AtbHLH29 of *Arabidopsis thaliana* is a functional ortholog of tomato *FER* involved in controlling iron acquisition in strategy I plants

You Xi YUAN[1,2], Juan ZHANG[1,2], Dao Wen WANG[1], Hong Qing LING[1,*]

[1]*The State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, China*
[2]*Graduate School of the Chinese Academy of Sciences, Yuquan Road, Shijingshan District, Beijing 100039, China*

ABSTRACT

AtbHLH29 of Arabidopsis, encoding a bHLH protein, reveals a high similarity to the tomato FER which is proposed as a transcriptional regulator involved in controlling the iron deficiency responses and the iron uptake in tomato. For identification of its biological functions, *AtbHLH29* was introduced into the genome of the tomato *FER* mutant *T3238fer* mediated by *Agrobacterium tumefaciencs*. Transgenic plants were regenerated and the stable integration of *AtbHLH29* into their genomes was confirmed by Southern hybridization. Molecular analysis demonstrated that expression of the exogenous *AtbHLH29* of Arabidopsis in roots of the *FER* mutant T3238*fer* enabled to complement the defect functions of FER. The transgenic plants regained the ability to activate the whole iron deficiency responses and showed normal growth as the wild type under iron-limiting stress. Our transformation data demonstrate that AtbHLH29 is a functional ortholog of the tomato FER and can completely replace FER in controlling the effective iron acquisition in tomato. Except of iron, FER protein was directly or indirectly involved in manganese homeostasis due to that loss functions of FER in T3238*fer* resulted in strong reduction of Mn content in leaves and the defect function on Mn accumulation in leaves was complemented by expression of *AtbHLH29* in the transgenic plants. Identification of the similar biological functions of FER and AtbHLH29, which isolated from two systematically wide-diverged "strategy I" plants, suggests that *FER* might be a universal gene presented in all strategy I plants in controlling effective iron acquisition system in roots.

***Keywords*:** tomato, *AtbHLH29*, iron uptake, Arabidopsis, *FER*, plant nutrition.

INTRODUCTION

Iron is essential for all the creatures. As a transition element it functions in the redox and many vital enzymatic reactions required for fundamental biological processes, such as photosynthesis and respiration. Both deficiency and excess of iron are harmful for organisms. Therefore, it is critical to maintain the iron concentration in organism [1]. Anemia caused by iron deficiency in human health is a severe problem afflicting more than two billion peoples in the world, especially in developing countries (http://www.harvestplus.org/iron.html). Biofortification of iron content and availability in plant food products is an economic, easy and basic method to reduce or solve this problem.

Although abundant in soil, iron is one of the most common nutrients limiting plant growth and development because it exists mostly in low-soluble oxidized form (Fe^{3+}), which is hardly available for plants. For meeting demand, plants have developed their unique mechanisms for effective acquisition of iron from soil [2, 3]. All plants except of Gramineae use an effective acquisition mechanism termed as 'strategy I' including (A) the release of proton into rhizosphere to increase the solubility of ferric iron in soil, (B) the induction of Fe^{3+}-chelate reductase activity to reduce ferric to ferrous iron on root surface and (C) the activation of the high-affinity Fe^{2+}-transport system to transfer Fe^{2+} across plasma membrane into cells as well as (D) accompanied morphologic changes in roots, such as formation of transfer cells, increased root hair formation. All these

*Correspondence: Hong Qing LING
Tel/Fax: +86-10-64860377;
E-mails: hqling@genetics.ac.cn

are called iron deficiency responses of the strategy I and are tightly regulated by iron status in plants. In past decade, a large progress has been achieved in studying molecular mechanisms of the strategy I. Three ferric-chelate reductase genes (*AtFRO2*, *PsFRO1* and *LeFRO1*) were isolated from Arabidopsis, pea and tomato, respectively [4-6]. Their transcriptions were induced in roots under iron deficiency. Many iron-regulated transporters (IRT) were identified and isolated from various plants, such as *AtIRT1* and *AtIRT2* from *Arabidopsis thaliana* [7, 8], *LeIRT1* and *LeIRT2* from tomato [9] and *RIT1* from pea [10] as well as *OsIRT1* from rice [11]. *AtIRT1* was an essential transporter for iron homeostasis in Arabidopsis. Knockout of *AtIRT1* led to a strong chlorosis and growth impairment and the defect functions in the *AtIRT1* mutant could not be complemented by overexpression of *AtIRT2* [12, 13]. The expression of *AtIRT1* was controlled both at transcriptional and posttranscriptional levels. Its transcription intensity was strongly enhanced under iron-limiting whereas the protein accumulation of AtIRT1 quickly diminished once supplying sufficient iron [14]. In addition to *IRTs*, some *NRAMP* (natural resistance-associated macrophage protein) genes were reported as metal transporters involved in iron homeostasis considering their increased transcription intensities under iron deficiency [15-17].

Apart from iron-chelate reductase and metal transporters, *FER* isolated from tomato by map-based cloning is proposed as a central regulatory gene involved in controlling the whole iron deficiency responses and iron uptake in roots of tomato [18, 19]. T3238*fer*, a mutant of *FER*, is inability to turn on the iron deficiency responses under iron-deficient stress, exhibits strong chlorosis and dies off at early stage under normal culture conditions [20]. Further characterization of T3238*fer* indicated that FER protein is involved in controlling the transcription of the ferric-chelate reductase *LeFRO1* and the metal transporters *LeIRT1* and *LeNRAMP1* [6, 19, 21]. *FER* encoding a bHLH protein is the first cloned regulatory gene in iron homeostasis of strategy I plants. It will be interesting to know whether all strategy I plants possess a functional ortholog of tomato *FER*, involved in the control of the iron deficiency responses and iron uptake. *Arabidopsis thaliana*, as a model plant for molecular biological studying, is a typical strategy I plants. The complete genome sequence of *Arbidopsis thaliana* is available [22]. We blasted the whole genome sequence of *Arabidopsis thaliana* with FER sequence at protein level and found that the AtbHLH29 (At2g28160) revealed the highest similarity to tomato FER among the 161 putative bHLH proteins in Arabidopsis [23]. Recently, it was reported that AtbHLH29 (FIT1 or FRU) was required for the iron deficiency responses in Arabidopsis [24, 25]. The knockout mutant of *AtbHLH29* displayed typical iron deficiency symptom (chlorosis) and strong growth impairment. The AtbHLH29 protein was involved in controlling of the ferric-chelate reductase *AtFRO2* at transcriptional level and the iron transporter AtIRT1 at protein level [24, 25]. However, it is not clear whether *AtbHLH29* is a functional ortholog of tomato FER. Here we provide our experiment results demonstrating that *AtbHLH29* is a functional ortholog of tomato *FER* in Arabidopsis and suggest that *FER* would be a universal gene controlling iron deficiency responses and iron acquisition from soil in all strategy I plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The iron-inefficient mutant T3238*fer* of tomato [20] and its wild type T3238 were used in this work. Unless otherwise stated, T3238, T3238*fer*, and transgenic plants were first grown on MS agar medium (Sigma, MO, USA) containing 100 μM Fe(III)-EDTA for two weeks in a culture chamber with 16 h light period, then shifted to a hydroponic culture system in Hoagland solution containing 10 μM Fe(III)-EDTA for growth. Four weeks later corresponding plant parts were harvested for further analysis.

Constructing plasmids for plant transformation

Two T-DNA constructs pBin35S-*AtbHLH29-HIS* (for expressing the cDNA of *AtbHLH29*) and pBin35S-*AtbHLH29-GUS* (for expressing the *AtbHLH29-GUS* fusion cistron) were prepared for the complementation experiments. The coding sequences of *AtbHLH29* were amplified from total RNAs of *Arabidopsis thaliana* by RT-PCR with primers 5'-cac cca tgg aag gaa gag tca ac-3' and 5'-act gga tcc tca gtg atg gtg atg gtg atg agt aaa tga ctt gat gaa-3' for constructing the vector pBin35S-*AtbHLH29-HIS* and 5'-cac cca tgg aag gaa gag tca ac-3' and 5'-tta gtc gac cta gta aat gac ttg atg-3' for the construct of pBin35S-*AtbHLH29-GUS* and cloned into pGEM T-easy vector (Promega, USA). After verification by sequencing, they were cleaved out with *Nco*I and *Sal*I and subcloned into the plasmid pJIT163 and pJIT166 (http://www.pgreen.ac.uk) to obtain expression cassettes of p35S-*AtbHLH29-HIS* and p35S-*AtbHLH29-GUS*. Then, the expression cassettes of 35S-*AtbHLH29-HIS* and 35S-*AtbHLH-GUS* were individually cut out with *Sac*I and *Sal*I and integrated into the T-plasmid pBINPLUS [26], generating plant transformation constructs pBin35S-*AtbHLH29* and pBin35S-*AtbHLH29-GUS*. The plasmids were finally introduced into *Agrobacterium tumefaciencs* strain GV3101 by electroporation.

Plant transformation and Southern analysis

Seeds of the iron-inefficient mutant *T3238fer* of tomato were surface sterilized and germinated on MS agar medium in a culture chamber with 16 h light period. The cotyledons of one-week-old seedlings were harvested and infected with *Agrobacterium tumefaciencs* strain GV3101 containing a corresponding construct for generation of transgenic plants. The processes of transformation, shoot regeneration and selection of putative transgenic plants were performed following the protocol described by Ling *et al*. [27]. For further identification at molecular level, total DNAs were extracted from transgenic plants, T3238*fer* and wild type according to the

microprep protocol of Fulton *et al*. [28]. For Southern analysis, approximately 10 μg total DNA were individually digested with *Eco*RI and with *Bam*HI and *Nco*I at 37ºC overnight. Gel separations, blotting of DNA fragments on membrane and hybridization with the probe *AtbHLH29* cDNA labeled with dCTP^{32} were carried out following the description of Ling *et al*. [18]. After washing in buffer (2×SSC, 0.1% SDS) twice (each time for 15 min), membranes were exposed to Kodak X-ray film for 2-3 d.

Elemental analysis

For determination of metal contents, leaves of transgenic lines, T3238*fer* and the wild type were harvested from plants grown four weeks in the hydroponic culture system with 10 μM Fe(III)-EDTA and dried overnight in an oven at 80°C. Elemental analysis was performed by the core facility center of Tsinghua University using an inductively coupled plasma atomic emission spectrometer (Prodigy, Leeman Labs, INC) according to methods published previously [29]. Data were analyzed by SIGMAPLOT (SYSTAT, CA, USA).

Expression profile analysis

For characterizing the expression profiles of *AtbHLH29* and the genes involved in iron homeostasis in transgenic plants, total RNAs were extracted using the Tri reagent (Sigma, USA) from leaves and roots collected from plants, which were treated under iron-limiting condition (10 μM Fe(III)-EDTA) in the hydroponic culture system for four weeks. After elimination of DNA contamination by treatment with RQ1 RNase-Free DNase (Promega, USA) at 37°C for 30 min, the mRNAs were then converted to cDNAs using M-MLV reverse transcriptase (Invitrogen, USA) according to the manufacture's brochure. Semiquantitative reverse transcription (RT)-PCR analysis was performed according to the protocol described by Li *et al*. [6] with 30 PCR cycles. *LeEF-1A* was used as an internal control with 20 PCR cycles. The gene-specific primers used for RT-PCR analysis are 5'-gag agt ggt aat gca tca atg ga-3' and 5'-gaa tcc att gag aga ctc aag-3' for *LeFER*, 5'-atg gaa gga aga gtc aac gct-3' and 5'-tca agt aaa tga ctt gat gaa-3' for *AtbHLH29*, 5'-gga gcc aga gaa aat cag tg-3' and 5'-cga agc cat agg agt tgc-3' for *LeFRO1*, 5'-tgg ctg tgg ctg gaa atc atg ttc-3' and 5'-aga att ttt ttg caa ctc cca ata ggt-3' for *LeIRT1*, 5'-gct ttg tcc tga ggc taa taa tg-3' and 5'-gtt tcg cgt tgt ttg tgt cc-3' for *LeNRAMP1* and 5'-act ggt ggt ttt gaa gct ggt atc tcc-3' and 5'-cct ctt ggg ctc gtt aat ctg gtc-3' for *LeEF-1A*.

Histochemical GUS-staining

For histochemical GUS-staining, the method described by Weigel and Glazebrook [30] was followed. The GUS activity in leaves, roots and flowers was assayed. Leaves and roots were collected from plants which grew in hydroponics with 10 μM Fe(III)-EDTA for 4 weeks and flowers were harvested from the transgenic plants growing in a greenhouse.

RESULTS

Phenotypic complementation of iron-inefficient mutant T3238*fer* of tomato with *AtbHLH29* of *Arabidopsis thaliana*

In the tomato mutant T3238*fer*, the iron deficiency responses in roots are disabled under iron-limiting condition owing to the insertion mutation of *FER* [18, 19]. Arabidopsis AtbHLH29 has 72% similarity and 42.5% identity to tomato FER at the protein level [19]. For testing if *AtbHLH29* was a functional ortholog of *FER,* the coding sequence of *AtbHLH29* and its fusion cistron with *GUS* (x-glucuronidase) driven by the CaMV35S promoter were introduced into the genome of T3238*fer* by *Agrobacterium*-mediated transformation. Seven kanamycin-resistant plants (two from transformation with the construct pBin35S-*AtbHLH29* and five from the transformation with pBin35S-*AtbHLH29-GUS*) were independently obtained from different transformation experiments. They, as putative transgenic lines, were further analyzed by Southern hybridization. Six of the seven lines (29-GUS2, 29-GUS10, 29-GUS12, 29-GUS13, 29-GUS14, 29-HIS1) displayed hybridization signals when probed with *AtbHLH29* cDNA whereas no signals were observed in the line 29-HIS2 and the negative controls T3238*fer* and T3238 (data not shown). A single probed band appeared in each of the 6 transgenic lines, indicating that a single copy of *AtbHLH29* was integrated in the genome of T3238*fer*. Three transgenic lines 29-GUS2, 29-GUS10 and 29-GUS13 were selected for further detailed analysis.

Fig. 1 Phenotypic characterization of the transgenic plants overexpressing Arabidopsis *AtbHLH29* together with the *FER* mutant T3238*fer* and the wild type T3238 in a hydroponics containing 10 μM Fe(III)-EDTA. The picture was taken four weeks after growing in the hydroponics. The transgenic line 29-GUS13 showed phenotypic complementation and grew as well as the wild type under iron-limiting stress whereas the mutant T3238*fer* revealed chlorotic phenotype.

Tomato T3238*fer* is an iron-inefficient mutant and shows strong chlorosis under iron-limiting condition. To check whether *AtbHLH29* can phenotypically complement T3238*fer*, the transgenic lines (29-GUS2, 29-GUS10 and 29-GUS13) together with *FER*-mutant T3238*fer* and its wild type T3238 were shifted from *in vitro* culture to a

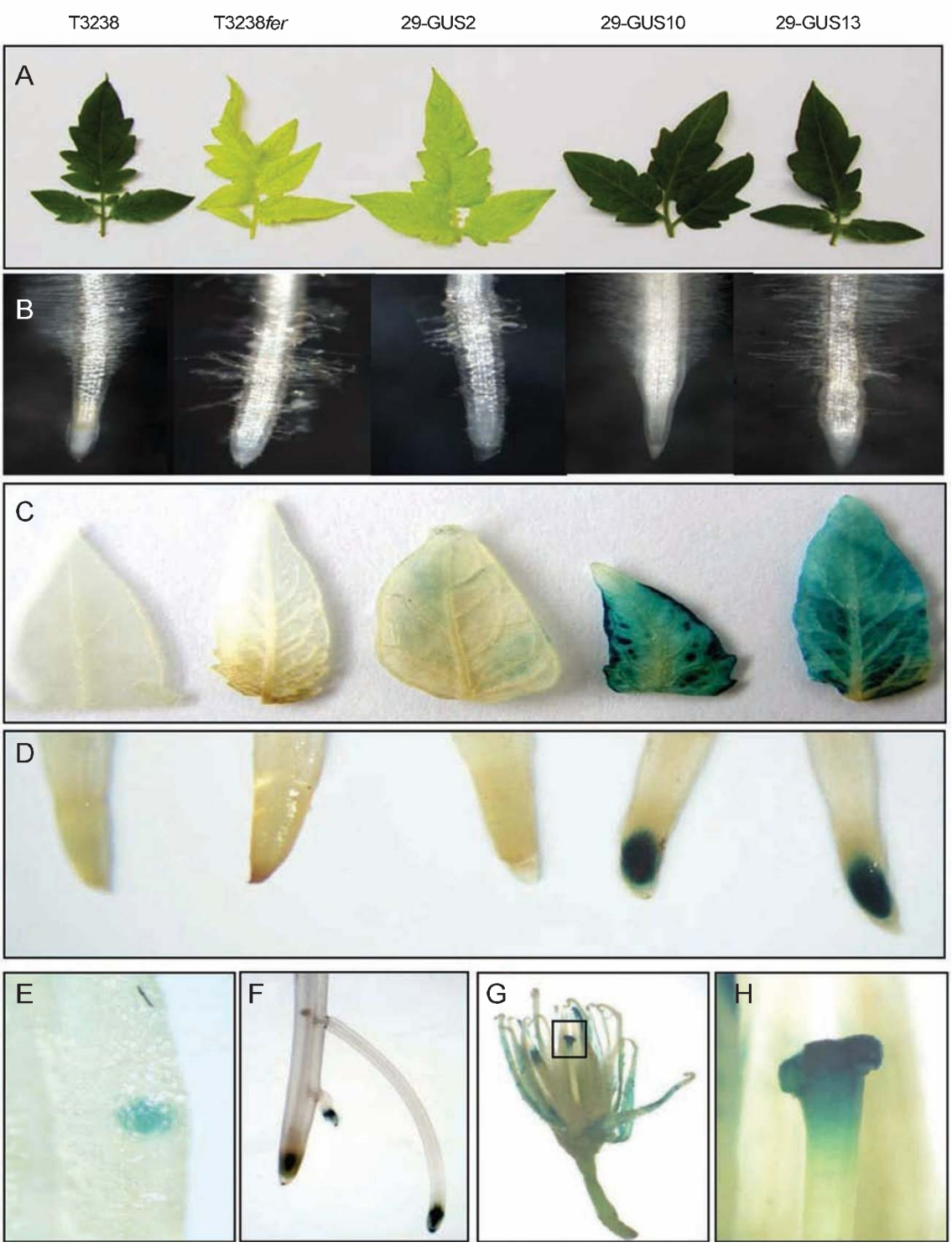

Fig. 2 Morphological and histological analysis of transgenic plants (29-GUS2, 29-GUS10 and 29-GUS13) with the positive (T3238) and negative (T3238*fer*) controls. **(A)** Leaf colors, the *FER* mutant T3238*fer* and the transgenic line 29-GUS2 revealed iron deficiency symptom (yellow) and the transgenic lines 29-GUS10 and 29-GUS13 displayed normal growth as the wild type T3238 (green) in greenhouse. **(B)** The morphology of root tips collected from plants which grew for two weeks in the hydroponics containing 10 μM Fe(III)-EDTA. Increased root hair formation was exhibited in 29-GUS10 and 29-GUS13 and T3238 under iron-limiting stress. C-H. Histochemical assay of GUS activity, blue color indicates present of the active fusion protein AtbHLH29-GUS. **(C)** GUS staining in leaves; **(D)** in roots; **(E)** GUS activity in lateral-root primordial of 29-GUS13; **(F)** GUS activity in tips of main and lateral roots of 29-GUS13; **(G)** GUS activity in filaments and stigmas of 29-GUS13; **(H)** an enlarged picture of the square-marked part of **(G)**.

hydroponic culture system with low iron concentration (10 μM Fe(III)-EDTA). The plants of 29-GUS2 and T3238*fer* began exhibition of chlorosis in young leaves two weeks after shifting into the iron-deficiency condition whereas 29-GUS10 and 29-GUS13 grew normally as the wild type till to four weeks (Fig. 1, Fig. 2A). The transgenic plants were then grown in soil in greenhouse. The plants exhibited same phenotypes observed as in the hydroponics, 29-GUS10 and 29-GUS13 revealed a normal growth and 29-GUS2 displayed chlorosis. The normalized growth of 29-GUS10 and 29-GUS13 under iron-limiting condition and in soil indicates that the defect functions of *FER* in T3238*fer* might be complemented by expression of exogenous *AtbHLH29*.

Increased root hair formation is a main morphological character of the iron deficiency responses in tomato. FER is directly or indirectly involved in the induced root hair formation by iron-limiting stress because the *FER* mutant T3238*fer* formed much less root hairs under iron deficiency condition than wild type [19]. Therefore, the root hair formation was investigated in the transgenic lines after treated under iron-deficient condition for two weeks. The root hair numbers in the two phenotypically complemented lines 29-GUS10 and 29-GUS13 were significantly increased under iron-limiting stress than that in T3238*fer* and the line 29-GUS2 which revealed chlorotic phenotype (Fig. 2B and Fig. 3). The increased root hair formation in the transgenic lines 29-GUS10 and 29-GUS13 under iron-limiting stress may be contributed by exogenously introduced *AtbHLH29*, supporting that AtbHLH29 of Arabidopsis has a similar biological function as FER in tomato in inducing root hair formation under iron-limiting stress.

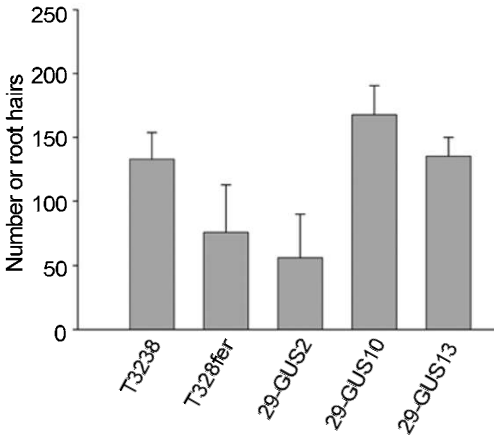

Fig. 3 Induced root hair formation under iron-deficient condition. The transgenic lines (29-GUS2, 29-GUS10 and 29-GUS13), the wild type T3238 and the mutant T3238*fer* were grown in the hydroponics supplementing 10 μM Fe(III)-EDTA for two weeks and root hairs were accounted at the lateral root tips (2 mm) under a microscope. The data shown are average value of 10 roots.

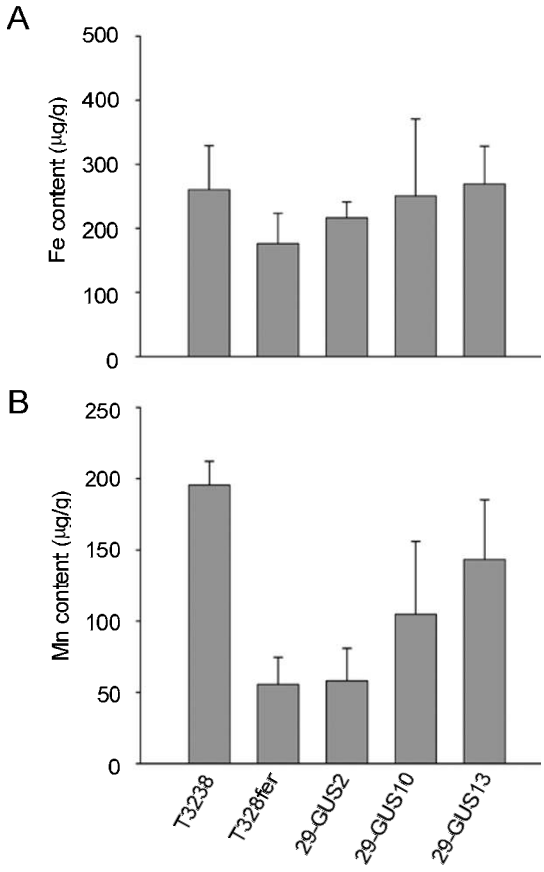

Fig. 4 Iron and manganese contents in leaves of the transgenic lines and the controls (T3238 and T3238*fer*). Leaves were collected from 29-GUS2, 29-GUS10, 29-GUS13, T3238 and T3238*fer* which grew in the hydroponics with 10 μM Fe(III)-EDTA for four weeks. Iron and manganese contents were determined by ICP-AES. **(A)** iron content, **(B)** Manganese content. The data shown are mean values of four individual experiments.

Metal content determination

The *FER*-mutant T3238*fer* is unable to activate the effective iron uptake system under iron-limiting stress, such exhibits iron deficiency induced chlorosis [20]. To determine iron contents, leaves from the transgenic lines, T3238 and T3238*fer* growing 4 weeks in a solution with 10 μM Fe(III)-EDTA were collected, the iron contents were measured by inductively coupled plasma atomic emission spectrometer and shown in Fig. 4. There is a clear correlation between the iron contents and the phenotypes. Lines 29-GUS10 and 29-GUS13, which grew normally as the wild type, contained significantly higher iron concentration in

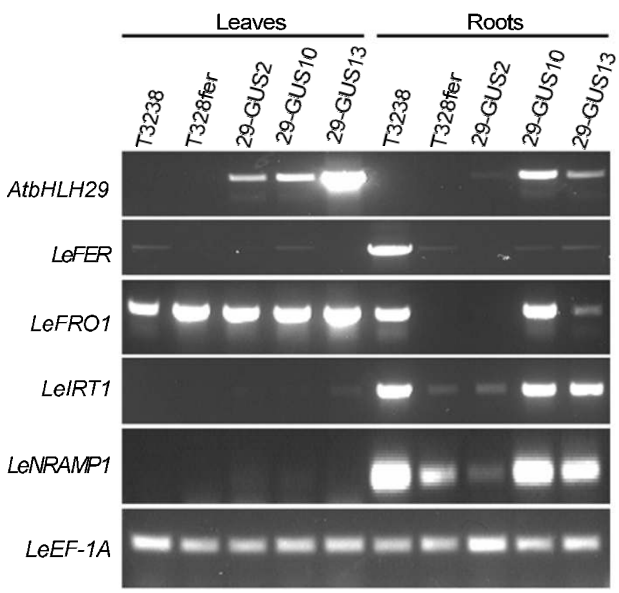

Fig. 5 Expression profiles of *AtbHLH29* and the genes involved in the effective iron acquisition system in tomato under iron-limiting stress. Wild-type (T3238), mutant (T3238*fer*) and three transgenic plants (29-GUS2, 29-GUS10, 29-GUS13) overexpressing *AtbHLH29* were grown under the hydroponic culture in Hoagland solution containing 10 μM Fe(III)-EDTA for four weeks. RNAs were prepared from leaves and roots and the expression profiles of *AtbHLH29*, *FER*, *LeFRO1*, *LeIRT1* and *LeNRAMP1* were analyzed by RT-PCR. Tomato elongation factor gene *LeEF-1A* was used as internal control.

their leaves than the mutant T3238*fer* whereas 29-GUS2 showed chlorotic phenotype similar to T3238*fer*, and contained low iron in leaves (Fig. 4A). In addition to iron, manganese contents in leaves were also determined. Interestingly, disability of *FER* in T3238*fer* resulted in the dramatic reduction of Mn content in leaves under iron-limiting stress, the Mn amount in the leaves of T3238*fer* was only approximately one fourth of the wild type (Fig. 4B). The transgenic line 29-GUS2 which showed chlorotic phenotype revealed a low Mn content in leaves same as T3238*fer* while the phenotype-normalized lines 29-GUS10 and 29-GUS13 contained significantly higher Mn content in leaves than the mutant and 29-GUS2. These results indicate that FER and its homologue AtbHLH29 are directly or indirectly involved in manganese homeostasis in tomato.

Expression profiles of *AtbHLH29* and the corresponding genes in transgenic plants

The ferric chelate reductase *LeFRO1* and the metal transporter *LeIRT1* and *LeNRAMP1* are proposed as main functional genes involved in the effective iron acquisition system in tomato and controlled by FER [6]. In the *FER* mutant T3238*fer*, the three genes lost their induced expression ability under iron deficiency [19, 21, 31]. To investigate the expression profiles of *AtbHLH29* and the three genes involved in the effective iron uptake in transgenic plants, the total RNAs of leaves and roots were extracted from plants which grew under iron-limiting condition for 4 weeks and characterized by RT-PCR analysis (Fig. 5). As shown in Fig. 5, *AtbHLH29* as an exogenous gene driven by CaMV35S promoter was exclusively expressed in the transgenic plants and its mRNA was detected in leaves and roots. Interestingly, the transcription level of *AtbHLH29* varied dramatically among the three transgenic lines and between tissues of leaves and roots. The line 29-GUS13 showed the most abundant mRNA in leaves whereas 29-GUS10 revealed the highest transcription intensity in roots. There is a very low level of *AtbHLH29* expression in the roots of 29-GUS2 that displayed a chlorotic phenotype, suggesting that the *AtbHLH29* transcripts in the roots of transgenic 29-GUS2 line is probably insufficient to rescue the phenotype.

In addition to AtbHLH29, expression patterns of *LeFRO1*, *LeIRT1* and *LeNRAMP1* were also analyzed. The transcript of *LeFRO1* was detected in roots of the transgenic lines 29-GUS10, 29-GUS13 and the wild type T3238, but not in roots of the mutant T3238*fer* and the transgenic line 29-GUS2. The expression intensity of *LeFRO1* was obviously correlated with the mRNA abundance of *AtbHLH29* (Fig. 5). However, such expression correlation was not observed in leaves (*LeFRO1* expressed strongly in leaves of all plants investigated). For the metal transporter genes *LeIRT1* and *LeNRAMP1*, their transcripts were only detected in roots. The transcription intensities of *LeIRT1* and *LeNRAMP1* were much less in the mutant and 29-GUS2 than that in 29-GUS10, 29-GUS13 and T3238 (Fig. 5). These dada indicate that AtbHLH29 of Arabidopsis is able to replace the tomato FER in the activation of downstream gene expression under iron-limiting condition. The gene expression dada also suggest that the chlorotic phenotype and low iron content in leaves of the transgenic line 29-GUS2 were contributed by insufficient expression of *AtbHLH29*.

Histochemical analysis

The *GUS* (β-glucuronidase) has widely been used as a reporter gene in studying plant molecular biology due to its stability and low activity in plants [32]. It was fused to the 3' end of *AtbHLH29*-coding sequence in order to analyze the expression profile of the AtbHLH29 protein in transgenic plants. Leaves, roots and flowers were collected and stained for GUS protein (Fig. 2C-H). The three

transgenic lines investigated revealed clear differences in the expression levels of the AtbHLH29-GUS protein. In leaves, GUS signal was strongly detected in 29-GUS10 and 29-GUS13 and very faint in 29-GUS2 (Fig. 2C). In roots, GUS staining was observed in the lines 29-GUS10 and 29-GUS-13, but not in 29-GUS2. Interestingly, GUS activity in the roots was delimited in the dividing zone and the elongation zone of root tips (Fig. 2D and 2F) and in lateral-root primordial (Fig. 2E) although it was driven by CaMV35S (a strong constitutive expression promoter). Additionally, the GUS activity was also detected in filaments and stigmas (Fig. 2G and 2H).

DISCUSSION

AtbHLH29 of Arabidopsis has high similarity to tomato *FER*. Both genes encode a bHLH protein and are proposed as transcriptional factors functioning in iron deficiency responses and iron uptake [19, 24, 25]. Loss function of AtbHLH29 in a T-DNA insertion line (SALK_126020) and FER in T3238*fer* disabled the effective iron uptake in the mutants. The mutant plants exhibit strong iron deficiency symptoms (chlorosis). Expression of exogenous *AtbHLH29* in T3238*fer* of tomato revealed functional complementation of *FER*. The transgenic plants regained the abilities to respond the iron deficiency in roots and exhibited normal growth as the wild type under iron-limiting stress. Considering the differences in expression and location pattern, Colangelo and Guerinot [24] suggested that *FER* in tomato and *AtbHLH29* in Arabidopsis were two genes governing the effective iron uptake system by different manner. However, our transformation data of *AtbHLH29* in T3238*fer* are clearly disagreed the speculation, supporting that AtbHLH29 in *Arabidopsis thaliana* is a functional ortholog of tomato FER and functions with similar manners as FER in controlling the effective iron uptake system in tomato. Isolation and functional identification of *FER* and *AtbHLH29* in tomato and Arabidopsis, which belong to systematically wide-diverged two families (Solanaceae and Cruciferae), suggesting that all strategy I plants (all higher plants except of grasses) might possess a similar central gene as *FER* controlling the iron deficiency responses and the effective iron acquisition from soil under iron-limiting stress.

Histological analysis by GUS staining revealed that the fusion protein AtbHLH29-GUS in transgenic plants was only detectable specifically in tissues of root tips, lateral-root primordial, leaves and filaments and stigmas (Fig. 2C to H) although the transcript of *AtbHLH29* was detectable in whole roots (data do not shown). These indicate that *AtbHLH29* expression in the genome of T3238*fer* will be controlled by a posttranscriptional regulation mechanism at protein level. The posttranscriptional regulation of *FER* in tomato [33] and *AtbHLH29* in Arabidopsis [24, 25] has already been observed. In Addition to *AtbHLH29* and *FER*, the posttranscriptional regulation of the ferric-chelate reductase *AtFRO2* and the Fe(II)-transporter *AtIRT1* was also reported [14, 34]. Taken together, it looks like that the posttranscriptional regulation of genes involved in iron uptake in strategy I plants is a common phenomenon.

For line 29-GUS2, it was shown that *AtbHLH29* was integrated into the genome of the transgenic plants by Southern analysis. There is decent transcription of *AtbHLH29* in leaves, however, due to unknown reasons, the transgenic roots revealed very faint *AtbHLH29* transcription (Fig. 5) and failed to show detectable AtbHLH29-GUS protein by histochemical analysis (Fig. 2D). The ferric chelate reductase *LeFRO1* required for plant response to low iron nutrient is absent in 29-GUS2 line (Fig. 5). Although there are weak transcripts for *LeIRT1* and *LeNRAMP1* in response to low iron growth condition, it is not known whether respective proteins are expressed and whether the proteins are active (Fig. 5). As mentioned above, the chlorotic phenotype in *FER* mutant cannot be complemented by the *AtbHLH29* transgene in line 29-GUS2, suggesting that expression of the transgene in the transgenic roots is required for activation of effective iron uptake pathway. The ferric chelate reductase *LeFRO1* and the metal transporters *LeIRT1* and *LeNRAMP1* are main functional genes involved in the effective iron uptake system in tomato. The transcription of the three genes in roots was directly or indirectly regulated by FER under iron-limiting stress. Of them, the transcription intensity of *LeFRO1* in roots of transgenic plants appeared to be tightest correlated with mRNA amount of *AtbHLH29* (Fig. 5), indicatting that *LeFRO1* will be directly targeted by AtbHLH29. Colangelo and Guerinot [24] recently reported that AtbHLH29 (FIT1) regulated the ferric chelate reductase *AtFRO2* at mRNA accumulation level and the ferrous transporter AtIRT1 at the level of protein accumulation in Arabidopsis.

Mn is an essential microelement for plant growth and development. It acts as a cofactor for enzymatic activities of many enzymes involved in oxidation-reduction, decarboxylation, hydrolytic reactions and so on. Mn(II) is the major form taken up by plants and is translocated from the root to the shoot through the xylem. In the *FER* mutant T3238*fer*, Mn content in the leaves reduced dramatically (approximately one fourth compared to the wild type). Interestingly, in the transgenic plants 29-GUS10 and 29-GUS13 the manganese amount in leaves was raised significantly (Fig. 4B), indicating Arabidopsis homologue of tomato FER is directly or indirectly required for manganese homeostasis in tomato. Previous studies showed that iron transporters AtIRT1 and LeIRT1 can deliver a broad

range of divalent metal substrates such as Fe, Mn, and Zn [9, 35]. Increased accumulation of Mn, Zn and Co as well as Cd in plants usually occurs under iron deficiency stress. Vert *et al*. [12] reported that *AtIRT1*-knockout Arabidopsis plants failed to accumulate Mn and Zn in the roots under iron deficient conditions, suggesting that Arabidopsis AtIRT1 might also be involved in the transport of manganese. In tomato, the expression of iron transporter *LeIRT1* is partially controlled by FER protein [6] and *LeIRT1* mRNA was significantly reduced in the roots of T3238*fer* mutant compared to the wild type under iron-deficient stress, suggesting the relevance of LeIRT1 function in Mn accumulation in tomato plants. Expression of the exogenous *AtbHLH29* in transgenic plant lines 29-GUS10 and 29-GUS13 enhanced Mn concentration, probably via stimulated LeIRT1 activity or other transporters involved in manganese homeostasis.

ACKNOWLEDGEMENTS

Authors thank Wen Juan ZHOU for technical assistance. This work was supported by grants from the Ministry of Science and Technology of China (Grant No. 2004AA222110), and the National Natural Science Foundation of China (Grant No. 30225029).

Received, May 8, 2005
Revised, July 25, 2005
Accepted, Aug 15, 2005

REFERENCES

1 Kampfenkel K, Van MM, Inzé D. Effects of iron excess on *Nicotiana plumbagnifolia* plants. Plant Physiol 1995; **107:**725-35.
2 Takagi S, Nomoto K, Takemoto T. Physiological aspect of mugineic acid, a possible phytosiderophore of *graminaceous* plants. J Plant Nutr 1984; **7:**469-77.
3 Römheld V. Different strategies for iron acquisition in higher plants. Physiol Plant 1987; 7**0:**231-34.
4 Robinson NJ, Procter CM, Connolly EL, *et al*. A ferric-chelate reductase for iron uptake from soils. Nature 1999; **397:**694-7.
5 Waters BM, Blevins DG, Eide DJ. Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol 2002; **129:**85-94.
6 Li L, Cheng XD, Ling H-Q. Isolation and characterization of Fe (III)-chelate reductase gene *LeFRO1* in tomato. Plant Mol Biol 2004; **54:**125-36.
7 Eide D, Broderius M, Fett J, *et al*. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci U S A 1996; **93:**5624-28.
8 Vert G, Briat JF, Curie C. Arabidopsis *IRT2* gene encodes a root-periphery iron transporter. Plant J 2001; **26:**181-9.
9 Eckhardt U, Mas MA, Buckhout TJ. Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. Plant Mol Biol 2001; **45:**437-48.
10 Cohen CK, Garvin DF, Kochian LV. Kinetic properties of a micronutrient transporter from *Pisum sativum* indicate a primary function in Fe uptake from the soil. Planta 2004; **218:**784-92.
11 Bughio N, Yamaguchi H, Nishizawa NK, *et al*. Cloning an iron-regulated transporter from rice. J Exp Bot 2002; **53:**1677-82.
12 Vert G., Grotz N, Dedaldechamp F, *et al*. IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell 2002; **14:**1223-33.
13 Henriques R, Jasik J, Klein M, *et al*. Knock-out of Arabidopsis metal transporter gene *IRT1* results in iron deficiency accompanied by cell differentiation defects. Plant Mol Biol 2002; **50:**587-97.
14 Connolly EL, Fett JP, Guerinot ML. Expression of the *IRT1* metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 2002; **14:**1347-57.
15 Curie C, Alonso JM, Le Jean M, *et al*. Involvement of *NRAMP1* from *Arabidopsis thaliana* in iron transport. Biochem J 2000; **347:**749-55.
16 Thomine S, Wang R, Ward JM, *et al*. Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to *Nramp* genes. Proc Natl Acad Sci U S A 2000; **97:**4991-6.
17 Thomine S, Lelievre F, Debarbieux E, *et al*. AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. Plant J 2003; **34:**685-95.
18 Ling H-Q, Pich A, Scholz G, *et al*. Genetic analysis of two tomato mutants affected in the regulation of iron metabolism. Mol Gen Genet 1996; 2**52:**87-92.
19 Ling H-Q, Bauer P, Bereczky Z. The tomato *FER* gene encoding a bHLH protein controls iron-uptake responses in roots. Proc Natl Acad Sci U S A 2002; **99:**13938-43.
20 Brown JC, Chaney RL, Ambler JE. A new tomato mutant inefficient in the transport of iron. Physiol Plant 1971; **25:**48-53.
21 Bereczky Z, Wang HY, Schubert V, *et al*. Differential regulation of *NRAMP* and *IRT* metal transporter genes in wild type and iron uptake mutants of tomato. J Biol Chem 2003; **278:**24697-704.
22 The Arabidopsis Initiative: Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 2000; **408:**796-815.
23 Toledo-Ortiz G, Huq E, Quail PH. The Arabidopsis basic/helix-loop-helix transcription factor family. Plant Cell 2003; **15:**1749-70.
24 Colangelo PE, Guerinot ML. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell 2004; **16:**3400-12.
25 Jakoby M, Wang HY, Reidt W, *et al*. FRU (BHLH029) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. FEBS Lett 2004; **577:**528-34.
26 Van Engelen FA, Molthoff JW, Conner AJ, *et al*. pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Res 1995; **4:**28890.
27 Ling H-Q, Kriseleit D, Ganal MW. Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium tumefaciens*-mediate transformation of tomato (*Lycopersicon esculentum*). Plant Cell Rep 1998; **17:**843-7.
28 Fulton TM, Chunwongse J, Tanksley SD. Microprep protocol for extraction of DNA from tomato and other herbaceous plants. Plant Mol Biol Rep 1995; **13:**207-9.
29 Herbik A, Bölling C, Buckhout TJ. The involvement of a multicopper oxidase in iron uptake by the green *algae Chlamy-*

domonas reinhardtii. Plant Physiol 2002; **130:**2039-48.
30 Weigel D, Glazebrook J. Arabidopsis, a laboratory manual. Cold spring harbor laboratory press: New York 2002:241-8.
31 Bauer P, Thiel T, Klatte M, *et al*. Analysis of sequence, map position, and gene expression reveals conserved essential genes for iron uptake in Arabidopsis and tomato. Plant Physiol 2004; **136:**4169-83.
32 Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 1987; **6:**3901-7.
33 Brumbarova T, Bauer P. Iron-mediated control of the basic helix-loop-helix protein FER, a regulator of iron uptake in tomato. Plant Physiol 2005; **137:**1018-26.
34 Connolly EL, Campbell NH, Grotz N, *et al*. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol 2003; **133:**1102-10.
35 Korshunova1 YO, Eide D, Clark WG, *et al*. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol Biol 1999; **40:**37-44.