Fe-induced inhibition of LR formation was aggravated in the presence of the ethylene synthesis inhibitor AVG in the wild type (Fig. 8D). The finding that the ethylene overproduction mutant *eto1-1* showed increased LR formation compared with the wild type under Fe stress (Fig. 8C) supports this notion. Also supportive is the observation that the externally applied ethylene inhibitor AVG prevented tolerance by elevated ethylene levels in *eto1-1* exposed to high Fe (Fig. 8E). Moreover, the inhibition of LR frequency was alleviated in the presence of ACC as well as in the ethylene overproduction mutant, while an inhibitor of ethylene biosynthesis (AVG) aggravated it (Tables I and II).

Together with PIN2, AUX1 is well known to form the basic machinery for basipetal auxin transport, which is critical for LR initiation (De Smet et al., 2007). Furthermore, in *aux1-7*, total LR initiation events and LR frequency were more sensitive to excess Fe than in the wild type (Fig. 4E; Table II; Supplemental Fig. S7), suggesting AUX1 is essential for the maintenance of LR initiation under excess Fe. Furthermore, Giehl et al. (2012) suggested that localized supply of iron regulated LR development in *Arabidopsis* by altering AUX1-mediated auxin distribution. Based on the present and previous findings, the hypothesis that ethylene induces tolerance in LR formation in the wild type partly through AUX1 mediation was borne out. The findings are also consistent with previous reports showing that ethylene enhances the expression of AUX1 reporters (Růžicka et al., 2007; Lewis et al., 2011). Strong support for this can be seen in the finding that excess Fe and AVG cotreatment markedly reduce AUX1 protein abundance in the root apex of wild-type seedlings compared with excess Fe alone (Fig. 9, C and D). More importantly, the *eto1-1* mutant, also lacking AUX1 (double mutant *eto1-1/aux1-7*), lost its previously documented higher LR tolerance characteristic under excess Fe stress (Fig. 9E), further supporting the hypothesis that AUX1 is involved in ethylene-mediated signaling under excess Fe stress. However, excess Fe and AVG cotreatment had no effect on PIN2 protein abundance (Fig. 9, A and B), although a small alteration in localization was noted after extending the master gain of the GFP channel (Fig. 9A). Together, we suggest that AUX1 is at least partially required for the tolerance effect of endogenous ethylene in Fe-dependent LR formation.

ABA is also often associated with LR development. ABA signaling pathways are recognized as essential for LR responses to salt and osmotic stresses (Deak and Malamy, 2005; Xiong et al., 2006). However, excess Fe does not exert its inhibition of LR number through these pathways. Firstly, excess Fe stress arrests LRP initiation without affecting subsequent LRP development, different from the inhibitory effect of ABA on meristem activation of LRP (Fig. 2, A and B; Signora et al., 2001;

De Smet et al., 2003). Secondly, ABA response and biosynthesis mutants, such as *abi1-1*, *aba2-1*, and *aba3-1*, still display LR growth sensitivity to excess Fe, similar to the wild type.

In conclusion, we propose the following model for LR formation under excess Fe stress in the distal portion of the root (Fig. 10). Our results show that the expression of PIN2 protein in root tips is dramatically reduced and involved in arresting LR initiation near the growing tip of the primary root in the early response to excess Fe. These modulations in hormone homeostasis can help root system architecture adjust rapidly to restrict excessive Fe absorption and avoid serious Fe toxicity. Meanwhile, the induced-ethylene and ethylene-related AUX1 functions, as a compensator modulating the process, can partially antagonize the reduction in excess Fe-mediated LR formation in the wild type. These compensatory effects of ethylene may prevent the excessive decline in LR number and maintain the absorption of other nutrients to maintain rigorously controlled plant acclimation to nutritional stress (Heil and Baldwin, 2002). Although the possible involvement of other pathways in Fe inhibition LR formation still needs to be explored, our results provide unique insight into how LR formation is regulated in response to early Fe toxicity. Further research into the interplay of excess Fe supply and auxin will enable a fuller understanding of how plants respond to Fe toxicity by regulating hormonal signaling and will be instrumental in the development of strategies to improve Fe toxicity tolerance in crops.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seedlings of the following lines were used in this study: *Arabidopsis thaliana* ecotype Columbia-0 (Col-0); the mutants *aba3-1*, *aba2-1*, *abi1-1*, *eto1-1*, *aux1-7*, *pin2-1*, *pin3-5*, *tir1-1*, *pin1-1*, and *eto1/aux1* (Strader et al., 2010) in the Col-0 background; the transgenic lines *DR5:GUS* and *DR5:GUS* in *aux1-7* and *pin2-1*; and *ProACS5:GUS* and *ProACS7:GUS* (Wang et al., 2005), *ProIAA2:GUS*, *ProPIN1:PIN1-GFP*, *ProPIN2:PIN2-GFP*, *ProPIN3:PIN3-GFP*, *ProAUX1:AUX1-YFP* (Swarup et al., 2004), and the *DR5:GFP* lines. Seeds were surface sterilized and cold treated at 4°C for 48 h prior to being sown on standard growth medium. The standard growth medium was as described previously (Li et al., 2013) and was composed of 2 mM KH₂PO₄, 5 mM NaNO₃, 2 mM MgSO₄, 1 mM CaCl₂, 50 μM Fe-EDTA, 50 μM H₃BO₃, 12 μM MnSO₄, 1 μM ZnCl₂, 1 μM CuSO₄, 0.2 μM Na₂MoO₄, 1% (w/v) Suc, 0.5 g L⁻¹ MES, and 0.8% (w/v) agar (adjusted to pH 5.7 with 1 M NaOH). The day of sowing was considered day 0. Seedlings were grown, oriented vertically on the surface of the culture plates in a growth chamber, and set to a 16-h-light/8-h-dark photoperiod, an irradiance of 100 μmol m⁻² s⁻¹, and a temperature of 23°C ± 1°C. Excess Fe was supplied as Fe-EDTA (FeSO₄·7H₂O plus EDTA [1:1 M ratio]). Fe availability and toxicity, and root growth itself, are strongly pH dependent (Koyama et al., 2001; Becker and Asch, 2005), and we thus set pH to 5.3, based on our previous report (Li et al., 2015), for further treatments. To study the effect of exogenous ABA, NAA, IAA, ACC, and AVG, seedlings were supplemented with varying concentrations of Fe-EDTA plus ABA (as indicated), 0.05 μM NAA, 0.01 μM IAA, 0.05 μM ACC, or 1 μM AVG. All chemicals were obtained from Sigma-Aldrich. For hydroponic culture, gravity stimulation, and qRT-PCR experiments, see Supplemental Materials and Methods S1.

Localized Fe Supply Experiments

Localized excess-Fe treatments were shown in Figure 2A, as described in our previous report (Li et al., 2015). It is briefly described here: For control or whole-supplied plants, seedlings were grown in the normal growth medium with or

without excess Fe following seedling transfer. For root tip-supplied plants, segmented agar plates were separated into upper and bottom parts with a 3-mm air gap using movable glass strips 3 mm in width. Normal growth medium was poured into the upper part, and control medium containing excess Fe-EDTA was poured into the bottom part. Plates on which only the primary root tip of the seedlings (approximately 2 mm) was in contact with the bottom of the medium are referred to as root tip-supplied Fe plants. For shoot-supplied Fe plants, normal growth medium was poured into the bottom part, and control medium containing excess Fe-EDTA was poured into the upper part. Plates on which only the shoot and mature primary root zone of the seedlings were in contact with the upper of the medium are referred to as shoot-supplied Fe plants, and treatment was for the times indicated.

Root Measurements

Root portions that were formed before or during the treatments were harvested separately for analysis. All initiation events, including emerged LRs and LRP, were quantified in cleared roots according to Ivanchenko et al. (2008) or using *DR5:GLUS* lines. The developmental stage of each LRP was classified according to Zhang et al. (1999): stage A, up to three cell layers; stage B, unemerged, more than three cell layers; stage C, emerged LRs, less than 0.5 mm in length; and stage D, LRs, greater than 0.5 mm. The emerged but not activated LRP are still referred to as LRP, and only mature LRs (exceeding 0.5 mm in length) are denoted as LRs. The lengths of at least 10 cortical cells were measured for each root along the entire root growth increment so as to exclude the possibility of differential response to the treatment. The average root length corresponding to 100 cortical cells in a file was estimated for each root based on the elongation increment and average cortex cell length of the root (Ivanchenko et al., 2008, 2010). The lengths of primary roots of individual seedlings were measured directly using Image J software from digital images captured with a Canon G7 camera.

Image Analysis

Histochemical analysis of GUS reporter enzyme activity was performed as described elsewhere (Weigel and Glazebrook, 2002). GUS staining patterns in roots were analyzed using an Olympus BX51 microscope with differential interference contrast optics. The *DR5:GFP*, *ProPIN1:PIN1-GFP*, *ProPIN2:PIN2-GFP*, *ProPIN3:PIN3-GFP*, and *ProAUX1:AUX1-YFP* reporters were analyzed using a Zeiss LSM710 confocal microscope, and image analysis was performed using Zeiss 2009 software. All staining and image analysis procedures were repeated at least twice. All the images and graphs were arranged in Adobe Photoshop.

Ethylene Measurements

After seedling exposure to various concentrations of Fe-EDTA for 4 d, roots were weighed separately and put into 5-mL gas-tight vials containing 1 mL of agar medium (0.7% agar). Headspace samples (1 mL) were withdrawn and analyzed using a GC-6850 gas chromatograph (Agilent Technologies Japan), which was equipped with a flame ionization detector.

Statistical and Graphical Analyses

For all experiments, data were statistically analyzed using the SPSS 13.0 program. Details are as presented in figure legends. Graphs were produced using Origin 8.0. All graphs and images were prepared using Adobe Photoshop 7.0.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *ABA3* (At1g16540), *ABA2* (At1g52340), *AB11* (At4g26080), *ACS5* (At5g65800), *ACS7* (At4g26200), *AUX1* (At2g38120), *ETO1* (At3g51770), *LA2* (At3g23030), *PIN1* (At1g73590), *PIN2* (At5g57090), *PIN3* (At1g70940), and *TIR1* (At3g62980).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effects of excess Fe on LR primordium proportion in Arabidopsis.

Supplemental Figure S2. Effects of Fe-citrate and K-citrate on LR initiation events in Arabidopsis.

Supplemental Figure S3. Effects of Fe excess on Arabidopsis LR initiation events in hydroponic condition.

Supplemental Figure S4. Effects of excess Fe on expression of *DR5:GFP* and *ProPIN1:PIN1-GFP*.

Supplemental Figure S5. Auxin activation of the *DR5* response in xylem-adjacent pericycle cells.

Supplemental Figure S6. Effects of excess Fe on the LR initiation of *pin3-5* and expression of *ProPIN3:PIN3-GFP* in Arabidopsis.

Supplemental Figure S7. Effects of excess Fe on the wild-type (Col-0), *pin2-1*, and *aux1-7* LR initiation.

Supplemental Figure S8. Auxin induction of *DR5:GUS* expression in Col-0, with or without excess Fe treatment.

Supplemental Figure S9. Effects of cadmium, paraquat, and glutathione on LR initiation in Arabidopsis.

Supplemental Figure S10. Effects of cadmium, paraquat, and glutathione on the expression of *ProPIN2:PIN2-GFP* in Arabidopsis.

Supplemental Figure S11. Effects of excess Fe treatment on *AtACS5* and *AtACS7* transcript levels in Arabidopsis.

Supplemental Figure S12. Experimental system for examining agravitropic response in response to excess Fe.

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

Supplemental Table S2. Comparison of the effects of excess Fe on total LR initiation events in the wild type and mutants in Arabidopsis.

Supplemental Materials and Methods S1. Hydroponic culture, gravity measurement, and qRT-PCR and auxin transport analysis.

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1 **Supplemental data**

2

3 **Supplemental Materials and Methods**

4 **Hydroponic growth conditions**

5 Seedlings at 8 days after germination (DAG) on agar medium were transferred to the control
6 Hoagland solution (Ctrl, 50 μ M Fe-EDTA, pH=5.3) or Hoagland solution supplemented with excess
7 Fe (+Fe, 250 μ M Fe-EDTA), and grown for an additional 3 d. The number of lateral root initiation
8 events was determined in the proximal root and distal portions. Hoagland solution was modified
9 accord with Xu et al. (2013) as the following nutrients: KNO₃, 0.5mM; Ca(NO₃)₂, 1.0mM; KH₂PO₄,
10 1.0mM; MgSO₄, 0.3mM; H₃BO₃, 13.3 μ M; MnCl₂, 3.0 μ M; CuSO₄, 0.5 μ M; ZnSO₄, 1.0 μ M;
11 Na₂MoO₄, 0.1 μ M; NaCl, 2 μ M; CoCl₂, 0.01 μ M; NiSO₄, 0.1 μ M; and Fe-EDTA, 50 μ M.

12

13 ***DR5:GUS*-based auxin transport assay**

14 The method, as described by Lewis and Muday (2009), was used to measure auxin transport. In
15 brief, to measure basipetal auxin transport, plates with the control seedlings (5 DAG *DR5:GUS*
16 plants) or IAA treated seedlings were incubated in the dark for 2 h. IAA treatment was conducted by
17 placing a solidified agar block containing 0.1 μ M IAA such that it overlapped with the root tip by
18 ~0.5 mm. The entire seedling was then subjected to GUS staining for 16 h at 37 °C. Auxin transport
19 was determined by comparing the distance of GUS staining from the site of IAA application of the
20 treated seedlings with that of the controls.

21

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

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

1 primers used in this study as following: *AtACS5*: 5'-GTTTTAGCGGCTGGTTCGACATCT-3' and
2 5'-CAACGCAGTGCCAAGTGGGTTA-3'; *AtACS7*: 5'-CCTGGGTTCGTTGAAAACGCATT-3'
3 and 5'-CGTCGTTAGGATCGGCGAGAATGA-3'; *CBP20*: 5'-ACCATCGGAAACGACAAAGAG
4 -3' and 5'-CTTCACCATCGTCATCGGAGT-3'.

5

6 **Gravity stimulation**

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

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15 **LITERATURE CITED**

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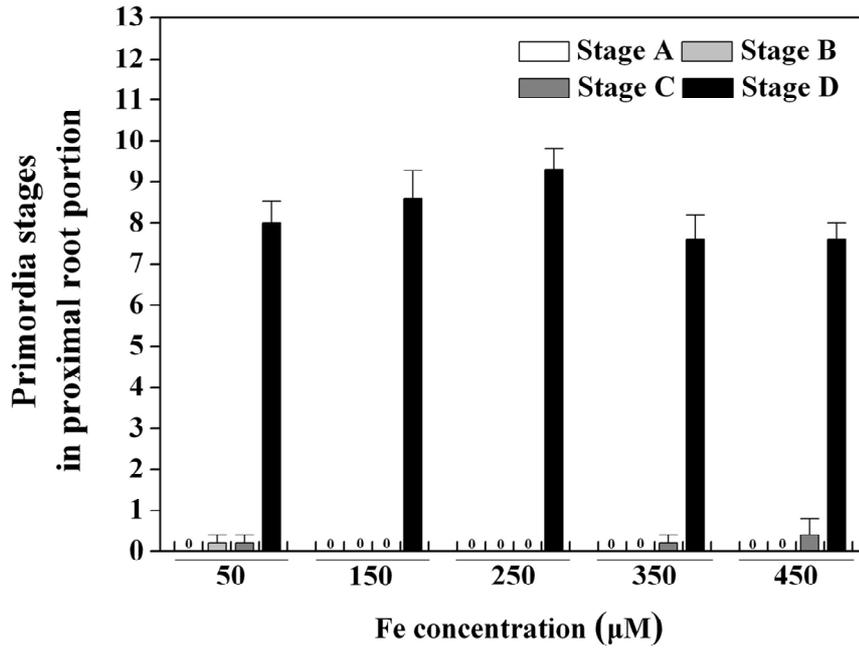
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19 ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*. *J*
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26 inhibition of root elongation in *Arabidopsis*. *J Exp Bot* **63**: 3777-3788

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

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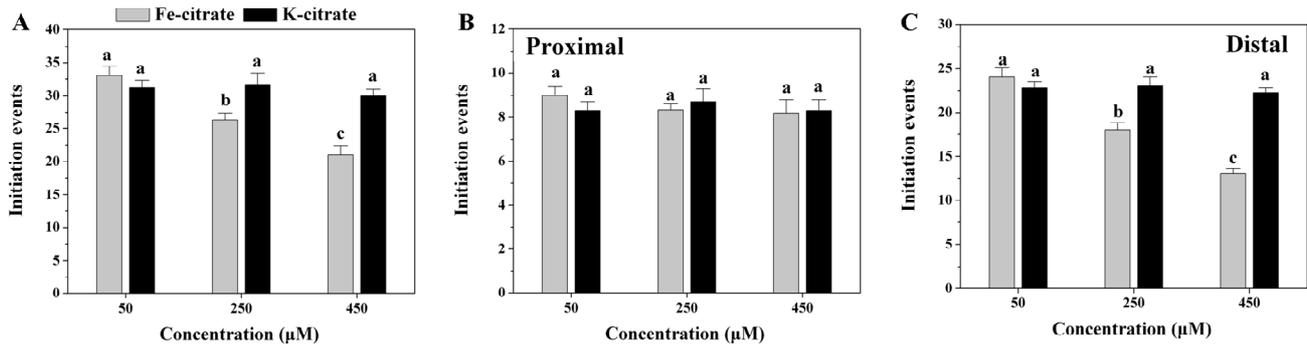
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2 **Supplemental Figure S2.** Effects of Fe-citrate and K-citrate on lateral root initiation events in
 3 *Arabidopsis*. Seedlings at 5 days after germination (DAG) were transferred to varying
 4 concentrations of Fe and K (supplied as Fe-citrate and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

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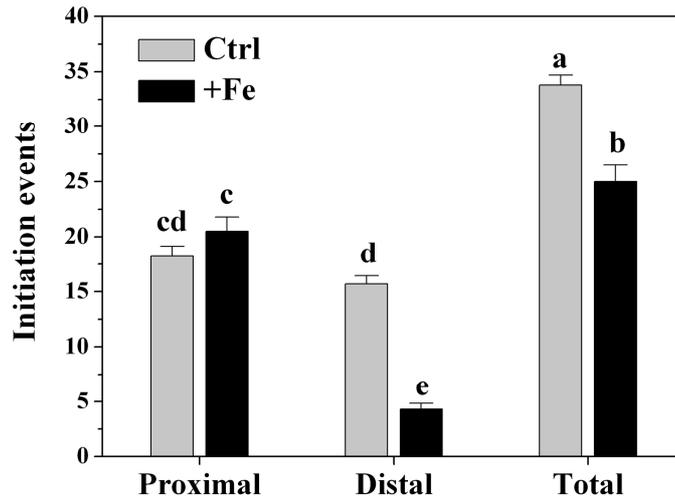
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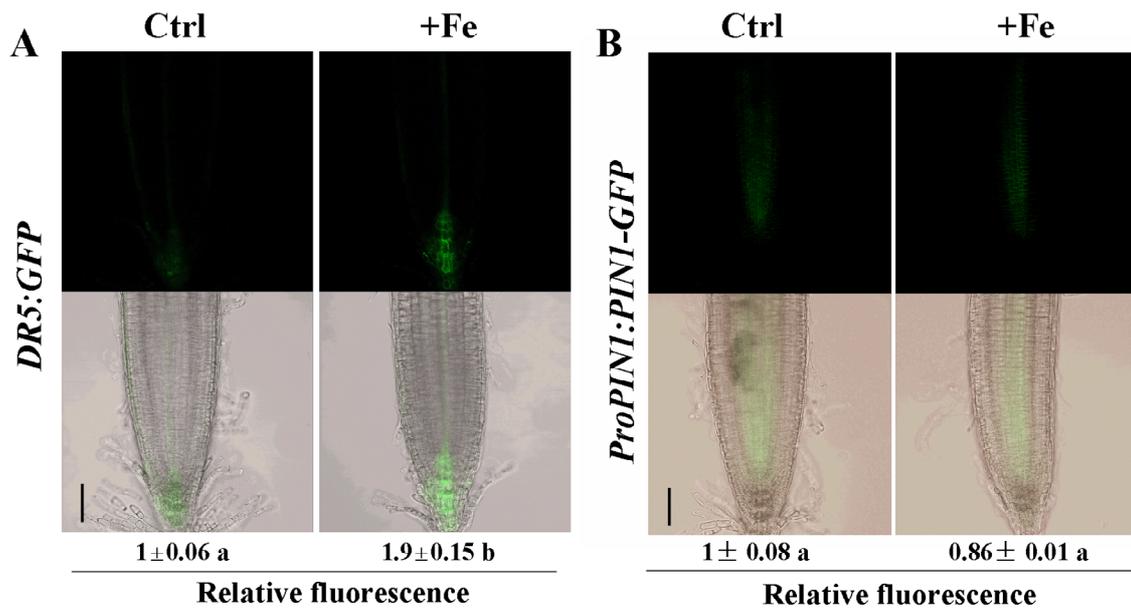
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2 **Supplemental Figure S3.** Effects of Fe excess on *Arabidopsis* lateral root initiation events in
 3 hydroponic condition. Seedlings at 8 days after germination (DAG) on agar medium were
 4 transferred to the control Hoagland solution (Ctrl, 50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

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

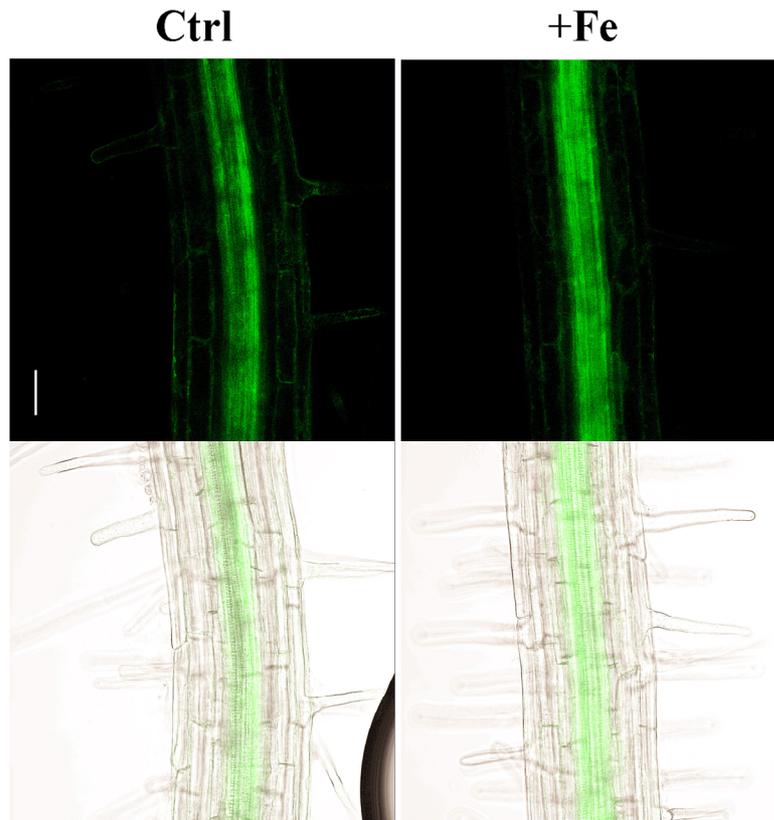
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2 **Supplemental Figure S5.** Auxin activation of the *DR5* response in xylem-adjacent pericycle cells.
3 Five-day-old WT seedlings, containing *DR5:GFP* construct, were transferred onto control medium
4 (Ctrl) or medium supplemented with excess Fe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

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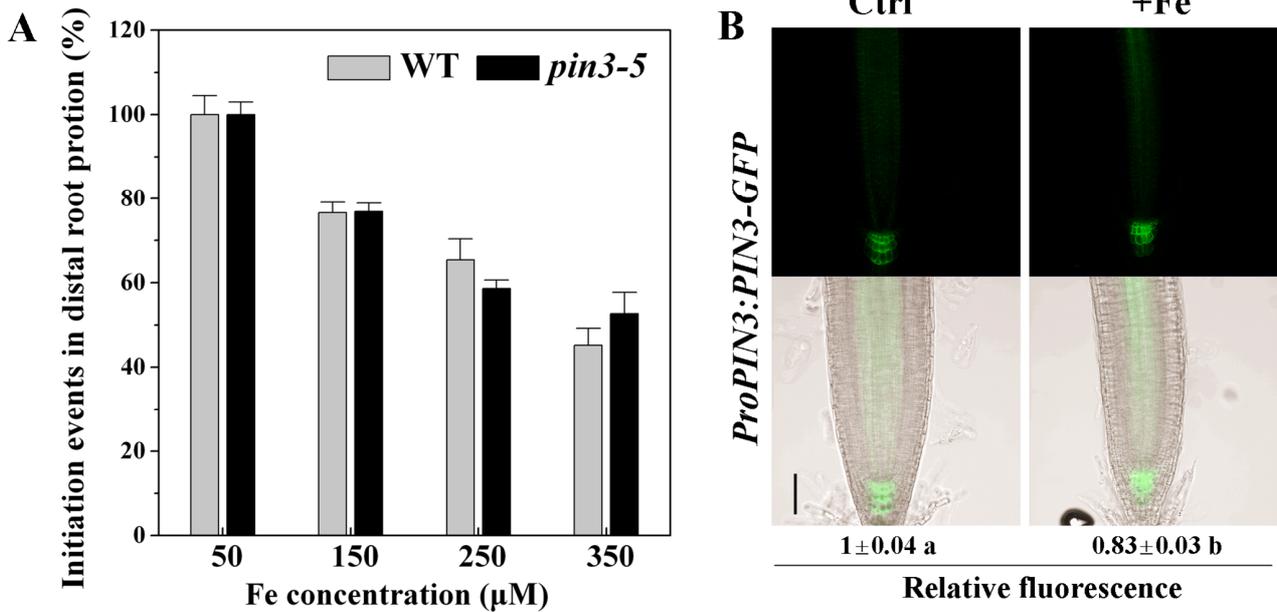
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2 **Supplemental Figure S6.** Effects of excess Fe on the lateral root initiation of *pin3-5* and expression

3 of *ProPIN3:PIN3-GFP* in *Arabidopsis*. (A) Effects of excess Fe on the lateral root initiation of

4 *pin3-5*. Five-day-old WT and *pin3-5* seedlings were transferred to medium, and roots were

5 supplemented with varying concentrations of Fe, for 5 d, after which LR initiation events were

6 quantified. Total LR initiation events in WT and *pin3-5* in control were 21.4 ± 0.88 and 18.2 ± 0.48 ,

7 respectively. Values are the means \pm SE, $n \geq 5$. (B) Effects of excess Fe on the expression of

8 *ProPIN3:PIN3-GFP* in roots. Five-day-old WT seedlings, containing *ProPIN3:PIN3-GFP*

9 constructs, were transferred onto control medium (Ctrl) or medium supplemented with excess Fe

10 (+Fe, 250 μ M). The fluorescence signal was detected with a Zeiss LSM710 confocal laser-scanning

11 microscope at the specified time points. One representative image for each experiment is shown.

12 Upper columns show the single GFP images; lower columns show the integration images of GFP

13 and bright field. The images were captured using the same confocal setting and are representative of

14 at least 10 roots obtained from at least three independent experiments. Scale of bars = 50 μ m.

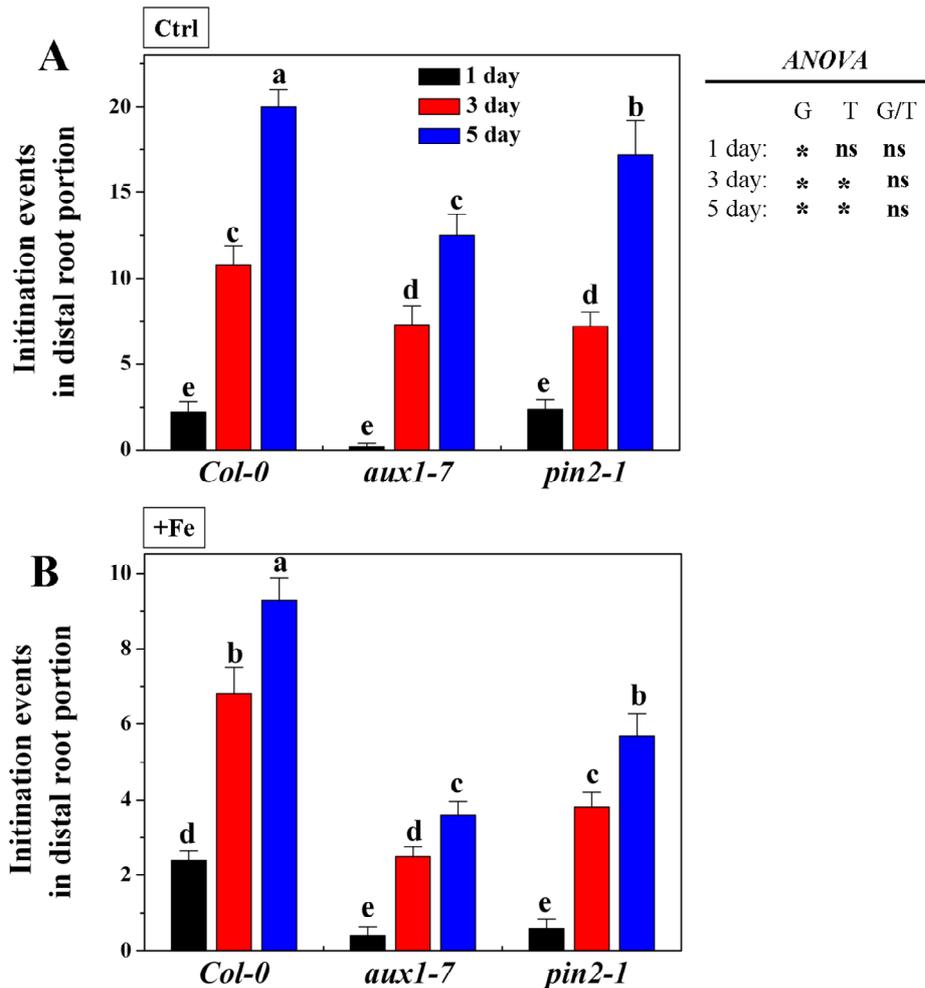
15 Means \pm SE are shown ($n \geq 5$). Different letters represent means statistically different at the 0.05

16 level (one-way ANOVA with Duncan post-hoc test).

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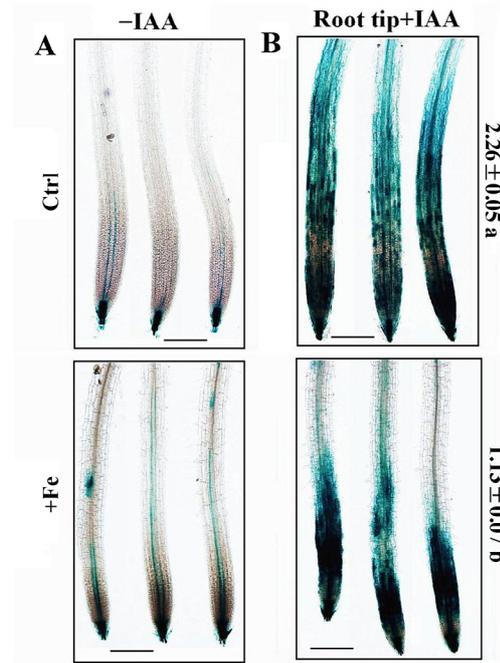
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2 **Supplemental Figure S7.** Effects of excess Fe on LR initiation in *Arabidopsis* WT (*Col-0*), *pin2-1*,
 3 and *aux1-7*. Total lateral root initiation at one, three, and five days in control (Ctrl, 50 μ M) and
 4 under excess Fe (+Fe, 350 μ M)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

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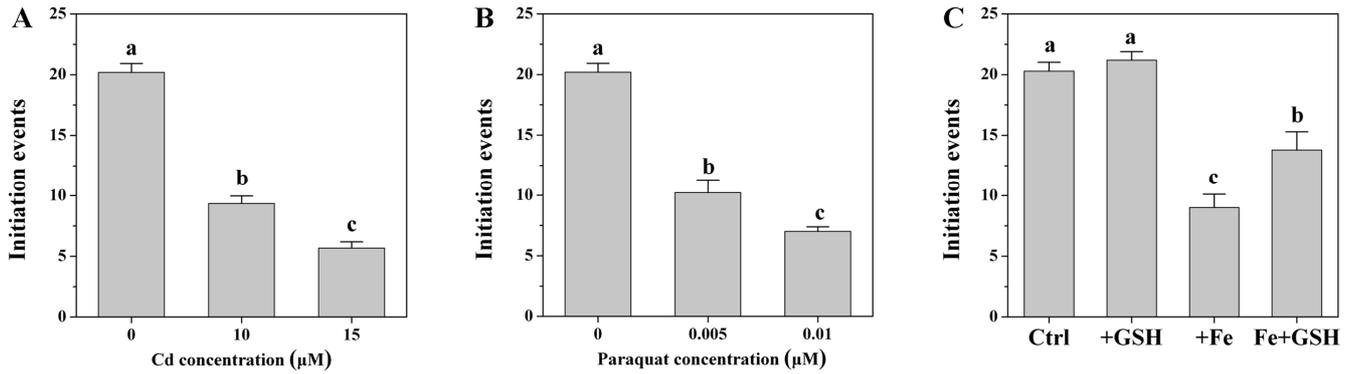


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2 **Supplemental Figure S8.** Auxin induction of *DR5::GUS* expression in *Col-0*, with or without
 3 excess Fe. Basipetal auxin transport was determined by comparing the distance of GUS staining
 4 from the site of IAA application of the treated seedlings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

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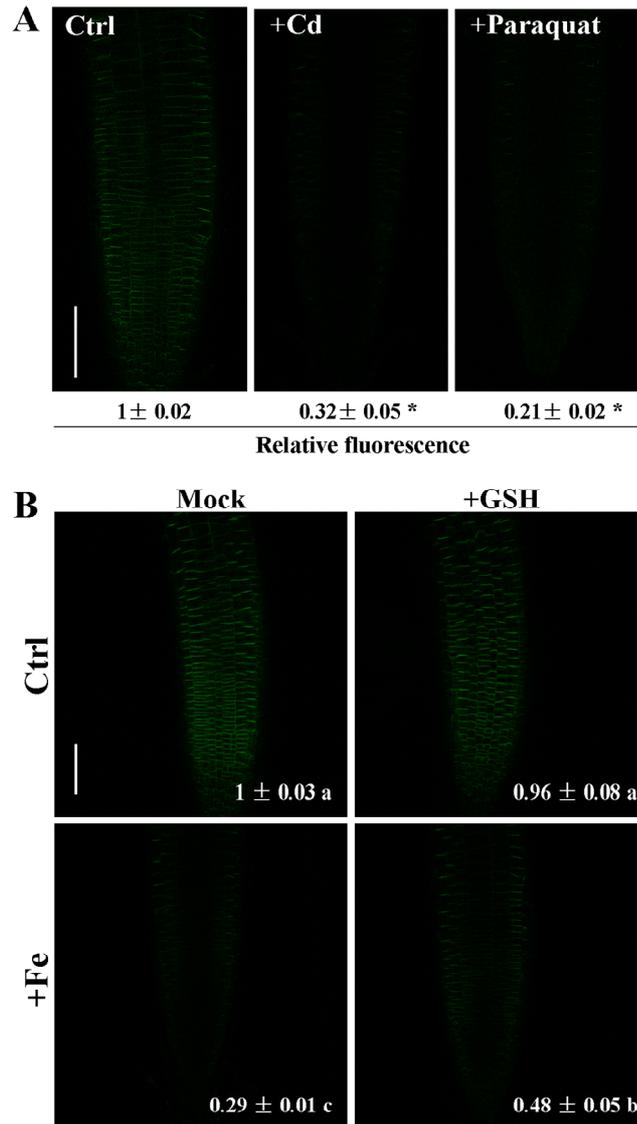
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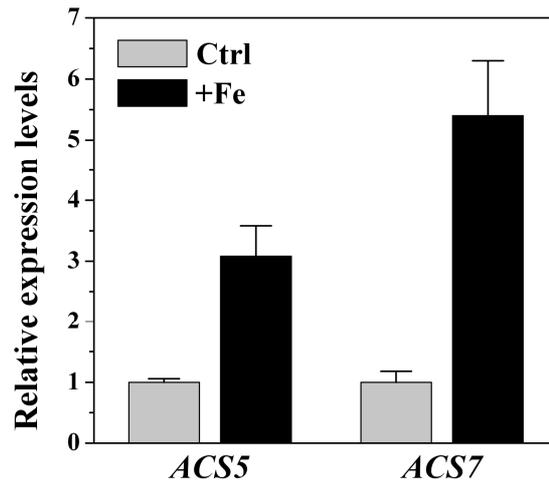
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2 **Supplemental Figure S9.** Effects of cadmium, paraquat and glutathione on lateral root initiation in
 3 *Arabidopsis*. (A) Effects of cadmium on the number of lateral root initiation events in the distal root
 4 portion. Seedlings (*DR5:GUS* lines) at 5 days after germination (DAG) were transferred to varying
 5 concentrations of cadmium (Cd) treatment medium and grown for an additional 5 d. (B) Effects of
 6 paraquat on the number of lateral root initiation events in the distal root portion. Seedlings
 7 (*DR5:GUS* lines) at 5 days after germination (DAG) were transferred to varying concentrations of
 8 paraquat treatment medium and grown for an additional 5 d. (C) Effects of glutathione (GSH) on
 9 lateral root initiation events in the distal root portion. Seedlings (*DR5:GUS* lines) at 5 days after
 10 germination (DAG) were transferred to control (Ctrl, 50μM) and excess Fe (+Fe, 350μM) treatment
 11 medium with or without GSH (50μM) and grown for an additional 5 d. Values are the means ± SE,
 12 $n \geq 5$. Different letters represent means statistically different at the 0.05 level (one-way ANOVA
 13 with Duncan post-hoc test).

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2 **Supplemental Figure S10.** Effects of cadmium, paraquat and glutathione on the expression of
3 *ProPIN2:PIN2-GFP* in *Arabidopsis*. Five-day-old WT seedlings, containing *ProPIN2:PIN2-GFP*
4 constructs, were transferred onto control medium (Ctrl) or medium supplemented with cadmium
5 (+Cd, 10 μ M), paraquat (+paraquat, 0.005 μ M) or excess Fe (+Fe, 250 μ M) with or without GSH
6 (50 μ M) and grown for 48h. (A) Effects of cadmium and paraquat on the expression of
7 *ProPIN2:PIN2-GFP* in roots. (B) Effects of GSH on the expression of *ProPIN2:PIN2-GFP* in roots.
8 The images were captured using the same confocal setting and are representative of at least 10 roots
9 obtained from at least three independent experiments. Scale of bars = 50 μ m. Means \pm SE are
10 shown ($n \geq 3$). Asterisks indicate statistical differences between control and the treatments
11 conditions (independent samples *t* test, $*P < 0.05$). Different letters represent means statistically
12 different at the 0.05 level (one-way ANOVA with Duncan post-hoc test).



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

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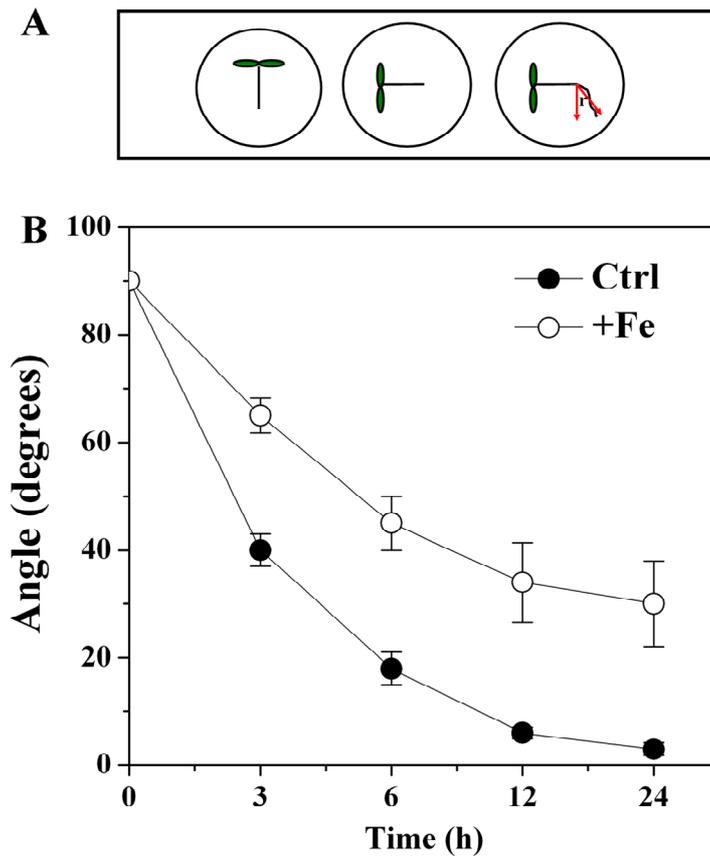
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2 **Supplemental Figure S12.** Experimental system for examining agravitropic response in response
 3 to excess Fe. (A) Diagram shows an experimental design for the study of interaction between Fe
 4 stress and gravitropism. Seedlings at 5 days after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

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

| | Background | LR initiation events |
|----|------------|----------------------|
| 5 | Ctrl | 21.5 ± 0.39 c |
| 6 | +Fe | 13.4 ± 0.67 d |
| 7 | +IAA | 25.2 ± 0.73 b |
| 8 | Fe +IAA | 25.5 ± 0.44 b |
| 9 | +ACC | 20.1 ± 0.89 c |
| 10 | Fe +ACC | 27.7 ± 0.31 a |
| 11 | +AVG | 20.8 ± 1.38 c |
| 12 | Fe +AVG | 5.33 ± 0.24 f |

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

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

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

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17 For growth conditions and treatments (+Fe, 250 μM Fe), see Table 2. The means ± SE are reported, with n ≥ 5 (*P*
18 ≤ 0.05).