AtCPL1 functions in the Fe deficiency response

CORRESPONDING AUTHOR:

Hisashi Koiwa

Molecular and Environmental Plant Sciences, Vegetable and Fruit Development Center, Department of Horticultural Sciences, Texas A&M University, College Station, TX-77843, USA

TEL: 979-845-5282

e-mail: koiwa@neo.tamu.edu

Research area: Environmental Stress and Adaptation
Loss of Function of *Arabidopsis* C-terminal Domain Phosphatase-like 1 (CPL1) Activates Iron Deficiency Responses at the Transcriptional Level¹

Emre Aksoy, Insil Jeong, Hisashi Koiwa*

Molecular and Environmental Plant Sciences (E.A., H.K.) Vegetable and Fruit Development Center (E.A., H.K.), Department of Horticultural Sciences (E.A., I.J., H.K.) Texas A&M University, College Station, TX-77843, USA
FOOTNOTES

1 This work was in part financially supported by NSF grant MCB 0950459 and USDA-CSREES “Designing food for health” grants 2008-34402-19195, 2009-34402-19831, and 2010-34402-20875.

*Corresponding author: Hisashi Koiwa (koiwa@neo.tamu.edu)
ABSTRACT

The expression of genes that control iron (Fe) uptake and distribution (i.e., Fe utilization-related genes) is tightly regulated. Fe deficiency strongly induces Fe utilization-related gene expression; however, little is known about the mechanisms that regulate this response in plants. Transcriptome analysis of an Arabidopsis thaliana mutant defective in RNA polymerase II CTD-phosphatase-like 1 (CPL1) revealed significant up-regulation of Fe utilization-related genes, e.g., IRON-REGULATED TRANSPORTER 1, suggesting the importance of RNA metabolism in Fe signaling. An analysis using multiple cpl1 alleles established that cpl1 mutations enhanced specific transcriptional responses to low Fe availability. Changes in protein level were less prominent than those in transcript level, indicating that cpl1-2 mainly affects the Fe deficiency response at the transcriptional level. However, Fe content was significantly increased in the roots and decreased in the shoots of cpl1-2 plants, indicating that the cpl1 mutations do indeed affect Fe homeostasis. Furthermore, root growth of cpl1-2 showed improved tolerance to Fe deficiency and cadmium (Cd) toxicity. cpl1-2 plants accumulated more Cd in the shoots, suggesting that Cd toxicity in the roots of this mutant is averted by the transport of excess Cd to the shoots. Genetic data indicate that cpl1-2 likely activates Fe deficiency responses upstream of both FE-DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT)-dependent and -independent signaling pathways. Interestingly, various osmotic stress/ABA-inducible genes were up-regulated in cpl1-2, and the expression of some ABA-inducible genes was controlled by Fe availability. We propose that the cpl1 mutations enhance Fe deficiency signaling and promote crosstalk with a branch of the osmotic stress/ABA signaling pathway.
INTRODUCTION

Iron (Fe) is an essential metal element for nearly all organisms. In plants, Fe is present as a cofactor in many metallo-proteins and is found in the active sites of photosynthetic and respiratory Fe-S clusters. Fe is also required for RNA and hormone biosynthesis, nitrogen fixation, sulfate assimilation, and chlorophyll biosynthesis (Broadley et al., 2012). On the other hand, Fe is highly reactive and excess Fe can produce reactive oxygen species via Fenton reactions (Moller et al., 2007). In soil, the predominant form of Fe is Fe(III), which is abundantly present as insoluble ferric oxides and ferric hydroxides in aerobic environments (Guerinot and Yi, 1994; Palmer and Guerinot, 2009). The solubility of Fe(III) in aqueous solution is an order of magnitude less than the Fe concentration needed for survival; therefore, a large portion of agricultural land is Fe deficient (Guerinot and Yi, 1994). Fe deficiency in plants induces intercostal/interveinal leaf chlorosis due to limited chlorophyll biosynthesis and results in significant yield loss of crops.

Plants have developed two distinct mechanisms, i.e., strategy I (reduction strategy) and II (chelation strategy), to mobilize insoluble Fe(III) in the rhizosphere and transport it through the plasma membrane (PM) (Romheld, 1987; Welch, 1995; Schmidt, 1999; Gross et al., 2003; Groz and Guerinot, 2006; Puig et al., 2007; Conte and Walker, 2011; Schmidt and Buckhout, 2011; Ivanov et al., 2012; White, 2012). Arabidopsis thaliana and other dicots rely on strategy I. In this strategy, the rhizosphere is first acidified by a PM-localized H+-ATPase, AHA2 (Santi and Schmidt, 2009). Then, FERRIC CHELATE REDUCTASE 2 (FRO2) reduces Fe(III) to soluble Fe(II) (Yi and Guerinot, 1996; Robinson et al., 1999). Finally, the reduced Fe is taken up by a high-affinity transporter, IRON-REGULATED TRANSPORTER 1 (IRT1) (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002), which is strongly expressed in root epidermal cells and is localized to the PM (Eide et al., 1996; Vert et al., 2002). Once the Fe(II) is absorbed, various transporters and chemicals mobilize it. Fe(II) can be chelated by nicotianamine and transported intercellularly by YELLOW STRIPE-LIKE (YSL) transporters (DiDonato et al., 2004; Waters et al., 2006; Waters and Grusak, 2008; Chu et al., 2010). In the vasculature, citrate, which is exuded by a FERRIC REDUCTASE DEFECTIVE 3 (FRD3) transporter (Durrett et al., 2007), forms a tri-Fe(III), tri-citrate complex for long-distance transport (Rogers and Guerinot, 2002; Green and...
Rogers, 2004; Rellan-Alvarez et al., 2010). In addition, OLIGOPEPTIDE TRANSPORTER 3 (OPT3) may also function as a transporter of Fe-chelates (Wintz et al., 2003; Stacey et al., 2008). In contrast, excess Fe is sequestered in plastids by Fe-binding proteins named ferritins (Waldo et al., 1995). Here, we collectively refer to genes that are involved in Fe uptake, mobilization, and signaling, as “Fe utilization-related genes”, according to Kobayashi et al. (2009).

The expression level of genes encoding components of the Fe acquisition mechanism, such as *FRO2* and *IRT1*, are under transcriptional regulation in Fe-limited conditions, as their mRNA levels increase in response to Fe deficiency (Eide et al., 1996; Robinson et al., 1999; Connolly et al., 2002; Vert et al., 2002). Central to this regulation are basic helix-loop-helix (bHLH) transcription factors, FER in tomato (Ling et al., 2002) and FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) in Arabidopsis (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Yuan et al., 2005; Bauer et al., 2007). In Arabidopsis, FIT regulates various Fe utilization-related genes, including *IRT1, FRO2, and NICOTINAMINE SYNTHASE 1 (NAS1)* (Colangelo and Guerinot, 2004; Jakoby et al., 2004). FIT dimerizes with group-Ib bHLH family transcription factors, bHLH38 or bHLH39, and directly activates the transcription of *IRT1* and *FRO2* (Yuan et al., 2008). While the induction of *FIT* expression during the Fe deficiency response is moderate (Bauer et al., 2004; Colangelo and Guerinot, 2004; Jakoby et al., 2004), *bHLH38, bHLH39, bHLH100, and bHLH101* are strongly up-regulated (Wang et al., 2007). It has been proposed that dimerization of FIT with different bHLHs determines the target specificity of Fe deficiency-induced transcriptional activation (Yuan et al., 2008). To date, little is known about the upstream mechanisms that sense cellular Fe levels and regulate the early signals that lead to the expression of *FIT* and group Ib *bHLH* genes. In addition to the central pathway, a recent study reported the existence of a cell-specific regulatory mechanism mediated by the bHLH transcription factors *POPEYE (PYE)* and *IAA-LEUCINE RESISTANT 3 (ILR3)*, and the E3 ligase *BRUTUS (BTS)* that operates in the root pericycle Fe response and controls root development (Long et al., 2010). Repression of *FERRITIN1 (FER1)* genes in the absence of excess Fe is mediated by the *cis*-element IDRS (iron-dependent regulatory sequence) (Petit et al., 2001). These regulators control specific branches of Fe deficiency signaling; however, the mechanisms that sense Fe availability and fine-tune the signal throughput of individual pathways have yet to be determined.
A wide range of physiological and molecular signals influence Fe signaling in Arabidopsis. Ethylene regulates the expression of *IRT1*, *FRO2*, and *FIT*, perhaps via the activity of EIN3 and EIL1, which stabilize FIT (Lingam et al., 2011). In contrast, cytokinin and ABA repress the expression of *IRT1*, *FRO2*, and *FIT* (Seguela et al., 2008). Environmental stresses, such as salt/osmotic stress, attenuate Fe deficiency responses (Seguela et al., 2008), whereas phosphate starvation triggers the Fe deficiency responses (Hirsch et al., 2006; Thibaud et al., 2010). Moreover, volatiles emitted by soil microbes can up-regulate the expression of Fe utilization-related genes (Zhang et al., 2009). The molecular mechanisms that facilitate multiple inputs into Fe signaling have not been determined; however, the plasticity of Fe signaling implies the presence of diverse regulatory components that are coordinated to achieve proper levels of cellular Fe.

Among the various genes that affect the expression of Fe utilization-related genes, *TRIDENT (TDT)* is unique as it encodes a subunit of an RNA decapping enzyme that is involved in RNA metabolism (Goeres et al., 2007). The RNA metabolic pathway is a major mechanism in the co-/post-transcriptional regulation of diverse developmental and environmental responses (Kuhn and Schroeder, 2003; Gregory et al., 2008; Nakaminami et al., 2012). While ABA signaling is a prototypical target of RNA metabolism-mediated regulation, many transcripts are regulated by small RNAs, such as miRNAs and siRNAs (Ramachandran and Chen, 2008). The *tdt* mutation has been shown to repress the expression of Fe utilization genes; however, whether this is due to severe growth defects in the mutant or to specific Fe signaling defects has not been elucidated (Goeres et al., 2007).

Here, we identify an isoform of RNA metabolism regulators, CPL1 [RNA polymerase II C-terminal domain (pol II CTD) phosphatase-like 1], as a novel regulator of the Fe deficiency responses. CPL family proteins are known to dephosphorylate pol II CTD (Koiwa et al., 2004), which is the regulatory domain of pol II. Of the more than 20 CPL isoforms present in the *Arabidopsis* genome, CPL1 is a negative regulator of stress-responsive gene expression under various osmotic stresses (Koiwa et al., 2002; Xiong et al., 2002). In this report, we determined that CPL1 also functions in the plant Fe deficiency response. Microarray analysis of the *cpl1* transcriptome found that both the osmotic stress/ABA response and the Fe deficiency response are activated in *cpl1*. *cpl1* mutant plants exhibited various hallmarks of the Fe deficiency response, such as
altered metal profiles, and increased tolerance to Fe deficiency and cadmium toxicity. These results suggest that CPL1 is a previously uncharacterized regulator of the Fe deficiency response. In addition, our data suggest that a subset of ABA/osmotic stress-induced genes are coregulated by Fe deficiency signals and are targets of CPL1 regulation.

RESULTS

The identification of genes uniquely regulated in cpl1

Arabidopsis CPL1 was previously shown to regulate gene expression under osmotic stress conditions through ABA signaling (Koiwa et al., 2002; Xiong et al., 2002). To obtain insight into the regulatory networks modulated by CPL1, the global transcript profile of cpl1-2 was determined using an Affymetrix ATH1 Gene Chip. To obtain unbiased gene expression profiles, cRNA probes were prepared from total RNA extracted from unstressed young (10-day-old) seedlings of the wild-type C24 or cpl1-2 lines grown on medium containing 1/4 x MS salts, 0.5% sucrose, and 0.8% agar. After probe-level data normalization using the Robust Multiarray Analysis (RMA) algorithm (Supplemental Fig. S1A), hybridization intensity data for 19597 out of 22810 probes, which were above the 20th percentile in at least one out of six hybridizations, were selected for further analysis. Next, consistency between the C24 and cpl1-2 replicate datasets was confirmed by Principal Component Analysis (PCA). Triplicate datasets of each genotype clustered together in a three-dimensional scatter plot (Supplemental Fig. S1), whereas there was a clear separation of the C24 and cpl1-2 datasets. This is indicative of data consistency among replicate datasets for each genotype, as well as of genotype differentiation. Differentially expressed genes were then determined by evaluating fold change (≥2 was considered as the cut-off) and one-way ANOVA (p <0.05 was considered significant), followed by the Tukey HSD (honestly significant difference) test and Benjamin Hochberg multiple corrections. A total of 114 and 132 genes were significantly up- and down-regulated by more than 2-fold, respectively, in the cpl1-2 mutant datasets relative to those of the control (Supplemental dataset). To confirm the microarray data, the expression of 41 up-regulated and 22 down-regulated genes was determined by RT-qPCR analysis. The up-regulation of 36 genes and down-regulation of 15 genes was confirmed (i.e., 81% of the genes tested by RT-qPCR), indicating that
the microarray results were generally reliable (Supplemental dataset). Genes that could not be confirmed as being differentially expressed were excluded in subsequent analyses. We refer to the resulting 109 genes that were up-regulated in cpl1-2 as CUTs (cpl1-Up Transcripts).

**CUTs encode ABA/Osmotic Stress- and Fe deficiency-responsive genes**

To determine the signaling pathway in which CPL1 is involved, we screened gene expression data in the AtGenExpress and Gene Expression Omnibus databases using the list of CUTs. The CUT expression profiles of four abiotic stress/ABA treatments showed overlap with that of cpl1-2, and were subjected to hierarchical clustering using Pearson correlation and the average linkage rule. Clustering results showed that no single treatment profile displayed overall similarity with the cpl1-2 profile; however, two clusters of genes were similarly up-regulated in cpl1-2 and in plants exposed to abiotic stresses and ABA (Fig. 1). Genes in cluster I (27 genes) were up-regulated by hyperosmotic stress and ABA. Cluster II contains 16 genes that were strongly induced under Fe deficiency stress, including At4g19690 (IRT1), a major Fe transporter. The differential regulation of clusters I and II by abiotic stresses was confirmed by Gene Set Enrichment Analysis (GSEA) using GeneTrail (Backes et al., 2007; Schuler et al., 2011). As shown in Supplemental Table S1, the genes in cluster I were significantly enriched by osmotic stress and ABA ($p<0.05$), but not by Fe deficiency. Conversely, those in cluster II were enriched only by Fe deficiency ($p<0.05$).

To obtain deeper insight into the regulation of CUTs by abiotic stresses, we examined the expression level of CUTs in public microarray datasets designed to analyze responses to Fe deficiency (Colangelo and Guerinot, 2004; Dinneny et al., 2008; Buckhout et al., 2009; Long et al., 2010; Yang et al., 2010; Schuler et al., 2011; Ivanov et al., 2012), ABA treatment (Seki et al., 2002; Nishimura et al., 2007; Goda et al., 2008; Mizoguchi et al., 2010), and osmotic stress treatment (mannitol or drought) (Kreps et al., 2002; Seki et al., 2002; Li et al., 2006; Kilian et al., 2007). Affymetrix CEL files for each experiment were processed as described above, and transcripts significantly up-regulated by 2 fold or more ($p<0.05$) were identified for each microarray experiment. Genes up-regulated in both cpl1-2 and in wild-type plants subjected to these abiotic stress treatments were identified and grouped (Fig. 2; Supplemental Table S2). In addition, the Fe-regulated genes described by Ivanov et al. (Ivanov et al., 2012)
were examined for overlaps. We identified 26 and 59 CUTs that were up-regulated in the Fe deficiency and osmotic stress datasets, respectively (p<0.05 by GSEA, Supplemental Table S3). Of these, 10 CUTs were regulated both in Fe and osmotic stress datasets. Some of the osmotically regulated CUTs, including nine CUTs regulated by Fe deficiency, were also up-regulated in ABA datasets, implying that ABA signaling plays a role in the dual regulation of some CUTs by osmotic stress and Fe deficiency. The remaining 27 CUTs were not up-regulated in either the Fe deficiency or osmotic stress experiments.

The IRT1 transcript showed the highest level of up-regulation (54.6-fold) in cpl1-2 microarray datasets, even though the plants used in the analysis were grown under Fe-sufficient conditions. Other CUTs in Fe-stress datasets include Fe utilization-related genes, such as OLIGOPEPTIDE TRANSPORTER 3 (OPT3), NICOTIANAMINE SYNTHASE 4 (NAS4), and ISOCITRATE LYASE (ICL). To confirm that the observed gene expression changes in cpl1-2 were due to the mutation in the CPL1 locus and not to a potential second-site mutation, the expression levels of select CUTs in both cpl1-1 and cpl1-2 alleles were determined by RT-qPCR. Of 26 CUTs tested, we confirmed the significant up-regulation of 19 Fe- and/or ABA/osmotic stress-regulated CUTs in both cpl1-1 and cpl1-2 plants. In addition, the expression of five more CUTs was significantly up-regulated in at least one cpl1 allele (Table I).

CPL1 is involved in Fe stress responses

Elevated basal expression levels of Fe utilization-related genes in cpl1-2 were unexpected, since cpl1-2 was isolated based on altered osmotic stress responses, and cpl1-2 does not exhibit the typical Fe deficiency symptoms (e.g., leaf chlorosis) seen in the irt1 or fit mutants (Connolly et al., 2002; Vert et al., 2002; Colangelo and Guerinot, 2004; Jakoby et al., 2004). To establish the causal link between cpl1 mutations and enhanced Fe deficiency responses, we tested the basal expression level of Fe utilization-related genes (IRT1, FRO2, FIT, and group Ib bHLHs) in several independent cpl1 mutants. In addition to cpl1-1 and cpl1-2 in the C24 background, two T-DNA insertion lines, cpl1-5 and cpl1-6, in the Col-0 background, were tested. In cpl1-5 and cpl1-6, T-DNAs were inserted at locus 11512171 and 11514092 of chromosome 4 (Supplemental Fig. S2A, B), respectively, and production of intact CPL1 mRNA was abolished (Supplemental Fig. S2C). The basal expression of Fe utilization-related genes
was elevated in all alleles, indicating that the cpl1 mutations caused the gene expression phenotypes observed in these plants (Supplemental Table S4). The phenotype was determined to be recessive, because F1 plants produced by crossing cpl1-2 and the wild type showed a wild-type level of gene expression (Supplemental Fig. S3). To compare the involvement of different CPL paralogs in this phenotype, we tested the gene expression levels in cpl2-2 and cpl3-1. The expression levels of IRT1, FRO2, and FIT (hereafter referred to as [IRT1, FIT, FRO2]) in cpl2-2 and cpl3-1 were similar to those of the wild type (Supplemental Table S4), indicating that CPL1 is uniquely associated with regulating Fe deficiency signaling.

The above results established that the basal expression of Fe utilization-related genes was elevated in cpl1 mutants. Next, we systematically analyzed the Fe deficiency responses of cpl1-2. For this purpose, we established Fe-sufficient conditions using basal growth medium containing 1/4x MS salts (modified to 50 µM Fe-EDTA) and 0.5% sucrose (Fig. 3). Fe deficiency stress was applied by transferring plants to the same medium lacking Fe-EDTA but containing 300 µM ferrozine, which chelates Fe in the medium and makes it unavailable to the plant (Colangelo and Guerinot, 2004). An increase in Fe-EDTA from 25 µM to 50 µM moderately decreased the basal expression level of Fe utilization-related genes in cpl1-2. Upon transfer to Fe-deficient media, cpl1-2 showed more rapid and greater expression of [IRT1, FIT, FRO2] and bHLH39 than did the wild type, as early as 12 h after transfer. The expression of bHLH38, bHLH100, and bHLH101 was greater in cpl1-2 than in the wild type, but the response kinetics of these genes in the mutant were similar to those of the wild type. FER1 (FERRITIN1) was repressed in both cpl1-2 and the wild type, albeit cpl1-2 showed lower basal expression levels. Similar genotype differences were observed when 1x MS medium was used as the basal medium (Supplemental Fig. S4). Further RT-qPCR analyses confirmed the enhanced basal and/or induced expression levels of many but not all Fe-regulated/Fe utilization-related genes in cpl1-2 (Supplemental Fig. S5). Overall, the rapid and strong induction of [IRT1, FIT, FRO2] suggests that the transcriptional response of cpl1-2 is more sensitive to Fe deficiency stress. To test this hypothesis, we determined if elevated external Fe could suppress the cpl1-2 phenotype. Basal [IRT1, FIT, FRO2] expression levels were determined in plants growing on medium containing various concentrations of Fe (Fig. 4). cpl1-2 maintained higher levels of [IRT1, FIT, FRO2], with the greatest difference between genotypes being observed at 25 µM; however, higher
Fe concentrations repressed the overall expression of \([\text{IRT1, FIT, FRO2}]\) in both the wild type and in \(cpl1\)-2. On media containing more than 100 µM Fe, the wild type and \(cpl1\)-2 showed similar, low-level expression of \([\text{IRT1, FIT, FRO2}]\). Together, these results support the hypothesis that the Fe deficiency response in \(cpl1\)-2 is more sensitive than that in the wild type, but is not constitutive.

The \(cpl1\)-2 mutation affects Fe homeostasis

To determine whether elevated \(\text{IRT1}\) and \(\text{FRO2}\) transcript levels result in an increase of the corresponding proteins in the \(cpl1\)-2 mutant, we tested the levels of root \(\text{FRO2}\) activity and \(\text{IRT1}\) protein accumulation. Consistent with the transcript levels, \(\text{FRO2}\) activity was 1.7- and 2-fold higher than in the wild type in Fe-sufficient and -deficient conditions, respectively (Fig. 5A). \(\text{IRT1}\) proteins were below the level of detection under Fe-sufficient conditions in both genotypes; however, upon exposure to Fe deficiency (0 µM Fe-EDTA + 300 µM ferrozine for 3 days), \(cpl1\)-2 accumulated a slightly but significantly higher level of \(\text{IRT1}\) than did the wild type (1.5 ± 0.24 fold, \(p<0.05\)) (Fig. 5B). The small difference in \(\text{IRT1}\) protein level between the wild-type and mutant plants is consistent with the tight regulation of \(\text{IRT1}\) by ubiquitin-mediated protein turnover (Kerkeb \textit{et al.}, 2008; Barberon \textit{et al.}, 2011).

To determine the physiological consequence of the molecular changes in \(cpl1\)-2, elemental analyses were conducted for plants grown under the same conditions as described above. Roots and shoots were harvested separately and metal contents were determined by inductively coupled plasma mass spectrometry (ICP-MS). As shown in Fig. 6, in Fe-sufficient conditions, \(cpl1\)-2 accumulated 34% more Fe in the roots than did the wild type, but 14% less Fe in the shoots. This suggests that although the \(\text{IRT1}\) protein level was below our detection limit, higher basal \(\text{IRT1}\) expression likely contributes to enhanced Fe acquisition in \(cpl1\)-2. It also suggests that translocation of Fe from the roots to the shoots is impaired in \(cpl1\)-2. As reported previously, exposure to Fe-deficient medium induced the accumulation of zinc (Zn) and manganese (Mn) in wild-type plants (Baxter \textit{et al.}, 2008). Interestingly, \(cpl1\)-2 roots accumulated 17.6% and 19.2% higher levels of Zn and Mn, respectively, under Fe-deficient conditions than did wild-type roots. Since \(\text{IRT1}\) is likely responsible for increases in Zn and Mn uptake during Fe deficiency (Korshunova \textit{et al.}, 1999; Vert \textit{et al.}, 2002), the elevated \(\text{IRT1}\) transcript and protein levels in \(cpl1\)-2 are likely to be biologically relevant. In addition,
we noted that the sodium (Na) and calcium (Ca) levels in the roots of cpl1-2 plants grown under Fe-sufficient conditions were elevated by 35% and 17.2%, respectively, compared to the wild type, and that potassium (K) levels were reduced by 22%. Overall, the cpl1-2 ion profile was consistent with the transcript profile of cpl1 mutants, and was distinct from that of prototypical Fe homeostasis mutants such as frd3, which showed constitutive Fe deficiency stress responses and accumulated Zn and Mn even under Fe-sufficient conditions (Rogers and Guerinot, 2002).

To determine if distribution of Fe in roots is affected by cpl1-2 mutation, Fe in root tissues was visualized by Perls staining (Supplemental Fig. S6). Unlike previously characterized frd3-1 mutant, which accumulates Fe in vascular tissues (Green and Rogers, 2004), root Fe profile of cpl1-2 was indistinguishable from wild type C24 and stained predominantly root hairs.

The root growth response of the cpl1 mutants to Fe deficiency stress was analyzed in vitro (Fig. 7). Plants were grown for 4 days on 1/4 x MS medium containing 50 µM Fe-EDTA and for another 5 days on Fe-deficient medium. Primary root growth of the wild type was inhibited by 28±3% upon transfer to Fe-deficient medium, whereas that of cpl1-1 and cpl1-2 was inhibited by only 13±4% and 6±2%, respectively. This indicates that the cpl1 mutants are more tolerant to Fe deficiency than is the wild type. The intermediate tolerance in cpl1-1 may be due to leaky expression of functional CPL1 transcript in this allele, which contains the T-DNA insertion in the third intron (Koiwa et al., 2002).

Since cadmium (Cd) ions can enter plant cells through Fe-uptake systems (Clemens, 2006), the cpl1-2 mutation may affect plant tolerance to Cd. To test this possibility, the cpl1-2 and C24 wild type were grown for 14 days on medium containing 60 µM CdCl₂ and various concentrations of Fe. In the absence of Fe, growth of both the wild type and cpl1-2 was severely inhibited by the presence of cadmium (Fig. 8A). With increasing amounts of Fe in the medium, both genotypes showed recovery; however, cpl1-2 plants showed greater tolerance to Cd on medium containing 25 µM or 50 µM Fe-EDTA. In the presence of 50 µM Fe-EDTA, wild-type plants showed substantial tolerance to Cd, albeit the tolerance was weaker than that of cpl1-2. Root Cd contents of wild-type and cpl1-2 plants were similar for all treatments, indicating that the basis for increased Cd tolerance in cpl1-2 does not likely involve exclusion of Cd from the roots
(Fig. 8B). Surprisingly, the shoots of cpl1-2 plants accumulated 54.2-73% more Cd than did those of the wild type, suggesting that the long-distance transport of Cd was enhanced in cpl1-2 plants (Fig. 8C).

**Genetic dissection of Fe signaling perturbation in cpl1**

It has been shown that the bHLH transcription factor FIT is a central regulator of Fe deficiency signaling, and that FIT regulates its own Fe deficiency-induced expression (Colangelo and Guerinot, 2004; Jakoby et al., 2004; Wang et al., 2007). To determine whether up-regulation of Fe utilization-related genes in the cpl1 mutants is dependent on FIT activity, the expression of these genes was analyzed in the fit cpl1 double mutant. Since a well-characterized fit-2 mutant is in the Col-0 genetic background (Colangelo and Guerinot, 2004), cpl1-6 in the Col-0 background was used in this analysis. Furthermore, to quantify FIT expression in the fit mutant background, lines with a FIT-LUC reporter gene were prepared in the wild type (Col-0), cpl1-6, and fit-2 single mutants, and in the fit-2 cpl1-6 double mutant by genetic crossing (Supplemental Fig. S7).

As shown in Fig. 9, FIT-LUC and endogenous FIT expression were both enhanced in the cpl1-6 mutant. On the other hand, the basal expression level of FIT-LUC in fit-2 plants was 40% that of the wild-type level. In fit-2 cpl1-6, however, the level of FIT-LUC expression was similar to that of the wild type and lower than that of the cpl1-6 single mutant. This suggests that the cpl1-6 mutation can activate the FIT-independent signal to promote basal FIT expression. Furthermore, positive feedback by the FIT autoactivation mechanism likely amplifies FIT expression. Interestingly, Fe deficiency treatment did not induce the expression of FIT-LUC in the absence of functional FIT. This may indicate that FIT induction by Fe deficiency is entirely dependent on a FIT autoregulation mechanism. However, a FIT-independent component may be activated in the fit-2 background even during growth on Fe-sufficient medium, due to the constitutive Fe-deficient status of the mutants resulting in the lack of further activation by Fe deficiency treatment.

The expression profiles of genes downstream of FIT were similar to that of FIT-LUC. Under Fe-sufficient conditions, IRT1 and FRO2 levels were 22% and 29% lower in
the fit-2 mutant than in the wild type, respectively; however, the expression of these genes was recovered (95% and 54% of wild-type expression, respectively) in fit-2 cpl1-6 plants. IRT1 and FRO2 expression levels increased in response to Fe deficiency treatment even in the fit-2 background, indicating that a FIT-independent pathway positively regulates the expression of these genes; however, similar to FIT-LUC, FIT is required for the full induction of IRT1 and FRO2. In contrast to IRT1, FIT-LUC, FRO2, the basal and induced levels of group Ib bHLH expression were higher in all mutant lines tested, and their expression levels were largely similar among mutant lines other than bHLH38. It appears that both the enhanced Fe deficiency signals in cpl1-6 plants and the Fe-deficient status caused by the fit-2 mutation activate group Ib bHLH expression by the same FIT-independent mechanism. Together, these results indicate that cpl1-2 likely activates Fe deficiency responses upstream of both FIT-dependent and -independent signaling pathways.

FIT is specifically expressed in the root epidermis (Colangelo and Guerinot, 2004); therefore, the direct regulation of FIT by CPL1 would require CPL1 expression in these cells. The tissue specificity of CPL1 expression was analyzed using a CPL1-GUS translational fusion construct. The GUS ORF was inserted in a 8.4-kbp CPL1 genomic fragment immediately before the stop codon of CPL1 ORF, whose expression was regulated by its native regulatory sequences. The expression of the reporter gene was monitored in the roots of transgenic Arabidopsis plants (Fig. 10). The expression profile of CPL1-GUS was similar in Fe-sufficient or -deficient conditions. CPL1-GUS expression was high in root tips; however, in the mature part of roots, CPL1-GUS expression was confined to the stele, particularly in the phloem, and virtually no activity was observed in the outer layers of root cells. Overall, except for a limited area of the root tip, CPL1 and FIT showed distinct tissue-specific expression patterns, suggesting that the regulation of FIT by CPL1 is likely indirect.

The cpl1-2 mutation promotes the Fe deficiency-mediated expression of ABA-osmotic stress-regulated genes

Our finding that cpl1-2 simultaneously enhances the expression of both osmotic stress/ABA-responsive and Fe deficiency-responsive genes is somewhat contradictory to the previous report that ABA and osmotic stress inhibit Fe deficiency signaling (Seguela et al., 2008). However, an observation similar to ours was reported in rice
overexpressing IDEF1 [iron deficiency element (IDE1)-binding factor 1] (Kobayashi et al., 2009). Because of the similarity between IDE1 (CATGA) and the ABA signaling cis-element (RY-motif, CATGCATG) (Kobayashi et al., 2009), we hypothesized that CPL1 regulates a branch of Fe deficiency and osmotic stress/ABA signaling through similar cis-regulatory elements. While ABA treatment inhibited [IRT1, FRO2, FIT] expression in both the wild type and cpl1-2 (data not shown), the expression of genes inducible by both Fe deficiency stress and ABA treatment was substantially enhanced in cpl1-2 (Fig. 11). This indicates that some ABA-responsive genes are also regulated by Fe through a common CPL1-repressed pathway. Consistently, the presence of higher concentrations of external Fe could repress not only the expression of prototypical Fe-regulated genes [IRT1, FRO2, FIT], but also the enhanced expression of LATE EMBRYONIC ABUNDANT (LEA) in cpl1-2 (Fig. 4). Interestingly, the repression of LEA expression by excess Fe (>50 µM) was less pronounced in cpl1-2 than in the wild type, and HVA22 expression was largely unaffected by increased Fe, suggesting that additional Fe-independent mechanisms up-regulate LEA expression in cpl1-2. To test the state of ABA signaling in cpl1, the expression levels of several ABA signaling components were determined. The expression of ABI1, ABI3, and AREB1/ABF2 was up-regulated 4.0-, 3.9-, and 5.4-fold in cpl1-2 under normal growth conditions. This result was consistent with the observation that cpl1-2 was hyperresponsive to ABA treatment (Fig. 11). Together, these results suggest that CPL1 regulates the signaling pathway upstream of the cross-talk between ABA and Fe signals.

DISCUSSION

RNA metabolism regulates diverse developmental and stress response signaling pathways in plants. This study shows that an upstream component of the Fe deficiency response was regulated by an RNA metabolism factor, CPL1. Although the Fe deficiency response signaling pathway is regulated by multiple mechanisms, such as protein-protein interactions (Yuan et al., 2008), differential promoter activation (Yuan et al., 2008; Long et al., 2010), protein ubiquitination (Kerkeb et al., 2008; Li and Schmidt, 2010), and proteasomal and non-proteasomal protein degradation (Barberon et al., 2011; Lingam et al., 2011; Sivitz et al., 2011), regulation at the level of RNA metabolism has not hitherto been established. CPL1 is a plant-specific isoform of the universal pol II
CTD phosphatase family of proteins, which harbor double-stranded RNA-binding motifs (DRMs) and can specifically dephosphorylate pol II CTD at Ser5-PO$_4$ in vitro (Koiwa et al., 2002; Xiong et al., 2002; Koiwa et al., 2004). We have shown that CPL1 localizes to the root stele and negatively regulates Fe signaling. cpl1 mutations result in enhanced expression of FIT and group Ib bHLH genes, which are essential for Fe deficiency sensing/signaling and affect metal homeostasis. This finding is consistent with a previous report showing that Fe utilization-related genes are down-regulated in the RNA decapping-deficient mutant, trident (tdt) (Goeres et al., 2007).

Since alteration of the pol II phosphorylation status can potentially trigger alterations in global transcription and RNA maturation, it could be argued that the observed up-regulation of Fe utilization-related genes resulted from a pleiotropic effect of the cpl1-2 mutation, similar to the down-regulation of Fe utilization-related genes in the tdt mutation (Goeres et al., 2007). However, several observations argue against the involvement of pleiotropic effects. First, in contrast to the tdt mutant, the cpl1 mutants do not exhibit any major defects in growth and development. Second, cpl1-2 affects the levels of metals associated with Fe homeostasis, i.e., Fe, Zn, Mn, and Cd. Third, cpl1-2 affects multiple regulons in Fe deficiency signaling, i.e., FIT-dependent and -independent Fe deficiency responses and repression of the basal-level of FER1. Fourth, the effect of cpl1-2 could be suppressed by increased Fe concentrations in the medium. Finally, the effect of cpl1-2 on the expression of [IRT1, FIT, FRO2] likely involves intercellular communication. These findings indicate that the cpl1 mutations affect Fe homeostasis and activate upstream signaling component(s) rather than causing misexpression of FIT, regardless of the presence or absence of Fe signals. On the other hand, levels of IRT1, which are regulated post-translationally by Fe status, are affected only moderately by cpl1 mutations under Fe deficiency stress. This suggests that CPL1 either specifically regulates branch(es) of Fe deficiency signaling, or that higher thresholds of Fe deficiency signals are required to activate post-translational Fe deficiency responses. Alternatively, the levels of IRT1 at the PM may be regulated by apoplastic Fe rather than by CPL1-mediated signaling.

How does CPL1 regulate the Fe status and deficiency responses? Our data indicate that CPL1 is strongly expressed in the root stele, and not at all in the epidermis, where FIT is expressed. It is also noteworthy that cpl1-2 induced the expression of
under Fe-sufficient conditions, even in the presence of elevated levels of root Fe, but did repress \([IRT1, FIT, FRO2]\) expression when additional Fe was supplied exogenously. One possibility is that CPL1 functions at the organ level to keep the root Fe level low, by repressing \([IRT1, FIT, FRO2]\) and promoting Fe loading into the xylem (Fig. 12A). This could be achieved either by promoting Fe transport from the epidermis to the xylem, by repressing sequestration/compartmentalization of Fe in the root cells, or by repressing xylem unloading of Fe in the roots. Upon Fe deficiency, Fe starvation signals generated in the shoot overcome repression of \([IRT1, FIT, FRO2]\) by CPL1. Upon mutation of \(CPL1\), both repression of \([IRT1, FIT, FRO2]\) and the promotion of xylem loading are impaired, resulting in elevated expression of \([IRT1, FIT, FRO2]\) and accumulation of Fe in the roots. At the same time, Fe in the shoots is reduced due to decreased root-to-shoot Fe transport, which in turn generates systemic Fe deficiency signals and promotes \([IRT1, FIT, FRO2]\) expression in the roots. This mutant phenotype can be ameliorated by the addition of Fe, because a basal level of xylem loading still occurs in \(cpl1\) mutants. The relatively large decrease in root Fe content in the \(cpl1-2\) mutant during Fe deficiency may be due to the continued growth of \(cpl1-2\) roots diluting the acquired Fe, and/or the greater mobilization of stored Fe by enhanced Fe deficiency signals. While the expression levels of \(FRD3\) and \(IRON\) \(REGULATED\) 1 (\(IREG1\)), which likely load citrate and Fe to the xylem, respectively, do not substantially differ between the WT and \(cpl1-2\), there is a moderate increase in the expression of some plastid Fe uptake genes in \(cpl1-2\). This implies that CPL1 attenuates Fe compartmentalization rather than regulates xylem loading activity (Supplemental Fig. S5B). However, we cannot exclude the possibility that \(cpl1-2\) regulates the expression of other xylem loading Fe transporters, since \(IREG1\) is likely not the sole/major xylem loading Fe transporter (Morrissey et al., 2009). Furthermore, the mode of CPL1 function in this process remains to be determined. Because the physicochemical nature of intercellular Fe deficiency signaling between the shoots and roots, and the root stele and epidermal tissues remains elusive, it is difficult to address this issue. Currently, we are not able to detect significant changes in overall CTD phosphorylation status during Fe deficiency, suggesting that bulk pol II CTD phosphorylation is not involved (data not shown). The presence of the dsRNA-binding motif in CPL1 suggests that CPL1 may regulate small RNA production and/or function. It is interesting that CPL1 is strongly expressed in the stele, likely in phloem companion cells, where the expression of small RNA production
machinery is enriched (Mustroph et al., 2009). Indeed, several miRNA are regulated by the Fe signal, (Kong and Yang, 2010), and the potential effect of cpl1-2 on the mobility of small RNAs may explain the systemic nature of this mutation as well as its impact on multiple pathways.

We initially expected that the enhanced levels of IRT1 in cpl1-2 would result in Cd oversensitivity, because IRT1 is responsible for Cd influx into root cells (Connolly et al., 2002). Instead, cpl1-2 showed Cd tolerance, and Cd levels in cpl1-2 roots were not greater than those in wild-type roots. Rather, cpl1-2 showed a substantial increase in shoot Cd levels, indicating an increase in the root-to-shoot transport of Cd. Since Cd transport and tolerance involves multiple mechanisms and has not been fully elucidated, there is no simple explanation for how cpl1-2 confers Cd tolerance. However, several genes that are up-regulated in cpl1-2 are related to Cd tolerance. For example, the elevated expression of Fe acquisition genes such as [IRT1, FIT, FRO2] and group Ib bHLH, and the resulting increase in root Fe levels can ameliorate toxicity of cellular Cd (Wu et al., 2012). In addition, Wu et al. (2012) also reported that co-expression of FIT and bHLH38/39 activates HMA3, MTP3, IREG2, and IRT2, which can enhance the sequestration of Cd into vacuoles in the root. In addition, OPT3 may mediate Cd tolerance in a similar manner to the yeast homolog, ScOPT2, which transports Cd into the vacuoles (Aouida et al., 2009). However, these mechanisms in general promote sequestration of Cd into vacuoles, resulting in higher levels of Cd in the roots. In contrast, the “overflow protection mechanism” proposed for phytochelatin-mediated long-distance Cd transport from roots to shoots (Gong et al., 2003) may better explain Cd tolerance of cpl1-2. Similar to cpl1 mutants, transgenic plants with root-specific expression of phytochelatin synthase have elevated levels of Cd in the shoots, but normal levels of Cd in the roots (Gong et al., 2003). However, since phytochelatin is not likely the sole chelator that mediates long-distance Cd transport (Clemens, 2006), we cannot exclude the contribution of other mechanisms.

The simultaneous up-regulation of Fe deficiency- and ABA/osmotic stress-regulated transcripts is a unique signature of the cpl1-2 transcriptome. This may indicate that the cpl1-2 mutation activates two distinct stress signaling pathways, and/or that these two pathways exhibit specific crosstalk. The first possibility is supported by our finding that both Fe-specific and ABA/osmotic stress-specific genes are up-regulated in
cpl1. The increase of Na (30%) and decrease of K (23%) in cpl1 are further indicators that cpl1 mutants indeed experience osmotic (salt) stress under normal growth conditions. While previous studies indicate that Fe deficiency and ABA/osmotic stress pathways exhibit crosstalk (Dinneny et al., 2008; Seguela et al., 2008; Kobayashi et al., 2009), the positive co-regulation of gene expression by ABA/osmotic stress and Fe deficiency has not been studied in dicots to date. In monocots, however, specific crosstalk between ABA/osmotic stress and Fe deficiency signaling occurs, as shown with rice overexpressing IDEF1, a B3 family transcription factor (Kobayashi et al., 2009). Although an obvious IDEF1 ortholog is not present in the Arabidopsis genome, IDE1 sequences are found in many Fe-responsive gene promoters, including those from Arabidopsis (Kobayashi et al., 2003; Kobayashi et al., 2005). Interestingly, the closest homolog of IDEF1 in Arabidopsis is ABI3, and the expression of this gene in cpl1-2 was elevated four-fold over wild-type levels. Furthermore, the recognition sequence of IDEF1 (CATGC) overlaps with that of ABI3/VP1-type B3 family transcription factors (CATGCA) (Kobayashi et al., 2009; Kobayashi et al., 2010). Therefore, co-regulation of genes by ABA and Fe deficiency in cpl1-2 may be caused by a yet unidentified B3 family transcription factor(s) (Fig. 12B). Another notable change in ABA signaling in cpl1-2 was the 5.4-fold up-regulation of AREB1/ABF2. Interestingly, plants overexpressing either ABI3 or an activated AREB1-ΔQT variant show enhanced expression of the LEA genes identified in our cpl1-2 microarray, and AREB1-ΔQT overexpression causes up-regulation of bHLH38 and bHLH39 as well (Fujita et al., 2005; Nakashima et al., 2006). It is tempting to speculate that B3 family transcription factor(s) function as an ancient form of IDEF1 in dicots and regulate both ABA and Fe deficiency signaling. However, higher-level integration of ABA and Fe signaling pathways may exist. Whereas the ABA signaling pathway and ABA-responsive promoter elements/transcription factors are well established (Hubbard et al., 2010), detailed information on specific Fe-responsive cis-elements in the dicot Fe deficiency-induced promoters and on the Fe signaling components that function upstream of FIT or group Ib bHLH is lacking. In addition, the role of LEA proteins in the Fe deficiency response has not been established, but some LEA proteins appear to bind metal ions and may function in metal transport (Kruger et al., 2002). Further genetic and molecular analyses are required to determine the role and the mode of action of the ABA-Fe deficiency signal crosstalk, which occurs in both strategy I and strategy II plants.
MATERIALS AND METHODS

Chemicals & Primer information

All chemicals were obtained from Sigma. Sigma agar E was used for all plant growth experiments. Primer sequences used in this study are shown in Supplemental Table S5.

Plant Materials

The Arabidopsis thaliana ecotypes Col-0 and C24 were used in this study. The cpl1-1 and cpl1-2 lines were described previously (Koiwa et al., 2002; Xiong et al., 2002). cpl1-5 (GK-590C07) and cpl1-6 (GK-165H09) were obtained from the Nottingham Arabidopsis Stock Center (NASC) and frd3-1 was obtained from Arabidopsis Biological Resource Center. fit-2 seeds were provided by Dr. Paul Paré.

For general growth and microarray analyses, seeds were sown on media containing 1/4 Murashige and Skoog (MS) salts, 0.5% sucrose, and 0.8% agar. After stratification for 2 days at 4°C, the plates were kept in a growth incubator under a long-day photoperiod (16 h light, 8 h darkness) at 25°C for 10 days.

RNA extraction

Total RNA was isolated using TRIzol reagent (Chomczynski and Sacchi, 1987) and treated with DNase I (Promega, WI) to remove genomic DNA contamination.

Microarray Analyses

Total RNA was extracted from 10-day-old wild-type C24 and cpl1-2 mutant seedlings. Quality tests of the RNA samples, complementary RNA (cRNA) synthesis, biotin labeling, hybridization to Affymetrix ATH1 GeneChips, and scanning were performed by the Affymetrix Service at the Nottingham Arabidopsis Stock Centre (NASC). After chip hybridization and scanning, triplicate data were obtained for each genotype and data were processed using GeneSpringGX 11.0.2 software (Agilent, CA). Raw intensity values, computed from .CEL files, were processed first by Robust Multiarray Analysis (RMA) (Irizarry et al., 2003). Filtering on expression levels and fold changes (≥2) were performed using the GeneSpring package and differentially expressed genes were determined. Statistical analyses were performed using one-way
ANOVA at $p < 0.05$ (with asymptotic $p$-value computation) followed by the Tukey HSD post hoc test and Benjamini Hochberg FDR multiple testing correction. A fold change of at least 2 was considered indicative of differential expression, where a $p$ value of 0.05 or under was considered indicative of significant alteration in expression. All microarray data from this study were deposited in the NASC Database under accession number 611.

For hierarchical clustering of up-regulated genes, raw gene expression data for abiotic stresses and hormone treatments were first obtained from the AtGenExpress project (http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). The CEL file names are NASCarray 176 (ABA), 140 (salt), and 139 (mannitol)]. The CEL file for the Fe deficiency experiment (GSE15189) was obtained from the GEO database (Buckhout et al., 2009). All data were clustered by Pearson correlation distance and the average linkage rule (Eisen et al., 1998).

**Gene Set Enrichment Analysis**

Gene Set Enrichment Analysis (GSEA) was performed using GeneTrail (Backes et al., 2007; Schuler et al., 2011). Briefly, 22811 probes on ATH1 microarray datasets obtained from public databases were ranked and sorted according to fold change from the most induced to the most suppressed by each stress treatment. Subsequently, GSEA analyses were performed for each sorted dataset, using gene sets created from an analysis of the cpl1-2 microarray. As a reference, gene sets consisting of constitutively expressed genes in Arabidopsis were analyzed (Czechowski et al., 2005). False Discovery Rate was used as the $p$-value adjustment (Benjamini and Hochberg, 1995).

**RT-qPCR Analysis**

Total RNA samples (2 μg) were reverse-transcribed using random hexamers and the Superscript III Reverse Transcriptase (Life Technology, CA) in a total volume of 20 μl. One-twentieth of the reverse transcription products was analyzed using ABI 7900 Sequence Detectors and SYBR Green Master Mix (Life Technologies, CA). The amplification reaction and data analysis were performed as described (Salzman et al., 2005). Each reaction was run in technical duplicate and the melting curves were analyzed by SDS2.2.2 software (Life Technologies, CA) to verify that only a single
product was amplified. TUB8 (At5g23860) was used as an internal control for normalization of data.

**Preparation and Analysis of the FIT-LUC Reporter Gene**

A 1324-bp genomic DNA fragment of the FIT promoter was amplified by PCR using a primer pair [1047,1048] and BAC clone F24D13 as template. The PCR product was digested by HindIII and EcoRV, and ligated into pEnEL2Ω-LUC (GenBank Accession No. JN570503) digested with HindIII and SnaBI. A Gateway LR reaction was performed according to the manufacturer’s instructions, using pBSVirHygGW as the destination vector (Ueda et al., 2008). Transformation of Agrobacterium tumefaciens GV3101(pMP90RK), floral transformation of cpl1-6, and hygromycin selection of transformants were performed as described previously (Ueda et al., 2008). The homozygous cpl1-6 FIT-LUC line was crossed with the Col-0 wild type and fit-2, and homozygous Col-0 FIT-LUC, fit-2 FIT-LUC, and cpl1-6 fit-2 FIT-LUC lines were identified in the segregating F2 population and confirmed in the F3 generation. The expression level of LUC in each line was determined by RT-qPCR.

**Preparation and Analysis of the CPL1-GUS Reporter Gene**

A genomic fragment (8.1 kb) of the CPL1 locus encoded by an 8.4-kb Blpl fragment of BAC clone F17L22 was cloned in Smal-digested pENTR2B (Life Technologies, CA). Subsequently, a β-glucuronidase-coding sequence of pBl101was inserted immediately before the stop codon of CPL1. The resulting entry clone was then recombined with pBSVirHygGW binary plasmid (Ueda et al., 2008) using LR recombinase (Life Technologies, CA) to produce pBSVirHygCPL1-GUS, which was introduced into Agrobacterium tumefaciens GV3101(pMP90RK) and then used for flower transformation of Col-0 plants (Koiwa et al., 2002). Hygromycin-resistant transgenic plants were selected on medium containing 1/4x MS salts, 30 µg/ml hygromycin, 100 µg/ml cefotaxime, and 0.8% agar.

Five-day-old Col-0 CPL-GUS plants germinated on basal medium were grown for an additional three days on Fe-sufficient or -deficient basal medium (see below). For high-resolution GUS staining, plants were fixed in ice cold 90% acetone for 15 min and then incubated at 37°C for 4 h in a solution containing 2 mM 5-bromo-4-chloro-3-indolyl glucuronide, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 100 mM sodium phosphate buffer (pH
7.0), and 0.1% Triton X-100. After incubation, the samples were rinsed with 100 mM phosphate buffer.

For embedding, GUS-stained plants were fixed in 2% glutaraldehyde using cold microwave technology in a BioWave™ microwave with a 6-min vacuum cycle (2 min on/2 min off) at 200 W. After washing in the 100 mM sodium phosphate buffer (pH 7.0), the sample was dehydrated in a MeOH/water graded series at 10% MeOH increments (from 50% to 90%) for 1 min (30 s on/30 s off) at 200 W followed by 3 cycles in 100% MeOH. Samples were rinsed in propylene oxide and then 2 ml of fresh propylene oxide was added to each sample. Samples were infiltrated with Quetol 651-low viscosity resin by the stepwise addition of 10% (V:V) resin to each sample (Ellis, 2006). After each addition of resin, samples were allowed to infiltrate for several hours to overnight before additional resin was added. After two changes of 100% resin, samples were again vacuum infiltrated for 6 min (2 min on/2 min off) at 200 W and then embedded. Five-micrometer sections were prepared and mounted onto slides, and stained with toluidine blue before observation.

Stress Treatments

For testing plant responses to various Fe regimens, seeds were sown on basal medium containing 1/4 x MS salts, 50 µM Fe-EDTA, 0.5% sucrose, and 1.5% agar. Fe deficiency was applied by transferring 7-day-old seedlings to basal medium without Fe-EDTA but containing 300 µM ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] (0FZN basal media). Total RNA was isolated at the indicated times. For growth analysis, 4-day-old seedlings grown on basal medium were transferred to the 0FZN basal medium and plants were photographed 5 days after the transfer. Primary root lengths were analyzed using Image J software.

To determine cadmium sensitivity, seedlings of the C24 wild type and cpl1-2 mutant were grown on basal medium adjusted to the indicated concentration of Fe-EDTA and in the presence or absence of 60 µM CdCl₂. Seedlings were photographed after 11 days of growth and primary root lengths were analyzed.

For ABA treatment, seven-day-old plants grown on basal medium were transferred to basal medium with or without 1 µM ABA.
Determination of Ferric Chelate Reductase Activity

Root ferric chelate reductase activity was measured spectrophotometrically as described (Yi and Guerinot, 1996). The assay solution was composed of 0.1 mM Fe(III)-EDTA and 0.3 mM ferrozine in distilled water. Roots of five plants were soaked in assay solution for 30 minutes in darkness and then the absorbance of the assay solution was determined. An identical assay solution without any plants was used as blank. Purple-colored Fe(II)-ferrozine complex formation was quantified using a molar extinction coefficient of 28.6 mM⁻¹ cm⁻¹ at 562 nm. The experimental results were the mean of three biological repeats with six technical replicates each.

IRT1 Accumulation

Total root protein was extracted from both C24 wild-type and cp1-2 mutant plants grown either in Fe-sufficient or Fe-deficient conditions. Extracts were prepared by grinding tissues in extraction buffer on ice, followed by centrifugation at 4°C for 15 min at 14,000 x g. Twenty micrograms of total protein extracts was resolved on a 10% SDS–polyacrylamide gel and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA). After blocking with 6% skim milk in Tris-buffered saline (TBS), the membrane was incubated with affinity-purified rabbit anti-IRT1 antibodies (diluted to 1/5000 in Tris-Tween buffered saline (TTBS)) prepared as described previously (Vert et al., 2002) (Genscript, CA) and then goat anti-rabbit IgG-HRP conjugate (Thermo Scientific, MA) diluted to 1/100,000 in TTBS. The blot was developed using a Supersignal West Femto Kit (Thermo Scientific, MA) and documented using a Cascade II CCD camera (Photometrics, AZ). Anti-actin antibody (A2066: Sigma, MO) was used as the loading control.

Determination of Metal Content

Tissue element analysis of Na, K, Ca, Fe, Mn, Zn, and Cd using inductively coupled plasma–mass spectrometry (ICP-MS) was performed as described (Baxter et al., 2007). Briefly, shoots were washed thoroughly with distilled water. Roots were washed first in 2 mM CaSO₄ and 10 mM EDTA for 10 minutes, and were then rinsed twice in distilled water. Then, tissues were blot-dried, divided into three replicates of about 100 mg fresh weight, dried in a 65°C oven for 48 h, and reweighed. The dried samples were digested completely using 0.6 ml of concentrated ultrapure grade HNO₃.
(JT Baker, Netherlands) at 110˚C for 4 h. Each sample was diluted to 6 ml with nanopure water (18.2 MΩ) and analyzed on a PerkinElmer NexION 300D ICP-MS in the reaction mode. Indium (EMD Millipore, Germany) was used as an internal standard. The National Institute of Standards and Technology traceable calibration standards (Alfa Aesar, MA) were used for the calibration. Each sample was read five times in the pulse detector mode. Three biological replicates were performed per analysis.

Perls Staining of Fe

Perls staining of Arabidopsis seedlings was performed as described (Stacey et al., 2008) with slight modifications. Ten-day-old wild type C24 and cpl1-2 mutant seedlings were first washed with 10 mM EDTA (pH 8.0) solution for 5 minutes, then with distilled water three times. Next, seedlings were vacuum infiltrated with equal volumes of 4% (v/v) HCl and 4% (w/v) potassium ferrocyanide (Perls solution) for 30 minutes at room temperature and then incubated for 30 min at 53°C. Seedlings were then rinsed several times with distilled water and observed under BX51 microscope (Olympus, PA).

Statistical Analysis

Statistical analyses were performed with MINITAB 16 software (Minitab, PA). Statistical significances of differences between mean values were determined using Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s HSD post hoc test. Differences were considered significant when the p-value was less than 0.05.
Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Normalization and principal component analysis of the cpl1-2 and C24 microarray.

**Supplemental Figure S2.** Molecular characterization of the cpl1-5 and cpl1-6 mutants.

**Supplemental Figure S3.** The expression levels of IRT1 and FIT in F1 plants produced after crossing cpl1-2 with the wild type.

**Supplemental Figure S4.** Time course analysis of expression levels of Fe utilization-related genes in the roots of cpl1-2 and C24 under Fe deficiency.

**Supplemental Figure S5.** The expression of Fe utilization-related genes in roots of cpl1-2 and C24 under Fe deficiency.

**Supplemental Figure S6.** Fe deposition in cpl1-2 and C24 roots.

**Supplemental Figure S7.** Molecular characterization of fit-2 and cpl1-6 mutations and the FIT-LUC transgene.

**Supplemental Table S1.** Gene Set Enrichment Analysis (GSEA) of Cluster I and Cluster II Gene Sets.

**Supplemental Table S2.** Categories of up-regulated genes in the cpl1-2 mutant and comparison with relevant published microarray datasets.

**Supplemental Table S3.** Gene Set Enrichment Analysis (GSEA) of Gene Sets Presented in Fig 2.

**Supplemental Table S4.** RT-qPCR analysis of Fe utilization-related gene expression levels in different cpl1 alleles, cpl2-2 and cpl3-1.

**Supplemental Table S5.** List of primers used in the study for genotyping of mutant and transgenic lines, and RT-qPCR.
ACKNOWLEDGEMENTS

The authors thank the Purdue Ionomics Information Management System for initial determination of the cpl1 ionome. We thank to Dr. William D. James for assistance in ICP-MS analyses at Texas A & M University Elemental Analysis Laboratory. We thank Dr. Greg Cobb and Ms. E. Ann Ellis for assistance in Microscopy. We thank Dr. Paul M. Hasegawa for helpful discussions and critical reading of the manuscript, and Dr. Xiaoqian Wu for technical assistance. We thank Dr. Paul Paré and Dr. Mary Lou Guerinot for providing fit-2 and NASC/GABI-kat, the Arabidopsis Biological Resource Center (ABRC) for providing T-DNA lines and a BAC clone, and Dr. Catherine Curie for anti-IRT1 antibodies, which were used earlier in this study.
LITERATURE CITED


Connolly EL, Fett JP, Guerinot ML (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell* 14: 1347-1357


Rogers EE, Guerinot ML (2002) FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. *Plant Cell* 14: 1787-1799

Romheld V (1987) DIFFERENT STRATEGIES FOR IRON ACQUISITION IN HIGHER-PLANTS. *Physiol Plantarum* 70: 231-234


FIGURE LEGENDS

Figure 1. Hierarchical clustering of CUTs (cpl1-Up Transcripts) based on their differential expression during abiotic stress. The expression level of 109 CUTs in cpl1-2 plants and in wild-type plants subjected to an Fe deficiency (Fe-free nutrient solution for 24 h; GEO accession GSE15189), ABA (1 µM for 3 h; NASCArray accession 176), salt (130 mM NaCl for 6 h, NASCArray accession 139), or mannitol (300 mM mannitol for 12 h, NASCArray accession 140) were subjected to hierarchical clustering using the Pearson correlation distance and average linkage rule.

Figure 2. Venn diagram representing the differential regulation of CUTs by abiotic stresses. The expression level of CUTs was examined in public microarray datasets for Fe deficiency, ABA, and osmotic stress (mannitol or drought) treatments (see text and Supplemental Table S3).

Figure 3. Time course of expression levels of Fe utilization-related genes in the roots of cpl1-2 and C24 under Fe deficiency. Plants were grown on basal medium for 7 days, and then transferred to Fe-deficient basal medium containing 300 µM ferrozine (see Materials and Methods). Root samples were collected at the time of transfer (0), or 12, 24, 48, or 72 h or 7 days after the transfer. The presented expression levels (relative to untreated C24 samples) are mean values of three biological replicates analyzed in duplicate. Bars indicate standard errors of the mean (SEM) of biological replicates. *p<0.05, Student's t-test between mean values of cpl1-2 and C24 for the same conditions.

Figure 4. Basal expression levels of IRT1, FIT, FRO2 and LEA family proteins under different Fe concentrations. Plants were grown for 10 days on medium containing 1/4 x MS salts adjusted to the indicated concentration of Fe-EDTA, 0.5% sucrose, and 1.5% agar. 0FZN indicates medium without Fe-EDTA, but containing 300 µM ferrozine. Total RNA was extracted from whole plants. The presented expression levels (relative to C24 samples collected from medium containing 50 µM Fe-EDTA) are mean values of three biological replicates analyzed in duplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student's t-test between mean values of cpl1-2 and C24 for the same conditions.

Figure 5. FRO activity and IRT1 protein accumulation in roots of cpl1-2 and C24 under Fe-sufficient or -deficient conditions. Plants were grown on basal medium for 7 days, and then transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or Fe-deficient (Fe-, 0 µM
Fe-EDTA + 300 µM ferrozine) basal medium. A, FRO2 activity after 3 days of treatment. The presented values are the means of three biological replicates, each consisting of six technical repeats. Bars indicate the SEM of biological replicates. B, IRT1 protein accumulation after 3 days of treatment. Twenty micrograms of total protein was analyzed by immunoblot using anti-IRT1 antibodies (αIRT1). An anti-actin antibody (αACT) (A2066: Sigma, MO) was used as the loading control. Average band intensities of three experiments (± SEM) are given in arbitrary units. *p<0.05, Student’s t-test between mean values of cpl1-2 and C24 for the same conditions.

Figure 6. Metal contents of cpl1-2 and C24 roots and shoots under Fe-sufficient or -deficient conditions. Plants were grown on basal medium for 7 days, and then transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or -deficient (Fe-, 0 µM Fe-EDTA + 300 µM ferrozine) basal medium. After 3 days, root and shoot tissues were collected separately and dried at 65ºC for 48 h, and elemental levels were determined from 100 mg of dried tissues by ICP-MS analysis. The presented elemental levels are the mean values of three biological replicates analyzed in triplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student’s t-test between mean values of cpl1-2 and C24 for the same conditions.

Figure 7. Primary root growth of C24 and cpl1 mutants under different levels of available Fe. Four-day-old seedlings of C24, cpl1-1, and cpl1-2 were subjected to Fe deficiency as described in Materials and Methods. A, Root growth after 5 days of treatment. Root tip positions are marked by arrowheads. Dashed line shows the position of the roots at the time of transfer. The scale bars represent 10 mm. Root length was quantified to calculate relative root growth of the wild type and cpl1 mutants (B). Bars indicate SEM of three biological replicates, each consisting of 20 seedling measurements. *p<0.05, Student’s t-test between mean values of C24 and each mutant under Fe deficiency conditions. Fe+: 50 µM Fe-EDTA and Fe-: 0 µM Fe-EDTA +300 µM ferrozine.

Figure 8. Cd resistance of cpl1-2. Primary root growth (A) and Cd levels in the roots (B) and shoots (C) of C24 and cpl1-2 growing on media containing various levels of Fe and Cd. Seeds were germinated and grown for 10 days on 1/4 x MS medium adjusted to the indicated concentrations of Fe-EDTA and CdCl₂. The presented root lengths are the means of three biological replicates, each consisting of 20 seedlings. The presented Cd levels are the means of three biological replicates analyzed in triplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student’s t-test between mean values of cpl1-2 and C24 for the same conditions.
**Figure 9.** Expression levels of FIT-LUC, FIT, IRT1, FRO2, and group lb bHLH transcription factors in Col-0, cpl1-6, fit-2, and fit-2 cpl1-6 plants containing the FIT-LUC reporter gene. Plants were grown on basal medium for 7 days, and transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or -deficient (Fe-, 0 µM Fe-EDTA + 300 µM ferrozine) basal medium. Total RNA was extracted from root tissue after three days of treatment. The presented expression levels (relative to untreated Col-0 samples) are mean values of biological duplicates analyzed in duplicate. Bars indicate the SEM of biological replicates. Different letters show significant differences between genotypes under Fe+ and Fe- conditions (*p*<0.05, one-way ANOVA followed by Tukey’s HSD post hoc test). *p*<0.05, Student’s *t*-test between mean values of Fe+ and Fe- for the same genotype.

**Figure 10.** CPL1 was expressed in the root tip and stele. Plants expressing the CPL1-GUS fusion protein were grown on basal medium for 7 days, and transferred to Fe-sufficient (50 µM Fe-EDTA) (a-c) or Fe-deficient (0 µM Fe-EDTA + 300 µM ferrozine) (d-f) basal medium. After three days, GUS activity was visualized and documented. Bars represent 10 mm in a and d; 2 mm in b,c,e, and f; and 0.05 mm in c, inset.

**Figure 11.** The expression of LEA family transcripts in response to ABA or Fe deficiency treatments. Plants were grown on basal medium for 7 days, and transferred to basal medium containing 1 µM ABA or to Fe-deficient basal medium (0 FZN, 0 µM Fe-EDTA + 300 µM ferrozine). The duration of treatment was 1 h for ABA and 72 h for Fe deficiency. Total RNA was extracted from roots. The presented expression levels (relative to untreated C24 samples) are mean values of biological triplicates analyzed in duplicate. Bars indicate the SEM of biological replicates. *p*<0.05, Student’s *t*-test between mean values of cp/l-2 and C24 for the same conditions.

**Figure 12.** Model for the role of CPL1. A. Role of CPL1 in root-shoot Fe distribution. CPL1 in the stele likely promotes the root-to-shoot transport of Fe and attenuates the Fe deficiency signal in shoots. Communication between different cell types in shoot tissues is omitted in this model. B. Co-regulation of a subset of osmotic stress/ABA response genes by ABA and Fe deficiency signals and its attenuation by CPL1. Solid and broken arrows indicate pathways that operate during Fe deficiency or in the presence of ABA, respectively. Open bars indicate the repression activity of CPL1, which is absent in cpl1 mutants. CPL1 attenuates the Fe deficiency signaling pathway upstream of FIT and group lb bHLH. Direct targets of CPL1 in the pathways are currently unknown. B3 family transcription factors up-regulate the expression of a subset of osmotic stress/ABA-responsive genes through the RY motif in response to Fe deficiency and ABA signaling. ABA also promotes gene expression via the ABRE (ABA-responsive element).
ABA levels are elevated, the enhanced expression of ABA-responsive genes causes slow growth, which inhibits the expression of Fe utilization genes.
Table I. Select CUTs confirmed as being up-regulated in cpl1-1 and cpl1-2.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Classa</th>
<th>Gene</th>
<th>Microarray</th>
<th>RT-qPCR (±SEM)</th>
<th>cpl1-2/C24</th>
<th>cpl1-1/C24</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT4G19690</td>
<td>F</td>
<td>Iron-responsive transporter 1 (IRT1)</td>
<td>54.57</td>
<td>57.68 (±0.88)*</td>
<td>37.79 (±0.56)*</td>
<td></td>
</tr>
<tr>
<td>AT3G21720</td>
<td>F</td>
<td>Isocitrate lyase, putative (ICL)</td>
<td>8.99</td>
<td>5.28 (±0.79)*</td>
<td>5.98 (±0.34)*</td>
<td></td>
</tr>
<tr>
<td>AT2G44460</td>
<td>F</td>
<td>Glycosyl hydrolase family 1 protein (BGLU29)</td>
<td>3.92</td>
<td>7.73 (±0.47)*</td>
<td>7.36 (±0.34)*</td>
<td></td>
</tr>
<tr>
<td>AT5G26220</td>
<td>F</td>
<td>ChaC-like family protein</td>
<td>3.46</td>
<td>2.64 (±0.17)*</td>
<td>2.71 (±0.06)*</td>
<td></td>
</tr>
<tr>
<td>AT4G16370</td>
<td>F</td>
<td>Oligopeptide transporter family protein (OPT3)</td>
<td>3.18</td>
<td>3.25 (±0.23)*</td>
<td>4.08 (±0.10)*</td>
<td></td>
</tr>
<tr>
<td>AT3G16360</td>
<td>F</td>
<td>HPT phosphotransmitter 4 (AHP4)</td>
<td>2.60</td>
<td>2.64 (±0.32)*</td>
<td>2.81 (±0.14)*</td>
<td></td>
</tr>
<tr>
<td>AT1G56430</td>
<td>F</td>
<td>Nicotiamine synthase 4 (NAS4)</td>
<td>2.35</td>
<td>1.87 (±0.08)*</td>
<td>1.79 (±0.26)</td>
<td></td>
</tr>
<tr>
<td>AT5G24660</td>
<td>F</td>
<td>Response to low sulfur 2 (LSU2)</td>
<td>2.22</td>
<td>1.87 (±0.43)*</td>
<td>2.44 (±0.40)*</td>
<td></td>
</tr>
<tr>
<td>AT1G08650</td>
<td>F</td>
<td>Phosphoenolpyruvate carboxylase kinase 1 (PPCK1)</td>
<td>2.03</td>
<td>3.43 (±0.37)*</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>AT1G17990</td>
<td>F/O</td>
<td>12-oxophytodienoate reductase, putative (OPR)</td>
<td>3.64</td>
<td>3.81 (±0.21)*</td>
<td>6.87 (±0.40)*</td>
<td></td>
</tr>
<tr>
<td>AT5G62490</td>
<td>F/A/O</td>
<td>ABA-responsive protein (HVA22b)</td>
<td>6.91</td>
<td>15.67 (±0.81)*</td>
<td>6.32 (±0.50)*</td>
<td></td>
</tr>
<tr>
<td>AT3G15670</td>
<td>F/A/O</td>
<td>Late embryogenesis abundant protein (LEA) family protein</td>
<td>6.32</td>
<td>8.89 (±0.11)*</td>
<td>4.29 (±0.08)*</td>
<td></td>
</tr>
<tr>
<td>AT2G35300</td>
<td>F/A/O</td>
<td>Late embryogenesis abundant protein 4-2 (LEA 4-2/LEA18)</td>
<td>4.82</td>
<td>5.28 (±0.52)*</td>
<td>3.29 (±0.32)*</td>
<td></td>
</tr>
<tr>
<td>AT5G06760</td>
<td>F/A/O</td>
<td>Late embryogenesis abundant protein 4-5 (LEA 4-5)</td>
<td>4.44</td>
<td>4.20 (±0.38)*</td>
<td>4.00 (±0.04)*</td>
<td></td>
</tr>
<tr>
<td>AT3G02480</td>
<td>F/A/O</td>
<td>ABA-responsive protein-related/ LEA family protein (ABAR)</td>
<td>4.11</td>
<td>3.43 (±0.40)*</td>
<td>2.08 (±0.07)*</td>
<td></td>
</tr>
<tr>
<td>AT1G67600</td>
<td>F/A/O</td>
<td>Acid phosphatase/vanadium-dependent haloperoxidase-related protein</td>
<td>2.99</td>
<td>2.62 (±0.32)*</td>
<td>1.89 (±0.02)*</td>
<td></td>
</tr>
<tr>
<td>AT3G60140</td>
<td>F/A/O</td>
<td>Beta glucosidase 30, dark inducible 2, senescence-related gene 2 (BGLU30/ DIN2/ SRG2)</td>
<td>2.81</td>
<td>2.35 (±0.28)*</td>
<td>N/D</td>
<td></td>
</tr>
<tr>
<td>AT4G34710</td>
<td>F/A/O</td>
<td>Arginine decarboxylase 2 (SPE2/ADC2)</td>
<td>2.19</td>
<td>3.73 (±0.27)*</td>
<td>3.46 (±0.10)*</td>
<td></td>
</tr>
<tr>
<td>AT3G17790</td>
<td>A/O</td>
<td>Purple acid phosphatase 17 (PAP17)</td>
<td>5.38</td>
<td>7.89 (±0.04)*</td>
<td>7.16 (±0.24)*</td>
<td></td>
</tr>
<tr>
<td>AT5G07330</td>
<td>A/O</td>
<td>unknown protein</td>
<td>4.93</td>
<td>4.26 (±0.13)*</td>
<td>2.68 (±0.04)*</td>
<td></td>
</tr>
<tr>
<td>AT5G45690</td>
<td>A/O</td>
<td>unknown protein</td>
<td>4.49</td>
<td>5.74 (±0.50)*</td>
<td>4.63 (±0.23)*</td>
<td></td>
</tr>
<tr>
<td>AT5G66780</td>
<td>A/O</td>
<td>unknown protein</td>
<td>4.41</td>
<td>3.46 (±0.41)*</td>
<td>2.06 (±0.04)*</td>
<td></td>
</tr>
<tr>
<td>AT1G05510</td>
<td>A/O</td>
<td>unknown protein</td>
<td>4.11</td>
<td>8.40 (±0.56)*</td>
<td>5.90 (±0.37)*</td>
<td></td>
</tr>
<tr>
<td>AT3G58450</td>
<td>A/O</td>
<td>Universal stress protein (USP) family protein/ Adenine nucleotide alpha hydrolases-like superfamily protein</td>
<td>2.55</td>
<td>6.73 (±0.62)*</td>
<td>5.86 (±0.17)*</td>
<td></td>
</tr>
</tbody>
</table>

aGene classes represent upregulation by Fe deficiency (F), ABA treatment (A), or Osmotic stress (O).

* p<0.05, Student’s t-test between mean values of cpl1 and C24. N/D not determined.
Figure 1. Hierarchical clustering of CUTs (cpl1-Up Transcripts) based on their differential expression during abiotic stress. The expression level of 109 CUTs in cpl1-2 plants and in wild-type plants subjected to an Fe deficiency (Fe-free nutrient solution for 24 h; GEO accession GSE15189), ABA (1 µM for 3 h; NASCArray accession 176), salt (130 mM NaCl for 6 h, NASCArray accession 139), or mannitol (300 mM mannitol for 12 h, NASCArray accession 140) were subjected to hierarchical clustering using the Pearson correlation distance and average linkage rule.
Figure 2. Venn diagram representing the differential regulation of CUTs by abiotic stresses. The expression level of CUTs was examined in public microarray datasets for Fe deficiency, ABA, and osmotic stress (mannitol or drought) treatments (see text and Supplemental Table S3).
Figure 3. Time course of expression levels of Fe utilization-related genes in the roots of cpl1-2 and C24 under Fe deficiency. Plants were grown on basal medium for 7 days, and then transferred to Fe-deficient basal medium containing 300 µM ferrozine (see Materials and Methods). Root samples were collected at the time of transfer (0), or 12, 24, 48, or 72 h or 7 days after the transfer. The presented expression levels (relative to untreated C24 samples) are mean values of three biological replicates analyzed in duplicate. Bars indicate standard errors of the mean (SEM) of biological replicates. *p<0.05, Students t-test between mean values of cpl1-2 and C24 for the same conditions.
Figure 4. Basal expression levels of *IRT1*, *FIT*, *FRO2* and *LEA* family proteins under different Fe concentrations. Plants were grown for 10 days on medium containing 1/4 x MS salts adjusted to the indicated concentration of Fe-EDTA, 0.5% sucrose, and 1.5% agar. 0FZN indicates medium without Fe-EDTA, but containing 300 µM ferrozine. Total RNA was extracted from whole plants. The presented expression levels (relative to C24 samples collected from medium containing 50 µM Fe-EDTA) are mean values of three biological replicates analyzed in duplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student’s $t$-test between mean values of *cpl1-2* and C24 for the same conditions.
Figure 5. FRO activity and IRT1 protein accumulation in roots of \textit{cpl1-2} and C24 under Fe-sufficient or -deficient conditions. Plants were grown on basal medium for 7 days, and then transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or Fe-deficient (Fe-, 0 µM Fe-EDTA + 300 µM ferrozine) basal medium. A, FRO2 activity after 3 days of treatment. The presented values are the means of three biological replicates, each consisting of six technical repeats. Bars indicate the SEM of biological replicates. B, IRT1 protein accumulation after 3 days of treatment. Twenty micrograms of total protein was analyzed by immunoblot using anti-IRT1 antibodies (\(\alpha\)IRT1). An anti-actin antibody (\(\alpha\)ACT) (A2066: Sigma, MO) was used as the loading control. Average band intensities of three experiments (±SEM) are given in arbitrary units. *\(p<0.05\), Student’s \(t\)-test between mean values of \textit{cpl1-2} and C24 for the same conditions.
Figure 6. Metal contents of cpl1-2 and C24 roots and shoots under Fe-sufficient or -deficient conditions. Plants were grown on basal medium for 7 days, and then transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or -deficient (Fe-, 0 µM Fe-EDTA + 300 µM ferrozine) basal medium. After 3 days, root and shoot tissues were collected separately and dried at 65°C for 48 h, and elemental levels were determined from 100 mg of dried tissues by ICP-MS analysis. The presented elemental levels are the mean values of three biological replicates analyzed in triplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student’s t-test between mean values of cpl1-2 and C24 for the same conditions.
Figure 7. Primary root growth of C24 and cpl1 mutants under different levels of available Fe. Four-day-old seedlings of C24, cpl1-1, and cpl1-2 were subjected to Fe deficiency as described in Materials and Methods. A, Root growth after 5 days of treatment. Root tip positions are marked by arrowheads. Dashed line shows the position of the roots at the time of transfer. The scale bars represent 10 mm. Root length was quantified to calculate relative root growth of the wild type and cpl1 mutants (B). Bars indicate SEM of three biological replicates, each consisting of 20 seedling measurements. *p<0.05, Student’s t-test between mean values of C24 and each mutant under Fe deficiency conditions. Fe+: 50 µM Fe-EDTA and Fe-: 0 µM Fe-EDTA +300 µM ferrozine.
Figure 8. Cd resistance of cpl1-2. Primary root growth (A) and Cd levels in the roots (B) and shoots (C) of C24 and cpl1-2 growing on media containing various levels of Fe and Cd. Seeds were germinated and grown for 10 days on 1/4 x MS medium adjusted to the indicated concentrations of Fe-EDTA and CdCl₂. The presented root lengths are the means of three biological replicates, each consisting of 20 seedlings. The presented Cd levels are the means of three biological replicates analyzed in triplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student's t-test between mean values of cpl1-2 and C24 for the same conditions.
Figure 9. Expression levels of FIT-LUC, FIT, IRT1, FRO2, and group lb bHLH transcription factors in Col-0, cpl1-6, fit-2, and fit-2 cpl1-6 plants containing the FIT-LUC reporter gene. Plants were grown on basal medium for 7 days, and transferred to Fe-sufficient (Fe+, 50 µM Fe-EDTA) or -deficient (Fe-, 0 µM Fe-EDTA + 300 µM ferrozine) basal medium. Total RNA was extracted from root tissue after three days of treatment. The presented expression levels (relative to untreated Col-0 samples) are mean values of biological duplicates analyzed in duplicate. Bars indicate the SEM of biological replicates. Different letters show significant differences between genotypes under Fe+ and Fe- conditions ($p<0.05$, one-way ANOVA followed by Tukey’s HSD post hoc test). *$p<0.05$, Student’s t-test between mean values of Fe+ and Fe- for the same genotype.
Figure 10. *CPL1* was expressed in the root tip and stele. Plants expressing the CPL1-GUS fusion protein were grown on basal medium for 7 days, and transferred to Fe-sufficient (50 µM Fe-EDTA) (a-c) or Fe-deficient (0 µM Fe-EDTA + 300 µM ferrozine) (d-f) basal medium. After three days, GUS activity was visualized and documented. Bars represent 10 mm in a and d; 2 mm in b,c,e, and f; and 0.05 mm in c, inset.
Figure 11. The expression of LEA family transcripts in response to ABA or Fe deficiency treatments. Plants were grown on basal medium for 7 days, and transferred to basal medium containing 1 µM ABA or to Fe-deficient basal medium (0 FZN, 0 µM Fe-EDTA + 300 µM ferrozine). The duration of treatment was 1 h for ABA and 72 h for Fe deficiency. Total RNA was extracted from roots. The presented expression levels (relative to untreated C24 samples) are mean values of biological triplicates analyzed in duplicate. Bars indicate the SEM of biological replicates. *p<0.05, Student’s t-test between mean values of col1-2 and C24 for the same conditions.
**Figure 12.** Model for the role of CPL1.  

A. Role of CPL1 in root-shoot Fe distribution. CPL1 in the stele likely promotes the root-to-shoot transport of Fe and attenuates the Fe deficiency signal in shoots. Communication between different cell types in shoot tissues is omitted in this model.  

B. Co-regulation of a subset of osmotic stress/ABA response genes by ABA and Fe deficiency signals and its attenuation by CPL1. Solid and broken arrows indicate pathways that operate during Fe deficiency or in the presence of ABA, respectively. Open bars indicate the repression activity of CPL1, which is absent in cpl1 mutants. CPL1 attenuates the Fe deficiency signaling pathway upstream of FIT and group Ib bHLH. Direct targets of CPL1 in the pathways are currently unknown. B3 family transcription factors up-regulate the expression of a subset of osmotic stress/ABA-responsive genes through the RY motif in response to Fe deficiency and ABA signaling. ABA also promotes gene expression via the ABRE (ABA-responsive element). When ABA levels are elevated, the enhanced expression of ABA-responsive genes causes slow growth, which inhibits the expression of Fe utilization genes.