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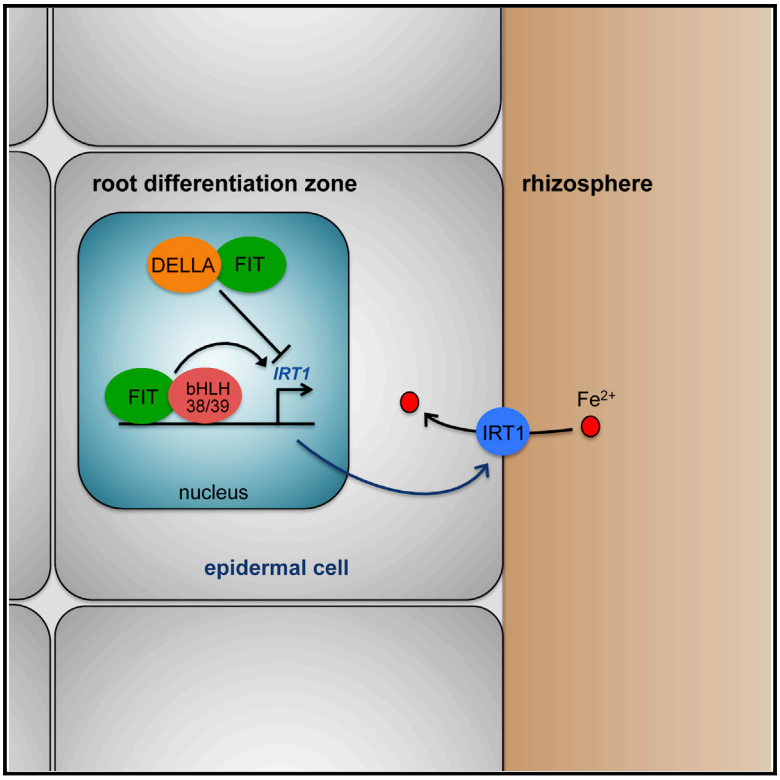
Developmental Cell

Tissue-Specific Regulation of Gibberellin Signaling Fine-Tunes *Arabidopsis* Iron-Deficiency Responses

Graphical Abstract



Authors

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In Brief

Iron deficiency is one of the most common nutrient shortages stunting plant growth. Wild et al. show that spatial distribution of the gibberellin-regulated DELLA growth repressors in *Arabidopsis* roots adapts the root system architecture and the iron-uptake machinery to the plant's iron demand.

Highlights

- Iron limitation slows primary root growth via a DELLA-dependent mechanism
- DELLAs interact and inhibit the activity of FIT and related transcription factors
- GA signaling controls the expression of FIT-regulated iron-uptake machinery genes
- Iron content regulates FIT and DELLA abundance in differentiated epidermal cells

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Tissue-Specific Regulation of Gibberellin Signaling Fine-Tunes *Arabidopsis* Iron-Deficiency Responses

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SUMMARY

Iron is an essential element for most living organisms. Plants acquire iron from the rhizosphere and have evolved different biochemical and developmental responses to adapt to a low-iron environment. In *Arabidopsis*, *FIT* encodes a basic helix-loop-helix transcription factor that activates the expression of iron-uptake genes in root epidermis upon iron deficiency. Here, we report that the gibberellin (GA)-signaling DELLA repressors contribute substantially in the adaptive responses to iron-deficient conditions. When iron availability decreases, DELLAs accumulate in the root meristem, thereby restraining root growth, while being progressively excluded from epidermal cells in the root differentiation zone. Such DELLA exclusion from the site of iron acquisition relieves FIT from DELLA-dependent inhibition and therefore promotes iron uptake. Consistent with this mechanism, expression of a non-GA-degradable DELLA mutant protein in root epidermis interferes with iron acquisition. Hence, spatial distribution of DELLAs in roots is essential to fine-tune the adaptive responses to iron availability.

INTRODUCTION

Iron deficiency is one of the most common nutrient shortages stunting plant growth and development, because soil iron often forms insoluble ferric (Fe^{3+}) hydroxide complexes that are not readily available for the plant (Kobayashi and Nishizawa, 2012). To sustain growth in such limited conditions, plants have developed specific morphological and physiological adaptive programs. Hence, limited iron availability triggers major root architectural changes, such as reduced primary root length and increased frequency of root hairs, associated with the induction of high-affinity iron-uptake mechanisms (Ivanov et al., 2012). Plants have evolved two distinct strategies to acquire iron from the soil. Graminaceous plants, such as barley, maize, and rice, extrude phytosiderophores (mugineic acids) that chelate Fe^{3+}, and the resulting complexes are taken up into root cells by the YELLOW STRIPE1 (YS1) transporter (Curie et al., 2001). In contrast, non-graminaceous plants, including *Arabidopsis thaliana*, take up iron in differentiated epidermal cells of the root by a three-step mechanism. Soil ferric iron is first solubilized through the action of H^+-ATPase (AHA) proton pumps that acidify the rhizosphere (Santi and Schmidt, 2009). Fe^{3+} is then reduced to Fe^{2+} by the ferric chelate reductase FERRIC REDUCTION OXIDASE2 (FRO2) (Robinson et al., 1999), and subsequently imported into root epidermal cells by the metal transporter IRON-REGULATED TRANSPORTER1 (IRT1) (Vert et al., 2002). *FRO2* and *IRT1* expression is induced upon iron deficiency and requires the activity of the basic helix-loop-helix (bHLH) transcription factor FER-like IRON-DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) and related bHLH from group Ib (Colangelo and Guerinot, 2004; Bauer et al., 2007; Yuan et al., 2008; Wang et al., 2013). Notably, under iron deficiency FIT forms heterodimers with bHLH38 or bHLH39, and these complexes are believed to directly activate the transcription of *FRO2* and *IRT1* in root epidermal cells (Yuan et al., 2008).

Various phytohormones such as auxin, cytokinin, jasmonate, and ethylene have been implicated in the regulation of iron-deficiency responses based on physiological observations in several plant species (Hindt and Guerinot, 2012). The molecular basis of these observations remains largely uncharacterized, except for the ethylene signaling for which the transcription factors ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 (EIN3/EIL1) were shown to interact with FIT, favoring its stabilization and, therefore, iron acquisition (Lingam et al., 2011).

Gibberellins (GAs) are plant growth-promoting hormones that play important roles throughout plant development, including seed germination, growth through cell expansion and division, floral transition, and in many aspects of the adaptation of plant growth in response to environmental variables (Colebrook et al., 2014). In particular, recent advances suggested that GAs substantially modulate the nutrient-deficiency responses. For example, phosphate (Pi) starvation causes a reduction in GA

levels, which in turn activates several adaptively significant plant Pi-deficiency responses, such as reduced primary root length and increased lateral root number (Jiang et al., 2007). More recently, exogenously applied GAs have been shown to induce the expression of several iron-uptake-related genes including *IRT1* and *FRO2*, although the mechanism and the role of GA in iron-deficiency responses remain largely unknown (Matsuoka et al., 2014).

GAs control a wide range of processes by opposing the function of the DELLA proteins, a family of nuclear growth repressors (Peng et al., 1997; Silverstone et al., 1998). While several plant species harbor a single *DELLA* gene, such as *SLENDER RICE1* (*SLR1*) in rice (Ikeda et al., 2001), the *Arabidopsis* genome encodes five DELLAs, GAI (GA-INSENSITIVE), RGA (REPRESSOR of GA1-3), RGL1 (RGA-LIKE1), RGL2, and RGL3, which display distinct but also overlapping functions (Davière and Achard, 2013). GA responses are mediated by the binding of bioactive GAs to the GA receptor GIBBERELLIN-INSENSITIVE DWARF1 (GID1), which triggers the specific destruction of DELLAs through the ubiquitin-dependent proteasome pathway (McGinnis et al., 2003; Sasaki et al., 2003; Dill et al., 2004; Fu et al., 2004; Ueguchi-Tanaka et al., 2005; Nakajima et al., 2006; Griffiths et al., 2006; Willige et al., 2007). When GA contents are low, DELLAs accumulate and modulate the activity of key regulatory proteins, including members of the bHLH family of transcription factors such as PHYTOCHROME INTERACTING FACTOR (PIF), ALCATRAZ (ALC), MYC2, or BRASSINAZOLE-RESISTANT1 (BZR1) (de Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Hong et al., 2012; Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). By doing so, DELLAs regulate the expression of a multitude of target genes involved in diverse pathways.

Here, we report that tissue-specific accumulation of the GA-regulated DELLA proteins is required for the full activation of the iron-deficiency responses. We show that iron deficiency asymmetrically regulates DELLA abundance in *Arabidopsis* seedling roots, favoring both root growth inhibition and iron acquisition. We demonstrate that DELLAs physically interact with FIT, bHLH38, and bHLH39 to prevent their binding to target promoters and inhibit their transcriptional activity. Limited iron availability antagonizes such repression by reducing DELLA accumulation in epidermal cells in the root differentiation zone and as a consequence fully activates FIT downstream target gene expression and, thus, iron uptake. Overall, our data highlight a dual function of GA signaling in the adaptation to fluctuating iron contents.

RESULTS

Iron Limitation Slows Root Growth via a DELLA-Dependent Mechanism

In response to low iron availability, the root system architecture undergoes major developmental changes that result in an increased absorption surface of the root hair zone. These include the development of lateral roots and root hairs, associated with reduced primary root length (Ivanov et al., 2012). To date, the molecular mechanisms driving morphological and physiological changes to iron deficiency remain elusive. We found that the primary root growth of *Arabidopsis* global-DELLA mutant seedlings (lacking the five DELLAs encoded by the *Arabidopsis* genome) is less inhibited in iron-deficient conditions than that of the wild-type, indicating that DELLAs contribute substantially to the root growth inhibition that is characteristic of plants experiencing iron deficiency (Figure 1A).

Next, we investigated whether the iron-deficiency-induced inhibition of root growth is associated with increased DELLA protein accumulation. To this end, we determined the accumulation levels of RGA, one of the main DELLAs restraining root growth (Fu and Harberd, 2003), in roots of seedlings transferred from iron-sufficient to iron-depleted media. Time-course analyses revealed that iron deficiency increases RGA protein abundance within 36 hr and maintains its accumulation at a high level over several days (Figure 1B), consistent with the above phenotypic data (Figure 1A). To further substantiate the role of DELLAs in restraining root growth in iron-limited conditions, we monitored the accumulation levels of a functional GFP-RGA fusion protein in *pRGA*:*GFP-RGA* primary root seedlings (Silverstone et al., 2001). GA signaling promotes root growth through cell proliferation and elongation by stimulating the degradation of DELLA proteins in endodermal cells of root meristem and elongation zone of the root, respectively (Ubeda-Tomas et al., 2008, 2009; Achard et al., 2009). We observed a significant increase of the GFP-RGA signal in every cell layer in both the tip and the elongation zone of the root of seedlings grown on iron-depleted media compared with control conditions (Figure 1C). Finally, we found that roots of wild-type seedlings grown on iron-depleted media contained reduced levels of the bioactive GA_4 and its precursors (GA_{12}, GA_{15}, GA_{24}, and GA_9) compared with those of seedlings grown on iron-sufficient media (Figure 1D). By contrast, their content in GA_{34} (the inactive 2β-hydroxylated product of GA_4) was not affected by iron deficiency, suggesting that GA_4 content is mainly regulated at the level of its synthesis. The fact that the 2-oxoglutarate-dependent dioxygenases (2-ODDs), catalyzing the conversion of GA_{12} into GA_4, require ferrous iron as co-factor for optimal activity (Prescott and John, 1996) may explain the overall reduction in GA synthesis and the relatively slow accumulation of RGA following iron limitation. Taken together, our results demonstrate that iron deficiency slows primary root growth, at least in part, via a DELLA-dependent mechanism that is associated with reduced accumulation of bioactive GA_4.

DELLAs Interact with FIT, bHLH38, and bHLH39 Transcription Factors

Given the fact that DELLAs preferentially interact with bHLH transcription factors to regulate plant development (Davière and Achard, 2013), we suspected that DELLAs might interact with FIT, bHLH38, and bHLH39 to control iron acquisition. To test this hypothesis, we performed yeast two-hybrid (Y2H) assays with the five *Arabidopsis* DELLAs. Because the full-length DELLAs show a high level of autoactivation in yeast, we used M5 truncated versions of DELLAs for the Y2H assays (Figure S1A; de Lucas et al., 2008). A strong interaction was observed for all the combinations between the DELLAs and FIT, bHLH38, and bHLH39 (Figure 2A).

To confirm these interactions in living plant cells, we next used fluorescence resonance energy transfer imaging (FRET-FLIM) assays in *Nicotiana benthamiana* leaves. FRET relies on the distance-dependent transfer of energy from a donor molecule (GFP)

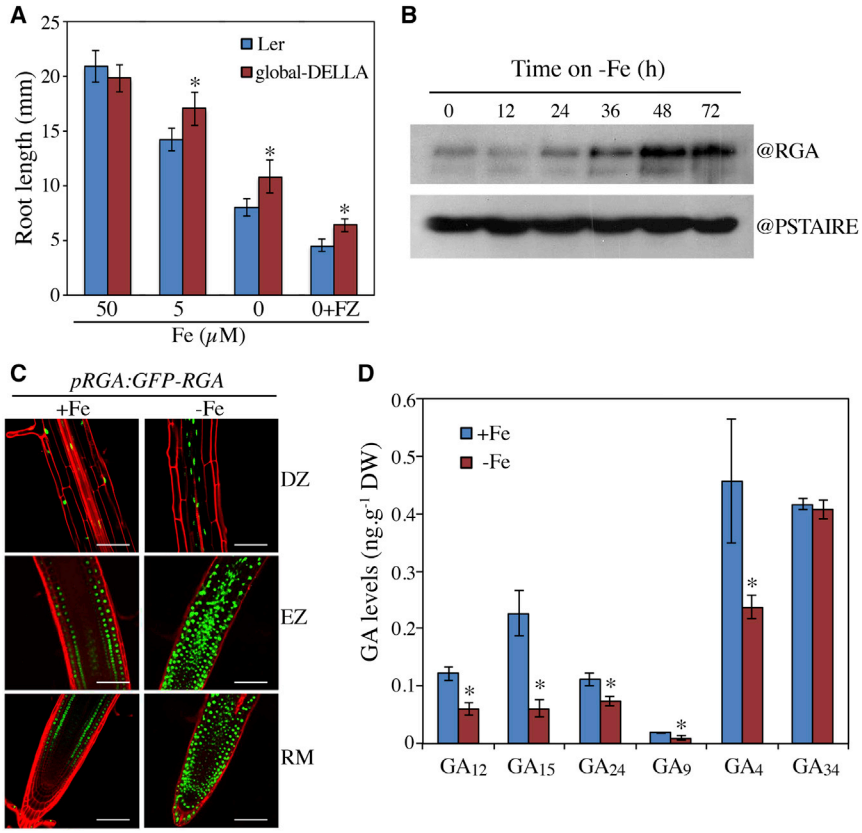

Figure 1. Iron Deficiency Restrains Root Growth via a DELLA-Dependent Mechanism

(A) Mean length (±SD; n > 20) of 7-day-old primary roots of wild-type (Ler; blue) and global-DELLA mutant (red) seedlings grown on media containing decreasing concentration of iron, as indicated. The asterisk indicates significant difference ($p < 0.05$) from the wild-type by Student's t test. FZ, iron chelator ferrozine (150 μM).

(B) Immunodetection of RGA in roots of 7-day-old wild-type (Ler) seedlings transferred to iron-deficient conditions for the times indicated. PSTAIRE serves as sample loading control. Similar results were obtained in three independent experiments.

(C) GFP fluorescence in nuclear cells of the root meristem (RM), elongating zone (EZ), and differentiation zone (DZ) of 7-day-old *pRGA:GFP-RGA* seedlings transferred to iron-deficient (−Fe) or iron-sufficient (+Fe) conditions for 2 days. Cell walls were stained with propidium iodide (red). All images were obtained with the same modifications and intensity parameters. Scale bars represent 100 μm.

(D) Concentration of GAs (ng g^{-1} dry weight) in roots of 7-day-old seedlings transferred to iron-deficient (−Fe; red) or iron-sufficient (+Fe; blue) conditions for 2 days. The values are means ± SD of three biological replicates. Asterisks indicate significant differences ($p < 0.05$) for −Fe versus +Fe roots by Student's t test.

to an acceptor molecule (RFP) if they are in close proximity (Bücherl et al., 2010). These assays revealed a direct interaction in the nuclei between FIT-GFP and RGA-RFP, with an FRET of 11.6% (Figure 2B), but also between GFP-RGA and bHLH38-RFP or bHLH39-RFP, with an FRET of 14.5% and 13.7%, respectively (Figure 2C). Moreover, co-immunoprecipitation studies from *N. benthamiana* agro-infiltrated leaves further corroborated this interaction (Figure 2D). Finally, interaction between RGA and FIT was confirmed in transgenic *Arabidopsis* roots co-expressing *GFP-RGA* and *FIT-RFP*, driven by their endogenous promoters (Figure 2E). Taken together, these results demonstrate that DELLAs do interact with FIT, bHLH38, and bHLH39.

To obtain insights into the molecular mechanism underlying these interactions, we mapped the interacting domains for DELLA and FIT proteins. Thus, we created a series of deletion constructs in both DELLA and FIT proteins that were tested for Y2H interactions. Deletion studies revealed that the first leucine heptad repeat in RGA is important for the interaction with FIT (Figures S1A and S1B). Moreover, we determined that two domains of FIT, including the bHLH DNA-binding domain, are responsible for the interaction of FIT with GAI (Figures S1C and S1D). This latter result suggests that the interaction between DELLA and FIT proteins may interfere with the DNA-binding activity of FIT, in a manner similar to that already reported for DELLA-bHLH interactions (de Lucas et al., 2008; Feng et al., 2008; Arnaud et al., 2010; Hong et al., 2012; Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012; Davière et al., 2014).

DELLA-FIT Interaction Inhibits FIT Transcriptional Activity

FIT directly binds in vivo to the promoters of the root iron-uptake genes *FRO2* and *IRT1* (Sivitz et al., 2012), and must interact with bHLH38 or bHLH39 to activate their transcription (Yuan et al., 2008). To shed light on the molecular mechanisms by which DELLA proteins impact on FIT-dependent iron-deficiency responses, we first evaluated the ability of RGA to interfere with the formation of FIT/bHLH38 and FIT/bHHLH39 heterodimers. To this purpose, the strength of interaction between FIT and bHLH39 was assessed in the presence and absence of RGA by FRET-FLIM (Figure 3A). Strikingly, the presence of RGA-HA (a non-fluorescent RGA protein) had no effect on the percentage of FRET between FIT-GFP and bHLH39-RFP, which was stable at about 11%. On the contrary, as positive control, the expression of RGA-HA decreased the percentage of FRET between FIT-GFP and RGA-RFP from 11.3% to 6.4%, indicative of competition between RGA-RFP and RGA-HA for FIT-GFP (Figure 3A). Thus, RGA does not alter the formation of FIT/bHLH39 heterodimer.

Since RGA interacts with the DNA-binding domain of FIT (Figure S1D), we next investigated whether RGA impairs the DNA-binding activity of FIT/bHLH38 and FIT/bHLH39 heterodimers on *IRT1* and *FRO2* target promoters, by electrophoretic mobility shift assay (EMSA) experiments using recombinant proteins. FIT, bHLH38, and bHLH39 are transcription factors that specifically bind to E-box (CANNTG) motifs (Colangelo and Guerinot, 2004). We respectively found two and one E-box motifs within the 1-kb promoter of *IRT1* and *FRO2* (Figure 3B). FIT/bHLH38

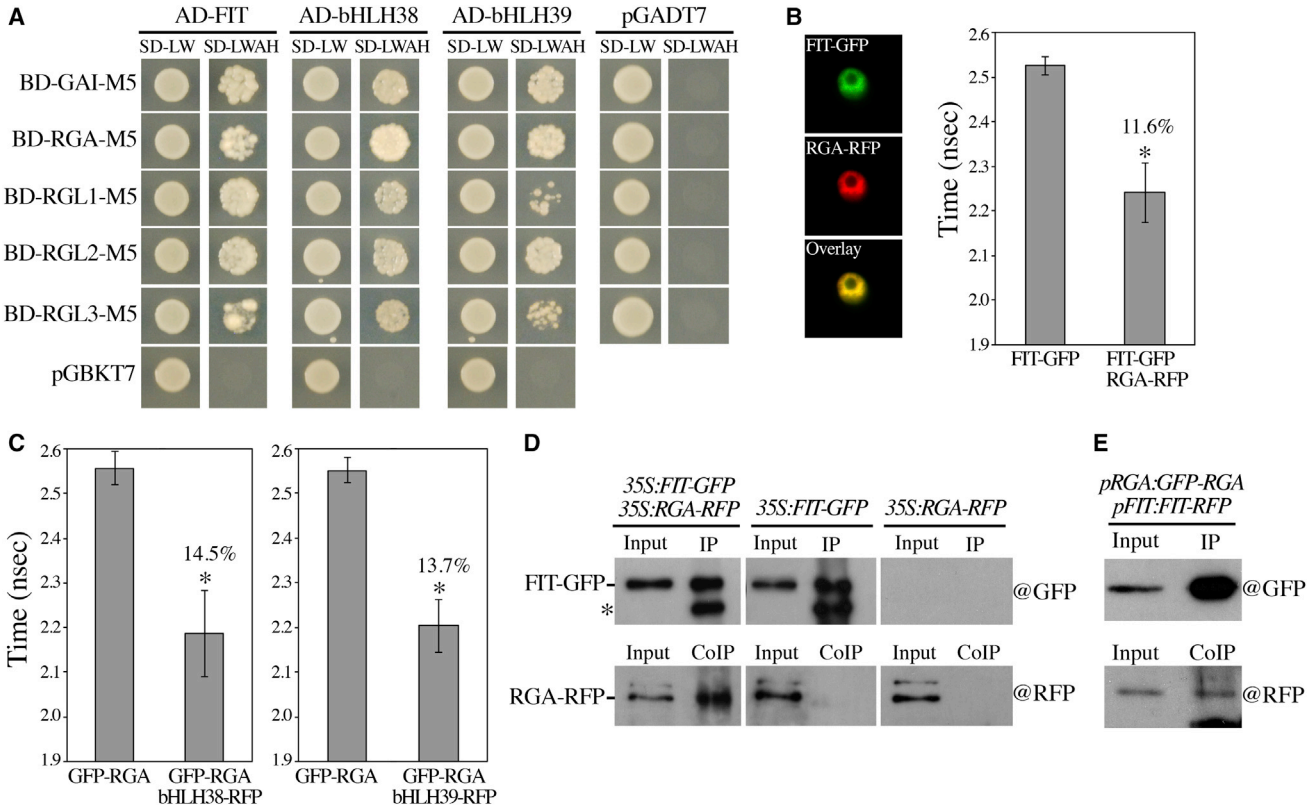

Figure 2. *Arabidopsis* DELLAs Interact with FIT, bHLH38, and bHLH39

(A) Yeast two-hybrid interactions. FIT, bHLH38, and bHLH39 were tested pairwise with the five *Arabidopsis* DELLA proteins GAI, RGA, RGL1, RGL2, and RGL3. Growth on selective plates lacking leucine, tryptophan, adenine, and histidine (SD-LWAH) and on control plates lacking leucine and tryptophan (SD-LW) is shown. Pictures of the plates were taken after 4 days at 30°C. See also Figures S1 and S6.

(B) Fluorescence resonance energy transfer imaging (FRET-FLIM) assays between FIT-GFP and RGA-RFP. Left: confocal images showing subcellular localization of FIT-GFP and RGA-RFP in transiently transformed *N. benthamiana* leaves. Right: fluorescence lifetime analyses in nanoseconds (mean ± SD; $n > 60$) of FIT-GFP expressed alone or together with RGA-RFP in *N. benthamiana* agro-infiltrated leaves. The fluorescence lifetime of the donor FIT-GFP decreases in presence of the acceptor RGA-RFP with an FRET of 11.6%, indicating that the two proteins interact. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test.

(C) Fluorescence lifetime analyses in nanoseconds of GFP-RGA alone or together with bHLH38-RFP or bHLH39-RFP, and mean (±SD; $n > 60$) FRET value (%) in *N. benthamiana* agro-infiltrated leaves. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test.

(D) Co-immunoprecipitation of transiently overexpressed FIT and RGA proteins. Total protein extracts from *N. benthamiana* agro-infiltrated leaves with *p35S:FIT-GFP* and *p35S:RGA-RFP* were immunoprecipitated with anti-GFP antibodies. The co-immunoprecipitated proteins were detected by anti-RFP antibodies. IP, immunoprecipitated proteins; CoIP, co-immunoprecipitated proteins. Asterisk indicates unspecific band.

(E) Interaction of RGA and FIT in stable transgenic *Arabidopsis* plants by co-immunoprecipitation. Total protein extracts from roots of 7-day-old *pRGA:GFP-RGA pFIT:FIT-RFP* seedlings transferred to iron-deficient conditions for 1 day were immunoprecipitated with anti-GFP antibodies. The co-immunoprecipitated proteins were detected by anti-RFP antibodies. IP, immunoprecipitated proteins; CoIP, co-immunoprecipitated proteins.

heterodimer was able to bind to each selected motif by EMSA, and competition experiments with unlabeled DNA probes showed that this binding activity requires an intact E box (Figure S2). As anticipated, the presence of RGA decreased the binding capacity of FIT/bHLH38 and FIT/bHLH39 heterodimers to selected E-box motifs (Figure 3C). Additional in vivo evidence for such a sequestration mechanism was obtained by chromatin immunoprecipitation (ChIP) assays using *p35S:FIT-GFP* roots transferred to iron-deficient conditions. After immunoprecipitation of protein-DNA complexes using an antibody against the GFP epitope, enriched DNA sequences were amplified by qPCR using primers that annealed near to and distant from (used as negative controls) the E-box motifs present in the *IRT1* and *FRO2* promoters (Figure 3B). Interaction of FIT with its target gene promoters was reduced in PAC-treated roots (paclobutrazol, an inhibitor of GA biosynthesis that enhances DELLA accumulation) in comparison with GA-treated roots (Figure 3D).

Finally, to examine the biological consequence of the interactions between RGA, FIT, and bHLH39 on the transcriptional activity of FIT/bHLH39 heterodimer, we performed transient transactivation assays in *N. benthamiana* leaves using a 1.4-kb promoter fragment of *IRT1* fused to the *GUS* gene as a reporter for FIT/bHLH39 transcriptional activity. As expected, expression of *FIT* alone or together with *RGA* had no effect on *GUS* expression (Figure 3E). Although FIT can interact with itself, FIT homodimers are not involved in controlling the expression of *IRT1* (Yuan et al., 2008). By contrast, co-expression of *FIT* and

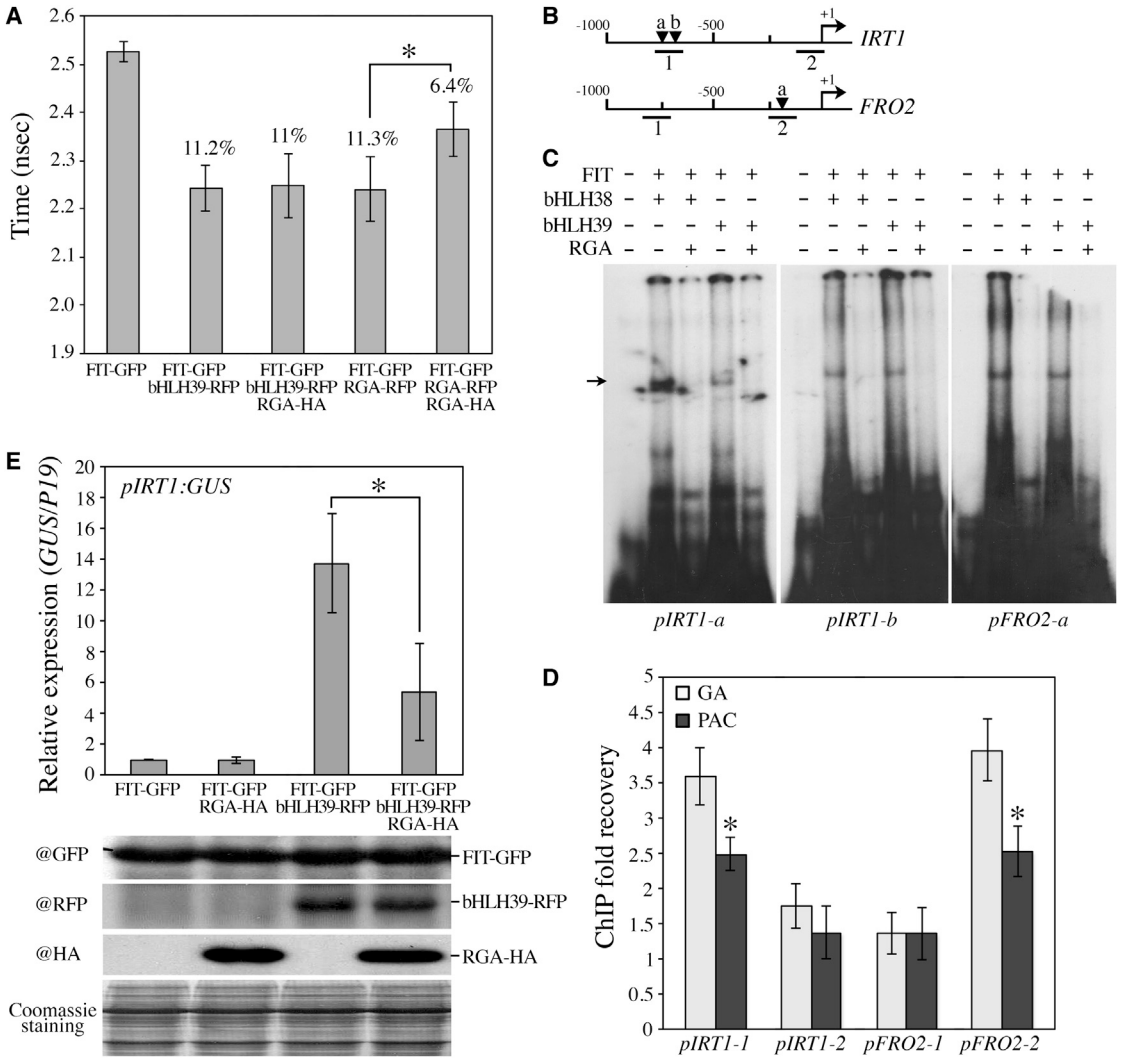

Figure 3. DELLA Function Inhibits FIT Transcriptional Activity

(A) FRET-FLIM analyses of RGA, FIT, and bHLH39. *p35S:FIT-GFP* was agro-infiltrated alone or together with various combinations of *p35S:bHLH39-RFP*, *p35S:RGA-RFP*, and *p35S:RGA-HA* in *N. benthamiana* leaves, as indicated. Data show the fluorescence lifetime (in nanoseconds) of FIT-GFP alone or in combination with bHLH39-RFP or RGA-RFP, with or without RGA-HA as competitor, and the values (%) represent the means (±SD; n > 60) of FRET. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test.

(B) Schematic representation of *IRT1* and *FRO2* promoters, including potential FIT-binding sites (referred to as a and b) and DNA fragments (1 and 2) used for ChIP analyses.

(C) EMSA studies using oligonucleotides encompassing potential FIT-binding sites in *IRT1* and *FRO2* promoters and recombinant FIT, bHLH38, bHLH39, and RGA proteins. ^{32}P-radiolabeled oligonucleotides were incubated with FIT and bHLH38 or bHLH39 alone or together with RGA (at 1:1:3 ratio), and the free and bound probes were separated in an acrylamide gel. Similar results were obtained in two independent experiments. The arrow indicates the bound probe. See also Figure S2.

(D) Fold enrichment of indicated *IRT1* and *FRO2* promoter fragments. Chromatin of 10-day-old primary roots of *p35S:FIT-GFP* seedlings transferred to iron-deficient conditions and treated with 10 μM GA_3 (GA) or 1 μM paclobutrazol (PAC) for 2 days were subjected to ChIP with anti-GFP antibodies followed by qPCR. Data represent means ± SD of two biological and two technical replicates. Asterisks indicate statistically significant differences in PAC-treated plants versus GA-treated plants using Student's t test ($p < 0.05$).

(E) Transient transactivation assays of *IRT1* promoter. *pIRT1:GUS* was agro-infiltrated with *p35S:FIT-GFP* alone or together with *p35S:bHLH39-RFP* and/or *p35S:RGA-HA* in *N. benthamiana* leaves, as indicated. Top: values represent the relative *pIRT1:GUS* expression (*GUS/P19*) determined by qRT-PCR, and are the average ± SD of three biological replicates. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test. Bottom: immunodetection of FIT-GFP, bHLH39-RFP, and RGA-HA from *N. benthamiana* agro-infiltrated leaves used for the transient transactivation assays. Coomassie staining of total proteins serves as sample loading control.

bHLH39 resulted in a 13.7-fold induction of *IRT1* reporter activity, indicating that the *pIRT1:GUS* reporter could be activated in *N. benthamiana* cells. More importantly, co-expression of *RGA* with *FIT* and *bHLH39* significantly altered *IRT1* reporter expression, demonstrating that RGA suppresses FIT/bHLH39 transcriptional activity (Figure 3E). Altogether, these results

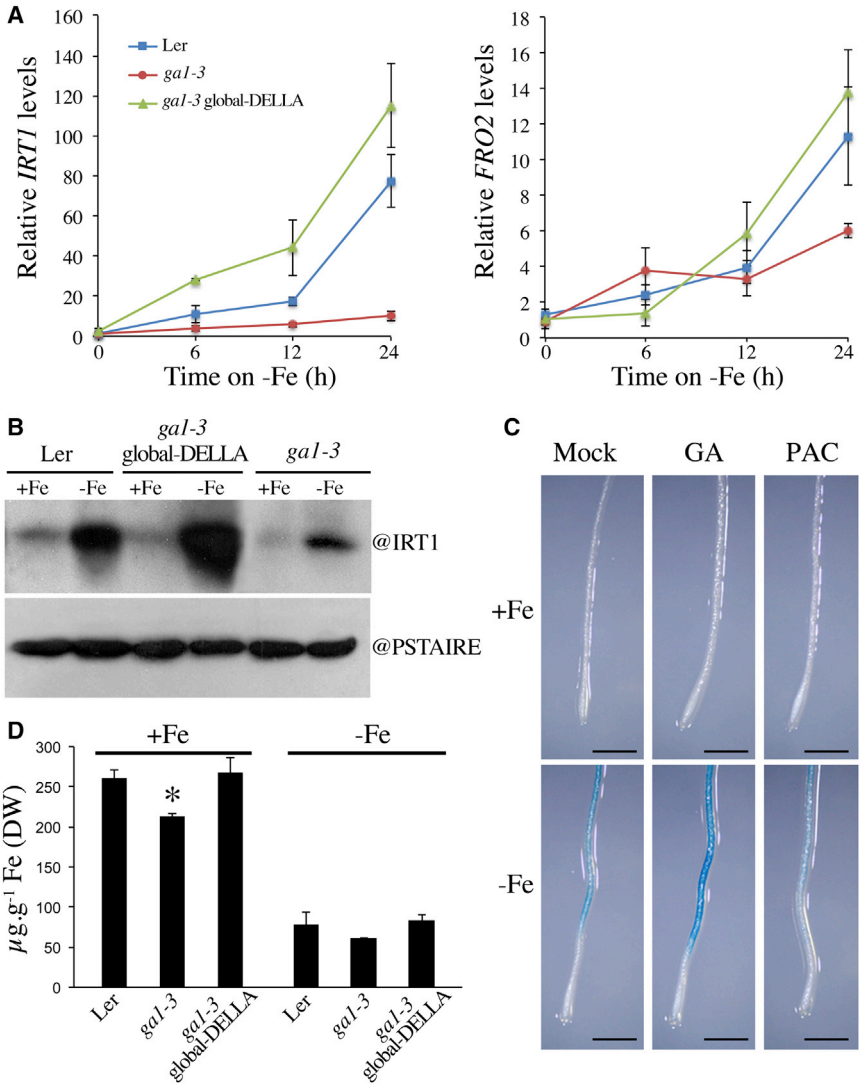

Figure 4. GA Signaling Controls the Expression of FIT-Regulated Iron-Uptake Genes

(A) Time course of *IRT1* and *FRO2* transcript accumulation in roots of 7-day-old wild-type (Ler; blue), *ga1-3* (red), and *ga1-3* global-DELLA (green) mutant seedlings transferred to iron-deficient conditions for the times indicated. Data are means ± SD of three biological replicates. See also Figure S3.

(B) Immunodetection of IRT1 protein in roots of 7-day-old wild-type (Ler), *ga1-3*, and *ga1-3* global-DELLA mutant seedlings grown in iron-deficient (−Fe) and iron-sufficient (+Fe) conditions. PSTAIRE serves as sample loading control. Similar results were obtained in two independent experiments.

(C) Effects of GA (5 μM GA_3) and PAC (1 μM) treatments on root *IRT1* promoter activity using 7-day-old *pIRT1:GUS* seedlings grown in iron-deficient (−Fe) and iron-sufficient (+Fe) conditions. Scale bars represent 500 μm.

(D) Iron content (μg g^{-1} dry weight) in 7-day-old wild-type (Ler), *ga1-3*, and *ga1-3* global-DELLA mutant seedlings grown in iron-deficient (−Fe) and iron-sufficient (+Fe) conditions. Data are means ± SD of three biological replicates. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test ($p < 0.05$).

highlight the role of DELLAs in blocking the ability of the master regulator complex FIT/bHLH38/bHLH39 to bind to and transactivate target genes driving iron-deficiency responses.

GA Signaling Modulates Iron Uptake

The presence of DELLA-FIT interaction suggests that GAs are involved in the regulation of iron acquisition. We therefore speculated that lack of DELLA activity would enhance the induction of FIT-regulated iron-uptake genes. To test this hypothesis, we compared the kinetics of induction of *IRT1* and *FRO2* transcripts in seedling roots of 7-day-old GA-deficient *ga1-3* mutant (which accumulates the DELLAs), *ga1-3* global-DELLA mutant, and wild-type control upon transfer to iron-depleted media. We observed that the induction levels of *IRT1* and *FRO2* transcripts are respectively reduced in *ga1-3* mutant and increased in *ga1-3* global-DELLA mutant in comparison with wild-type (Figure 4A). Accordingly, roots of *ga1-3* global-DELLA mutant had higher levels of IRT1 protein than those of *ga1-3* mutant under iron deficiency, paralleling transcript accumulation profiles (Figure 4B). Thus, DELLAs repress the iron-deficiency-mediated induction of *IRT1* and *FRO2* expression, whereas GAs enhance their induction by overcoming DELLA-mediated repression. This observation is consistent with a previous work showing that application of exogenous GAs to GA-deficient mutants enhanced the expression of iron-related genes (Matsuoka et al., 2014). It is noteworthy that among these iron-related genes are found *bHLH38* and *bHLH39*, ensuring a feedforward regulation in response to iron deficiency (Figure S3; Matsuoka et al., 2014). Finally, to visualize the effects of GA on *IRT1* expression in roots, we monitored how changes in GA levels affected the expression of *pIRT1:GUS* reporter. As already shown, iron-deficient plants showed strong GUS staining in roots, except for the meristematic and elongating zones (Figure 4C; Vert et al., 2002). In addition, we observed that GA treatment enhanced GUS activity in iron-deficient roots, whereas PAC reduced it compared with the control (Figure 4C). We conclude from these experiments that GA signaling contributes substantially to the expression of FIT-regulated iron-uptake machinery genes and should be critical in adapting iron acquisition to the demand of the plant.

To determine whether GAs control iron acquisition, we measured the iron content in 7-day-old wild-type, *ga1-3*, and *ga1-3* global-DELLA mutant seedlings grown on iron-sufficient and iron-deficient media (Figure 4D). Whereas *ga1-3* mutant seedlings grown in iron-sufficient conditions accumulated significantly less iron than those of wild-type, lack of DELLAs suppressed the reduced accumulation of iron conferred by *ga1-3* (in *ga1-3* global-DELLA mutant). Moreover, the concentration

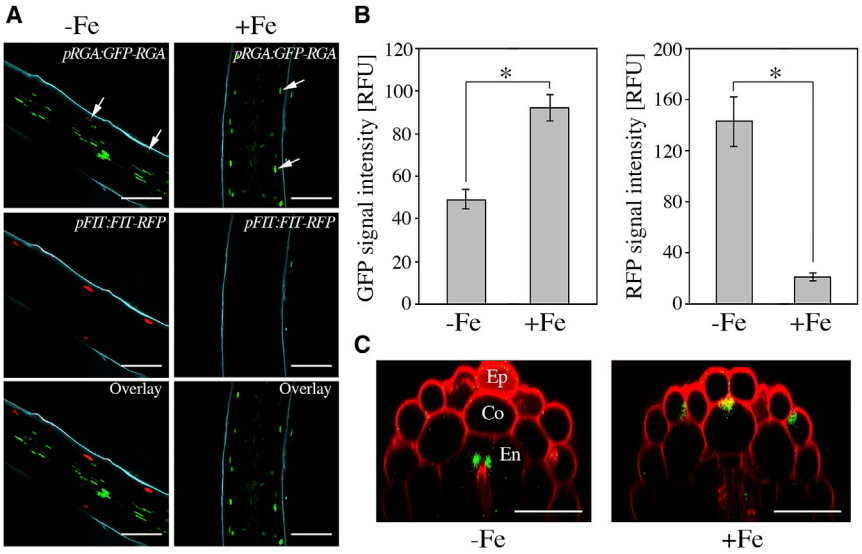

Figure 5. Iron Availability Controls DELLA Abundance in Root Epidermis

(A) Confocal image of GFP-RGA and FIT-RFP signals in the root differentiation zone of 7-day-old *pRGA:GFP-RGA pFIT:FIT-RFP* seedlings transferred to iron-deficient (−Fe) or iron-sufficient (+Fe) conditions for 2 days. White arrows mark the nuclei where fluorescence signal intensities were quantified. Root edges were stained with calcofluor white (blue). All images were obtained with the same modifications and intensity parameters. Scale bars represent 100 μm. See also Figure S4.

(B) Quantification of GFP-RGA and FIT-RFP fluorescence intensities in the nucleus of epidermal and cortical cells of the differentiation zone of seedling roots grown as in (A). Data shown are averages ± SE (10 root images, n > 40). RFU, relative fluorescence units. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test.

(C) Optical cross section from z-series image stacks through the differentiation zone of the root of 7-day-old *pRGA:GFP-RGA* seedlings transferred to iron-deficient (−Fe) or iron-sufficient (+Fe) conditions for 2 days. Cell walls were stained with propidium iodide (red). z-Stack images were obtained with the same modifications and intensity parameters. Ep, epidermis; Co, cortex; En, endodermis. Scale bars represent 50 μm.

of iron was very low (<100 μg g^{-1}) when seedlings were grown in iron-deficient conditions, and as a consequence no significant differences were found between all the genotypes. Hence, GA signaling is an important regulator of the iron-acquisition responses.

Iron Availability Regulates DELLA Abundance in Root Epidermis

The previous observations lead to a contradictory situation; under iron limitation, DELLAs accumulate in root meristem and restrain root elongation but, on the other hand, DELLAs also attenuate the induction of the root-epidermis-expressed iron-uptake machinery genes. One possible explanation to overcome this paradox would be that iron deficiency regulates DELLA abundance in a tissue-specific manner. To investigate this hypothesis, we analyzed the distribution of both GFP-RGA and FIT-RFP proteins in the root differentiation zone of *pRGA:GFP-RGA pFIT:FIT-RFP* seedlings grown in iron-sufficient and iron-deficient conditions (Figures 5A and S4). Quantitative comparison between the different tissue layers revealed a significant decrease of the GFP-RGA signal in the epidermis and cortex of iron-deficient roots compared with that of iron-sufficient roots (Figures 5A–5C). Reciprocally, FIT-RFP signal increased in the epidermis of the same roots after transfer to iron-deficient conditions (Figures 5A–5C and S4; Colangelo and Guerinot, 2004). Taken together, these results demonstrate that iron availability tightly controls the abundance of both DELLA and FIT proteins in root epidermal cells in the root differentiation zone, likely to adjust the absorption of iron from the soil to the demand of the plant.

Expressing *gai* in Root Epidermis Disrupts Iron Uptake

We have shown that DELLAs have a dual function in the adaptation to iron availability. Under iron limitation, DELLAs accumulate in all tissues (including the endodermis) in the root meristem and elongation zone to inhibit root growth, while their accumulation levels decrease in the epidermis in the differentiation zone to activate iron uptake. To specifically assess the biological relevance of the physical interactions between the DELLAs and FIT, bHLH38, and bHLH39 for the activation of the iron-uptake machinery, we targeted the expression of *gai*, a non-GA-degradable mutant form of GAI (Peng et al., 1997), solely in the epidermis in the root differentiation zone. To this end, *gai-GFP* coding sequence was expressed under the *FIT* promoter (in *pFIT:gai-GFP*). The corresponding lines showed strong chlorosis when grown on soil, a phenotype reminiscent of loss-of-function mutants affected in iron uptake (Yi and Guerinot, 1996; Vert et al., 2002; Colangelo and Guerinot, 2004), with a clear correlation with the expression level of *gai-GFP* (Figures S5A and S5B). Among all independent transgenic lines analyzed, we selected the *pFIT:gai-GFP* line #36, which triggers a strong induction of *gai-GFP* in epidermal cells of the root hair zone upon iron deficiency without affecting the expression level of endogenous *FIT* (Figures S5C and S5D). Noteworthy, the primary root growth of *pFIT:gai-GFP* seedlings was as sensitive as that of the wild-type to iron deficiency (Figure S5E), hence confirming that GA signaling in the epidermis of the root differentiation zone does not contribute in the regulation of root elongation (Ubeda-Tomas et al., 2008).

We next addressed whether expressing *gai-GFP* in epidermal cells alters the expression of the root iron-uptake machinery genes. Consistent with our model, the kinetics of *IRT1* and *FRO2* induction in response to iron deficiency was reduced in *pFIT:gai-GFP* roots compared with control roots (Figure 6A). As a consequence, IRT1 protein level was also reduced in iron-deficient *pFIT:gai-GFP* roots in comparison with wild-type roots (Figure 6B). Finally, roots of *pFIT:gai-GFP* seedlings grown under iron-sufficient conditions accumulated less iron than those of wild-type, similarly to *ga1-3* roots (Figure 6C), consistent with their severe chlorotic phenotype (Figures S5A and 6D). Taken

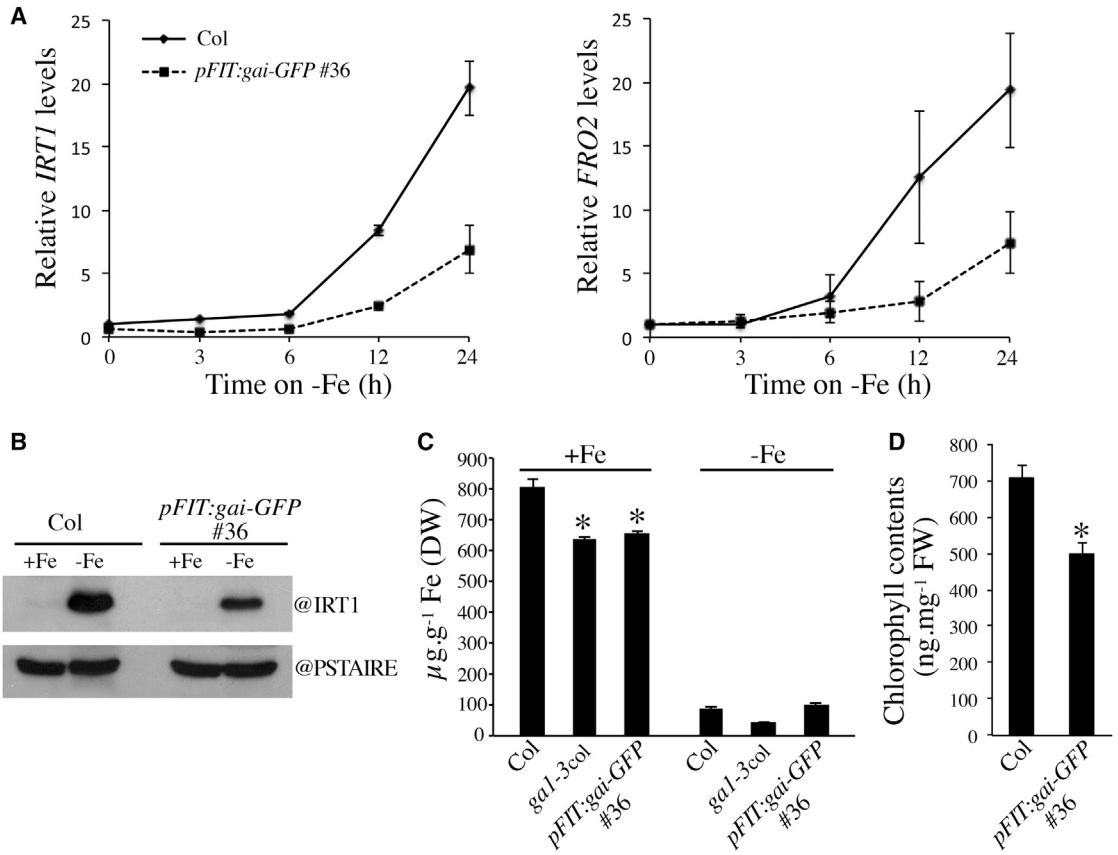

Figure 6. GA Signaling in the Root Epidermis Regulates Iron Uptake

(A) Influence of epidermis-expressed *gai* on iron-deficiency responses. Time course of *IRT1* and *FRO2* transcript accumulation in roots of 7-day-old wild-type (Col; solid line) and *pFIT:gai-GFP* (line #36; dashed line) seedlings transferred to iron-deficient conditions for the times indicated. Data are means ± SD of three biological replicates.

(B) Immunodetection of IRT1 protein in roots of 7-day-old wild-type (Col) and *pFIT:gai-GFP* (line #36) seedlings grown in iron-deficient (−Fe) and iron-sufficient (+Fe) conditions. PSTAIRE serves as sample loading control. Similar results were obtained in two independent experiments.

(C) Iron contents (ng g^{-1} dry weight) in roots of 7-day-old wild-type (Col), *ga1-3*col, and *pFIT:gai-GFP* (line #36) seedlings grown in iron-deficient (−Fe) and iron-sufficient (+Fe) conditions. Data are means ± SD of three biological replicates. The asterisk denotes statistical significance ($p < 0.05$) using one-way ANOVA followed by Tukey's test for multiple comparisons with the wild-type.

(D) Chlorophyll contents (ng mg^{-1} fresh weight) in 2-week-old wild-type (Col) and *pFIT:gai-GFP* (line #36) plants grown in soil. Data shown are the mean ± SD of three replicates and range of two biological repeats. The asterisk denotes statistical significance ($p < 0.05$) using Student's t test.

See also Figure S5.

together, these observations indicate that although the endodermis in the root elongation zone represents the primary GA-responsive tissue regulating root growth, GA signaling in the epidermis in the root differentiation zone is critical for the control of iron acquisition from the soil.

DISCUSSION

Plant development, yield, and overall fitness are dependent on the optimal root architecture. The root system plays essential roles in the anchorage of the plant to the soil but also in nutrient and water uptake (Malekpoor Mansoorkhani et al., 2014). Given the importance of iron for plant growth, it is not surprising that roots have developed unique capabilities to sense and respond to iron availability in the soil. Hence, changes in the internal and external concentrations of iron trigger important developmental processes, such as primary root growth and lateral root and root hair formation, associated with biochemical adaptive programs (Ivanov et al., 2012). Here, we demonstrated that the GA-signaling pathway represents a key regulator of both morphological and physiological root iron-deficiency responses.

The phytohormones GAs play a central role in the regulation of plant growth and development with respect to environmental variability (Colebrook et al., 2014). GAs regulate root growth by controlling both cell proliferation and expansion rates in meristem and elongation zone of the root (Ubeda-Tomas et al., 2008, 2009; Achard et al., 2009). Previous work has indicated that the endodermis in the root elongation zone represents the primary responsive tissue for GA-regulated growth (Ubeda-Tomas et al., 2008, 2009). Although GAs are needed in other tissues, including the epidermis and cortex, for appropriate coordination of their growth with that of the endodermis, GA signaling in these tissues does not regulate overall root length (Ubeda-Tomas et al., 2008). Here we showed that tissue-specific

localization of the GA-regulated DELLA proteins is essential in adapting the root system architecture and the iron-uptake machinery to the demand of the plant in iron. First, we have shown that iron limitation reduces bioactive GA levels in roots, which in turn enhances DELLA accumulation and thus stunts root growth. Accordingly, GFP-RGA accumulation was enhanced in every tissue including the endodermis in the root meristem and the elongation zone of the root under iron-deficient conditions, and lack of DELLA activity in global-DELLA mutant reduced the iron-deficiency-induced root growth inhibition. Second, we have demonstrated that DELLAs are able to interact with FIT and other iron-regulated bHLHs at the site of iron uptake in root epidermal cells in the root differentiation zone to prevent DNA binding and transactivation of iron-uptake genes. Iron deficiency counteracts such repression by reducing the abundance of DELLAs in root epidermis, and thereby fully activates the iron-uptake gene expression. Thus DELLAs exert a dual function in the adaptation to fluctuating iron contents, depending on their tissue-specific accumulation. Consistent with this model, expressing *gai* solely in the epidermis altered the activity of the iron-uptake machinery but had no effect on the overall root length.

It remains unclear how this asymmetric distribution in DELLA protein abundance in the different root tissues is achieved upon iron deficiency. If it becomes clear that iron deficiency diminishes bioactive GA levels in the root tip, two possible mechanisms could explain the observed change in the abundance of DELLAs in root epidermis. First, GA biosynthesis could be specifically upregulated in the epidermis in the differentiation zone of the root under iron deficiency. Although the quantification of GA contents in decapitated roots (after removal of the root tip) exposed to different iron conditions might give a more precise picture of the amount of GA_4 in the root differentiation zone, cell-type-specific transcriptional profiling performed on seedlings grown under iron-deficient conditions did not reveal alterations in the expression of key GA biosynthetic genes in root epidermis (Dinneny et al., 2008). Second, GAs could be transported from the endodermis toward the epidermis in the root differentiation zone. For example, ethylene has recently been shown to inhibit the accumulation of fluorescent-labeled GA (GA_3-Fl) in the elongating endodermal cells, consistent with the negative effect of ethylene on root growth (Shani et al., 2013). Unfortunately, although the GA_3-Fl is a valuable tool to monitor changes in GA distribution in the elongation zone of the root, the fluorescence intensity is very low in the differentiation zone (Shani et al., 2013), making it impossible to compare the effects of iron availability on GA_3-Fl distribution in this part of the root.

Intriguingly, several studies reported that external iron availability modifies primary root growth under low-Pi conditions (Ward et al., 2008). Pi deficiency triggers apoplastic iron and callose deposition in root meristem, which in turn inhibits symplastic cell-to-cell communication in the stem-cell niche that ensures precise coordination of cell division and differentiation (Müller et al., 2015). FIT-regulated iron-uptake machinery is excluded from the root meristematic and elongating zones (Vert et al., 2002; Colangelo and Guerinot, 2004), and thus is unlikely to mediate the developmental responses to Pi availability in root tips. Nevertheless, because both iron and Pi deficiency enhance DELLA accumulation in root meristem (Figure 1C; Jiang et al., 2007), the role of GA signaling in the antagonistic interactions between iron and Pi availability needs to be further investigated.

In contrast to *Arabidopsis*, graminaceous plants, which include the majority of varieties of agricultural plants, use a chelation strategy to overcome iron-deficient growth conditions (Kobayashi and Nishizawa, 2012). Despite the differences in their iron-uptake strategy, the molecular mechanism by which graminaceous and non-graminaceous plants control iron acquisition is partially conserved. In rice, *OsIRO2* encodes an iron-deficiency-inducible bHLH transcription factor, which shows a high degree of sequence similarity with *AtbHLH38* and *AtbHLH39* (Ogo et al., 2006; Hindt and Guerinot, 2012). Under iron-deficient conditions, IRO2 positively regulates iron acquisition through induced expression of mugineic acid family of phytosiderophore biosynthesis genes and iron-uptake transporter genes (Ogo et al., 2007). Strikingly, we found that the rice DELLA, SLR1, could interact with IRO2 in Y2H assays (Figure S6). Hence, our results suggest that DELLA/FIT/IRO2 interactions represent a conserved regulatory module that enables plants to adjust iron acquisition to their needs. In this circumstance, manipulation of these components would be an efficient means to produce crops with enhanced tolerance to iron deficiency and/or increased iron content.

In the 1960s and 1970s during the so-called green revolution, the introduction of semi-dwarf alleles, less sensitive to GA action, into most modern varieties led to large increases in crop yields, due to improvements in both harvest index and lodging resistance (Peng et al., 1999; Hedden, 2003). Although it remains unclear whether the DELLAs accumulate in root epidermal cells of these semi-dwarf varieties, a re-evaluation of their potential agronomic value in low-iron environments needs to be addressed.

EXPERIMENTAL PROCEDURES

Standard procedures, such as plasmid constructs and plant transformation, Y2H assays, co-immunoprecipitation assays, histology, microscopy and FRET-FLIM analyses, qRT-PCR, EMSAs, immunoblotting, and GA and chlorophyll determinations are described in Supplemental Experimental Procedures.

Plant Material and Growth Conditions

Mutants and transgenic lines were derived from Landsberg *erecta* (*ga1-3*; *gai*; *ga1-3* global-DELLA; global-DELLA; *pRGA*:*GFP-RGA*) or Columbia-0 (*ga1-3*col; *pIRT1*:*GUS*; *p35S*:*FIT-GFP*) ecotypes as previously described (Silverstone et al., 2001; Vert et al., 2002; Tyler et al., 2004; Cheminant et al., 2011; Sivitz et al., 2011). Plants were grown on soil or on plates containing 0.5× Murashige-Skoog (MS) medium (Sigma), 0.5% sucrose, and 1% ultrapure agar (Merck) under a 16-hr photoperiod at 22°C. For iron-deficiency experiments, 7-day-old seedlings grown on 0.5× MS medium containing 50 μM Fe-EDTA (Sigma) were transferred on iron-depleted medium without Fe and containing 150 μM ferrozine (FZ; Sigma), a strong iron chelator. As GA-deficient *ga1-3* mutants do not germinate without exogenous GA, the seeds were pretreated at 4°C with 5 μM GA_3 (Sigma-Aldrich) for 3 days to synchronize germination, washed thoroughly three times, then surface sterilized before sowing.

ChIP Assays

ChIP assays were performed on roots of 10-day-old *p35S*:*FIT-GFP* seedlings that had been transferred to iron-deficient conditions and treated with 10 μM GA_3 (GA) or 1 μM PAC for 2 days, as previously described (Wild et al., 2012). In brief, chromatin was immunoprecipitated with anti-GFP antibodies (Abcam) together with protein A magnetic beads (Millipore). ChIP experiments on *p35S*:*TAP-GFP* were carried out as negative controls. The resulting ChIP DNA was subjected to qPCR analysis. Enrichment of promoter regions

(*p35S:FIT-GFP/p35S:TAP-GFP*) was averaged over three replicates and normalized using *ACTIN2*. ChIP-qPCR analyses were performed on two independent biological repeats with two technical repeats. qPCR primers used are listed in Supplemental Experimental Procedures.

Transient Transactivation Assays

Transactivation assays were performed on *N. benthamiana* agro-infiltrated leaves with the effectors *p35S:FIT-GFP* and *p35S:bHLH39-RFP* alone or together with *p35S:RGA-HA*, as indicated. The relative *pIRT1:GUS* expression (used as reporter) was calculated by normalizing against *P19* expression, using Lightcycler 480 software, and averaged over three replicates, as previously described (Davière et al., 2014). qPCR primers used are listed in Supplemental Experimental Procedures.

Determination of Iron Contents

Iron concentration in 7-day-old roots and whole seedlings grown under iron-sufficient and iron-deficient conditions was determined by atomic absorption spectrometry, using an AA240FS flame spectrometer (Agilent Technologies). In brief, seedlings were washed for 5 min in a solution containing 5 mM $CaSO_4$ and 10 mM EDTA, dried overnight at 70°C, and weighed. Tissues were digested completely in 70% HNO_3 at 120°C before elemental analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.03.022.

AUTHOR CONTRIBUTIONS

M.W., J.M.D., T.R., L.S.A., E.C., I.L.D., A.C., G.D., G.V., and P.A. conceived and performed experiments. M.W., J.M.D., G.V., and P.A. wrote the manuscript. G.V. and P.A. secured funding.

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