**FRU (BHLH029) is required for induction of iron mobilization genes in Arabidopsis thaliana**

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**Abstract** Iron mobilization responses are induced by low iron supply at transcriptional level. In tomato, the basic helix-loop-helix gene **FER** is required for induction of iron mobilization. Using molecular-genetic techniques, we analyzed the function of **BHLH029**, named **FRU** (FER-like regulator of iron uptake), the *Arabidopsis thaliana* homolog of the tomato **FER** gene. The **FRU** gene was mainly expressed in roots in a cell-specific pattern and induced by iron deficiency. **FRU** mutant plants were chlorotic, and the **FRU** gene was found necessary for induction of the essential iron mobilization genes **FRO2** (ferric chelate reductase gene) and **IRT1** (iron-regulated transporter gene). Overexpression of **FRU** resulted in an increase of iron mobilization responses at low iron supply. Thus, the **FRU** gene is a mediator in induction of iron mobilization responses in Arabidopsis, indicating that regulation of iron uptake is conserved in dicot species.

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**Keywords:** Arabidopsis; bHLH; Iron; Mobilization; Plant; Root

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1. **Introduction**

The basic helix-loop-helix (bHLH) domain proteins are a family of transcription factors that have been characterized as important regulatory components controlling a diversity of biological processes. The *Arabidopsis* genome contains 162 predicted bHLH genes, which can be further divided into eight groups [1,2]. Outside of the conserved bHLH domain, little sequence similarity is found between the 162 members especially between those belonging to different groups.

Recently, we described *LeFER*, a gene encoding a bHLH protein that is required for regulation of the molecular responses to iron deficiency in tomato [3]. Iron deficiency is a serious problem on alkaline or calcareous soils because Fe\(^{3+}\), the main form of iron in soils, occurs as insoluble ferrihydroxide. To cope with iron deficiency and increased requirement for iron, plants need to mobilize iron in the soil. Dicot and monocot plants with the exception of grasses mobilize iron through iron reduction (strategy I) as opposed to grasses that utilize a phytosiderophore-based iron chelation mechanism (strategy II). Strategy I responses also include rhizosphere acidification and secretion of small iron chelating substances like citrate. Reduced iron is subsequently imported into the roots via divalent iron transporters [4,5].

The tomato *LeFER* gene is required for regulation of the iron transporter gene *LeIRT1* and the iron reductase gene *LeFRO1* in tomato roots [6,7]. The relevance of *LeIRT1* and *LeFRO1* for iron homeostasis in tomato has not yet been investigated in genetic or transgenic studies. In *Arabidopsis*, *IRT1* and *FRO2* have been shown to encode the main components for iron mobilization and iron uptake [8–11].

In *Arabidopsis*, an ortholog of *LeFER* was found as a potential iron-regulated transcription factor gene, named **FRU** (FER-like regulator of iron uptake) that was mainly expressed in roots [12]. The analysis of iron signaling components in the model species *Arabidopsis thaliana* is eased by the availability of the complete genome sequence of this plant. Regulatory networks involved in iron regulation can be unravelled not only by classical genetic analysis but also by the use of reverse genetic approaches.

Here, we analyzed the function of **FRU** at the genetic level. We describe that **FRU** was necessary for upregulation of iron mobilization genes, suggesting that iron uptake is controlled by conserved regulatory genes in dicots.

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2. **Materials and methods**

2.1. **Plant material and genotyping**

Wild type *A. thaliana* was Col-0. The *fru-G108* T-DNA insertion Arabidopsis mutant line, namely GABI\(_{108C10}\), was identified from the T-DNA mutagenized GABI-Kat collection [13]. Wild type and *fru-G108* mutant alleles were distinguished by PCR using the primer combinations 5’-cgcctttcatagacgagaacc-3’/5’-agctctgttcgaagcatgtc-3’ (FRU) and 5’-attattgaccatcatactcattgc-3’ (C) (FRU). The sequence adjacent to the T-DNA insert was deposited at Genbank (Accession No. AJ704989). The EMS allele *fru-C497T* was obtained through Arabidopsis TILLING [14]. Pyrosequencing was performed for genotyping using the manufacturer’s protocols (Biotage AB, Sweden). A biotinylated PCR product was sequenced was performed for genotyping using the manufacturer’s protocols (Biotage AB, Sweden). A biotinylated PCR product was sequenced by bisulfite sequencing (Biotage AB, Sweden). A biotinylated PCR product was sequenced by bisulfite sequencing (Biotage AB, Sweden). A biotinylated PCR product was sequenced by bisulfite sequencing (Biotage AB, Sweden).
generated using the primers 5’-agcaagatggcgcaaaag-3’ and 5’-tggaagcaggaggagccg-3’. Pyrosequencing was performed with primer 5’-gatgctagctttggttagtt-3’. Both PCR-based methods were suitable for distinguishing homozygous and heterozygous individuals in the two lines.

FRU overexpression (OX) constructs were generated as follows: a FRU cDNA generated with primers 5’-ggagcaggtgttctggaaatgagagctccttgcaagctgagacatcgtc-3’ and 5’-ggagcaggtgttctggaaatgagagctccttgcaagctgagacatcgtc-3’ was cloned into pDONR201 (Gateway system, Invitrogen, USA). After sequencing the FRU cDNA, it was transferred behind the double enhancer cauliflower mosaic virus 35S promoter in vector pLELA, which is a derivative of pLahw1-3RNAi (GenBank AF404854) containing a GATEWAY cassette introduced into the HpaI site. Promoter-reporter gene (GUS) transgenic lines were constructed as follows: 20 bp of genomic sequence upstream of the FRU ATG start codon were amplified and cloned into binary vector pBI101 containing a promoterless GUS gene [15]. Transgenic lines were generated by Agrobacterium tumefaciens-mediated transformation of Arabidopsis plants (Col-0) according to the floral dip protocol [16]. All transgenic lines were selected on Kanamycin (GUS line) or BASTA (OX) selection medium and self-fertilized. The T2 plants were analyzed.

2.3. Gene identities


2.4. Analysis of GUS activity

Arabidopsis plants were washed in sodium phosphate buffer (pH 7.0) briefly and incubated in GUS staining buffer (50 mM sodium phosphate, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% Trition X-100, and 2 mM GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) at 37 °C for 4 h according to the methods of Jefferson et al. [15]. Then, the plants were incubated in 100% ethanol for de-staining and kept in 70% ethanol before observation. Cross-sections (50–100 μm) of GUS-stained roots were obtained after embedding of GUS-stained roots in 6% agarose and dissection using a vibrating microtome.

For GUS activity measurement, roots and leaves were separately collected. Protein was isolated with 200 μl of GUS extraction buffer. Two mM 4-methylumbeliferyl-β-D-glucuronide was used as a substrate for GUS measurement. The formation of fluorescent product methylumbelliferone was quantified by fluorimetry. GUS activity was normalized to the protein concentration in the extract determined according to the methods of Bradford [18].

2.5. Ferric chelate reductase assay and chlorophyll measurement

Four plants were pooled in a sample and the roots incubated in 2 ml Hoagland medium containing 40 μM FeEDTA and 170 μM sodium bathophenanthroline disulfonate (BPDS). Reduction rates were calculated from the absorption of Fe3+/BPDS at 540 nm in 1 h in the medium and per gram root material (molar extinction coefficient 22.5). Each value represents the mean of four independent samples.

For chlorophyll measurement, shoots from plants per sample were incubated with 100% ethanol at 80 °C for 1 h. The extinction of total chlorophyll was measured at 652 nm according to the methods of Lichtenthaler [19]. Each value represents the mean of four independent samples.

2.6. Gene expression analysis

Gene expression was measured by semi-quantitative reverse transcription-PCR according to the methods of Bereczky et al. and Bauer et al. [6,20]. Briefly, total RNA was isolated from leaf and root material using the Invisorb Spin Plant RNA Mini Kit (Invitek Germany). One ug total RNA was DNase-treated (Fermentas, Lithuania) and reverse transferrered into cDNA using oligo(dT) primer (RevertAid first strand cDNA synthesis kit, Fermentas, Lithuania). Specific oligonucleotides were used to amplify FRU (5’-ctctgatctgacaagaa-3’ and 5’-ccagggaggagggtaac-3’) and 5’-ctctgatctgacaagaa-3’, FRT1 (5’-cagcaactgtggctgtt-3’ and 5’-atccagatgtcctggttaag-3’), and FRT2 (5’-ctctgatctgacaagaa-3’ and 5’-aactgttgaggaagggagtaac-3’) served as control. Ex Tag enzyme was used for amplification (TaKaRa, Japan). The number of cycles was adjusted so that PCR products were analyzed in the exponential phase of amplification, where no or only weak bands were detectable on a gel by ethidium bromide staining, generally after 20–25 cycles (details in figure legends). All primers surrounded an intron so that genomic DNA was clearly distinguished from cDNA-derived products. PCR products were separated by agarose gel electrophoresis, blotted to a Nylon filter and hybridized with specific probes.

3. Results

3.1. Arabidopsis FRU is the sequence homolog of the tomato LeFER gene

The bHLH transcription factor family is defined by the presence of a conserved bHLH signature domain, which consists of ~60 amino acids [2]. Outside of the bHLH domain, bHLH domain proteins can vary substantially. A2g28160 was identified by BLASTP searches as the closest sequence relative of the tomato iron uptake regulator bHLH protein LeFER [3]. We named A2g28160 (BHLH029) FRU (= FER-like regulator of iron uptake; the abbreviation FER was already in use for ferritins in Arabidopsis). A full-length cDNA sequence of FRU was identified [2]; see Section 2 for accession and gene identification numbers). At amino acid level, Arabidopsis FRU and tomato LeFER shared 42% identity and 72% similarity (Fig. 1). The bHLH domain sequences were almost identical. FRU and LeFER contain both the T–E–R motif in the basic domain whereas most other bHLH proteins contain an H–E–R motif for which binding to DNA was shown [2].

3.2. Expression analysis of FRU

To investigate whether FRU might be involved in iron uptake responses, we studied the expression of FRU in different tissue and iron supply conditions of Arabidopsis plants. We found that in wild type plants, FRU transcripts were present in roots and inflorescence, and to a low level in leaves (Fig. 2). No expression signals were detected in cotyledons and siliques. Upon low iron supply, FRU expression signals were induced in roots about 2–4 fold (see about 2-fold induction in Fig. 5B and C; see about 4-fold induction in Fig. 6A and [12]).

FRU promoter activity was further analyzed in transgenic GUS reporter lines. GUS activity measurements in protein extracts indicated that the FRU promoter was more active in roots than in leaves. GUS activity was increased in roots about in average 4-fold by iron deficiency (Fig. 3A). Since the GUS staining results confirmed RNA expression studies, we regarded FRU promoter-GUS staining as representative for FRU gene expression. Taken together, the results indicated that FRU might play a predominant role upon iron deficiency in roots.
Histochemical staining confirmed that the FRU promoter was mainly active in roots and increased upon low iron supply (Fig. 3B–E). In leaves, GUS activity was occasionally detected in trichomes. In roots, FRU promoter-GUS activity was present in a cellular pattern in the developing root zones (Fig. 3D–I). Upon iron deficiency, GUS activity was found mainly in the epidermis and to a lower level in the inner not yet differentiated cells of the meristematic root zone (Fig. 3G). In the elongation root zone, intense staining of all epidermal cells occurred at iron deficiency (Fig. 3H). However, upon iron supply, GUS staining was alternately strong or weak in epidermal cells (Fig. 3E and F) and presumably identified non-hair and root hair cells, respectively. Similar staining patterns were observed for root epidermal patterning genes (for review, see [21]). In the maturation root zone, GUS staining patterns did not depend on iron supply. GUS activity was then found in the epidermis with the exception of a few unstained cells. Moreover, GUS staining was detected in the endodermis and inner tissues of the central cylinder (Fig. 3I).

3.3. Identification and analysis of fru knockout lines

To reveal the function of FRU in plants, we identified two knockout lines using reverse genetic resources provided by Arabidopsis functional genome initiatives (Section 2). Allele fru-G108 was identified from the GABI-Kat T-DNA collection [13]. Sequencing demonstrated that the T-DNA was inserted into the first exon of FRU (Fig. 4A). EMS allele fru-C497T was identified through Arabidopsis TILLING [14]. The C/T transition point mutation resulted in a premature stop codon in the second exon (Fig. 4B). The progeny of heterozygous mutant plants obtained with either of the two fru alleles segregated 1:3 for chlorotic individuals on Hoagland plant medium (chlorotic fru-G108 individuals grown with 10 μM Fe shown in Fig. 4C and without iron in Fig. 4C top). Genotyping of the segregating individuals of both alleles revealed that homozygous plants were all chlorotic, whereas green plants were heterozygous or wild type (not shown). Transfer of the chlorotic individuals to Hoagland medium containing 200 μM Fe rescued the mutants (not shown). In the greenhouse, chlorotic individuals only survived with exogenous spraying of iron chelators (Fe-EDTA). These findings indicate that fru mutants might not be able to efficiently utilize iron through the root and that FRU function might be similar to that of the tomato LeFER gene.

To confirm that the chlorotic fru mutant plants had a defect in iron mobilization, we studied the molecular and physiological responses that serve as indicator for iron mobilization. First, we analyzed whether fru mutant plants were capable of reducing iron on the root surface. We found that fru-G108 and fru-C497T homozygous plants even when grown upon low iron were not able to reduce iron to the same level as wild type (Fig. 5A). At the molecular level, we analyzed the expression of ferric chelate reductase FRO2 gene and iron transporter IRT1 gene. Previously, it was established that FRO2 and IRT1 expressions are co-regulated and induced by iron deficiency [9,17,22]. Although basic levels of FRO2 and IRT1 mRNA plants were all chlorotic, whereas green plants were heterozygous or wild type (not shown). Transfer of the chlorotic individuals to Hoagland medium containing 200 μM Fe rescued the mutants (not shown).

Fig. 1. Amino acid alignment of A. thaliana FRU and Lycopersicon esculentum FER bHLH domain proteins. Sequences were aligned using CLUSTALW. Black shading indicates identical amino acid positions, gray shading indicates similar residues. The basic (b), helical (H) and loop (L) regions of the bHLH domain are highlighted by gray boxes. Asterisks indicate DNA contacting amino acids in the basic region [2].
could be detected in mutant plant roots, there was no or low induction (0–2 fold) of the two genes upon low iron supply, whereas wild type roots showed 3–10 fold induction of the two genes (Figs. 5B, C and 6A). Thus, both physiological and molecular responses of iron mobilization were reduced in the two fru mutant lines.

3.4. Characterization of FRU overexpression lines

It was hypothesized that overexpression of FRU might result in enhanced iron mobilization if FRU was indeed a regulatory gene involved in controlling iron starvation responses. Transgenic plants expressing FRU under control of the double enhancer cauliflower mosaic virus 35S promoter were generated (OX lines). All identified independent and homozygous transgenic lines did not show a visible phenotype when grown on 10 μM Fe Hoagland medium (data not shown). We selected for further analysis two to three lines that expressed FRU at high level in leaves. We found that FRU mRNA levels were increased in leaves and roots of OX lines compared to wild type, irrespective of iron supply (Fig. 6A, shown for lines OX-1 and OX-7). Ectopic or increased expression of FRU did not generally result in ectopic or increased expression of IRT1 and FRO2. At all conditions tested, IRT1 was expressed at higher level in OX plants than in wild type plants. IRT1 expression

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**Fig. 3.** Analysis of promoter pFRU-GUS reporter activity. (A) GUS activity in root and leaf protein extracts of plants grown under 0 or 50 μM iron supply. The mean values were derived from 12 plants and three independent lines. Standard deviations are indicated. (B)–(I) GUS histochemical staining. (B, C) Whole plants grown in 0 and 50 μM Fe, respectively. (D, E) Root tips from lateral roots grown in 0 and 50 μM Fe, respectively. (F)–(I) 40 μm cross-sections of GUS stained roots derived from different root zones. (F) Developing elongation zone, 50 μM Fe. (G) Root tip zone at the approximate level of the meristem, 0 μM Fe. (H) Developing elongation zone, 0 μM Fe. (I) Root hair zone, 0 μM Fe.
exceeded the expression level of wild type at iron deficiency only in OX plants that were also exposed to iron deficiency. However, at iron supply, *IRT1* expression was significantly lower in OX roots than in iron-starved wild type roots. *FRO2* was expressed at similar level in iron-starved roots of wild type and OX plants. At iron supply, *FRO2* expression was hardly detectable. Interestingly, both *IRT1* and *FRO2* were ectopically expressed in iron-starved OX leaves. This finding was clearly different from the previous overexpression results obtained with the tomato *LeFER* gene, which did not result in ectopic *LeIRT1* expression in leaves [12]. However, after iron supply leaf expression of *IRT1* and *FRO2* was weak or hardly detectable.

Iron reductase activity was significantly induced in wild type and OX roots by approximately twice the level when plants were exposed to iron deficiency compared to iron supply (Fig. 6B). At both iron conditions, average iron reductase activity was about 20% higher in OX plants than in wild type. At iron deficiency this increase was significant, whereas at iron supply the increase of iron reductase activity in OX plants compared to wild type was not significant.

In a further experiment, we analyzed whether *FRU* overexpression might lead to increased tolerance of the transgenic plants to iron deficiency. For this purpose, 2-week-old OX-1 and OX-6 plant seedlings were transferred to Hoagland medium without iron and supplemented with ferrozine. After 7 days, OX plants were significantly darker green than wild type plants (Fig. 6C). *fru-G108* mutant plants served as negative control and were highly chlorotic. Chlorophyll measurements indicated that OX plants had about two times higher chlorophyll concentrations than wild type plants (Fig. 6D).
mutant plants had only about 25% of chlorophyll concentrations than wild type plants.

Therefore, FRU was able to significantly enhance iron mobilization responses at low iron supply, whereas upon high iron supply FRU expression was not able or not sufficient to induce iron mobilization responses to the same level as at low iron.

4. Discussion

4.1. FRU activity is required for induction of iron mobilization gene expression

The BHLH gene FRU was required for the onset of essential iron mobilization genes, IRT1 and FRO2, in roots. In fru knockout plants, IRT1 and FRO2 gene expression was downregulated. In contrast, in FRU overexpression lines these two iron mobilization marker genes were induced upon low iron supply in roots and/or leaves. Gene expression was paralleled by low root iron reduction capacity and low chlorophyll shoot contents in fru knockout mutants, indicating low uptake of iron. On the other hand, increased root iron reduction and increased chlorophyll concentrations were observed in FRU overexpression lines compared to wild type, indicating increased iron uptake.

It is interesting to note that in the chlorotic knockout mutants irtl, frd1 (fro2) or frd3 molecular iron mobilization responses were upregulated compared to the wild type [11,17,23,24]. Increased upregulation of iron mobilization responses at the molecular level was however not sufficient to overcome the mutation-dependent iron deficiency in these chlorotic mutants. In this respect, fru mutants plants were clearly unique as they exhibited reduced molecular iron mobilization responses despite being chlorotic.

Therefore, we propose that FRU is a mediator in regulating iron uptake in plants.

FRU promoter-GUS activity patterns were consistent with the mRNA gene expression analysis, so that we regarded the GUS signals as representative for FRU gene expression sites. The GUS expression signals suggested that FRU was active all along the root in epidermal cells. Epidermal cells were previously shown to express IRT1 and FRO2 [11,25]. A fairly universal DNA motif recognized by bHLH proteins is the E-box (CANNTG) [1,2]. Several E-boxes were detected in FRO2 and IRT1 promoters (not shown). FRU and LeFER do not contain the canonical H–E–R motif in the bHLH amino acid sequences but a T–E–R motif. It is not known whether bHLH factors with alterations in the DNA contacting amino acids bind to the E-box in plants [1,2]. Since neither IRT1 nor FRO2 were constitutively induced in FRU overexpression lines upon high iron supply, we take this as an indication that FRU action was post-transcriptionally downregulated by iron supply. Additional iron-dependent factors might be required for FRU action. Such iron-dependent factors may act post-transcriptionally through protein–protein interaction, by influencing protein stability or protein modification.

The presence of pFRU-GUS signals in the inner root cells close to the vascular cylinder suggested additional functions of FRU in regulating internal root iron homeostasis processes. Gene expression of iron homeostasis genes in inner roots cells was previously reported for LeFER [3] as well as the iron-regulated metal transporter gene LeNRAMP1 in tomato roots [6] and AtNRAMP3 in Arabidopsis roots [26].

The analysis of FRU showed that conserved iron regulatory mechanisms were present in Arabidopsis and tomato. We predict that these regulatory mechanisms are similar in other dicot plants as well. Despite the similarities between LeFER and FRU action, we could also detect differences in the mode of action of the two genes in the two species. For example, FRU expression was iron-regulated in Arabidopsis, however, in tomato this was not previously observed [3]. We are currently testing if LeFER expression in tomato might perhaps be downregulated by generous (more than sufficient) iron supply. The cellular expression patterns of FRU mRNA at the root tip were also distinct between Arabidopsis and tomato roots (compare with [3]). Furthermore, our results showed that upon iron deficiency FRU overexpression was able to direct IRT1 and FRO2 expression in leaves, which was not the case for LeFER effects on LeIRT1 [6]. Moreover, LeFRO1, a homolog of Arabidopsis FRO2, was generally expressed in tomato wild type leaves upon low iron supply, despite LeFER not being expressed in leaves at all [7].

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References


