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Further insights into the role of bHLH121 in the regulation of iron homeostasis in *Arabidopsis thaliana*

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ABSTRACT
Iron (Fe) is an important micronutrient for plant growth and development but any excess of Fe is toxic because of the Fe-dependent generation of reactive oxygen species (ROS). Thus, Fe homeostasis must be tightly regulated. In Arabidopsis thaliana, a cascade of transcription factors has been identified as involved in the regulation of this process by modulating the expression of genes related to Fe uptake, transport, and storage. Recently, it was demonstrated that in response to Fe deficiency, bHLH121/URI (UPSTREAM REGULATOR OF IRT1) directly activates the expression of several genes involved in this regulatory network. It was also shown that bHLH121 interacts with ILR3 (bHLH105) and its homologs. Herein it is shown that bHLH121 is necessary for the expression of the main markers of the plant responses to Fe excess, the ferritin genes (i.e. FER1, FER3, and FER4). bHLH121 regulates ferritin genes expression by directly binding to their promoters, at the same locus than the ILR3-PYE repressive complex. Therefore, this study highlight that bHLH121, PYE, and ILR3 form a chain of antagonistic switches that regulate the expression of ferritin genes. The implication of this finding is discussed.

Iron (Fe) is an important micronutrient for plant growth and development, as it serves as cofactors for numerous enzymes involved in various cellular processes such as photosynthesis, respiration, or the synthesis of amino acids. However, an excess of Fe is deleterious for plants because of its capacity to generate ROS. Thus, the level of Fe in plant cells must be tightly regulated to avoid both Fe deficiency and Fe excess.

Fe uptake is the first limiting step for the maintenance of Fe homeostasis in plants. Non-grass species have evolved a reduction base mechanism allowing taking up the Fe present in the soil in the form of Fe(III)-chelates (Figure 1a). Once into the plant, Fe is transported to the different organs to be assimilated in several metalloproteins or stored in different cell compartments. Part of the Fe is transiently stored into ferritins whose expression is strongly induced in response to Fe excess. In Arabidopsis thaliana, there are four ferritin genes; three are expressed in vegetative tissues (i.e. FER1, FER3, and FER4) and one in seeds (i.e. FER2).

Maintaining Fe homeostasis necessitates the activity of several transcription factors (TFs) organized into an intricate regulatory network. In Arabidopsis, it involves 17 bHLH TFs, among which ILR3/bHLH105 (IAA-LEUCINE RESISTANT3) plays a dual role. ILR3 physically interacts with its orthologs from the bHLH clade IVc (i.e. bHLH34, bHLH104, and bHLH115) to activate the Fe deficiency responses (Figure 1a). ILR3 also acts as a transcriptional repressor when it interacts with PYE/bHLH47 (POPEYE). Among the ILR3-PYE targets are the ferritin genes (i.e. FER1, FER3, and FER4), the main markers of the plant response to Fe excess.

bHLH121 (URI, UPSTREAM REGULATOR OF IRT1) was identified as a novel TF that acts upstream the Fe homeostasis regulatory network, together with ILR3 and its orthologs, as a transcriptional activator. bhlh121 mutants display severe Fe deficiency symptoms, even in control Fe condition, that can be rescued by providing extra Fe supply (Figure 1b). In agreement with the upstream position of bHLH121 in the Fe homeostasis network, bhlh121 mutants are affected in all the aspects of the Fe-deficiency responses and the expression of several regulatory proteins and peptides involved in this network is impaired.

Since bHLH121 controls Fe uptake, one may hypothesize that bHLH121 might directly or indirectly regulate the expression of ferritin genes. In order to validate this hypothesis, the expression of FER1, FER3, and FER4 was analyzed in wild type and three independent bhlh121 mutant lines by qRT-PCR. For this purpose, seedlings were first grown for 7 days in both Fe sufficient (50 µM Fe) and Fe deficiency (0 µM Fe) conditions. Under Fe deficiency condition, expression analysis revealed that FER1 and FER4 mRNA accumulation was similar between the wild type and the mutants whereas FER3 mRNA accumulation was higher in the wild type than in the mutants (Figure 2a). These results suggest that bHLH121 has a positive effect on FER3 expression when Fe availability is low. In contrast, under Fe sufficient condition, the mRNA levels of the three ferritin genes were lower in the mutants than in the wild type. These results indicate that bHLH121 is necessary to maintain ferritin gene expressions when Fe availability is not limiting. These observations are in contrast with a previous study, based on the analysis of microarray data, showing that ferritin genes expression is not affected in bhlh121 mutants grown under Fe replete condition. This apparent discrepancy
mRNA levels of the three ferritin genes were lower between both genotypes (i.e. a reduction of about one-third in the mutant when compared to the wild type). Therefore, similar experiments were conducted with seedlings grown under two different Fe excess conditions: 200 µM Fe (mid-Fe excess condition) and 500 µM Fe (high Fe excess condition). Under mid excess condition, the mRNA levels of the three ferritin genes were lower...
in the bhlh121 mutants than in the wild type (Figure 2b) whereas Fe accumulation was similar between all genotypes (Figure 2c). This later result confirms that when Fe is not limiting bHLH121 is necessary for ferritin genes expression. Interestingly, under high Fe excess, the expression of ferritin genes was no longer different between the wild type and the bhlh121 mutants (Figure 2b). Fe accumulation was also similar between all genotypes (Figure 2c). In addition, at the rosette stage, no chlorosis was observed on the leaves of bhlh121 mutants submitted to Fe excess as it was previously reported for Arabidopsis plants deprived of ferritins (Figure 1b). Taken together, these results indicate that bHLH121 is required to induce ferritin genes expression when Fe availability is in adequacy with the plant physiological needs. These data also suggest that under high Fe excess, ferritin genes expression relies on the IDR5 (IRON-DEPENDENT REGULATORY SEQUENCE) signaling pathway.

In order to confirm whether the regulation of ferritin genes expression by bHLH121 is direct or not, ChIP-qPCR experiments were conducted using two independent bhlh121 mutant lines complemented with the whole bHLH121 locus translationally fused to the GFP reporter gene (i.e. pbHLH121:gbHLH121-GFP in bhlh121-2). This assay was centered on the promoter regions of the ferritin genes that contain the G-box motifs (CACGTG) known to be recognized by bHLH TFs. These loci were chosen for two reasons. First, because these G-boxes are directly targeted by the ILR3-PYE complex. Second, because these G-boxes are located in nucleosome-free regions, suggesting the binding of regulatory proteins at the G-box loci. ChIP-qPCR assays supported the in vivo binding of bHLH121 to the promoter of FER1, FER3, and FER4 (Figure 3a), confirming previous results obtained by ChIP-seq analysis. ChIP-qPCR experiments also showed that bHLH121 binds to the promoter

Figure 2. bHLH121 is an activator of the Arabidopsis thaliana FER1, FER3 and FER4 expression. (A) Relative expression levels of FER1, FER3, and FER4. Relative expression was determined by quantitative reverse transcription PCR (qRT-PCR) in 1-week-old Arabidopsis seedlings grown on iron-sufficient (50 µM Fe) or iron-deficient (0 µM Fe) medium. Seedlings were germinated and grown under long-day conditions (8h/16h light/dark; light intensity: 120 mmol/cm2/s provided by Osram 18-W 840 Lumilux neon tubes) on half-strength Murashige and Skoog medium containing 0.05% (w/v) MES, 1% (w/v) sucrose and 0.7% (w/v) agar. Iron was provided as Fe(III)-EDTA. (B) Relative expression levels of FER1, FER3, and FER4 (qRT-PCR) in 1-week-old Arabidopsis seedlings grown as in A on two iron excess regimes: 200 µM Fe (mid-Fe excess) and 500 µM Fe (high Fe excess regime). (C) Fe contents of the wild type (WT) and the bhlh121 mutants grown as in B. (A-C) Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, p < .05 (n = 3 technical repeats). Error bars show ±SD.
of ferritin genes at the same locus than the ILR3-PYE repressive complex.\textsuperscript{10}

While the transcript level and protein abundance of bHLH121 are not significantly affected by the Fe status, Fe availability affects the cellular localization of bHLH121 in Arabidopsis roots.\textsuperscript{15,16} Interestingly, the patterns of ILR3 and PYE accumulation in Arabidopsis root cells also depend on Fe availability. When Fe availability is not limiting, most bHLH121 accumulates in the stele, the site of ferritin gene expressions.\textsuperscript{20} In this condition, ILR3 localizes in all root cells with higher abundance in the stele than in the epidermis and the cortex, whereas only traces of PYE are found in the stele.\textsuperscript{5,21} These observations indicate that, under Fe sufficient condition, the repressive activity of the ILR3-PYE complex on ferritin genes expression is low.\textsuperscript{10,22} It also supports that bHLH121 is a direct activator of ferritin genes expression. In contrast, when Fe availability is low, bHLH121 mainly localizes at the root epidermis and the cortex, the sites for Fe uptake, and ILR3 and PYE in all root cell types.\textsuperscript{5,16,21} These observations are consistent with the repressive activity of the ILR3-PYE complex on ferritin genes expression and the positive role of bHLH121 on Fe uptake (Figure 3b).

The identification of the protein that interacts with bHLH121 to regulate ferritin genes expression is one of the main questions that remain to be solved (Figure 3b). It is unlikely that one of the clade IVc bHLH interact with bHLH121 to activate the expression of ferritin genes since ILR3 is a repressor of ferritin gene expressions and since the expression of ferritin genes is not lowered in the bhlh34, bhlh104, and bhlh115 loss-of-function mutants.\textsuperscript{10} It is also unlikely that bHLH121 acts as homodimers since in vivo experiments suggest that bHLH121 cannot interact with itself.\textsuperscript{16}

Interestingly, the phosphorylation state of bHLH121 also depends on Fe availability.\textsuperscript{15} Under Fe deficiency, the phosphorylated form of bHLH121 accumulates in roots. It is proposed that this mechanism allows the heterodimerization of bHLH121 with ILR3 and its three homologs, and thus the transcriptional activation of their target genes to activate the Fe deficiency responses.\textsuperscript{15} Whether the phosphorylation of bHLH121 is necessary to activate the expression of ferritin genes will have to be demonstrated. Firstly, because bHLH121 activates the expression of ferritin genes in the stele when Fe availability is not limiting, and thus when bHLH121 phosphorylation is low. Second, because the phosphorylated form of bHLH121 is degraded when plants are recovering from Fe deficiency, a growth condition that strongly induces ferritin genes expression.\textsuperscript{10,15} The phosphorylation states of bHLH121 might rather be necessary to modify bHLH121 pattern of accumulation within the root cells in a Fe-dependent manner. In this hypothesis, which remains to be tested, there would be a balance between the phosphorylated bHLH121 form in the epidermis and the cortex and the non-phosphorylated form in the stele. The activity of FIT is also modulated by

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**Figure 3.** bHLH121 is a direct activator of the Arabidopsis thaliana FER1, FER3 and FER4 expression. (A) Left panels: chromatin immunoprecipitation coupled with qPCR (ChiP-qPCR) analysis of the binding of bHLH121 to the FER1, FER3, and FER4 promoters (ProFER1, ProFER3, and ProFER4). Seedlings were germinated and grown under long-day conditions (8h/16h light/dark; light intensity: 120 mmol/cm2/s provided by Osrum 18-W 840 Luminlux neon tubes) on half-strength Murashige and Skoog medium containing 0.05% (w/v) MES, 1% (w/v) sucrose and 0.7% (w/v) agar. Iron was provided as Fe(III)-EDTA. After 1 week of growth, seedlings were exposed to Fe deficiency for 3 days prior analysis. Chromatin from two complemented bhlh121-2 lines expressing the ProbHLH121:gbHLH121-GFP construct was extracted using anti-GFP antibodies. Seedlings expressing GFP under the control of the ILR3 promoter (ProILR3:GFP) were used as a negative control. qPCR was used to quantify enrichment of bHLH121 on ferritin gene promoters. Means within each condition with the same letter are not significantly different according to one-way ANOVA followed by post hoc Tukey test, p < .05 (n = 3 to 4 technical repeats). Error bars show ±SD. Right panel: Promoter structure diagrams for the three ferritin genes assayed in ChiP-qPCR experiments. Grey boxes, E-box (CANNTG); black boxes, G-box (CAGGTG). Lines under the boxes indicate sequences detected by ChiP-qPCR assays and correspond to known binding sites of ILR3 and PYE. (B) Schematic representation of the transcriptional regulation of ferritin genes expression in the stele by bHLH transcription factors. When Fe is not limiting (+Fe), bHLH121 localizes into the stele where it directly activates the expression of FER1, FER3, and FER4 (FERs). The bHLH121 protein partner remains to be characterized. Under Fe deficiency condition (-Fe), bHLH121 localizes in the epidermis and the cortex where it activates the Fe deficiency response whereas the ILR3-PYE complex directly represses the expression of FER1, FER3, and FER4 in the stele.
phosphorylation via the activity of CIPK11 (CBL-INTERACTING PROTEIN KINASE 11).\textsuperscript{23,24} Whether or not CIPK11 or closely related kinases, might also phosphorylate bHLH121 and thus function as a coordinating factor for different aspects of Fe homeostasis is an appealing hypothesis that will have to be investigated.

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**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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