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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, abscissic 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

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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.*, 1999; 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.

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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-Benzyladenine (6-BA) or zeatin (Z), representatives of the two structural groups of aromatic CKs and isoprenoid CKs were supplied at a concentration of 1 μM 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 μM 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).

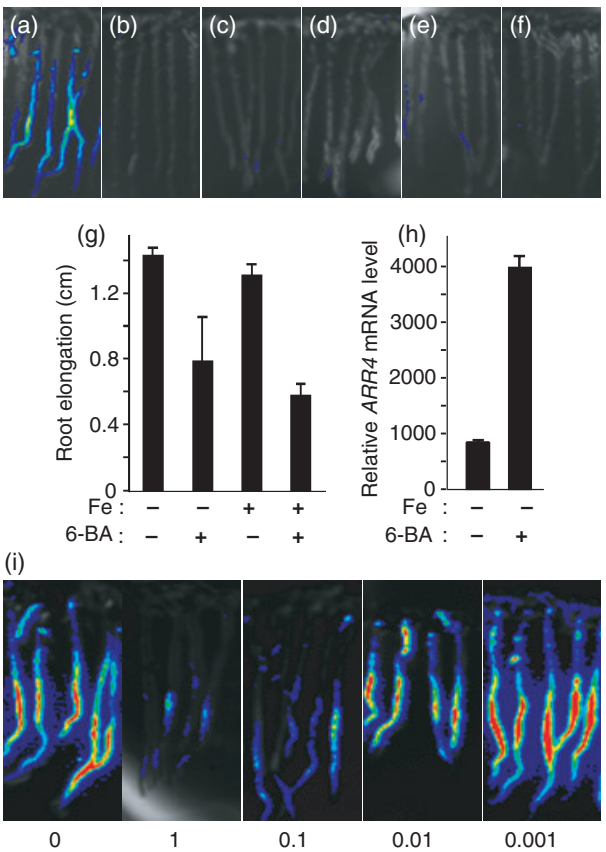


Figure 1. Effects of cytokinins on *IRT1-LUC* expression.
(a–f) LUC bioluminescence imaging of *IRT1-LUC* plants grown for 7 days on iron-sufficient medium (50 μM), and then transferred for 2 days on iron-deficient (300 μM ferrozine, panels a, c, e) or iron-sufficient (50 μM Fe-EDTA, panels b, d, f) medium supplemented or not with plant hormones. (a, b): Control, no hormone; (c, d): 1 μM N^6-benzyladenine (6-BA); (e, f): 1 μM Zeatin. Plants were sprayed with 1 mM luciferin prior to imaging.
(g) Primary root elongation during the 2-day-treatment.
(h) *ARR4* mRNA accumulation in response to 6-BA in iron-deficient conditions. Transcript accumulation was determined by real-time RT-PCR on total RNA extracted from the roots of plants cultivated as described in (c and d).
(i) Dose-dependent inhibition of LUC activity in response to decreasing 6-BA concentrations. Numbers refer to the 6-BA concentration in μM.

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,

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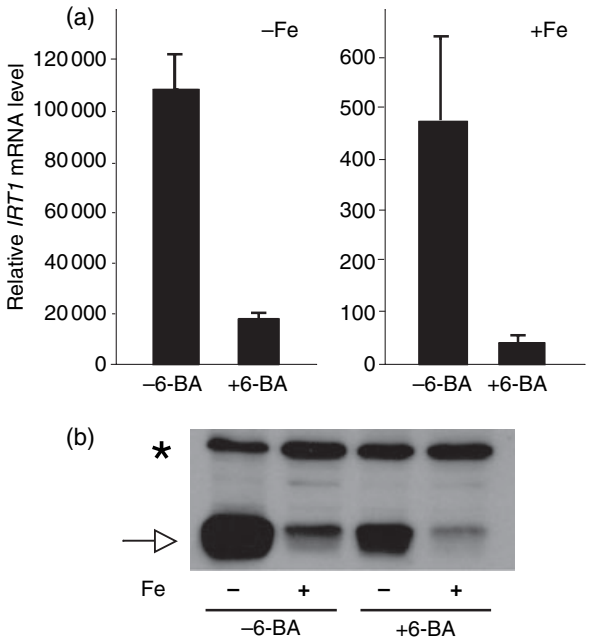


Figure 2. Cytokinins repress *IRT1* at the level of transcript accumulation.
(a) *IRT1* mRNA accumulation in response to cytokinin in iron-deficient and iron-sufficient conditions. Plants were grown for 7 days on iron-sufficient medium (50 μM), and were then transferred to iron-deficient (300 μM ferrozine) or iron-sufficient medium supplemented or not with 1 μM N^6-benzyladenine (6-BA). The transcript level was determined by real-time RT-PCR in roots harvested 2 days after transfer.
(b) IRT1 protein accumulation in response to cytokinin in iron-deficient and iron-sufficient conditions. Plants were grown as described in (a). Roots were harvested 2 days after transfer. IRT1 protein was detected using an IRT1 affinity purified peptide antibody. The asterisk labels a non-specific signal that is used as a loading control. The IRT1 protein signal is indicated by an arrow.

and IRT1 protein, in an Arabidopsis transgenic line expressing *IRT1* coding sequence, under the control of the Cauliflower mosaic virus (CaMV) *35S* promoter (Connolly *et al.*, 2002). This experiment indicates that 6-BA does not significantly modify the expression of the transgene, neither at the level of mRNA (Figure S2a) nor at the level of the protein (Figure S2b) accumulation, which is consistent with the CKs control being mediated by *IRT1* upstream sequences.

Cytokinins repress genes of the root high-affinity uptake machinery

In addition to *IRT1*, known components of the root iron uptake machinery include *FRO2*, encoding a ferric-chelate reductase, and *FIT*, encoding a bHLH transcription factor. These genes are coordinately upregulated by iron deficiency in roots (Colangelo and Guerinot, 2004; Connolly *et al.*, 2003; Vert *et al.*, 2003). We have tested whether CKs also repress the expression of *FRO2* and *FIT* by monitoring the effect of the addition of 6-BA on transcript accumulation in roots. Upon 6-BA exposure, the *FRO2* mRNA level decreased by about ninefold compared with untreated plants, in both iron-deficient and iron-sufficient conditions (Figure 3a), which is similar to the effect observed for *IRT1* (Figure 2a). We next assayed ferric-chelate reductase activity in response to CKs (Figure 3c). When challenged with 6-BA, iron-starved roots show a threefold decrease in ferric-chelate reductase activity. As the reduction of ferric iron is a rate-limiting step during iron uptake (Yi and Guerinot, 1996), CKs clearly affect the plant response to iron deficiency. In iron-replete conditions, the rate of iron reduction reached basal level, as observed with the activity of the *FRO2* null mutant *frd1* (Figure 3c), preventing us to conclude on the ability of CKs to repress the activity of the root ferric-chelate reductase. Similar to *IRT1* and *FRO2* transcripts, *FIT* mRNA accumulation was decreased in response to CKs in both iron-deficient and iron-sufficient conditions (Figure 3b). These results show that *IRT1*, *FRO2* and *FIT*, which encode essential components of the root iron uptake machinery, are similarly downregulated by CKs, irrespective of the iron status of the plant.

Cytokinins specifically control the iron-regulated genes involved in uptake from the soil

To test whether other iron-regulated genes are similarly regulated, we investigated the effect of exogenous CK application on the expression of two well-characterized iron starvation induced genes, At*NRAMP3* and At*NRAMP4*. Both genes encode iron-regulated metal transporters in vascular tissues that mobilize iron stores from the vacuole to the cytoplasm in response to iron deficiency (Lanquar *et al.*, 2005). Although At*NRAMP3* and At*NRAMP4* were upregulated by 4- and 2.5-fold, respectively, by iron deficiency, exogenous application of 6-BA did not affect their transcript 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 twofold 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

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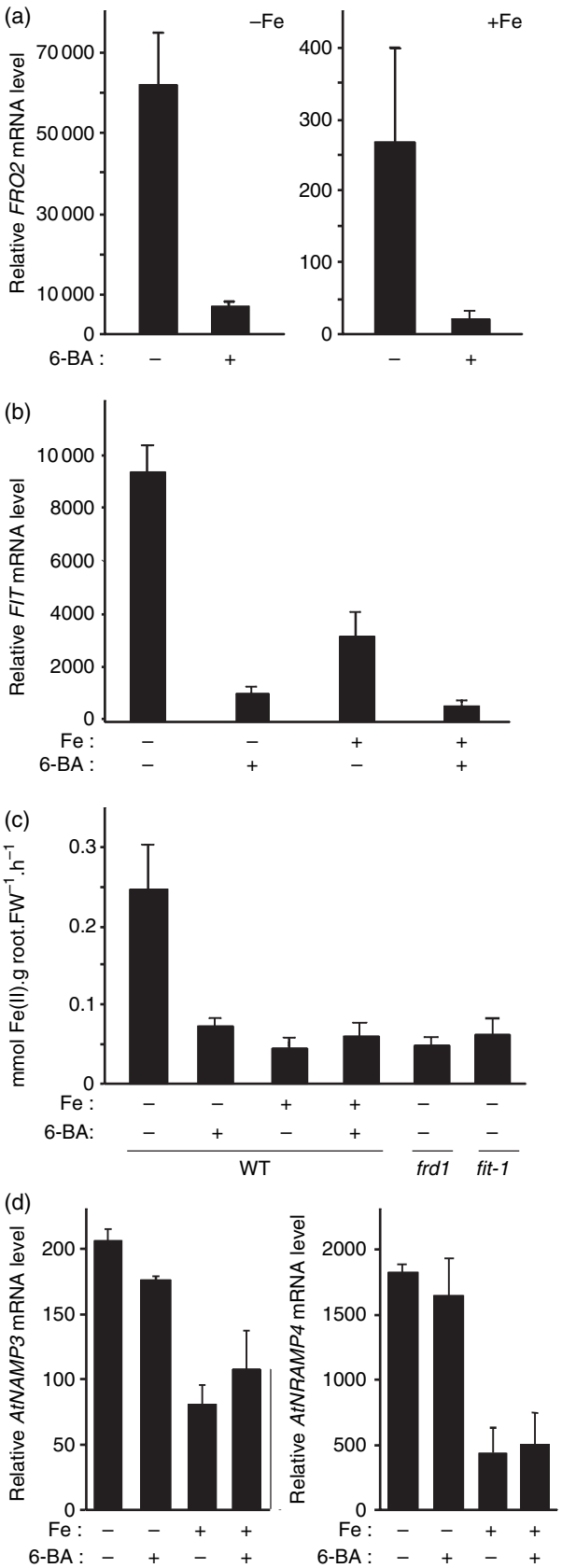


Figure 3. Differential control of iron starvation induced genes by exogenous application of cytokinins (CKs).
(a–c) *FRO2* and *FIT* expression and root ferric reductase activity are repressed by N^6-benzyladenine (6-BA). Iron-replete plants (7-days old) were transferred for 2 days on iron-deficient (300 μM ferrozine, –Fe) or iron-sufficient (50 μM, +Fe) medium supplemented or not with 1 μM 6-BA, as indicated. *FRO2* (a) and *FIT* (b) transcript levels, assayed by real-time RT-PCR, and root ferric-chelate reductase activity (c), were determined from roots.
(d) *AtNRAMP3* and *AtNRAMP4* expression in roots is not regulated by exogenous application of CKs. Transcript accumulation was determined by real-time RT-PCR on the roots of plants grown as described in (a–c).

Guerinot (2004). In *fit-1*, however, both *IRT1* and *FRO2* mRNA levels were strongly decreased by CKs in the two iron regimes (Figure 4a–d), indicating that FIT is not required for CK-mediated repression of *IRT1*. Immunoblot analysis showed that IRT1 protein accumulation, which was greatly reduced in *fit-1*, became undetectable upon CK treatment (Figure 4e), confirming that FIT is not required for CK-dependent repression. These results reveal that CKs act independently of the FIT transcription factor to negatively regulate *IRT1* and *FRO2* expression.

CRE1/Wol/AHK4 *and* AHK3 *cytokinin receptors mediate* IRT1 *downregulation by cytokinins*

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.

Cytokinins 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

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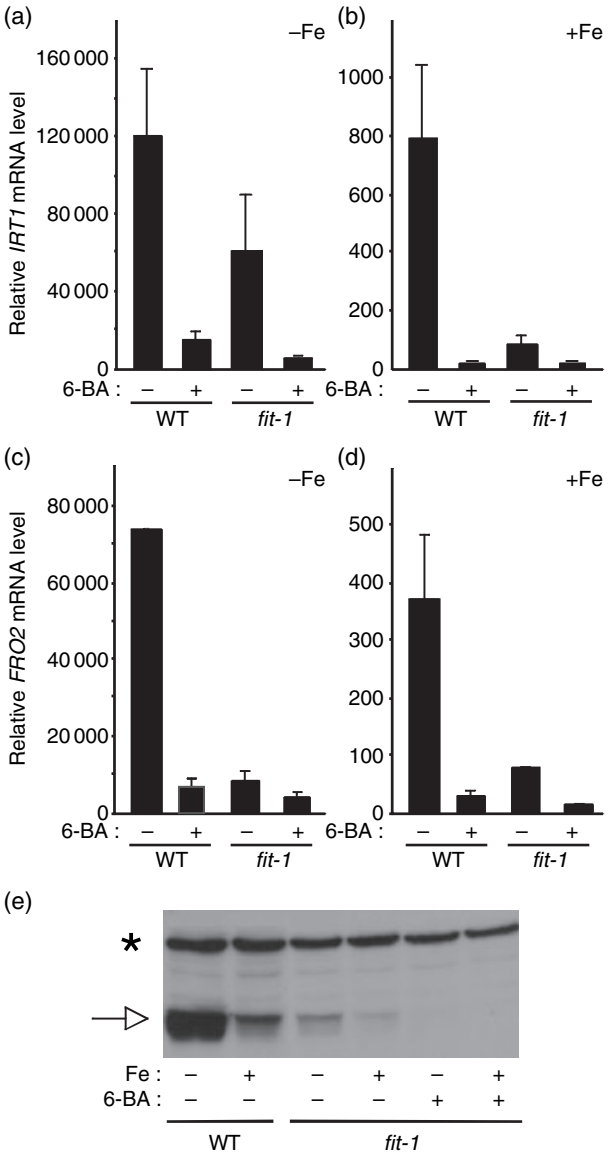


Figure 4. *IRT1* and *FRO2* downregulation in response to cytokinins is independent of *FIT1*.
(a–d) Effect of cytokinin on *IRT1* (a, b) and *FRO2* (c, d) mRNA accumulation in wild-type (WT) and *fit-1* plants. Plants were grown for 7 days on iron-sufficient medium (50 μM), and were then transferred for 2 days on iron-deficient (300 μM ferrozine, a and c) or iron-sufficient (b and d) medium supplemented or not with 1 μM N^6-benzyladenine (6-BA), as indicated. The level of root transcript was determined by real-time RT-PCR.
(e) IRT1 protein accumulation in response to cytokinins in iron-deficient and iron-sufficient conditions in wild-type and *fit-1* plants. The plants were grown as described for (a–d). IRT1 protein was detected using an IRT1 affinity purified peptide antibody on total protein extract of roots harvested 2 days after transfer. The asterisk labels a non-specific signal that is used as a loading control. The IRT1 protein signal is indicated by an arrow.

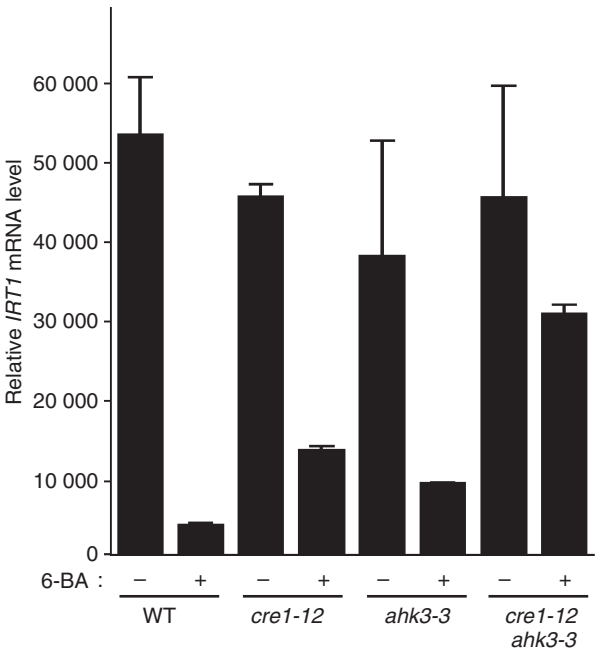


Figure 5. Repression of *IRT1* expression by cytokinins (CKs) is inhibited in CK receptors mutants.
Wild-type, *cre1-12*, *ahk3-3* and *cre1-12 ahk3-3* plants were grown for 7 days on iron-sufficient medium (50 μM), and were then transferred for 2 days on iron-deficient medium (300 μM ferrozine) supplemented or not with 1 μM N^6-benzyladenine (6-BA), as indicated. The *IRT1* transcript level was determined in roots by real-time RT-PCR.

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

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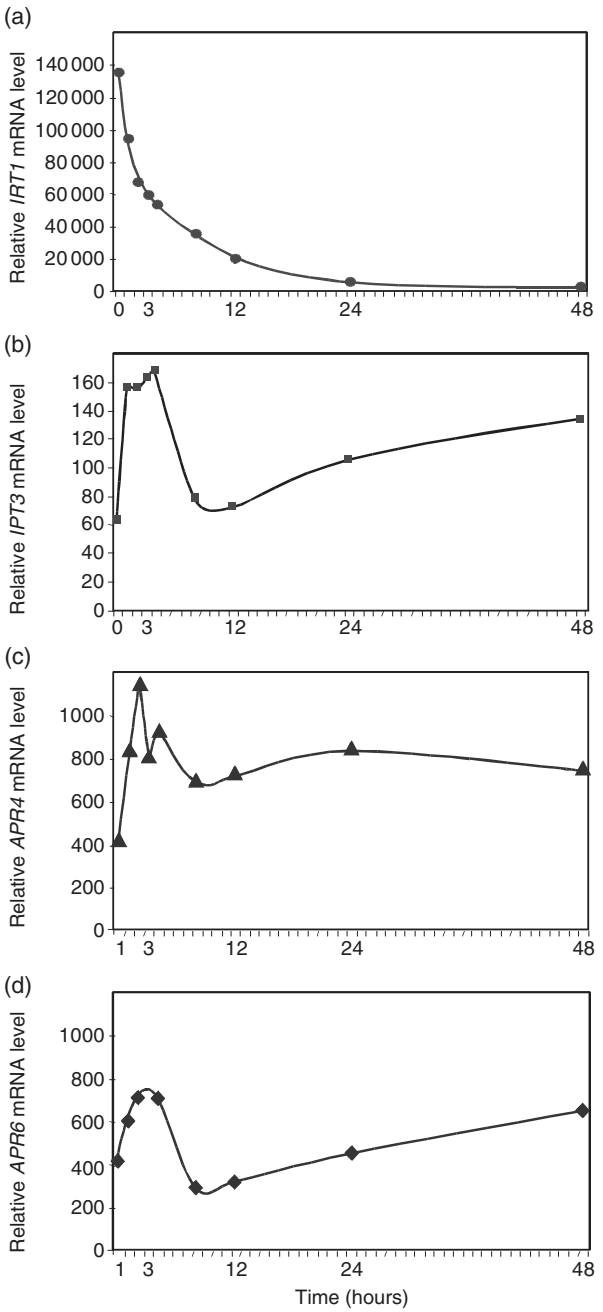


Figure 6. Role of cytokinins in the iron re-supply promoted repression of the iron starvation responsive genes.
The kinetics of mRNA accumulation of *IRT1* (a), *IPT3* (b), *ARR4* (c) and *ARR6* (d) in roots following an iron re-supply was determined using real-time RT-PCR on plants grown for 7 days in standard conditions, which were then transferred for 2 days on iron-deficient (300 μM ferrozine) medium to induce expression of *IRT1*, and were then transferred back to 100 μM iron-containing medium.

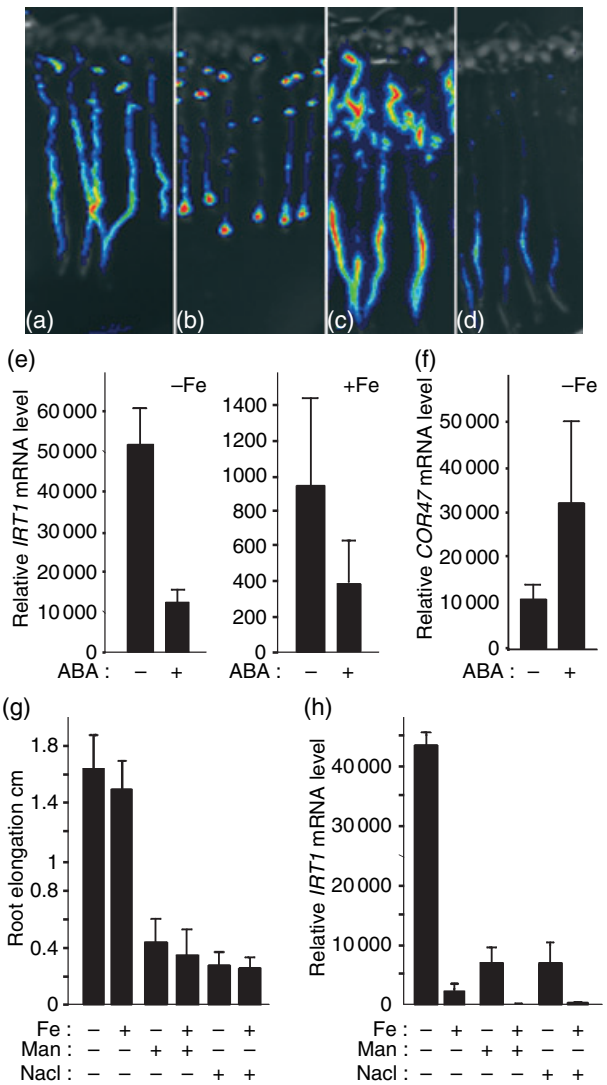


Figure 7. *IRT1* downregulation by root growth inhibitors.
(a–d) LUC activity in *IRT1-LUC* transgenic plants exposed to indole-3-acetic acid (IAA) and ABA. Plants were cultivated for 7 days (IAA) or 9 days (ABA) in iron-sufficient conditions (50 μM), and were then transferred for 2 days to iron-deficient (300 μM ferrozine) medium supplemented either with 1 μM IAA (b) or 1 μM ABA (d).
(e, f) *IRT1* and *COR47* mRNA accumulation in response to ABA. The transcript levels were determined by real-time RT-PCR in roots (e) or entire plants (f).
(g, h) Effects of mannitol and NaCl on *IRT1* expression. Plants were grown for 9 days on iron-sufficient medium (50 μM) and were then transferred for 2 days to iron-deficient (300 μM ferrozine) or iron-sufficient medium supplemented or not with 300 mM mannitol or 150 mM NaCl, as indicated.
(g) Primary root elongation of plants exposed to mannitol or NaCl, scored by measuring the position of root tips (n = 30) at the time of transfer and 2 days later on vertically-grown plants.
(h) Relative *IRT1* transcript level in roots grown as described in (g), determined by real-time RT-PCR.

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 (Deak and Malamy, 2005). 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. Séguéla, 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 *FIT1*, 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 *AtNRAMP3* and *AtNRAMP4*, 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 (Ioio *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), respectively, induce and reduce expression of phosphate (Pi) starvation responsive genes. This study and ours provide evidence that CKs influence nutrient uptake by affecting the rate of growth. Iron is the first micronutrient reported to be under the control of CKs. Although our knowledge of the coordinate regulation of macro- and micronutrient acquisition and distribution is still limited, CKs represent a good candidate for integrating the various nutrient uptake systems to plant growth.

Experimental procedures

Plant material and growth conditions

The *Arabidopsis thaliana* ecotypes used in this study were all Columbia (Col-0). The 35S::IRT1 overexpressing line, *frd1*, *cre1-12*, *ahk3-3* and *cre1-12 ahk3-3* mutants were in the Col-0 background (Connolly *et al.*, 2002; Higuchi *et al.*, 2004; Yi and Guerinot, 1996), and the *fit-1* mutant was in the Wassileskija 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′-CATGCCATGGGAAGTG-CTGAATTTGAAGCC-3′, and OCC133, 5′-GGGTCGACTATTTGT-CATTATCCTTTCCC-3′, containing a *Nco*I and a *Sal*I restriction site, respectively. The amplified fragment was cloned into the *Sal*I and *Nco*I 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 *Sal*I-*Kpn*I 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 T_3 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

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Table 1 Primers used for real-time RT-PCR

*IRT1*for	5′-CGGTTGGACTTCTAAATGC-3′
*IRT1*rev	5′-CGATAATCGACATTCCACCG-3′
*FRO2*for	5′-GCGACTTGTAGTGCGGCTATG-3′
*FRO2*rev	5′-CGTTGCACGAGCGATTCTG-3′
*FIT1*for	5′-GGAGAAGGTGTTGCTCCATCTC-3′
*FIT1*rev	5′-GTCTCGAATTTGAACGGATTGG-3′
*AtNramp3*for	5′-ACAATGGGAGTCTCATTCGC-3′
*AtNramp3*rev	5′-ATGCAACCCACAACTCCAAC-3′
*AtNramp4*for	5′-CTTGGATGTTTGGTCAGACC-3′
*AtNramp4*rev	5′-TCGACTTCTCCTGGATTGCAC-3′
*ARR4*for	5′-CTGTATGCCTGGAATGACT-3′
*ARR4*rev	5′-AATAAGAAATCTTGAGCACCT-3′
*ARR6*for	5′-GTCTACCCTGTTCACTCG-3′
*ARR6*rev	5′-AGAATCATCAGTGTAGGCT-3′
*IPT3*for	5′-CATGGCGAATCTCTCCATTGA-3′
*IPT3*rev	5′-AGTTGGAACCTCCAACGATGA-3′
*COR47*for	5′-ACACCACGACAAGACAGCAG-3′
*COR47*rev	5′-CCAAAATCCCCTTCTTCTCC-3′

Hamamatsu C4880 high-performance CCD camera (Hamamatsu Photonics K.K., http://www.hamamatsu.com). Image acquisition and processing were performed using the WINVIEW software provided by the camera manufacturer. Exposure lasted for 5 min.

RNA extraction and real-time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen, http://www.invitrogen.com) as described by the manufacturer. A DNAse (Promega) treatment was performed to avoid genomic DNA contamination. The integrity of DNA-free RNA was verified on agarose gel electrophoresis, and equal quantities (3 μg) were used for reverse transcription (MMLV-RT; Promega) with anchored oligo(dT_{23}). Real-time PCR was performed with LightCycler® Fast-Start DNA Master^{PLUS} SYBER GREEN I (Roche, http://www.roche.com) using the gene-specific primers listed in Table 1. 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 *Clathrin* gene (At4g24550), as follows: $RTL = 1000 \times 2^{-\Delta C_t}$. The change in cycle threshold (ΔC_t) values were calculated as follows: $\Delta C_t(IRT1) = C_t(IRT1) - C_t(Clat)$. In all the figures, bars represent mean relative transcript levels and error bars represent the SE of two independent experiments.

The primers used for real-time RT-PCR are listed in Table 1.

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, 0.1% orthophenantroline and 0.1% phenylmethylsulfonyl fluoride) to tissues, followed by centrifugation at 4°C for 15 min at 14 000 ***g***. The protein concentration was estimated according to the method described by Schaffner and Weissmann (1973), using bovine serum albumin as a standard. Thirty micrograms of total proteins was separated on a 13% polyacrylamide 0.1% SDS gel and transferred onto a Hybond-P membrane (Amersham, http://www.amersham.com) by electroblotting. Membranes were blocked overnight in PBST (PBS 1X and 0.1% Tween 20), with 0.2% blocking reagent (Aurora™ Western blot Chemiluminescence Detection System; ICN Biochemicals Inc., http://www.bioscorpio.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-CONH2 (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 alcaline 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. Cytokinins do not affect the expression of *IRT1* when driven by the Cauliflower mosaic virus (CaMV) *35S* promoter.

This material is available as part of the online article from http://www.blackwell-synergy.com

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