Cytokinins negatively regulate the root iron uptake machinery in Arabidopsis through a growth-dependent pathway

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Summary

Plants display a number of biochemical and developmental responses to low iron availability in order to increase iron uptake from the soil. The ferric-chelate reductase FRO2 and the ferrous iron transporter IRT1 control iron entry from the soil into the root epidermis. In Arabidopsis, expression of IRT1 and FRO2 is tightly controlled to maintain iron homeostasis, and involves local and long-distance signals, as well as transcriptional and post-transcriptional events. FIT encodes a putative basic helix-loop-helix (bHLH) transcription factor that regulates iron uptake responses in Arabidopsis. Here, we uncover a new regulation of the root iron uptake genes. We show that IRT1, FRO2 and FIT are repressed by the exogenous addition of cytokinins (CKs), and that this repression acts at the level of transcript accumulation, and depends on the AHK3 and CRE1 CK receptors. The CKs and iron-deficiency signals act through distinct pathways to regulate the soil iron uptake genes, as (i) CK repression is independent of the iron status, (ii) IRT1 and FRO2 downregulation is unchanged in a fit loss-of-function mutant, indicating that FIT does not mediate CK repression, and (iii) the iron-regulated genes AtNRAMP3 and AtNRAMP4 are not downregulated by CKs. We show that root growth-inhibitory conditions, such as abiotic stresses (mannitol, NaCl) and hormonal treatments (auxin, abscisic acid), repress the iron starvation response genes. We propose that CKs control the root iron uptake machinery through a root growth dependent pathway in order to adapt nutrient uptake to the demand of the plant.

Keywords: iron, signaling, hormones, cytokinins, transport, root growth.

Introduction

Iron is a micronutrient required in small quantities for plant growth, but can become very limiting in alkaline conditions such as in calcareous soils. In response to decreasing iron in the environment, plants develop specific morphological and physiological adaptive programs. Limited iron availability triggers the production of root hairs to increase the absorption surface of the root, and the induction of a high-affinity iron uptake system. To assist the mobilization of sparingly soluble iron particles, iron-starved roots of higher plants, except grasses, acidify the soil by enhancing the extrusion of organic acids and by activating H⁺-ATPase(s). Solubilized ferric-chelates are then reduced, releasing ferrous iron that is subsequently taken up into the root by a high-affinity transporter. Genes encoding the root ferric-chelate reductase and ferrous iron uptake activities have been identified in several dicotyledonous plants (Curie and Briat, 2003). Sequence similarity with the yeast ferric-chelate reductases Fre1-Fre2 proteins enabled the cloning of the ferric reductase oxidase FRO2 gene from Arabidopsis (Robinson et al., 1999). IRT1, encoding a root high-affinity iron transporter of the ZIP family, was initially isolated from an Arabidopsis cDNA expression library in yeast screened for the ability to restore growth of a yeast mutant defective in iron uptake (Eide et al., 1996). FRO2 and IRT1 provide a major entry route for iron because the Arabidopsis fRO2 and irt1 loss-of-function mutants are dramatically chlorotic, and do not develop into mature plants unless supplied with a high concentration of iron (Henriques et al., 2002; Robinson...
et al., 1999; Varotto et al., 2002; Vert et al., 2002). In addition to iron, IRT1 was shown to mediate the entry of zinc (Zn), manganese (Mn), cobalt (Co) and cadmium (Cd) in iron-starved plants (Vert et al., 2002).

Unlike animals that control the expression of their iron homeostasis genes post-transcriptionally through their Iron Responsive Element (IRE)/Iron Responsive Protein (IRP) system, plants regulate the root iron-regulated genes primarily at the level of transcription (Curie and Briat, 2003). Both promoter activity and transcript accumulation of FRO2 and IRT1 are observed specifically in the outer layers of the root (epidermis and cortex), where they are strongly enhanced in response to iron deficiency (Connolly et al., 2002; Vert et al., 2002). Grafting and split-root experiments, in which only half of the root system is iron limited, indicate that both FRO2 and IRT1 mRNA accumulation are dually controlled by local input, which is likely to reflect the availability of iron in the medium, and by long-distance signals, originating from the shoots (Vert et al., 2003). Such a complex regulatory network, also shared by other nutrients such as nitrate, sulfate and phosphate (Burleigh and Harrison, 1999; Gansel et al., 2001; Lappartient et al., 1993; Liu et al., 1998), ensures a close match between root absorption and plant demand, in relation with the element availability in the rhizosphere. In addition, adaptation to the ever-changing nutrient availability involves a rapid downregulation of the root iron uptake system when iron becomes suddenly available to the plant. Experimentally, the addition of iron to iron-starved plants abolishes the accumulation of FRO2 and IRT1 transcripts (Connolly et al., 2002, 2003; Vert et al., 2003). Interestingly, FRO2 and IRT1 are further regulated post-transcriptionally under such conditions. Although the exact mechanism is still unclear for FRO2, IRT1 appears to be regulated at the protein level. 35S-IRT1 transgenic lines accumulate IRT1 protein only in roots of iron-deficient plants, in spite of a high and constitutive level of the corresponding transcript (Connolly et al., 2002, 2003). Such a combination of positive and negative controls of IRT1 and FRO2 gene expression are likely to contribute to the fine tuning of the root response to iron deprivation.

To date, the only component that has been identified in the regulatory cascade leading to activation of the root iron deficiency response, is a transcription factor of the basic helix-loop-helix (bHLH) family. The fer gene was originally isolated, by map-based cloning, from the tomato FER mutant T3238fer that fails to activate physiological and developmental iron-deficiency responses in the root (Ling et al., 2002). The Arabidopsis ortholog of the tomato FER gene was simultaneously identified by several laboratories, and is called FIT1/FRU/bHLH29 or FIT (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). Expression of FIT occurs in the same outer cell layers of iron-deficient roots as IRT1 and FRO2, making these iron starvation inducible genes potential targets of FIT. FIT is necessary for the accumulation of FRO2, and to a lesser extent of IRT1, mRNA in response to iron starvation (Colangelo and Guerinot, 2004; Jakoby et al., 2004); however, no direct binding of FIT to FRO2 and IRT1 promoters has been reported to date. Interestingly, FIT is required for the IRT1 protein to accumulate under iron deficiency (Colangelo and Guerinot, 2004). Consistent with reduced IRT1 and FRO2 levels overall, the fit loss-of-function mutant is severely chlorotic, and dies unless supplemented with iron (Colangelo and Guerinot, 2004; Jakoby et al., 2004).

Hormones have been implicated in the regulation of iron-deficiency responses in different species. Exogenous applications of auxin, ethylene and ABA mimic the morphological responses to iron deficiency, including enhanced formation of root hairs and induction of transfer cells in the epidermis (Landsberg, 1986; Romera and Alcantara, 1994; Schmidt and Bartels, 1996). The existence of a link between iron-deficiency responses and ethylene has been clearly established, but whether or not the physiological response to limiting iron is mediated by ethylene is still controversial. Ethylene production was shown to increase upon iron deprivation in several dicots (Romera et al., 1999). The expression of Arabidopsis IRT1, FRO2 and FIT is repressed when plants are treated with ethylene inhibitors, and conversely, the addition of a precursor of ethylene biosynthesis, although moderately, enhances their expression (Lucena et al., 2006). However, analysis of the root ferric reductase activity in genetic backgrounds affected in ethylene perception and signaling failed to confirm these pharmacological observations (Schmidt et al., 2000).

The phytohormones cytokinins (CKs) control growth and developmental processes, such as cell proliferation, seed germination and nutrient mobilization. CKs have recently been shown to repress the responses to nitrate, phosphate and sulfate deficiencies in Arabidopsis, including the uptake of the corresponding nutrients (Brenner et al., 2005; Franco-Zorrilla et al., 2002, 2005; Maruyama-Nakashita et al., 2004). The local and long-distance dual control shared by the uptake machineries of nitrate and iron in Arabidopsis prompted us to investigate the involvement of CKs in controlling root iron uptake. Here, we show that CKs repress IRT1, FRO2 and FIT expression at the level of transcript accumulation, regardless of the plant iron status. This downregulation is independent of FIT, but requires the two cytokinin receptors CRE1/WOL/AHK4 and AHK3. Similar to what is observed for CKs, other root growth inhibitory conditions, including indole-3-acetic acid (IAA), ABA, mannitol or salt treatments, also suppress the iron starvation response genes, supporting the view that CKs are likely to control the uptake of iron and various elements through a root growth signaling cascade, in order to adapt uptake to growth reduction.
Results

Cytokinins downregulate IRT1 expression at the transcriptional level

To test whether CKs regulate the iron uptake machinery, we first analyzed the effect of CK treatment on IRT1 promoter activity. A 1-kb region located upstream of the IRT1 open reading frame had previously been shown to efficiently drive GUS expression specifically in iron-deficient roots (Vert et al., 2002), we therefore generated transgenic Arabidopsis lines expressing the luciferase (LUC) reporter gene under the control of the same region of the IRT1 promoter. Mono-insertional T-DNA homozygous lines were selected, and the inducibility of LUC activity in response to iron starvation was quantified by a classical enzymatic assay (data not shown), as well as by visualization of the imaging bioluminescence emission with a CCD camera (Figure 1). Bioluminescence analysis of IRT1-LUC transgenic plants showed a typical IRT1 expression pattern (Vert et al., 2002), with the LUC activity detected in the roots of iron-deficient plants (Figure 1a), but not in the roots of iron-sufficient plants (Figure 1b), thereby providing us with a suitable system to analyze changes in IRT1 promoter activity.

$N^6$-Benzylationine (6-BA) or zeatin (Z), representatives of the two structural groups of aromatic CKs and isoprenoid CKs were supplied at a concentration of 1 $\mu$M for 48 h, either in the absence (Figure 1c,e) or in the presence (Figure 1d,f) of iron. Such conditions triggered the inhibition of the primary root elongation (Figure 1g), and increased transcript accumulation of the root-expressed CK-responsive gene ARR4 (Figure 1h), indicating that treatment with CKs is biologically active. In iron-starved plants, treatment with 6-BA (Figure 1c) and Z (Figure 1e) abolished root bioluminescence, whereas in iron-sufficient conditions, no change in luciferase activity could be observed after treatment with CKs, in either roots or shoots (Figure 1d,f). We concluded from this experiment that CKs strongly repress the luciferase activity driven by the IRT1 promoter. Treatment of the IRT1-LUC plants with decreasing concentrations of 6-BA, from 1 $\mu$M down to 1 nM (Figure 1i), showed a progressive reduction of the 6-BA inhibitory effect, indicating that the activity of the IRT1 promoter responds to CKs in a dose-dependent manner.

To test whether CKs regulate IRT1 endogenous gene expression, we measured IRT1 mRNA accumulation in response to 6-BA using real-time quantitative RT-PCR. IRT1 mRNA accumulation was approximately sevenfold lower in 6-BA-treated plants in both iron-deficient and iron-sufficient conditions (Figure 2a). CK inhibition of IRT1 mRNA accumulation therefore appears to be independent of the iron status of the plant. Repression occurs very fast, as a 50% reduction in IRT1 transcript accumulation was observed less than 3 h after 6-BA treatment (Figure S1).

Finally, to test whether CKs could further regulate IRT1 expression by modulating its protein accumulation, we assessed the level of IRT1 protein following a 6-BA treatment by western blot using antibodies raised against Arabidopsis IRT1 (see Experimental procedures). 6-BA treatment reduced IRT1 protein accumulation in both iron-deficient and iron-sufficient conditions (Figure 2b). This reduction paralleled the decrease in transcript accumulation (Figure 2a), thus indicating that CKs do not alter IRT1 protein accumulation. To emphasize the importance of IRT1 upstream sequences in CK repression, we also investigated the effect of 6-BA on the accumulation of IRT1 transcript,
Cytokinins repress the expression of the transgene, neither at the level of mRNA accumulation, in either iron-sufficient (+Fe) or iron-deficient (−Fe) conditions (Figure 3d). Thus, it appears that only a subset of iron-regulated genes, corresponding to genes expressed in the root epidermis and involved in iron acquisition from the soil, is under the control of CKs.

IRT1 and FRO2 downregulation by cytokinins is independent of FIT1

FIT is the only transcription factor identified to date in the iron signaling pathway that leads to the expression of the root iron deficiency responsive genes, IRT1 and FRO2 (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005). To test whether FIT mediates the CK-dependent downregulation of IRT1 and FRO2, we monitored IRT1 and FRO2 mRNA accumulation in response to 6-BA in the fit-1 loss-of-function mutant. IRT1 mRNA levels showed a two-fold reduction in iron-starved fit-1 compared with wild type (Figure 4a), whereas FRO2 was dramatically affected (Figure 4c), as was previously reported by Colangelo and
In Arabidopsis, the CK signal is mediated by three plasma membrane receptors of the two-component histidine kinase family, AHK2, AHK3 and CRE1/Wol/AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). As AHK3 and CRE1/Wol/AHK4 are expressed in roots (Higuchi et al., 2004), expression of IRT1 in response to CKs was investigated in mutant alleles of these two genes. In cre1-12 and ahk3-3 (Higuchi et al., 2004), the repression mediated by 6-BA was lower than in wild-type plants (Figure 5). Because of the functional redundancy between those receptors, we investigated the influence of the combined mutations on IRT1 expression. The cre1-12 ahk3-3 double mutant was greatly impaired in the CK-dependent downregulation of IRT1 transcript accumulation (Figure 5), indicating that the repression observed upon CK treatment is to a large extent mediated by these two CK receptors.

Cytokinin mediate iron resupply induced repression of the iron uptake genes

Previously, the resupply of inorganic nitrogen sources to previously starved plants has been shown to correlate positively with the expression of IPT3, which encodes an enzyme of the CK biosynthesis pathway, and with CK accumulation (Takei et al., 2004). In the case of iron, it has been reported that resupplying iron-starved plants with iron rapidly switches off IRT1 and FRO2 (Connolly et al., 2002). To uncover a role of CKs in mediating the iron resupply response, we investigated its effect on the expression of
IRT1 and CK biosynthetic and signaling genes. Following iron resupply, IRT1 mRNA levels dropped dramatically, 50% in 4 h and 99% in 24 h (Figure 6a). At the same time, the level of IPT3 mRNA doubled in the first 4 h of iron resupply, before decreasing to level off at approximately 50% above the level in untreated plants (Figure 6b). Thus, iron resupply is likely to provoke a transient induction of CK production, and triggers the AHK3/CRE1-dependent CK signaling cascade in the root. Next, we examined the response of root CK responsive type A ARR genes that encode downstream components of the CK signaling pathway (To et al., 2004). Both ARR4 and ARR6 transcripts transiently accumulated, approximately twofold, in the first 3 h following iron resupply (Figure 6c,d), indicating that CKs and CK signaling are likely to contribute directly or indirectly to the downregulation of the iron uptake genes. However, as we have shown that CKs negatively regulate IRT1 independently of the iron supply, its effect in iron resupply is likely to be indirect.

**Root growth inhibition represses IRT1 expression**

As in the case for iron, responses to starvation of other nutrients are suppressed by CKs in the root epidermis (Martin et al., 2000; Maruyama-Nakashita et al., 2004; Sakakibara et al., 2006). Considering the effects of both CKs and nutrient availability on root growth, we hypothesized that the general downregulation of various nutrient root uptake systems, including iron, may reflect the effect of CKs on...
growth. We reasoned that if 6-BA exerts its effect by reducing the rate of root growth, other treatments affecting root growth should downregulate the iron uptake machinery. At elevated concentrations, the natural auxin IAA and the stress hormone ABA slow down primary root growth, and have antagonistic effects on the elongation of secondary roots, which is promoted by IAA and inhibited by ABA (De Smet et al., 2006; Rahman et al., 2007). We found that applying

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1 μM IAA for 2 days to iron-starved IRT1-LUC transgenic plants significantly reduced the bioluminescence of the primary root, compared with similar conditions without IAA (Figure 7a,b). As expected, IAA-treated roots developed numerous lateral roots showing a high level of LUC activity that correlates with the high meristematic activity (Figure 7b). We next exposed plants to 1 μM ABA. As a 2-day-long ABA treatment only produces slight changes in the root architecture, we verified that it efficiently triggered the induction of the ABA-responsive gene COR47 (Robert et al., 2006). ABA application reduced LUC activity in primary roots, and abolished it in lateral roots (Figure 7c,d). ABA also repressed endogenous IRT1 mRNA accumulation, both in sufficient and insufficient iron supply (Figure 7e). Therefore, IRT1 expression correlates with the modification of the root architecture in response to hormonal treatments.

To further strengthen the hypothesis that IRT1 downregulation is specified by the growth rate of the root, we examined the effect of non-hormonal supplements known to affect root growth. High osmolarity environments inhibit root and plant growth. To increase osmolarity, we supplemented the medium with either 300 mM mannitol or 150 mM NaCl. A 2-day exposure of Arabidopsis seedlings to mannitol or NaCl inhibited root growth by 75 and 85%, respectively (Figure 7g). Both treatments repressed the accumulation of IRT1 transcripts by about sixfold in iron-deficient conditions, and up to 10-fold in iron-replete conditions (Figure 7h), further supporting the idea that IRT1 expression, and thus iron demand, is determined at least in part by the growth rate of the root.

Discussion

Analysis of iron starvation responsive gene expression in roots of plants supplemented with various hormones revealed for the first time that CKs negatively regulate the root iron uptake machinery in Arabidopsis. Exogenous applications of 6-BA repressed IRT1, FRO2 and FIT mRNA accumulation, the root reductase activity and IRT1 protein levels. The inhibitory effect of CKs was observed with different CK variants, as zeatin also repressed IRT1, and this inhibition operated quickly and in a dose-dependent manner. We found CK-mediated repression to be dependent on the CK signaling cascade: repression of the adaptive genes to iron starvation was reduced in cre1 and ahk3, two CK-insensitive mutants, and was almost abolished in the double mutant cre1 ahk3 (Figure 5). Indeed, AHK3 and CRE1/Wol/AHK4 have overlapping functions in development, as well as in the control of phosphate starvation induced genes (Franco-Zorrilla et al., 2005; Nishimura et al., 2004).

CKs affect IRT1 expression at the level of its transcript accumulation, as 6-BA inhibited IRT1 mRNA accumulation, as well as the accumulation of a reporter gene mRNA transcribed from the IRT1 promoter, but did not affect the level of IRT1 transcripts driven by the constitutive CaMV 35S promoter (Figure 2c). Although the IRT1 5’ untranslated region (5’UTR) region is present in the IRT1-LUC construct and absent in the 35S-IRT1 construct (E. Connolly, personal communication), a role for the 5’UTR in mediating CK regulation can be ruled out based on deletion analyses of the IRT1 upstream regulatory sequences (M. Séguela, J.-F. Briat, G. Vert, C. Curie, unpublished data). This indicates that CK-dependent inhibition is mediated by the IRT1 promoter sequence. The repression triggered by CKs is reflected, but not enhanced, at the protein level, as IRT1 mRNA and protein levels are similarly decreased upon treatment with 6-BA. Likewise, the decrease of root iron reduction capacity strictly paralleled the decrease of FRO2 transcripts. Therefore, CKs do reduce the activity of the root iron starvation response proteins by specifically inhibiting their expression at the level of transcription. An in silico search (http://www.dna.affrc.go.jp/PLACE/) for cis-acting elements in the promoters of IRT1, FRO2 and FIT, that could be involved in CK-mediated repression, led to numerous ARR1 motifs in each promoter, which may not be of significance, considering that these motifs are 4-bp sequences.

Our data indicate that the CK-induced repression of iron starvation response genes is independent of the iron nutritional status of the plants. We found IRT1, FRO2 and FIT expression to be similarly downregulated by 6-BA in iron-sufficient and iron-deficient conditions. CKs therefore modulate the expression of IRT1, FRO2 and FIT, but do not control their iron starvation specific expression. This result is reminiscent of the high-affinity sulfate transporter genes SULTR1;1 and SULTR1;2, the expression of which were also shown to decrease in response to CKs, regardless of the sulfate growth conditions (Maruyama-Nakashita et al., 2004). In addition, the CK inhibition does not involve the transcriptional regulator FIT, as full repression of IRT1 and FRO2 by 6-BA was observed in the fit-1 mutant (Figure 4).

A previous report indicated that FIT mediates the induction of FRO2 and IRT1 specifically in response to iron deficiency (Colangelo and Guerinot, 2004). However, using the more sensitive real-time RT-PCR technique to monitor IRT1 and FRO2 transcript levels, we showed that their levels were also reduced in fit-1 grown with sufficient iron (Figure 4b,d). The influence of FIT on ferric reductase activity could not be addressed because fit-1 displayed a ferric reductase activity close to basal level, as compared with the frd1 mutant (Figure 3b). However, we found that the IRT1 protein, which was reported to be drastically less abundant in fit-1 grown in iron-deficient conditions (Colangelo and Guerinot, 2004), also accumulated several fold less in fit-1 grown in iron-replete conditions (Figure 4e, compare WT +Fe with fit-1 +Fe). Consequently, although IRT1 and FRO2
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are less expressed in fit-1 than in the wild type, they are still regulated by iron availability. We concluded that although Arabidopsis FIT is important in controlling the iron uptake machinery, it does so by contributing to the general level of gene expression, but neither mediates their CK-dependent downregulation nor controls their iron starvation induced response. The latter point is consistent with the fact that, in our experimental conditions, the iron starvation induction factor reached over 200-fold for IRT1 and FRO2, albeit that it reached less than threefold for FIT. It is likely, therefore, that the specificity of the iron regulation is in fact provided by the interaction of FIT with other transcription factors, which might confer the induction by iron deficiency to IRT1 and FRO2.

Our data establish that both CKs and FIT regulate IRT1 and FRO2; however, they act through distinct signaling pathways. The uncoupling of iron-deficiency- and CK-signaling pathways is further supported by the fact that AtNRRAMP3 and AtNRRAMP4, two iron starvation regulated genes expressed in the root central cylinder, are not repressed by exogenous 6-BA treatment (Figure 3d). Among the iron-regulated genes, therefore, CKs only regulate genes expressed in the root epidermis controlling iron entry into the plant. As CKs downregulate transporters involved in the acquisition of several macronutrients in the root epidermis (Brenner et al., 2005; Martin et al., 2000; Maruyama-Nakashita et al., 2004; Sakakibara et al., 2006), these findings are consistent with the hypothesis that CKs co-regulate these different nutrient uptake systems through their effect on growth.

CKs inhibit root growth by controlling meristem size and cell differentiation at the transition zone (Iorio et al., 2007). CK-treated plants with altered root growth are likely to limit root nutrient uptake to satisfy the decrease in the whole plant demand. Our data support this hypothesis because we found that growth inhibitory conditions, caused either by hormonal treatments (CK, IAA and ABA) or by osmotic stress (mannitol and NaCl), repressed IRT1 expression, regardless of the iron supply. The effect of growth-promoting conditions on the expression of IRT1 remains to be established. However, publicly available microarray data indicate that a treatment with high sucrose, known to promote root growth, triggers a fourfold induction of IRT1 expression (https://www.genevestigator.ethz.ch/at/). Our finding that markers of CK production correlate with the IRT1 response to iron re-supply is also consistent with a growth signaling pathway. Indeed, plants experiencing iron starvation respond by elongating their primary root to favor foraging, whereas iron re-supply has the opposite effect (data not shown). Thus, we can speculate that the transient production of CKs signals root elongation arrest, and results in a decrease in nutrient demand. Finally, a recent report indicates that the magnitude of phosphate starvation responses is determined by cell division activity (Lai et al., 2007). Growth-promoting and growth-inhibiting conditions (such as CK treatment), respec-

**Experimental procedures**

**Plant material and growth conditions**

The Arabidopsis thaliana ecotypes used in this study were all Columbia (Col-0). The 35S::I RT1 overexpressing line, frd1, cre1-12, ahk3-3 and cre1-12 ahk3-3 mutants were in the Col-0 background (Connon et al., 2002; Higuchi et al., 2004; Yi and Guerinot, 1998), and the fit-1 mutant was in the Wassilewskija background (Colangelo and Guerinot, 2004). Plants were grown in sterile conditions on vertical plates at 21°C with 16-h light/8-h dark cycles. 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 its pH was adjusted to 5.7 with KOH. Plants were cultivated in the conditions described above for 7 days (or 9 days for ABA, osmotic and salt treatments), and were then transferred to iron-sufficient (50 µM FeEDTA) or iron-deficient (300 µM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate], a strong iron chelator) medium, with or without exogenous treatment as indicated in the figure legends. All reagents were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com).

**Production of IRT1-LUC transgenic plants**

The 1011-bp-long IRT1 promoter fragment, located directly upstream of the ATG initiation codon of the IRT1 gene, was amplified with the Pfu DNA polymerase (Promega, http://www.promega. com) on genomic DNA prepared from Arabidopsis Col-0 plants. The following primers were used: OCC6, 5'-CATGCGCTGGAGAGTGGTCAGAATTGAGCG-3', and OCC133, 5'-GGGTCGACTTTGTCAATTACCTTCC-3', containing a Ncol and a SalI restriction site, respectively. The amplified fragment was cloned into the SalI and Ncol sites of a pBKS-LUC vector (Eyal et al., 1995) to create a translational fusion with the Luciferase open reading frame. The [IRT1 promoter-LUC-CaMV 35S terminator] cassette thus generated was then subcloned as a SalI-KpnI fragment into the pGreen029 vector (http://www.pgreen.ac.uk). The Agrobacterium tumefaciens MP90 strain transformed with the resulting plasmid was used to stably introduce the construct into Arabidopsis Col-0 plants by the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected on 1/2 MS containing 50 µg ml⁻¹ kanamycin. Homozygous kanamycin resistant T₃ progenies were obtained. Single T-DNA insertion plants were screened by Southern blot (data not shown).

**LUC imaging**

One millimolar luciferin (Promega) in 0.01% Triton X-100 was sprayed uniformly on vertically grown seedlings. Plants were then kept for exactly 10 min in the dark prior to imaging with a
Chemiluminescence Detection System; ICN Biochemicals Inc., http://www.bioscricomp.com/ (ICN-biochemicals.html). 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 raised in rabbits against a synthetic peptide H2N-CPANDVTLPIKEDDSS-COOH (Eurogentec, http://www.eurogentec.com), as previously described (Connolly et al., 2002; Vert et al., 2002). Next, the membrane was washed in blocking buffer three times for 15 min each, and was then incubated for 1 h with anti-rabbit IgG conjugated to alkaline phosphatase (Promega) diluted 1:20 000 in blocking buffer. Three different washes of 15 min each were performed: first in blocking buffer, second in PBS 1X and then in ‘Aurora assay buffer’. The membrane was next incubated for 5 min in the presence of ‘Aurora Chemiluminescence Substrate solution’. Chemiluminescence was revealed on KODAK BioMax XAR film (http://www.kodak.com).

**Root length measurements**

Root elongation was scored by measuring the position of the primary root tip on Petri dishes grown vertically, both at the time of transfer and 2 days later. Root growth was analyzed by using the optimas image analysis software (MediaCybernetics, http://www.mediacy.com).

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**Supplementary Material**

The following supplementary material is available for this article online:

**Figure S1.** Kinetics of cytokinin (CK)-mediated inhibition of IRT1 mRNA accumulation.

**Figure S2.** Cytokinin do not affect the expression of IRT1 when driven by the Cauliflower mosaic virus (CaMV) 35S promoter.

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