Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses.

Alicia Sivitz, Claudia Grinvalds, Marie Barberon, Catherine Curie, Grégory Vert

the plant journal

SEB Society for Experimental Biology

The Plant Journal (2011) doi: 10.1111/j.1365-313X.2011.04565.x

Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses

Alicia Sivitz, Claudia Grinvalds, Marie Barberon, Catherine Curie and Grégory Vert*

Biochimie et Physiologie Moléculaire des Plantes, Centre National de la Recherche scientifique, Unité Mixte de Recherche 5004, Institut de Biologie Intégrative des Plantes, 2 Place Viala, 34060 Montpellier Cedex 1, France

Received 23 December 2010; revised 15 February 2011; accepted 2 March 2011.
*For correspondence (fax +33 (0) 4 67 52 57 37; e-mail Gregory.Vert@supagro.inra.fr).

SUMMARY

Plants display a number of responses to low iron availability in order to increase iron uptake from the soil. In the model plant *Arabidopsis thaliana*, the ferric-chelate reductase FRO2 and the ferrous iron transporter IRT1 control iron entry from the soil into the root epidermis. To maintain iron homeostasis, the expression of *FRO2* and *IRT1* is tightly controlled by iron deficiency at the transcriptional level. The basic helix–loop–helix (bHLH) transcription factor FIT represents the most upstream actor known in the iron-deficiency signaling pathway, and directly regulates the expression of the root iron uptake machinery genes *FRO2* and *IRT1*. However, how FIT is controlled by iron and acts to activate transcription of its targets remains obscure. Here we show that *FIT* mRNA and endogenous FIT protein accumulate in Arabidopsis roots upon iron deficiency. However, using plants constitutively expressing *FIT*, we observed that FIT protein accumulation is reduced in iron-limited conditions. This post-transcriptional regulation of *FIT* is perfectly synchronized with the accumulation of endogenous FIT and IRT1 proteins, and therefore is part of the early responses to low iron. We demonstrated that such regulation affects FIT protein stability under iron deficiency as a result of 26S proteasome-dependent degradation. In addition, we showed that *FIT* post-translational regulation by iron is required for *FRO2* and *IRT1* gene expression. Taken together our results indicate that *FIT* transcriptional and post-translational regulations are integrated in plant roots to ensure that the positive regulator FIT accumulates as a short-lived protein following iron shortage, and to allow proper iron-deficiency responses.

Keywords: Arabidopsis, iron, proteasome, degradation, transcription factor, iron transporter.

INTRODUCTION

As sessile organisms, plants have developed sophisticated mechanisms to respond to ever changing environmental conditions. Iron is an essential element for most organisms, including plants, where it is required for cellular functions including photosynthesis and respiration. Although abundant in nature, iron is often available in limited quantities because it is mostly present in rather insoluble Fe(III) complexes in soil. The model plant *Arabidopsis thaliana* copes with iron deficiency by increasing root H^+-ATPase activity, encoded by the *AHA2* gene in Arabidopsis, to lower the rhizosphere pH and increase Fe(III) solubility (Santi and Schmidt, 2009). The inducible root ferric-chelate reductase activity encoded by the *FRO2* gene reduces Fe(III) to the more soluble Fe(II) form (Robinson *et al.*, 1999). Iron is subsequently taken up into root peripheral cell layers by the specialized root Fe(II) iron transporter IRT1 (Henriques *et al.*, 2002; Vert *et al.*, 2002).

The expression of *FRO2* and *IRT1* genes is regulated by iron at the transcriptional level. Iron deficiency rapidly triggers strong *FRO2* and *IRT1* promoter activation, and corresponding mRNAs accumulate in root epidermal and cortical cells where iron uptake occurs (Vert *et al.*, 2001, 2002; Connolly *et al.*, 2003). The FIT basic helix–loop–helix (bHLH) transcription factor controls the transcription of *FRO2* and partly that of *IRT1* in response to iron starvation in Arabidopsis (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005). FIT directly binds to *FRO2* and *IRT1* promoters in yeast, presumably through E-box motifs presents in both promoters (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2008). Consistently, a *fit* loss-of-function mutant displays low *FRO2* and *IRT1* expression in roots (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005). Similar to *irt1*, *fit* shows a chlorotic phenotype that is rescued by the

massive exogenous application of iron. Microarray analyses revealed that FIT is a master regulator of iron-deficiency responses in roots. Indeed, 72 genes out of the 179 genes that are upregulated by iron deficiency in roots are affected in the *fit-1* knock-down mutant (Colangelo and Guerinot, 2004). However, constitutive expression of *FIT* is not sufficient to further upregulate *FRO2* and *IRT1* in iron-deficient roots, or to induce *FRO2* and *IRT1* mRNA accumulation under iron-sufficient conditions (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005). FIT has been shown to heterodimerize with two other iron-regulated bHLH transcription factors, bHLH38 and bHLH39, to induce expression of the iron-uptake machinery (Yuan *et al.*, 2008). Interestingly, the master regulator FIT is itself transcriptionally regulated by iron starvation, leading to an increase in *FIT* mRNA level in the root epidermis (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004). Here, we identify a new level of regulation of *FIT* by iron starvation, and shed light on the underlying mechanisms. *FIT* is post-translationally regulated by iron deficiency, leading to a decrease in FIT protein stability mediated by proteasome-dependent degradation. This additional level of regulation is integrated with *FIT* transcriptional control to fine-tune iron-deficiency responses. We propose a model where FIT post-translational regulation allows the sustained transcription of target genes by clearing exhausted FIT off its target promoters.

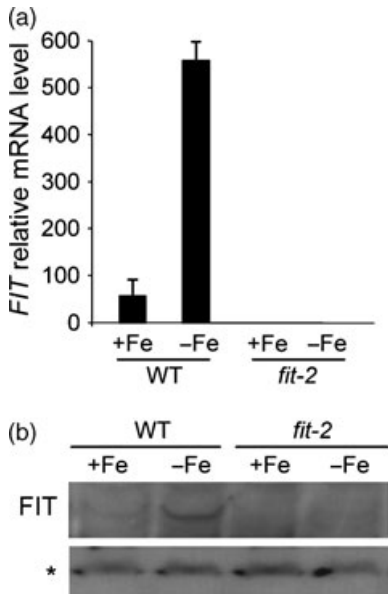

Figure 1. The transcription factor FIT is upregulated at the mRNA and protein levels in roots following iron starvation.
(a) *FIT* mRNA accumulation in plant roots in response to iron-deficient and iron-sufficient conditions. Wild-type or *fit-2* loss-of-function mutant plants were grown for 7 days on iron-sufficient medium (100 μM Fe-EDTA), and were then transferred to iron-deficient (no added iron, 300 μM ferrozine) or iron-sufficient medium for another 3 days. The transcript level of *FIT* was determined in roots by real-time RT-PCR. Error bars indicate standard errors.
(b) FIT protein accumulation from iron-starved or iron-sufficient plant root extracts. Plants were grown as described in (a). FIT protein was detected using an anti-FIT peptide antibody. *Non-specific signal used as a loading control.

RESULTS

FIT protein accumulates under iron-depleted conditions

FIT was previously reported to be transcriptionally regulated by iron deficiency, with iron starvation slightly increasing *FIT* promoter activity and *FIT* mRNA accumulation mostly in root epidermal cells (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004). We first verified that in our hands, *FIT* mRNA accumulates in roots after a 3-day period of iron starvation, compared with standard growth conditions. Real-time quantitative RT-PCR indicates that *FIT* transcripts accumulate about 10-fold in roots subjected to a 3-day period of iron deficiency, compared with standard conditions (Figure 1a). A similar experiment revealed that *FIT* mRNA accumulation is abolished in the *fit-2* knock-out mutant background carrying a T-DNA insertion 70 bp downstream of the start of the third exon (Colangelo and Guerinot, 2004). To validate this observation, we developed a FIT-specific antibody targeted against two specific 15-amino-acid peptides found in the FIT protein. Western blot analysis of iron-deficient roots detected a band around 55 kDa in wild-type plants, but not in the *fit-2* mutant (Figure 1b). Although FIT has a predicted molecular weight of 36 kDa, we observed that both recombinant FIT protein in *Escherichia coli* and radiolabeled FIT produced by *in vitro* transcription/translation migrate at 55 kDa on PAGE gels (Figure S1). Taken together, these observations indicate that iron starvation triggers a modest accumulation of FIT protein in Arabidopsis roots.

FIT is post-transcriptionally regulated by iron deficiency

To investigate the existence of post-transcriptional events regulating *FIT* gene expression, we generated transgenic plants expressing the FIT-GFP translational fusion under the control of the constitutive CaMV 35S promoter, both in wild-type (Col/35S::FIT-GFP) and in *fit-2* Arabidopsis plants (*fit-2*/35S::FIT-GFP). When gown on soil, *fit-2* is extremely chlorotic (Figure 2a), and dies rapidly after germination unless fertilized with massive quantities of iron. Expression of FIT-GFP in the *fit-2* mutant background complemented its severe chlorosis and growth defects (Figure 2a), indicating that the FIT-GFP fusion protein is functional. Knowing that FIT-GFP is functional, we performed most of the following analyses on Col/35S::FIT-GFP transgenic plants in the wild-type background to concomitantly monitor endogenous FIT, IRT1 and FIT-GFP protein levels during the establishment of iron-deficiency responses in the root. We first evaluated the influence of iron nutrition on the FIT-GFP protein accumulation pattern by western blot analysis using an anti-GFP antibody. Whereas no signal was detected in root protein extracts from wild-type plants, several independent

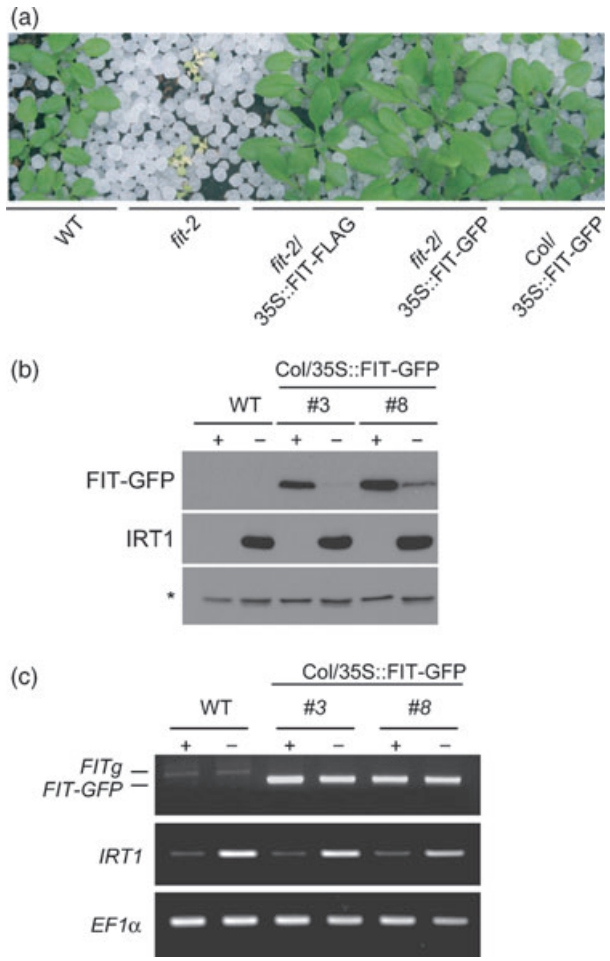

Figure 2. *FIT* is post-transcriptionally regulated by iron.
(a) Phenotype of plants constitutively overexpressing FIT-GFP in the wild-type (Col/35S::FIT-GFP) or *fit-2* loss-of-function mutant backgrounds (*fit-2*/FIT-GFP) and *fit-2*/FIT-FLAG. Plants were grown on soil for 3 weeks before assessing the phenotypic complementation of *fit-2* chlorosis.
(b) Western blot analyses of FIT-GFP and IRT1 protein accumulation patterns in wild-type and Col/35S::FIT-GFP transgenic plants. Plants were grown for 7 days on iron-sufficient medium (100 μM), and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient medium for another 3 days. The FIT-GFP protein level was determined in roots by western blot analysis using an anti-GFP antibody. Two representative independent transgenic lines from the T_2 generation are shown. *Non-specific signal that is used as a loading control.
(c) Semi-quantitative RT-PCR was performed to monitor FIT-GFP, FIT and IRT1 mRNA levels in wild-type and Col/35S::FIT-GFP transgenic plants. Plants were grown for 7 days on iron-sufficient medium (100 μM), and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient medium for another 3 days. The primers used are FIT seq F and qPCR GFP R (Table S1). EF1α amplification is used as a loading control.

transgenic lines showed a FIT-GFP-specific signal around 85 kDa (Figure 2b). Interestingly, a marked decrease in the FIT-GFP protein level was observed in different independent transgenic lines when plants were subjected to a 3-day period of iron deficiency, compared with standard conditions. Iron deficiency was controlled by hybridizing the same protein extracts with an antibody specifically recognizing IRT1 that only accumulates in roots following iron starvation (Vert *et al.*, 2002). The strong IRT1 protein accumulation observed in iron-deficient conditions confirmed that FIT-GFP accumulates less when plants are subjected to iron shortage (Figure 2b). It is worth noticing that *FIT* overexpression does not lead to *IRT1* upregulation, consistent with previous reports (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2008).

To rule out the possibility that the lower accumulation of FIT-GFP results from the presence of the large GFP tag, we confirmed these observations by constitutively expressing the FIT coding sequence alone or fused to three copies of the eight-amino-acid FLAG tag in the *fit-2* background (*fit-2*/35S::FIT and *fit-2*/35S::FIT-FLAG, respectively). Such transgenic plants showed the exact same decrease in constitutively expressed FIT or FIT-FLAG protein accumulation under iron-deficient conditions (Figure S2), confirming what has been observed with FIT-GFP overexpression in wild-type plants (Figure 2b). The fact that the different transgenic lines behaved similarly led us to focus, in the following experiments, on tagged versions of FIT to take advantage of the highly specific anti-tag antibodies. Taken together, our observations using constitutively-expressed FIT indicate that *FIT* is post-transcriptionally regulated by iron starvation, leading to a decreased accumulation of FIT protein. Notably this is in contrast to the overall accumulation of endogenous FIT observed in iron-deficient conditions, using the anti-FIT antibody (Figure 1b), and suggests that iron regulates *FIT* expression at different levels.

Such post-transcriptional regulation by iron starvation may target *FIT* mRNA stability, FIT protein translation or degradation. To decipher between these different mechanisms, we next examined the influence of iron on *FIT-GFP* mRNA levels in Col/35S::FIT-GFP transgenic plants. To avoid the possible amplification of small *FIT-GFP* mRNA degradation products that may result from post-transcriptional gene silencing (PTGS), we first amplified large *FIT-GFP* mRNA fragments by semi-quantitative RT-PCR using a FIT- and a GFP-specific primer. This experiment shows that *FIT-GFP* transcripts are present, regardless of iron nutrition, in two representative Col/35S::FIT-GFP lines, whereas wild-type plants showed no specific amplification (Figure 2c). In parallel, *IRT1* mRNA levels dramatically increased following iron starvation, proving that plants were actually deficient in iron. To further confirm these results, we performed real-time quantitative RT-PCR and observed no or little difference in *FIT-GFP* transcript levels following iron starvation, whereas the two iron-deficiency markers *IRT1* and *FRO2* were clearly upregulated under iron starvation (Figure S3). Thus, *FIT* mRNA stability is not affected by iron nutrition. This suggests that FIT is subjected to either translational or post-translational regulation by iron starvation, leading to a decrease in FIT-GFP protein accumulation in Col/35S::FIT-GFP lines.

FIT post-transcriptional control is synchronized with early iron-deficiency responses

The role of *FIT* post-transcriptional regulation may be diverse, from triggering the termination of iron-deficiency responses, by facilitating the removal of the positive regulator FIT to prevent a high accumulation of iron, to anticipating a sudden change in iron nutrition by means of a short-lived transcriptional activator. To evaluate the biological significance of such a regulatory mechanism, we determined the timing of *FIT* post-transcriptional regulation visualized by the decrease in constitutively expressed FIT-GFP in Col/35S::FIT-GFP plants, compared with other known responses to iron starvation. The most commonly used and robust marker for the establishment of these early molecular responses is the accumulation of IRT1 protein in roots (Connolly *et al.*, 2002). Time-course analyses of protein accumulation by western blot revealed that in our conditions, IRT1 protein starts to accumulate 24–48 h after transfer to iron-limiting conditions, and keeps increasing until day 5 (Figure 3a). Endogenous FIT protein levels detected with the anti-FIT antibody rose earlier than IRT1, consistent with FIT acting upstream and being necessary for the expression of *IRT1* (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005; Seguela *et al.*, 2008). Strikingly, the post-transcriptional regulation of *FIT*, monitored by the decrease in FIT-GFP detected by the anti-GFP antibody, is set up at the onset of iron-deficiency responses, when endogenous FIT and IRT1 proteins first appear (Figure 3a). We could not detect the FIT-GFP fusion protein with the anti-FIT antibody because it migrates around 85 kDa, in a zone where the anti-FIT antibody gives a very high background. We performed the opposite experiment, where Col/35S::FIT-GFP plants grown in the absence of iron, and thus harboring low FIT-GFP levels, were subjected to short-term iron re-supply. FIT-GFP protein levels rose as early as 6 h after the addition of iron, concomitantly with endogenous FIT protein decrease, and preceding the drop in the stable IRT1 protein (Figure 3b). Taken together, these observations indicate that the post-transcriptional control of *FIT* is perfectly synchronized with early responses to changes in iron availability.

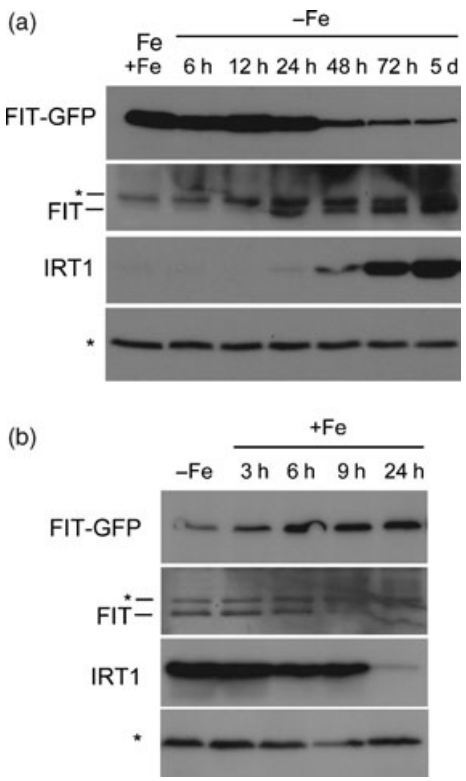

Figure 3. *FIT* post-transcriptional regulation is an early response to iron deficiency.
(a) Time-course analyses of FIT-GFP, FIT and IRT1 protein accumulation in plants in response to iron deficiency. Col/35S::FIT-GFP#8 plants were grown for 7 days on iron-sufficient medium (100 μM), and were then transferred to iron-deficient medium (300 μM ferrozine) before harvesting at several time points. Protein levels were determined using anti-GFP, FIT and IRT1 antibodies, respectively. The asterisk labels a non-specific signal that is used as a loading control.
(b) Time-course analyses of FIT-GFP and IRT1 protein accumulation in plants in response to iron re-supply following iron starvation. Col/35S::FIT-GFP#8 plants were grown for 7 days on iron-sufficient medium, and were then transferred to iron-deficient medium (300 μM ferrozine) and grown for an additional 3 days before adding 100 μM iron to the medium. Protein levels were determined using anti-GFP and IRT1 antibodies, respectively. The asterisk labels a non-specific signal that is used as a loading control.

FIT is dually regulated by transcriptional and post-transcriptional regulatory mechanisms

Although endogenous FIT protein accumulates in iron-starved roots from wild-type plants (Figure 1b), we demonstrated that *FIT* is post-transcriptionally downregulated under the same growth conditions (Figures 2b and S2) and timing (Figure 3), suggesting that the two regulatory levels are likely to co-exist. To better visualize the simultaneous dual regulation of *FIT* expression at the transcriptional and post-transcriptional levels, we generated Col/35S::FIT transgenic plants in the wild-type background. The FIT protein levels in such Col/35S::FIT plants therefore result from the addition of endogenous FIT and constitutively expressed FIT that both migrate at 55 kDa on PAGE gels. Western blot analyses using an anti-FIT antibody revealed that the total FIT protein levels are largely unaffected by iron limitation in Col/35S::FIT (Figure 4), in contrast to what has been observed for *fit-2*/35S::FIT, *fit-2*/35S::FIT-GFP and *fit-2*/35S::FIT-FLAG plants, where FIT levels decreased upon iron starvation as the sole consequence of post-transcriptional regulation (Figures 2b and S2). These observations therefore support the fact that the post-transcriptional decrease in FIT protein occurs concomitantly and antagonizes *FIT* transcriptional activation by iron starvation (Figures 2b and S2), further supporting the synchronization of the two regulatory

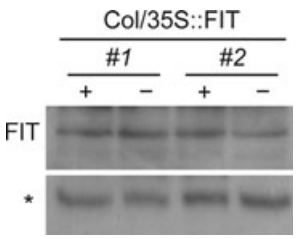

Figure 4. Integration of *FIT* transcriptional and post-transcriptional regulations by iron.
Western blot analyses of FIT total protein accumulation patterns in Col/35S::FIT transgenic plants in the wild-type background. Plants were grown for 7 days on iron-sufficient medium, and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient medium for another 3 days. The FIT total protein level was determined in roots by western blot analysis using an anti-FIT antibody. Two representative independent transgenic lines from the T_2 generation are shown. The asterisk labels a non-specific signal that is used as a loading control.

mechanisms observed (Figure 3). Therefore, *FIT* gene regulation by iron results from the integration of several simultaneous iron-dependent regulatory mechanisms acting at the transcriptional and post-transcriptional levels.

FIT protein is destabilized upon iron starvation

To shed light on the regulatory mechanism underlying *FIT* post-transcriptional regulation, we examined the differential stability of FIT-GFP protein, taking advantage of the translation inhibitor cycloheximide (CHX) to follow the fate of a pool of FIT-GFP in the absence of *de novo* protein synthesis. Consistent with what has been observed, FIT-GFP accumulates to slightly higher levels in standard conditions compared with plants subjected to iron deficiency for 2 days (Figure 5a). However, CHX treatment revealed different behavior of constitutively expressed FIT-GFP in the two iron regimes. FIT-GFP accumulation was only modestly affected by CHX in iron-replete conditions, whereas the FIT-GFP protein level severely dropped in iron-starvation conditions (Figure 5a). Quantification of the chemiluminescence signal intensity showed a roughly twofold decrease in FIT-GFP protein accumulation under iron-replete conditions, compared with a roughly ninefold drop in iron-starved plants over the course of the CHX experiment (Figure 5b). The stability of FIT protein therefore appears to be regulated in an iron-dependent manner, with iron starvation triggering a roughly fivefold decrease in FIT stability.

To test which cellular mechanism is responsible for the observed destabilization of FIT, we studied the effect of MG132, a *26S* proteasome inhibitor, on FIT-GFP accumulation. MG132 treatment led to a greater accumulation of FIT-GFP under iron-limited conditions (Figure 5c), whereas the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was ineffective in this respect (Figure 5d). This indicates that FIT undergoes *26S* proteasome-dependent degradation under such growth conditions. Collectively, these data

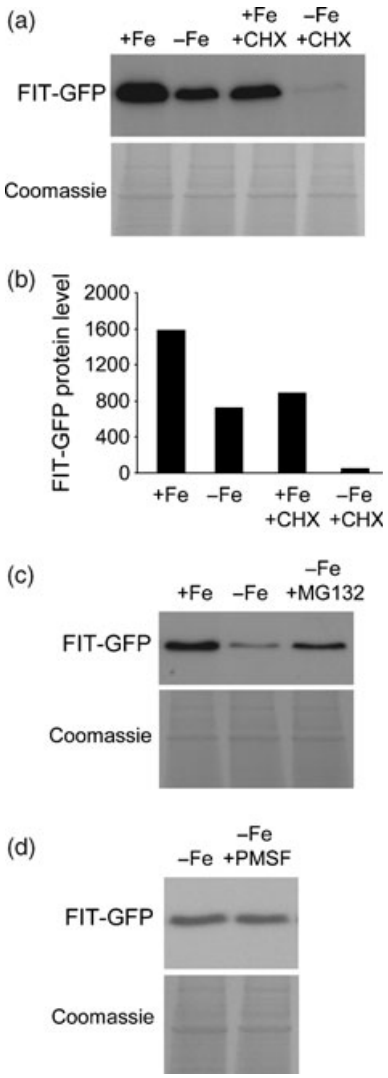

Figure 5. Iron regulates FIT post-translationally by proteasome-mediated degradation.
(a) Western blot analyses of FIT-GFP protein accumulation in plants treated with cycloheximide. Col/35S::FIT-GFP#8 plants were grown for 7 days on iron-sufficient medium (100 μM), and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient medium for another 2 days before mock or 100 μM cycloheximide treatment for 6 h. The FIT-GFP protein level was determined in roots by western blot analysis using an anti-GFP antibody. Coomassie staining is used as a loading control.
(b) Quantification of the FIT-GFP destabilization by iron measured in (a).
(c) Western blot analyses of FIT-GFP protein accumulation in plants treated with MG132. Plants were grown as in (a) but treated with 50 μM MG132 for 2 h before root harvest. Coomassie staining is used as a loading control.
(d) Western blot analyses of FIT-GFP protein accumulation in plants treated with phenylmethylsulfonyl fluoride (PMSF). Plants were grown as in (a), but were treated with 1 mM PMSF for 2 h before root harvest. Coomassie staining is used as a loading control.

demonstrate that the turnover rate of FIT, a positive regulator of iron-deficiency responses, is higher under iron-limited conditions, despite the fact that FIT endogenous protein accumulates.

Proteasome activity is required for FIT transcriptional activity

The establishment of iron-deficiency responses, and more precisely of the high-affinity iron-uptake system, requires the positive regulator FIT (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005). Therefore, the rationale for degrading FIT under iron-limited conditions is unclear. To clarify the biological significance of FIT destabilization upon iron shortage, we investigated the influence of the *26S* proteasome inhibitor MG132, which stabilizes FIT (Figure 5c), on FIT target gene expression: i.e. *FRO2* and *IRT1*. However, to avoid competition between the endogenous FIT and the tagged FIT proteins, that may harbor different DNA-binding or transcriptional activity, we performed this experiment in the *fit-2* mutant background. Shortly after transfer from standard to iron-limited conditions, *FRO2* and *IRT1* transcripts started to accumulate in roots (Figure 6a,b). Stabilization of the positive regulator FIT by MG132 treatment in iron-limited conditions did not yield a greater expression of *FRO2* and *IRT1*, as one would expect if FIT protein levels correlated with its target gene expression. Rather, stabilizing FIT, which is known to be required for *FRO2* and *IRT1* expression (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005), hampered the induction of *FRO2* and *IRT1* (Figure 6a,b). Collectively, these data suggest that *26S* proteasome activity and FIT destabilization are required for optimal FIT activity, and for the establishment of the iron-deficiency response.

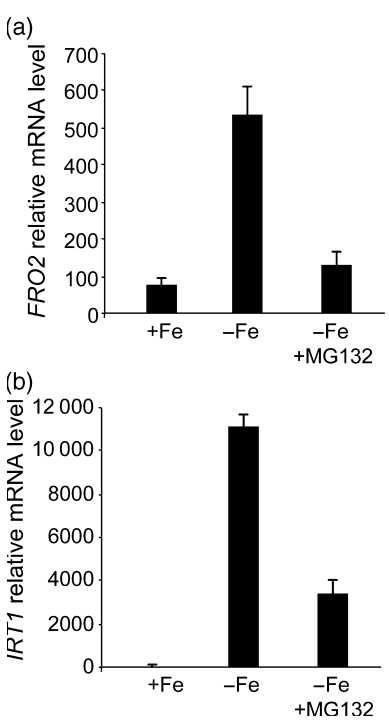

Figure 6. Proteasome-mediated degradation of FIT is required for iron-deficiency responses.
FRO2 (a) and *IRT1* (b) mRNA accumulation in roots was measured by real-time quantitative RT-PCR in transgenic plants *fit-2*/35S::FIT-FLAG#3. Plants from the T_2 generation were grown for 7 days on iron-sufficient medium (100 μM), and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient media for 36 h before mock or 50 μM MG132 treatment for an additional 6 h. Error bars indicate standard errors.

DISCUSSION

Plants respond to iron shortage by increasing the expression of the root iron uptake machinery that is under the control of the positive regulator FIT (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004; Yuan *et al.*, 2005). Here, we show that the *FIT* gene is dually regulated by iron nutrition. *FIT* is transcriptionally regulated by iron starvation, leading to the accumulation of *FIT* mRNA and FIT protein in iron-deficient roots (Figure 1a,b). However, using plants constitutively expressing *FIT*, we uncovered another iron-dependent regulatory mechanism targeting *FIT* acting at the post-transcriptional level. We demonstrated that under conditions of iron starvation, FIT is actively destabilized and turned over by *26S* proteasome-dependent degradation, with the consequence that FIT accumulates in a rather unstable form.

Proteasome-mediated protein degradation plays a pivotal role in many plant growth and developmental processes, including photomorphogenesis and plant hormone signaling (Smalle and Vierstra, 2004). The proteasome acts by either degrading activators to stop transcription of target genes or by degrading repressors to activate gene expression. The role of bHLH turnover by proteasome-mediated degradation has been extensively studied in the case of the transcription factor PIFs that control light-regulated gene expression in Arabidopsis. PIF family members are rapidly phosphorylated and degraded by the *26S* proteasome upon exposure to light (Castillon *et al.*, 2007). Our work sheds light on a new regulatory mechanism driving the root high-affinity iron-uptake system in plants, and unravels the essential role of the proteasome in degrading the FIT bHLH transcription factor and stimulating gene expression during iron-deficiency responses. In the presence of iron, *FIT* is not expressed due to the low *FIT* promoter activity. This transcriptional regulation prevents iron-deficiency responses, so as to avoid the toxic accumulation of iron and metals associated with constitutive uptake. Iron shortage increases *FIT* mRNA and protein levels in roots as a result of the iron-mediated transcriptional regulation of *FIT* (Figure 1a,b). Interestingly, we showed that such transcriptional control of *FIT* strictly coexists with FIT protein destabilization (Figures 3 and 4). The overall increase in FIT protein observed under iron-limited conditions in wild-type plants therefore reflects the integration of the two regulatory mechanisms acting simultaneously. This raises the question of the biological significance of increasing FIT destabilization under conditions where its presence is most needed for the establishment and maintenance of the iron-deficiency responses. The dual control of *FIT* allows FIT protein to

accumulate as a short-lived form during iron starvation, a prerequisite to rapidly anticipate sudden changes in iron availability. However, one would expect FIT stabilization to reinforce the iron-deficiency responses. The fact that the inhibition of transcriptionally active FIT degradation by the proteasome under iron limitation led to decreased iron-deficiency responses suggests that FIT destabilization is also required for proper iron-deficiency responses (Figure 6). Although we cannot exclude that MG132 prevents the turnover of a negative regulator of *IRT1* and *FRO2* expression, we favor the model where the level of transcriptionally active FIT directly impacts the transactivation of *FRO2* and *IRT1*. Recent findings in yeast and humans indicate that transcription factors are often unstable and their instability correlates with their ability to transactivate target gene transcription (Kim *et al.*, 2003; Reid *et al.*, 2003; Lipford *et al.*, 2005; Muratani *et al.*, 2005). For example, the Dsg1-dependent ubiquitination and proteolysis of the yeast transcriptional activator Gal4 in the presence of galactose is specifically required for optimal Gal4 protein activity (Muratani *et al.*, 2005). It is thought that limiting the half-life of transcription factors promotes gene expression by continuously delivering 'fresh' activators to their target promoters. This may be necessary to sustain a high ratio of transcriptionally active transcription factors compared with 'fatigued' inactive factors (Collins and Tansey, 2006). The negative effect of MG132-mediated FIT stabilization on FIT target gene expression is consistent with the proteasome being required for FIT activity. We therefore propose a model where iron deficiency triggers FIT destabilization to constantly clear 'exhausted' FIT protein from target gene promoters (Figure 7), thereby allowing further cycles of transcription and a high level of expression of FIT target genes. Such a role for the proteasome in promoting high rates of transcription was recently proposed in plants for the co-activator of systemic acquired resistance NPR1 (Spoel *et al.*, 2009).

Proteasome degradation has been shown to require polyubiquitination of lysine residues in the target protein by E3 ubiquitin ligases (Moon *et al.*, 2004). It would be of great interest to determine if FIT is ubiquitinated *in vivo* in response to iron deficiency, and to identify the E3 ligase(s) controlling FIT ubiquitination and stability. The Arabidopsis genome encodes over 1000 E3 ligases, highlighting the necessity for targeted approaches to identify E3 ubiquitin ligases involved in a particular process. However, the synchronization of IRT1 protein accumulation and FIT destabilization prompted us to look at publicly available microarray analysis to identify iron-regulated E3 ligases expressed in roots, and possibly involved in FIT post-translational regulation. The different studies identified about 20 E3 ubiquitin ligases upregulated by iron starvation in Arabidopsis roots (Colangelo and Guerinot, 2004; Dinneny *et al.*, 2008; Buckhout *et al.*, 2009). These candidate proteins are currently under investigation, and should help confirm the direct role played by proteasome-mediated turnover of FIT in the control of iron-deficiency responses.

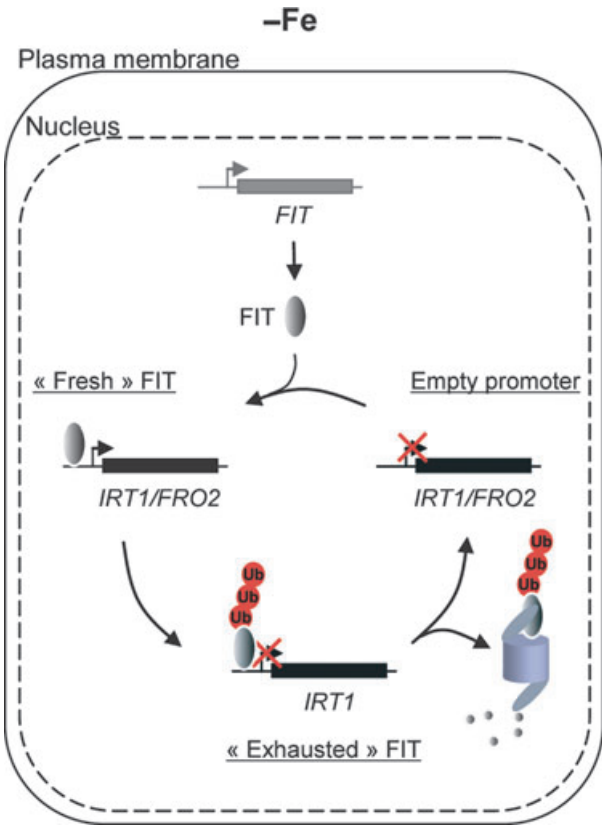

Figure 7. Model for the dual regulation of *FIT* by iron deficiency.
Iron starvation triggers *FIT* mRNA and protein accumulation in root cells to initiate the root iron-deficiency responses, including the upregulation of the root high-affinity iron uptake system. In parallel, FIT protein stability drops as a consequence of increased FIT turnover by the *26S* proteasome, probably via FIT polyubiquitination. Clearance of 'exhausted' FIT from the target gene promoters allows 'fresh' FIT to reinitiate further transcription cycles, thereby directly linking the rate of FIT degradation to the amplitude of target gene transcription.

Previously, a unified model for how transcription factors are regulated by ubiquitination has been difficult to propose. In some instances, such as p53 (Haupt *et al.*, 1997), ubiquitination leads to proteolysis and the inhibition of activator function. In other cases, however, ubiquitination leads to proteolysis and enhanced activator function (Salghetti *et al.*, 2001). The complex code of ubiquitin in regulating transcription factors also seems to be shared by plants. Further work will determine whether this mechanism is important for other plant transcription factors.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Columbia wild-type plants and *fit-2* knock-out mutants, and the different transgenic lines and mutants obtained in this study, were grown in sterile conditions on vertical plates at 21°C with a 16-h

light/8-h dark cycle. Seeds were surface-sterilized and sown on half-strength Murashige and Skoog medium (1/2 MS) containing 1% sucrose and 1% agar. The medium was buffered with 0.5 g L^{-1} 2-(*N*-morpholino-ethanesulfonic acid (MES), and the pH was adjusted to 5.7 by KOH. Plants were cultivated in the conditions described above for 7 days and then transferred onto iron-sufficient (100 μM FeEDTA) or iron-deficient media {no added iron and 300 μM Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate], a strong iron chelator}. For inhibitor studies (cycloheximide, CHX; MG132; PMSF) plants were transferred to liquid 1/2 MS medium before mock or drug treatment. All reagents were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com).

The *fit-2* knock-out mutant (SALK_126020, Columbia ecotype) was isolated from the SALK Collection (http://signal.salk.edu/cgi-bin/tdnaexpress). Homozygous mutants were isolated by PCR using the FIT seq F and FIT seq R primers (Table S1).

Construction of plasmids

FIT was amplified by RT-PCR on RNA extracted from young seedlings using FIT F and FIT R primers (Table S1), and cloned in pCHF3 at the *Bam*HI-*Sal*I sites in frame with GFP or 3xFLAG. FIT alone was also cloned in pCHF3 downstream of the *35S* promoter using FIT F and FIT R2 primers. The different constructs were transferred to *Agrobacterium tumefaciens* strain GV3101, and transformed into wild-type or *fit-2* plants by floral dipping (Clough and Bent, 1998).

For recombinant protein production and *in vitro* transcription/translation, FIT was amplified by PCR using FIT F and FIT R3 primers, and cloned into pET28a at *Bam*HI–*Sal*I restriction sites.

Recombinant His-tagged FIT protein was produced according to the manufacturer's recommendations (Novagen, http://www.emdchemicals.com/life-science-research). In parallel, FIT was subjected to [^{35}S]Met TNT T7 *in vitro* transcription/translation using a wheat-germ lysate system (Promega, http://www.promega.com). Both recombinant protein extracts and *in vitro* transcribed/translated FIT protein were boiled with 4X SDS buffer and loaded onto PAGE gels and subjected to Coomassie staining, or fixing drying and signal detection using KODAK BioMax XAR film (Sigma-Aldrich), respectively.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using the RNeasy kit (Invitrogen, http://www.invitrogen.com) and treated with DNAse to avoid genomic DNA contamination. The integrity of DNA-free RNA was verified by agarose gel electrophoresis, and equal quantities of total RNA (2 μg) were used for reverse transcription with anchored oligo(dT23). Semi-quantitative PCR for FIT-GFP (22 cyles) and IRT1 (26 cycles) were performed using the primers listed in Table S1. Amplification of EF1α (25 cycles) served as an internal control. Real-time PCR was performed with LightCycler® Fast-Start DNA MasterPLUS SYBER GREEN I (Roche Applied Science, http://www.roche-applied-science.com) using gene-specific primers (Table S1). The primer specificity was confirmed by analysis of melting curves and agarose gel electrophoresis of the PCR products. Relative transcript levels (RTLs) were calculated relative to the transcript level of the constitutively expressed *EF1α* gene, as follows: $\mathrm{RTL} = 100 \times 2^{-\Delta C_t}$ The change in the cycle threshold (ΔC_t) values were calculated as follows: $\Delta C_t(\mathrm{gene}) = C_t(\mathrm{gene}) - C_t(EF1\alpha)$. The experiments were performed in duplicate. The primers used for real-time RT-PCR are listed in Table S1.

Protein extraction and western blot

Total protein was extracted from 100 mg of root tissues grown in the conditions described above. Extracts were prepared by adding 300 μL of extraction buffer (50 mM Tris–HCl, pH 8, 5% SDS, 5% β-mercaptoethanol, 25 mM EDTA and 0.1% phenylmethylsulfonyl fluoride) to tissues, followed by centrifugation at 4°C for 15 min at 14 000 ***g***. Total proteins were separated on a 13% polyacrylamide 0.1% SDS gel and transferred onto a Hybond-P membrane (GE Healthcare, http://www.gelifesciences.com) by electroblotting. Membranes were blocked overnight in PBST (1X PBS and 0.1% Tween-20), with 0.2% blocking reagent (AuroraTM Western Blot Chemiluminescence Detection System; MP Biomedicals, http://www.mpbio.com). Immunodetection of IRT1 protein was performed using an affinity-purified anti-peptide IRT1 antibody diluted 1:5000 in blocking buffer for 1 h. The IRT1 polyclonal antibody was previously described (Seguela *et al.*, 2008). Commercially available monoclonal anti-GFP (Roche Applied Science) and polyclonal anti-FLAG (Sigma-Aldrich) antibodies were used to detect FIT-GFP and FIT-FLAG translational fusions, respectively. The FIT polyclonal antibody was raised in rabbits against two synthetic peptides (EDEEDYNDGDDSSAT and APDAQKTQPFRGINP) that correspond to amino acids 101–115 and 204–218, respectively, of the FIT deduced protein sequence, and that are unique to FIT (Eurogentec, http://www.eurogentec.com). After primary antibody incubation, the membrane was washed in blocking buffer four times for 15 min each, and was then incubated for 1 h with the appropriate secondary antibodies conjugated to alkaline phosphatase (Promega) (diluted 1:20 000 in blocking buffer). After three different washes, the membrane was incubated for 5 min in the presence of 'Aurora Chemiluminescence Substrate solution'. Chemiluminescence was revealed on BioMax XAR film (Kodak, http://www.kodak.com) or using the LAS3000 Imaging System (Fujifilm, http://www.fujifilm.com) for accurate quantification.

ACKNOWLEDGEMENTS

Stéphane Mari, Pierre Czernic, Fanchon Divol and Enric Zelazny are acknowledged for their critical reading of the manuscript. Work was supported by a European Molecular Biology Organization long-term fellowship to AS, a PhD fellowship from the French Ministry of National Education, Research and Technology (MENRT) to MB, and grants to GV from the Human Frontier Science Program Organization (CDA0005/2007) and the Agence Nationale de la Recherche (ANR-08-JCJC-0058).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Migration of recombinant and *in vitro* transcribed/translated FIT.

Figure S2. FIT and FIT-FLAG overexpressors also reveal post-transcriptional control of *FIT* expression by iron starvation.

Figure S3. Accumulation of *FIT-GFP* mRNA in Col/35S::FIT-GFP transgenic plants monitored by quantitative RT-PCR.

Table S1. Primer list.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Buckhout, T.J., Yang, T.J. and Schmidt, W. (2009) Early iron-deficiency-induced transcriptional changes in Arabidopsis roots as revealed by microarray analyses. *BMC Genomics*, **10**, 147.

Castillon, A., Shen, H. and Huq, E. (2007) Phytochrome Interacting Factors: central players in phytochrome-mediated light signaling networks. *Trends Plant Sci.* **12**, 514–521.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

Colangelo, E.P. and Guerinot, M.L. (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell*, **16**, 3400–3412.

Collins, G.A. and Tansey, W.P. (2006) The proteasome: a utility tool for transcription? *Curr. Opin. Genet. Dev.* **16**, 197–202.

Connolly, E.L., Fett, J.P. and Guerinot, M.L. (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell*, **14**, 1347–1357.

Connolly, E.L., Campbell, N.H., Grotz, N., Prichard, C.L. and Guerinot, M.L. (2003) Overexpression of the FRO2 Ferric Chelate Reductase Confers Tolerance to Growth on Low Iron and Uncovers Posttranscriptional Control. *Plant Physiol.* **133**, 1102–1110.

Dinneny, J.R., Long, T.A., Wang, J.Y., Jung, J.W., Mace, D., Pointer, S., Barron, C., Brady, S.M., Schiefelbein, J. and Benfey, P.N. (2008) Cell identity mediates the response of Arabidopsis roots to abiotic stress. *Science*, **320**, 942–945.

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296–299.

Henriques, R., Jasik, J., Klein, M., Martinoia, E., Feller, U., Schell, J., Pais, M.S. and Koncz, C. (2002) Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects. *Plant Mol. Biol.* **50**, 587–597.

Jakoby, M., Wang, H.Y., Reidt, W., Weisshaar, B. and Bauer, P. (2004) FRU (BHLH029) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. *FEBS Lett.* **577**, 528–534.

Kim, S.Y., Herbst, A., Tworkowski, K.A., Salghetti, S.E. and Tansey, W.P. (2003) Skp2 regulates Myc protein stability and activity. *Mol. Cell*, **11**, 1177–1188.

Lipford, J.R., Smith, G.T., Chi, Y. and Deshaies, R.J. (2005) A putative stimulatory role for activator turnover in gene expression. *Nature*, **438**, 113–116.

Moon, J., Parry, G. and Estelle, M. (2004) The ubiquitin-proteasome pathway and plant development. *Plant Cell*, **16**, 3181–3195.

Muratani, M., Kung, C., Shokat, K.M. and Tansey, W.P. (2005) The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell*, **120**, 887–899.

Reid, G., Hubner, M.R., Metivier, R., Brand, H., Denger, S., Manu, D., Beaudouin, J., Ellenberg, J. and Gannon, F. (2003) Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell*, **11**, 695–707.

Robinson, N.J., Procter, C.M., Connolly, E.L. and Guerinot, M.L. (1999) A ferric-chelate reductase for iron uptake from soils. *Nature*, **397**, 694–697.

Salghetti, S.E., Caudy, A.A., Chenoweth, J.G. and Tansey, W.P. (2001) Regulation of transcriptional activation domain function by ubiquitin. *Science*, **293**, 1651–1653.

Santi, S. and Schmidt, W. (2009) Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. *New Phytol.* **183**, 1072–1084.

Seguela, M., Briat, J.F., Vert, G. and Curie, C. (2008) Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway. *Plant J.* **55**, 289–300.

Smalle, J. and Vierstra, R.D. (2004) The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* **55**, 555–590.

Spoel, S.H., Mou, Z., Tada, Y., Spivey, N.W., Genschik, P. and Dong, X. (2009) Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. *Cell*, **137**, 860–872.

Vert, G., Briat, J.F. and Curie, C. (2001) Arabidopsis IRT2 gene encodes a root-periphery iron transporter. *Plant J.* **26**, 181–189.

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M.L., Briat, J.F. and Curie, C. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell*, **14**, 1223–1233.

Yuan, Y.X., Zhang, J., Wang, D.W. and Ling, H.Q. (2005) AtbHLH29 of *Arabidopsis thaliana* is a functional ortholog of tomato FER involved in controlling iron acquisition in strategy I plants. *Cell Res.* **15**, 613–621.

Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D. and Ling, H.Q. (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. *Cell Res.* **18**, 385–397.