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ORIGINAL PAPER

Molecular characterization of the basic helix-loop-helix (*bHLH*) genes that are differentially expressed and induced by iron deficiency in *Populus*

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Abstract
Key message **Two *Populus bHLH* genes (*PtFIT* and *PtIRO*) were cloned and characterized. The iron deficiency tolerance may be regulated by the *PtFIT*-dependent response pathway in *Populus*.**
Abstract Five orthologs of eight *Arabidopsis* basic helix-loop-helix (*bHLH*) genes responding to iron deficiency in *Populus* were analyzed. Open reading frame (ORF) regions of two *bHLH* genes (*PtFIT* and *PtIRO*) were isolated from the iron deficiency tolerant (PtG) and susceptible (PtY) genotypes of *Populus tremula* 'Erecta'. Gene sequence analyses showed that each of the two genes was identical in PtG and PtY. Phylogenetic analysis revealed that *PtFIT* was clustered with the *bHLH* genes regulating iron deficiency responses, while *PtIRO* was clustered with another group of the *bHLH* genes regulating iron deficiency responses in a *FIT*-independent pathway. Tissue-specific expression analysis indicated that *PtFIT* was only detected in the root among all tested tissues, while *PtIRO* was rarely detected in all tested tissues. Real-time PCR showed that *PtFIT* was up-regulated in roots under the iron-deficient condition. A higher level of *PtFIT* transcripts was detected in PtG than in PtY. Pearson Correlation Coefficient calculations indicated a strong positive correlation ($r = 0.94$) between *PtFIT* and *PtIRT1* in PtG. It suggests that the iron deficiency tolerance of PtG may be regulated by the *PtFIT*-dependent response pathway. The *PtFIT*-transgenic poplar plants had an increased expression level of *PtFIT* and *PtIRT1* responding to iron deficiency. One *PtFIT*-transgenic line (TL2) showed enhanced iron deficiency tolerance with higher chlorophyll content and Chl a/b ratio under iron deficiency than the control plants, indicating that *PtFIT* is involved in iron deficiency response in *Populus*. The results would provide useful information to understand iron deficiency response mechanisms in woody species.

Keywords Iron chlorosis · Poplar tree · Transcription factor · Iron transport · Tissue-specific expression

Introduction

Iron deficiency-induced chlorosis causes yield loss and poor quality of crops, particularly in calcareous and high pH soils. Iron deficiency is not caused by the lack of iron in the soil, but rather the low availability for plants to absorb (Römheld and Marschner 1986; Guerinot and Yi 1994). Higher plants acquire iron from the soil through two strategies, reduction-based and chelation-based strategies (Römheld and Marschner 1986; Kobayashi and Nishizawa 2012). Non-graminaceous plants (known as Strategy I species) acquire iron through the reduction-based strategy, which is accomplished by reducing Fe(III) to absorbable Fe(II) and then the absorbable Fe(II) is transported into the root by the *IRT1* transporter (Eide et al. 1996; Robinson, et al. 1999). Graminaceous plants (known as Strategy II species) produce Fe(III) chelators, mugineic acid family phytosiderophores (MAs), to form a complex of Fe(III)-MAs that is transported into the root by *YS1* (*Yellow stripe 1*) or YSL (*Yellow stripe 1-like*) transporter (Takagi 1976;

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Curie et al. 2001; Murata et al. 2006; Inoue et al. 2009). Research has revealed that two groups of genes, *Ferric reductase oxidase* (*FRO*) and *Iron-regulated transporter* (*IRT*), are involved in iron reduction and transport in Strategy I species (Eide et al. 1996; Robinson, et al. 1999). When plants are exposed to the iron deficiency condition, the *FRO* and *IRT* genes are induced to reduce Fe(III) to Fe(II) and to regulate the transport of Fe(II) in plants (Eide et al. 1996; Vert et al. 2001; Jeong and Connolly 2009).

Plants can survive under various stressful conditions by regulating the expression of genes that are involved in many biological changes. Gene regulation could be achieved by many ways, such as DNA methylation, transcriptional and post-transcriptional regulation, or regulation of translation (Latchman 2007). Transcriptional regulation refers to the change at gene expression level via altering the transcription rate. Transcription factors are proteins that bind to a specific region of a promoter to control the transcription rate. As a large family of transcription factors, the basic helix-loop-helix (*bHLH*) proteins play an important role in regulating genes involved in iron deficiency responses in plants. A *bHLH* transcription factor (*LeFER*) isolated from tomato offered the first clue to know how iron deficiency responses are regulated by plants (Ling et al. 2002). The *fer* mutant in tomato failed to induce *LeIRT1* expression under the iron-deficient condition, indicating the direct role of *LeFER* in regulating the *LeIRT1* gene. In addition, *LeFER* expression was suppressed by iron sufficiency at the post-transcriptional level (Brumbarova and Bauer 2005). An ortholog of *LeFER* named *AtFIT1* (*FER-like iron deficiency-induced transcription factor 1*, also known as *AtbHLH29* or *AtFRU*) is required for inducing the iron mobilization genes in *Arabidopsis* (Jakoby et al. 2004; Yuan et al. 2005). The *AtFIT1* gene regulates several iron deficiency inducible genes with known or putative functions in iron homeostasis, including *AtFRO2* and *AtIRT1* (Colangelo and Guerinot 2004; Ivanov et al. 2012). Colangelo and Guerinot (2004) reported that *AtFIT1* regulated *AtFRO2* at the level of mRNA accumulation and *AtIRT1* at the level of protein accumulation. Further study demonstrated that with the co-expression of another two *AtbHLH* transcription factors, forming *AtFIT1/AtbHLH38* and *AtFIT1/AtbHLH39* complexes, *AtFRO2* and *AtIRT1* could be constitutively expressed even under the iron-sufficient condition (Yuan et al. 2008). Additionally, another two *AtbHLH* transcription factors (*AtbHLH100* and *AtbHLH101*) within the same subgroup of *AtbHLH38* and *AtbHLH39* were strongly induced by iron deficiency in the root and leaf of *Arabidopsis* (Wang et al. 2007). Sivitz et al. (2012) proposed that rather than the *AtFIT1* target genes, *AtbHLH100* and *AtbHLH101* likely regulate genes involved in the distribution of iron within the plant, suggesting that *AtbHLH100* and *AtbHLH101* play an *AtFIT1*-independent regulation role in iron deficiency responses. Maurer et al. (2014) reported that the triple defective mutant of *bHLH39/100/101* showed an up-regulated expression of *AtIRT1*, *AtFRO2*, and *AtFIT* as the wild type under iron deficiency. With a different opinion, Wang et al. (2013) proposed that both *AtbHLH100* and *AtbHLH101* could interact with *AtFIT1* according to the yeast two-hybrid analysis and bimolecular fluorescence complementation assay. Furthermore, Long et al. (2010) reported that in addition to *AtFIT1* acting as a master regulator in the iron deficiency response, another *AtbHLH* transcription factor, *POPEYE* (*AtPYE,* also known as *AtbHLH047*) expressed in the pericycle, was also responding to iron deficiency. *AtPYE* helps maintain iron homeostasis by regulating the expression of known iron homeostasis genes and other genes involved in transcription, development, and stress response according to microarray data. Additionally, *AtPYE* interacts with *AtPYE* homologs, including *IAA–Leu Resistant3* (*AtILR3*, also named as *bHLH105*) or *AtbHLH115* that is involved in metal ion homeostasis to regulate the downstream target genes. In other species, Legay et al. (2012) proposed that in potato, the expression of the *FER*-like transcription factor that shares 90 % identities with the *LeFER* gene was also influenced by iron status and a strong positive correlation between the expressions of the *FER*-like transcription factor and *IRT1* was observed. In woody plants, three *bHLH* genes (*MxbHLH01*, *MxIRO2*, and *MxFIT*) were isolated and characterized in *Malus xiaojinesis*. The *MxbHLH01* expression was restricted to the root and up-regulated under the iron-deficient condition and *MxbHLH01* might interact with other proteins to regulate genes in response to iron deficiency (Xu et al. 2011). The *MxIRO2* gene (*iron-related transcription factor*) was induced in the root and leaf of *Malus xiaojinesis* under iron deficiency. It might form a heterodimer or multimer with other transcription factors to control the expression of genes related to iron absorption (Yin et al. 2013). The *MxFIT* gene was up-regulated in roots under iron deficiency at both mRNA and protein levels, while almost no expression was detected in leaves irrespective of iron supply. The transgenic *Arabidopsis* plants with *MxFIT* had increased *AtIRT1* and *AtFRO2* transcripts in roots under the iron-deficient condition, showing a stronger resistance to iron deficiency (Yin et al. 2014).

Plant hormones were considered as the upstream signaling molecules that involve in the iron deficiency response by regulating the *FIT* gene (Brumbarova et al. 2014). The expression of *AtIRT1* and *AtFRO2* was positively regulated by auxin (Giehl et al. 2012) and ethylene (Lingam et al. 2011). In addition, cytokinin and jasmonic acid showed the suppression of the iron deficiency responses (Seguela et al. 2008; Maurer et al. 2011).

In a previous study (Huang and Dai, personal communication), the *PtIRT1* gene from *Populus tremula* was cloned and its expression was strongly induced by iron deficiency; particularly, the increment of *PtIRT1* transcripts was much greater in the iron deficiency tolerant clone (PtG) than in the iron deficiency susceptible clone (PtY) of *Populus tremula*. However, overexpression of the *PtIRT1* gene in other transgenic poplar species did not enhance Fe accumulation compared to the wild type regardless of iron status. It indicated that some transcriptional control mechanisms might be involved in regulating *PtIRT1* in iron uptake and transport in poplar. Therefore, we cloned and characterized the *bHLH* genes from both iron deficiency tolerant and susceptible clones of *Populus tremula* and overexpressed them in other poplar species. The results would offer a view of how transcription factors regulate genes in response to iron deficiency and further strengthen the understanding of iron deficiency response mechanisms in woody species.

Materials and methods

Discovery of the *bHLH* candidate genes in response to iron deficiency in *Populus*

To identify *bHLH* proteins that may be in response to iron deficiency in *Populus*, the expression profile of the *Arabidopsis bHLH* genes in the root of *Arabidopsis* under iron deficiency at various time points was analyzed based on the published microarray data in NCBI. A total of 167 *bHLH* genes in *Arabidopsis* predicted by Carretero-Paulet et al. (2010) were used in this study. Microarray data were downloaded from the Gene Expression Omnibus under the series entry GSE10502 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10502). The expression data of the *AtbHLH* genes were extracted from GSE10502 using BRB-Array Tools software (Simon et al. 2007). All extracted data were normalized based on the mean expression value of each gene and analyzed and graphed using the mean of Multi-Experiment Viewer software (Saeed et al. 2006). Genes were hierarchically clustered based on 'Pearson's correlation' distance matric and 'average linkage' method. Analysis of variance (ANOVA) was performed to identify the *AtbHLH* genes that expressed at a significantly different level in each treatment at the level of $P \leq 0.01$. The output genes were used as queries to BlastP their orthologs in *Populus trichocarpa* in NCBI. The putative orthologous genes showing high similarity to the *AtbHLH* genes in response to iron deficiency were considered as the candidates that may also respond to iron deficiency in *Populus.*

Plant materials and growth conditions

Iron deficiency tolerant (PtG) and susceptible (PtY) clones of *Populus tremula* 'Erecta' were used in this study. Plants were maintained and grown in a hydroponic system comprised a 30-hole PVC plate, black plastic container (42 × 34 × 13 cm), Hoagland's solution, and an air pump. The PVC plate was floated on the solution that was prepared according to Hoagland and Arnon (1939). Each container contained seven liters of Hoagland's solution that was aerated with an air pump (TOPFIN Aquarium Air Pump, Model: AIR-8000) and refreshed every week. Each hole on the plate held one plant. The container was then covered by plastic film to maintain the moisture. For acclimation, film was gradually removed from the container after 1 week. For the iron-sufficient treatment, plants were grown in full strength Hoagland's solution containing 30 μM Fe(II)-ethylenediaminetetraacetic acid (EDTA). For the iron-deficient treatment, Fe(II)-EDTA was removed from the Hoagland's solution and 200 μM ferrozine was added.

RNA extraction and cDNA preparation

Total RNA was isolated using the QIAGEN RNeasy Plant Mini Kit (QIAGEN Inc, Valencia, CA, USA) according to the manufacturer's instructions. RNA was isolated from three biological replicates of each treatment. Prior to cDNA synthesis, the RNA was quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and agarose gel electrophoresis. A total of 1 μg RNA was treated with gDNA wipeout buffer to eliminate possible contaminating genomic DNA and then subjected to reverse transcription with RT primer mix (oligo-dT and random primers) and unique QIAGEN Omniscript and Sensiscript reverse transcriptases according to the manufacturer's instructions of the QuantiTect Reverse Transcription Kit (QIAGEN Inc, Valencia, CA, USA).

Gene cloning and sequence analysis

The open reading frame (ORF) region of each candidate gene was cloned from *Populus tremula*. Primers were designed using the PrimerSelect module of the DNASTAR Lasergene® software package (DNASTAR, Inc., Madison, WI, USA). All primers used for gene cloning and RT-PCR are listed in Table 1. The PCR was performed according to the instructions of the Elongase® Enzyme Mix (Invitrogen™, Carlsbad, CA, USA). Target PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN Inc, Valencia, CA, USA) and then ligated into the pGEM-T

Table 1 Sequence information and applications of the primers used in this study

Primer	Sequence (5′–3′)	Application
PtFIT-F3	GTATCTTCTAGAAAAGAATGGATAGGATGGATGA	*PtFIT* cloning
PtFIT-R3	CATAGAGAGCTCAGGGCTAAACAGATGGATT	
PtIRO-F2	TAATAACCCTCCAACTAATCCACA	*PtIRO* cloning
PtIRO-R2	GAAGGTTTTTGCGACAGTATCTAA	
PtFIT-F1	ACCGCCACAACGACTAAGAAGAC	Semi-quantitative/real-time quantitative RT-PCR for *PtFIT*
PtFIT-R1	AACCAAGGACCGCAAAGCATA	
PtIRO-F2	TAATAACCCTCCAACTAATCCACA	Semi-quantitative/real-time quantitative RT-PCR for *PtIRO*
PtIRO-R6	CATCTCTAAAGCTGCACTGTTCAT	
PtAct1-F7	ATGGTTGGAATGGGGCAGAAG	Semi-quantitative RT-PCR internal control *PtAct1*
PtAct1-R7	CGAAGGATGGCGTGTGGA	
PtTIF5α-F	GACGGTATTTTAGCTATGGAATTG	Real-time quantitative PCR for reference *PtTIF5α*
PtTIF5α-R	CTGATAACACAAGTTCCCTGC	
T-DNA-F	ACGTCGCATGCTCCCGG	Gene transfer confirmation
T-DNA-R	CATATGGTCGACCTGCAG	
PtIRT1-F50	TCTCGGAGCCTCAAACAACACT	Real-time quantitative PCR for *PtIRT1*
PtIRT1-R50	AAAAATGCCATGACTGCCTTCTT	

easy vector (Promega, Madison, WI, USA). Plasmid DNA was extracted from the white colonies grown on indicator plates containing X-gal and IPTG, using a PerfectPrep™ Spin Mini Kit (5 PRIME Inc., Gaithersburg, MD, USA) and sent for sequencing at the Iowa State University DNA Facility (Ames, IA, USA). The domains of putative proteins were analyzed using SMART (http://smart.embl-heidelberg.de/). A phylogenetic tree was constructed using the predicted amino acid sequences of the *bHLH* genes from *Populus* and other species by the MegAlign module of the DNASTAR Lasergene® software package.

Evaluation of the expression profile of the *bHLH* genes

The expression profile of the *bHLH* genes in various poplar tissues including the root tip, root, phloem, xylem, mature leaf, young leaf, and shoot tip was analyzed using semi-quantitative RT-PCR. Samples were collected from PtG and PtY grown under the iron-sufficient condition. Primers corresponding to the *PtbHLH* genes and the *Populus* actin gene (NCBI accession no: XM_002298674.1) were designed using the PrimerSelect module of the DNASTAR Lasergene® software package and are listed in Table 1. Prior to RT-PCR, the quality of cDNA was assessed by PCR using actin-specific primers designed to span introns to detect genomic DNA contamination. PCR amplification was carried out in a 16 µl reaction that consisted of 5 ng cDNA template, 0.375 µM of each primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1×Green GoTaq® Flexi buffer, and 5 U *Taq* DNA Polymerase. The amplification conditions were denaturing for 30 s at 94 °C (3 min before the first cycle), annealing for 40 s at 56 °C, and extension for 50 s at 72 °C (5 min after the final cycle) for 30 cycles. PCR products were separated in a 2 % agarose gel at 110 volts (V) for 30 min. The gel was visualized under UV light and images were captured using an AlphaImager® Gel Documentation System (Proteinsimple Inc., Santa Clara, California, USA).

Expression quantification of the *bHLH* genes responding to iron deficiency

The expression of the *bHLH* genes in the root of the poplar plant responding to iron deficiency was evaluated by real-time quantitative PCR that was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a PerfCTa® SYBR Green SuperMix (ROX) kit (Quanta BioSciences, Inc, Gaithersburg, MD, USA). The gene expression was detected in both PtG and PtY plants after they were transferred to the iron-deficient solution for 0, 0.5, 1, 3, and 6 days. Each treatment had three biological replicates with 10 individual plants per replicate. Primers of the *PtbHLH* genes and internal control (*PtTIF5α*, NCBI accession no: CV251327.1) used for real-time quantitative PCR are listed in Table 1. The absolute quantity of the target gene was determined based on the standard curves constructed for each gene according to the method of Larionov et al. (2005). Values of expression level were presented as a ratio of the copy number of *PtbHLHs* to that of *PtTIF5α*. To evaluate amplification specificity, melting curve analysis was performed at the end of each PCR run according to the manufacturer's recommendation. Two technical replicates were used for each PCR.

Expression vector construction and plant transformation

The Suppro::*PtbHLH* expression cassette was constructed using the pCAMBIA S1300 expression vector system (Supplementary Fig. 1) and introduced into *P. canescens* × *P. grandidentata* 'Cl6' using an *Agrobacterium*-mediated method according to Dai et al. (2003). Positive transgenic plants were screened on rooting medium (1/2 MS medium with 0.5 μM NAA) supplemented with 5 μM hygromycin. Verified transgenic lines were proliferated in vitro (Dai et al. 2003) and grown in the hydroponic culture system.

Molecular characterization of transgenic events

Transgenic lines were confirmed by PCR with the specific primers located in the flanking regions of the insertions according to the method of Dai et al. (2003) (Table 1). The expression level of *PtFIT* and *PtIRT1* was determined in the root of transgenic plants grown under either iron-sufficient or iron-deficient conditions by real-time quantitative PCR as described above.

Physiological analysis of transgenic plants grown under the iron-deficient condition

The content of chlorophyll and carotenoids, Chl a/b ratio, and the content of Mn, Zn, Fe, and Cu in the control and transgenic plants grown in either iron-sufficient or iron-deficient condition were determined. Leaves were collected at day 9 after the treatment. Pigments were extracted from liquid nitrogen grinded leaf tissues by 80 % acetone and the content of chlorophyll a, chlorophyll b, and carotenoids was determined following the method of Lichtenthaler (1987). The chlorophyll and carotenoid concentration was expressed as mg/kg FW (fresh weight). For determining the content of mineral elements, leaf samples were oven-dried at 65 °C for 2 days and subjected to leaf tests in the North Dakota State University Cereal Science lab.

Statistical analysis

Data were expressed as mean ± SE. An unpaired two-tailed *t* test was used to analyze significant differences between the treatment and the control.

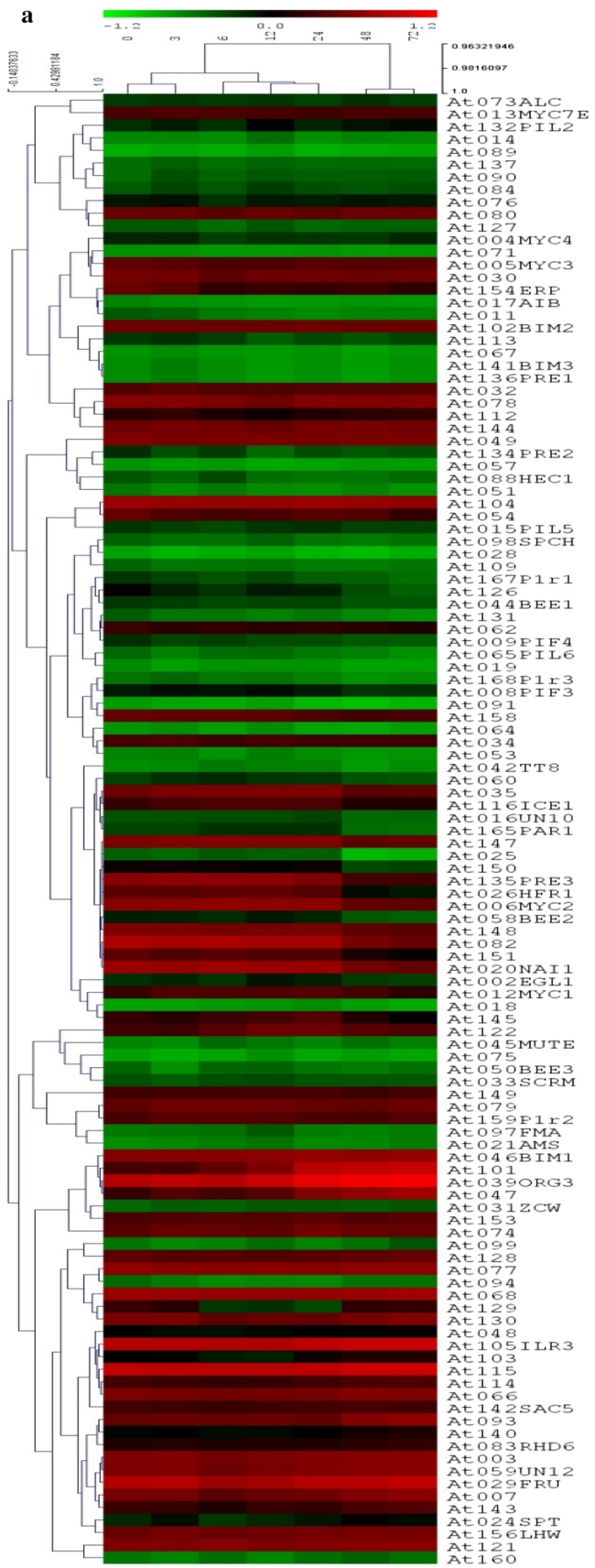
Results

Discovery of the *bHLH* candidate genes in response to iron deficiency in *Populus*

A total of 117 *AtbHLH* genes had the corresponding probes in the array of GSE10502 containing 22,810 genes based on the microarray gene annotation database. The expression pattern of individual *AtbHLH* genes is illustrated in Fig. 1. The seven time points of the iron deficiency treatment (0, 3, 6, 12, 24, 48, and 72 h) were clustered into three groups: Group 1 included 0 and 3 h treatment; group 2 included 6, 12, and 24 h treatment; and Group 3 contained 48 and 72 h treatment. A significant difference in gene expression was observed between group 1 and 3, indicating that the *bHLH* genes might respond to iron deficiency at 3 h after the treatment and such a response could be clearly observed at 48 h after the treatment. According to the ANOVA analysis, 25 *AtbHLH* genes showed significant differences in the level of expression at seven time points (Fig. 1). Of these genes, nine (*At007*, *At039ORG3*, *At047*, *At059UN12*, *At093*, *At101*, *At105ILR3*, *At115*, and *At143*) were up-regulated and the rest of genes were down-regulated by iron deficiency. Previous researches confirmed that *At105ILR3*, *At101*, *At115*, *At039ORG3*, and *At047* were transcription factors that positively regulate iron deficiency responses in plants (Wang et al. 2007, 2013; Yuan et al. 2008; Long et al. 2010). In other research, the *At093* gene was found to be involved in stomatal development (Ohashi-Ito and Bergmann 2006) and *At059UN12* may be related to fertilization (Pagnussat et al. 2005). No research was reported regarding the function of *At007*and *At143*. Therefore, the orthologs of *At105ILR3*, *At101*, *At115*, *At039ORG3*, and *At047* in *Populus trichocarpa* were considered as the candidate genes that may regulate responses to iron deficiency in *Populus*. In addition, *At029FRU*, *At038ORG2*, and *At100* likely play a role in regulation of iron deficiency (Jakoby et al. 2004; Yuan et al. 2005, 2008; Wang et al. 2007, 2013; Sivitz et al. 2012). However, no corresponding probes of *At038ORG2* and *At100* in the microarray were identified and the *P* value of *At029FRU* was 0.024 (>0.01) according to the ANOVA analysis. All eight orthologs in *Populus* are listed in Table 2.

Cloning and sequence analysis of *PtFIT* and *PtIRO*

The open reading frame (ORF) regions of two candidate genes were amplified from *Populus tremula* (PtG and PtY). The PCR primers were designed according to the corresponding nucleotide sequence information of XP_002313541.2 and XP_002323250.2. Nucleotide sequence alignment revealed that either of two genes was identical in both PtG and PtY. Based on their similarity to the *bHLH* genes, two genes were named *PtFIT* and *PtIRO*, respectively (Supplementary Fig. 2). The 939 bp ORF of *PtFIT* encoded a deduced protein of 313 amino acid residues with a molecular weight of 34.9 kDa and an isoelectric point of 4.74. The 795 bp ORF of *PtIRO* encoded a deduced protein of 265 amino acid residues with a



b

	Expression line
At006MYC2	
At007	
At020NAI1	
At025	
At026HFR1	
At035	
At039ORG3	
At047	
At059UN12	
At082	
At093	
At101	
At105ILR3	
At115	
At116ICE1	
At126	
At129	
At135PRE3	
At143	
At147	
At148	
At150	
At151	
At154ERP	
At158	

◀**Fig. 1** Expression profiles of the *AtbHLH* genes in response to iron deficiency at different time points after the iron deficiency treatment. **a** Microarray analyses of the 117 *AtbHLH* genes. Genes and samples are ordered based on a hierarchical clustering analysis; **b** Expression patterns of 25 *AtbHLH* genes showing significant differences at different time points

molecular weight of 30.0 kDa and an isoelectric point of 6.55. Deduced amino acid sequence analysis revealed that *PtFIT* showed 14.0–57.1 % identity to other *bHLH* genes (57.15 % to *AtbHLH29* and 55.7 % to *MxFIT*). The *PtIRO* also showed 14.3–54.7 % identity to other *bHLH* genes (54.7 % to *MxIRO2*). Alignment analysis of amino acid sequences showed that *PtFIT* and *PtIRO* contained the conserved bHLH domain (Pfam accession: PF00010) (Fig. 2). Phylogenetic analysis suggested that the bHLH proteins could be divided into two subgroups (Fig. 3). *PtFIT* was clustered with *AtbHLH29*, *LeFER*, *MxbHLH01*, and *MxFIT*, while *PtIRO* was clustered with the rest of *bHLHs*. The distinct structures between these two subgroups might lead to the divergence in their functions. A similar result was reported by Carretero-Paulet et al. (2010) where *AtbHLH29* and *AtbHLH38/AtbHLH39* belong to two different subfamilies and play different roles.

Expression analysis of *PtFIT* and *PtIRO*

The expression of *PtFIT* and *PtIRO* in the root tip, root, phloem, xylem, mature leaf, young leaf, and shoot tip of both PtG and PtY was determined using semi-quantitative PCR. As shown in Fig. 4, *PtFIT* was detected only in the root tip and root, while a weak expression of *PtIRO* was detected in the shoot tip. A real-time quantitative PCR was conducted to evaluate the expression profile of *PtFIT* in the root responding to iron deficiency at different times after the iron deficiency treatment (Fig. 5). Results showed that a slight decrease of the *PtFIT* transcript was detected at day 0.5, then gradually increased and peaked at day 6 in PtG. In PtY, the expression of *PtFIT* showed the first peak at day 1, and then decreased until day 3. The second expression peak of *PtFIT* in PtY was detected at day 6. The expression level of *PtFIT* at day 6 was more than 2-fold higher in PtG than in PtY.

Overexpression of the *PtFIT* gene in *P. canescens* × *P. grandidentata* 'Cl6'

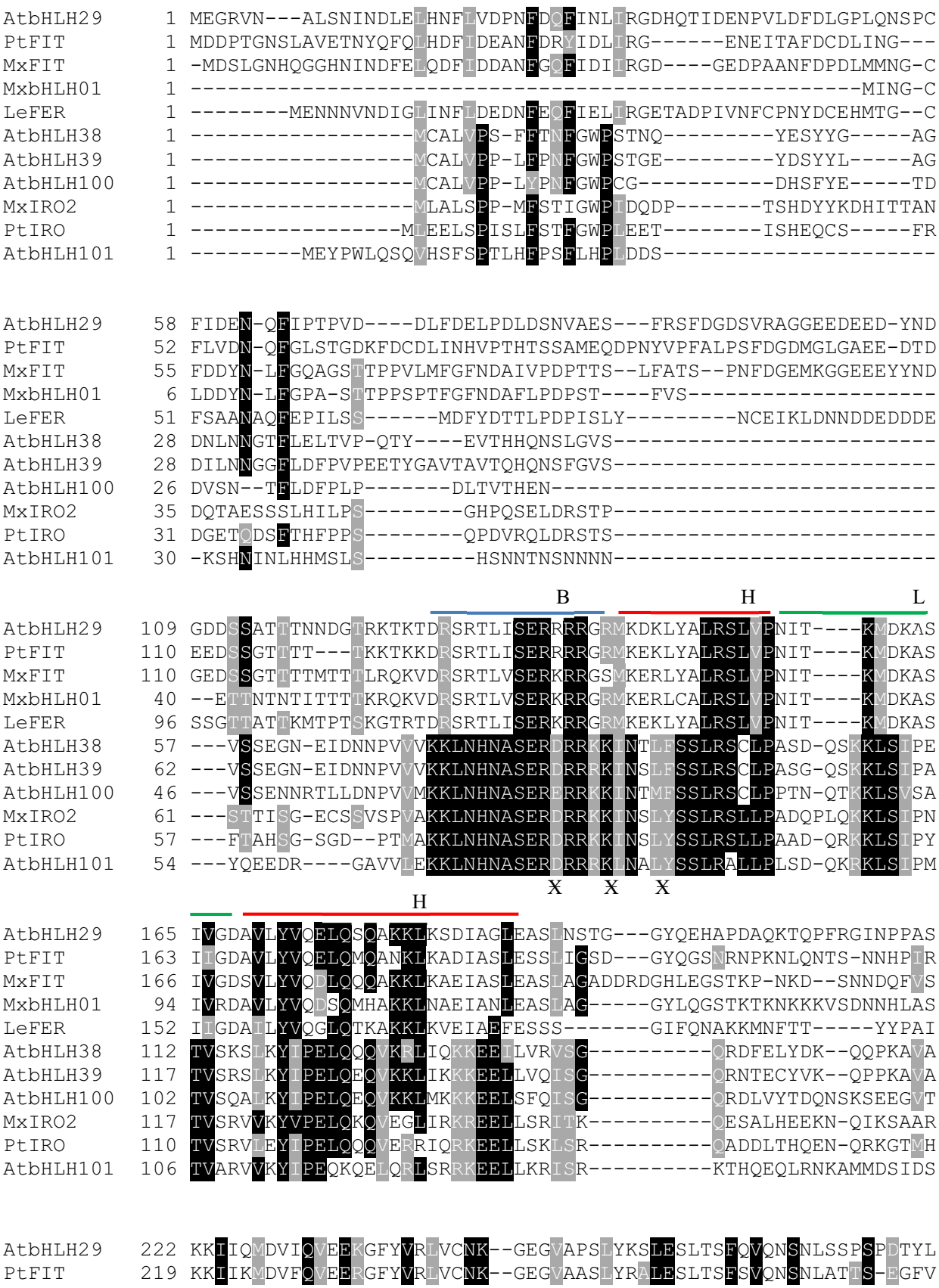
Overexpression of the *PtFIT* gene was evaluated in five independent transgenic lines of *P. canescens* × *P. grandidentata* 'Cl6'. One line with the empty vector (no *PtFIT*) was the control (NC). As shown in Fig. 6, the expression level of *PtFIT* in transgenic lines decreased under the iron-sufficient condition except in TL5 that was similar to NC. Under the iron-deficient condition, the expression of *PtFIT* significantly increased except in TL13. Two transgenic lines (TL12 and TL5) showed a significantly higher expression level of *PtFIT* than the control under iron deficiency. The expression pattern of *PtIRT1* in *PtFIT*-transgenic lines was also evaluated. Similar to that of *PtFIT,* the expression of *PtIRT1* was also inhibited under iron sufficiency and dramatically enhanced under iron deficiency (Fig. 6). Compared to NC, *PtIRT1* transcripts in transgenic plants were reduced more than half under the iron-sufficient condition, while *PtIRT1* transcripts were significantly increased in TL10 compared to NC under iron deficiency. On the other hand, *PtIRT1* transcripts were increased 2.44 times in NC and 3.38–13.43 times in transgenic plants under the iron-deficient condition (Supplementary Table 1).

Physiological analysis of the *PtFIT*-transgenic lines responding to iron deficiency

The contents of chlorophyll and mineral elements in the *PtFIT*-transgenic leaves were determined. As shown in Figs. 7 and 8, no significant increase of chlorophyll (Chl

Table 2 The BlastP identified candidates of the *bHLH* genes related to iron deficiency response in *Populus*

Arabidopsis accessions	Populus accessions	Query cover (%)	*E* value	Identity (%)
At029FRU (At2G28160)	XP_002313541.2	95	5.00E−86	50
At038ORG2 (At3G56970)	XP_002307969.2	97	7.00E−51	48
	XP_002323250.2	97	3.00E−49	47
At039ORG3 (At3G56980)	XP_002307969.2	93	1.00E−45	47
	XP_002323250.2	94	3.00E−41	43
At047 (At3G47640)	XP_002303343.1	91	1.00E−59	51
At100 (At2G41240)	XP_002307969.2	94	2.00E−39	41
	XP_002323250.2	94	3.00E−36	42
At101 (At5G04150)	XP_002307969.2	93	2.00E−39	44
At105ILR3 (At5G54680)	XP_002316971.1	100	1.00E−93	68
At115 (At1G51070)	XP_002316971.1	64	1.00E−75	69


AtbHLH29 1 MEGRVN---ALSNINDLELHNFLVDPNFDQFINLIRGDHQTIDENPVLDFDLGPLQNSPC
PtFIT 1 MDDPTGNSLAVETNYQFQLHDFIDEANFDRYIDLIRG------ENEITAFDCDLING---
MxFIT 1 -MDSLGNHQGGHNINDFELQDFIDDANFGQFIDIIRGD----GEDPAANFDPDLMMNG-C
MxbHLH01 1 ----------MING-C
LeFER 1 --------MENNNVNDIGLINFLDEDNFEQFIELIRGETADPIVNFCPNYDCEHMTG--C
AtbHLH38 1 ----------MCALVPS-FFTNFGWPSTNQ---------YESYYG-----AG
AtbHLH39 1 ----------MCALVPP-LFPNFGWPSTGE---------YDSYYL-----AG
AtbHLH100 1 ----------MCALVPP-LYPNFGWPCG----------DHSFYE-----TD
MxIRO2 1 ----------MLALSPP-MFSTIGWPIDQDP-------TSHDYYKDHITTAN
PtIRO 1 ----------MLEELSPISLFSTFGWPLEET--------ISHEQCS-----FR
AtbHLH101 1 ---------MEYPWLQSQVHSFSPTLHFPSFLHPLDDS----------
AtbHLH29 58 FIDENQFIPTPVD----DLFDELPDLDSNVAES---FRSFDGDSVRAGGEEDEED-YND
PtFIT 52 FLVDNQFGLSTGDKFDCDLINHVPTHTSSAMEQDPNYVPFALPSFDGDMGLGAEE-DTD
MxFIT 55 FDDYNLFGQAGSTTPPVLMFGFNDAIVPDPTTS--LFATS--PNFDGEMKGGEEEYYND
MxbHLH01 6 LDDYNLFGPA-STTPPSPTFGFNDAFLPDPST----FVS----------
LeFER 51 FSAANAQFEPILSS------MDFYDTTLPDPISLY---------NCEIKLDNNDDEDDDE
AtbHLH38 28 DNLNNGTFLELTVP-QTY----EVTHHQNSLGVS----------
AtbHLH39 28 DILNNGGFLDFPVPEETYGAVTAVTQHQNSFGVS----------
AtbHLH100 26 DVSN--TFLDFPLP-------DLTVTHEN----------
MxIRO2 35 DQTAESSSLHILPS--------GHPQSELDRSTP----------
PtIRO 31 DGETQDSFTHFPPS--------QPDVRQLDRSTS----------
AtbHLH101 30 -KSHNINLHHMSLS---------HSNNTNSNNNN----------
B
H
L
AtbHLH29 109 GDDSSATTTNNDGTRKTKTDRSRTLISERRRRGRMKDKLYALRSLVPNIT----KMDKAS
PtFIT 110 EEDSSGTTTT---TKKTKKDRSRTLISERRRRGRMKEKLYALRSLVPNIT----KMDKAS
MxFIT 110 GEDSSGTTTMTTTLRQKVDRSRTLVSERKRRGSMKERLYALRSLVPNIT----KMDKAS
MxbHLH01 40 --ETTNTNTITTTTKRQKVDRSRTLVSERKRRGRMKERLCALRSLVPNIT----KMDKAS
LeFER 96 SSGTTATTKMTPTSKGTRTDRSRTLISERKRRGRMKEKLYALRSLVPNIT----KMDKAS
AtbHLH38 57 ---VSSEGN-EIDNNPVVVKKLNHNASERDRRKKINTLFSSLRSCLPASD-QSKKLSIPE
AtbHLH39 62 ---VSSEGN-EIDNNPVVVKKLNHNASERDRRKKINSLFSSLRSCLPASG-QSKKLSIPA
AtbHLH100 46 ---VSSENNRTLLDNPVVMKKLNHNASERERRKKINTMFSSLRSCLPPTN-QTKKLSVSA
MxIRO2 61 ---STTISG-ECSSVSPVAKKLNHNASERDRRKKINSLYSSLRSLLPADQPLQKKLSIPN
PtIRO 57 ---FTAHSG-SGD--PTMAKKLNHNASERDRRKKINSLYSSLRSLLPAAD-QRKKLSIPY
AtbHLH101 54 ---YQEEDR----GAVVLEKKLNHNASERDRRRKLNALYSSLRALLPLSD-QKRKLSIPM
X X X
H
AtbHLH29 165 IVGDAVLYVQELQSQAKKLKSDIAGLEASLNSTG---GYQEHAPDAQKTQPFRGINPPAS
PtFIT 163 IIGDAVLYVQELQMQANKLKADIASLESSLIGSD---GYQGSNRNPKNLQNTS-NNHPIR
MxFIT 166 IVGDSVLYVQDLQQQAKKLKAEIASLEASLAGADDRDGHLEGSTKP-NKD--SNNDQFVS
MxbHLH01 94 IVRDAVLYVQDSQMHAKKLNAEIANLEASLAG-----GYLQGSTKTKNKKKVSDNNHLAS
LeFER 152 IIGDAILYVQGLQTKAKKLKVEIAEFESSS-------GIFQNAKKMNFTT-----YYPAI
AtbHLH38 112 TVSKSLKYIPELQQQVKRLIQKKEEILVRVSG----------QRDFELYDK--QQPKAVA
AtbHLH39 117 TVSRSLKYIPELQEQVKKLIKKKEELLVQISG----------QRNTECYVK--QPPKAVA
AtbHLH100 102 TVSQALKYIPELQEQVKKLMKKKEELSFQISG----------QRDLVYTDQNSKSEEGVT
MxIRO2 117 TVSRVVKYVPELQKQVEGLIRKREELLSRITK----------QESALHEEKN-QIKSAAR
PtIRO 110 TVSRVLEYIPELQQQVERRIQRKEELLSKLSR----------QADDLTHQEN-QRKGTMH
AtbHLH101 106 TVARVVKYIPEQKQELQRLSRRKEELLKRISR----------KTHQEQLRNKAMMDSIDS
AtbHLH29 222 KKIIQMDVIQVEEKGFYVRLVCNK--GEGVAPSLYKSLESLTSFQVQNSNLSSPSPDTYL
PtFIT 219 KKIIKMDVFQVEERGFYVRLVCNK--GEGVAASLYRALESLTSFSVQNSNLATTS-EGFV

◀**Fig. 2** Amino acid alignment of *PtFIT* and *PtIRO* proteins with other *bHLH* proteins from *Arabidopsis thaliana*, *Malus xiaojinensis*, and *Lycopersicon esculentum*. The *bHLH* conserved domain is indicated with *lines*. *Shaded* areas represent identical residues (*black*) or similar residues (*gray*) found in most of the proteins. ''X'' indicates the motifs

a + b), Chl a/b ratio, or carotenoids was observed in transgenic lines under the iron-sufficient condition. Under the iron-deficient condition, the chlorophyll content decreased in TL10 and TL13 as well as in NC, but no significant changes in TL12, TL2, and TL5 were detected. The Chl a/b ratio also decreased under iron deficiency in all transgenic plants as well as in NC. TL2 had the highest Chl a/b ratio in all transgenic lines (Fig. 7). Similarly, iron deficiency significantly decreased the content of carotenoids in NC, while no significant decrease in transgenic plants except TL2 and TL10 was found (Fig. 8). The contents of Mn, Zn, Cu, and Fe in leaves of the transgenic plants under the iron-deficient or iron-sufficient condition were determined (Fig. 9). In transgenic plants, iron deficiency showed no effect on Mn content, but slightly lowered the Zn content. Moreover, iron content was significantly decreased and Cu content was significantly increased under iron deficiency. Under the iron-sufficient condition, transgenic lines accumulated more Zn and Fe

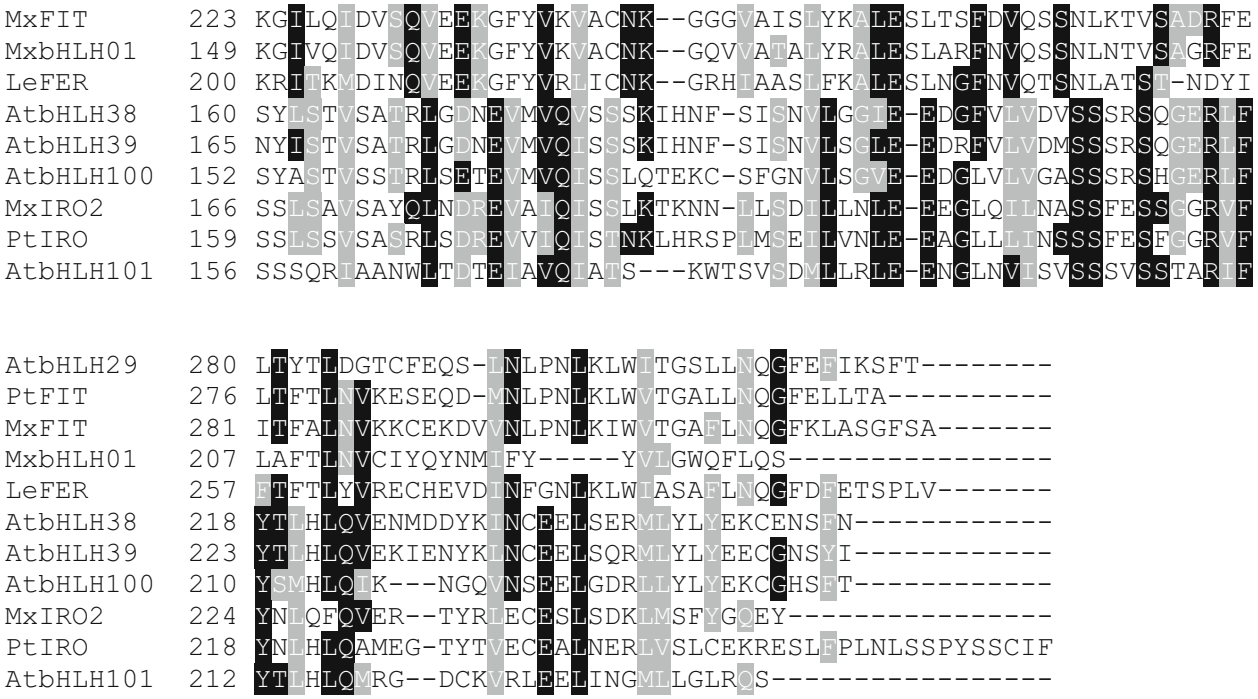


Fig. 2 continued

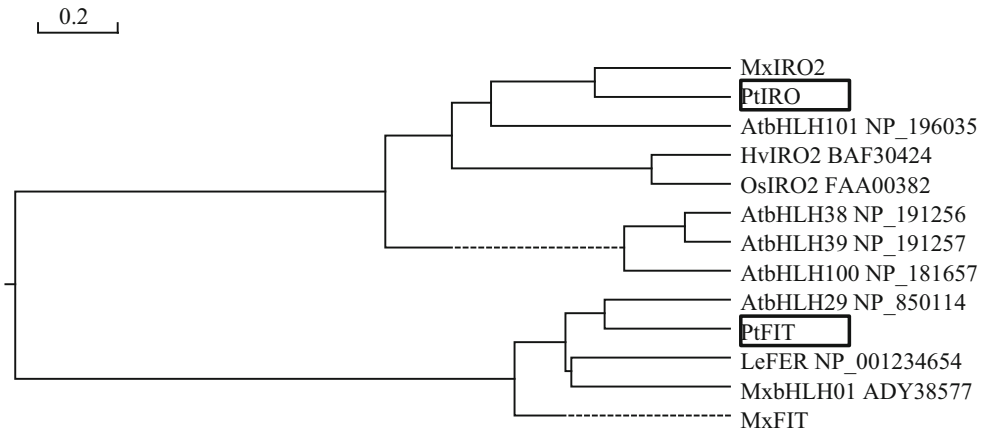


Fig. 3 A phylogenetic tree of the *PtFIT, PtIRO2*, and other *bHLH* genes constructed by ClustalW. The accession numbers of the studied proteins are following the gene name. *PtFIT* and *PtIRO* are framed. The corresponding sources are *Lycopersicon esculentum* (*Le*), *Malus xiaojinensis* (*Mx*), *Arabidopsis thaliana* (*At*), *Oryza sativa* (*Os*), and *Hordeum vulgare* (*Hv*). The *scale bar* represents 0.2 amino acid substitutions per site

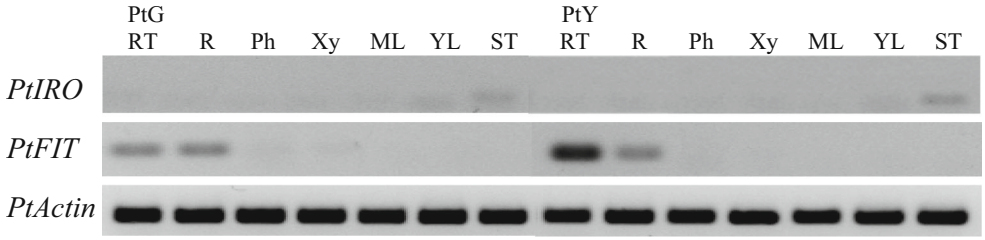


Fig. 4 Expression levels of *PtFIT* and *PtIRO* in various tissues of PtG and PtY by semi-quantitative PCR. *PtG* iron deficiency tolerant clone and *PtY* iron deficiency susceptible clone of *Populus tremula*; *RT* root tip, *R* root, *Ph* phloem, *Xy* xylem, *ML* mature leaf, *YL* young leaf, *ST* shoot tip

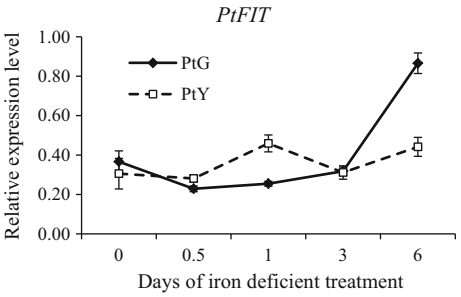


Fig. 5 *PtFIT* expression in response to iron deficiency at 0, 0.5, 1, 3, and 6 days in the root of PtG and PtY. The relative expression is quantified by real-time PCR and normalized to the *PtTIF5α* gene. Data represent means and standard errors (*vertical bar*) of three replicates

with the exception that TL5 had a similar Fe content to NC. Under iron deficiency, TL2 and TL5 accumulated more Zn than other lines and NC, while no significant difference in Fe, Mn, or Cu content was found among all transgenic and NC plants.

Discussion

The basic helix-loop-helix (*bHLH*) is an important family of transcription factors (Massari and Murre 2000). In this study, the orthologs of the *AtbHLH* genes responding to iron deficiency in *Populus* were discovered using microarray data. Microarray is a useful tool to explore the genes responding to changes of the environmental conditions. In *Arabidopsis*, the microarray method has been used to analyze the genome-wide responses of genes to iron deficiency, such as the differential expression in different tissues (shoots, roots, or leaves) or under different Fe supply conditions (Dinneny et al. 2008; Buckhout et al. 2009; Long et al. 2010; Schuler et al. 2011; Sivitz et al. 2012; Waters et al. 2012). We analyzed the expression profile of the *AtbHLH* genes in response to iron deficiency in the root using the microarray data submitted by Dinneny et al. (2008). Twenty-five *AtbHLH* genes showed the responses to iron deficiency (Fig. 1). Among them, three up-regulated genes (*At039ORG3*, *At047*, and *At101)* were also confirmed by other microarray analyses that involved in iron deficiency responses (Sivitz et al. 2012; Waters et al. 2012). Five orthologs of *AtbHLH* were identified in *Populus trichocarpa* according to the BlastP result and two out of five (*PtFIT* and *PtIRO*) that are homologs of XP_002313541.2 and XP_002323250.2 were cloned from *Populus tremula*.

Amino acid sequence analyses revealed that the *bHLH* domain sequences of *PtFIT* and *PtIRO* had the highest similarity with *AtbHLH29* and *MxIRO2*, respectively (Supplementary Fig. 2). *AtbHLH29* and *MxIRO2* functioned as regulators of the genes responding to iron deficiency (Colangelo and Guerinot 2004; Jakoby et al. 2004; Yuan et al. 2005; Yin et al. 2013). Phylogenetic analysis showed that the two iron deficiency regulators were clustered into two groups (Fig. 3). The *PtFIT* protein contained the typical threonine–glutamate–arginine (T–E–R) motif in the basic region of the *bHLH* domain at positions 5-9-13, as other members in the same subgroup. Differently, the feature showed in the *PtIRO* protein was histidine–glutamate–arginine (H–E–R) (Fig. 2). Research showed that H–E–R could bind to the G-box (5′-CACGTG-3′) of a promoter and T–E–R was bound to a variation of the E-box (5′-CANNTG-3′) hexanucleotide sequence, revealing the different roles of the *FIT* and *IRO* genes in regulating iron deficiency response in plants (Heim et al. 2003; Li et al. 2006). Information in this study suggests that *PtFIT* and *PtIRO* cloned from *Populus tremula* belong to the *bHLH* family and may function differently in iron deficiency response in *Populus*.

The up-regulated expression of *PtFIT* was observed and more transcripts were detected in roots under iron deficiency (Figs. 5, 6). Similar responses of *AtbHLH29* and M*xFIT* were also reported (Jakoby et al. 2004; Yin et al. 2014). However, very weak expression of *PtIRO* was detected in the shoot tip of *Populus tremula* (Fig. 4), which is different from the *MxIRO2* gene that expressed in the leaf and root of *Malus xiaojinensis* under iron sufficiency (Yin et al. 2013). It indicates that *PtIRO* may have a different function from *MxIRO2* and further research is needed to

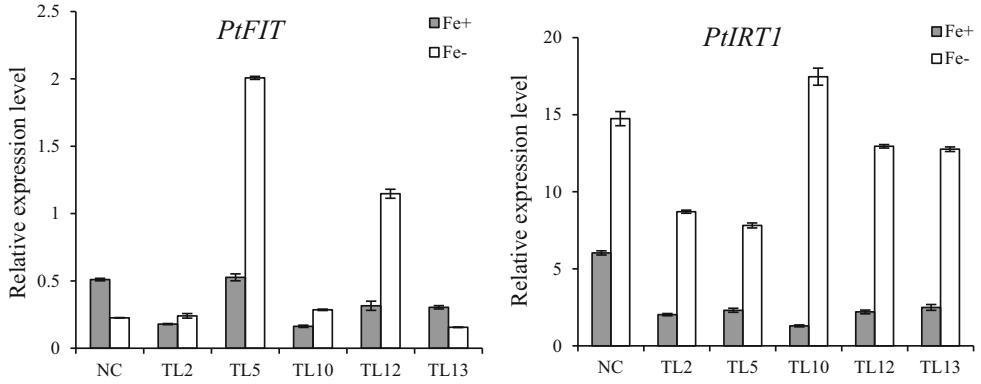


Fig. 6 Quantitative assay of *PtFIT* and *PtIRT* expression in the control and transgenic poplar plants. Plants were grown under iron sufficiency (Fe+) or deficiency (Fe−). *NC* the transgenic line with the empty vector (no target gene). The relative expression is quantified by real-time PCR and normalized to the *PtTIF5α* gene. Values are means of three replicates and standard errors are indicated as a *vertical line* on the top of each *bar*

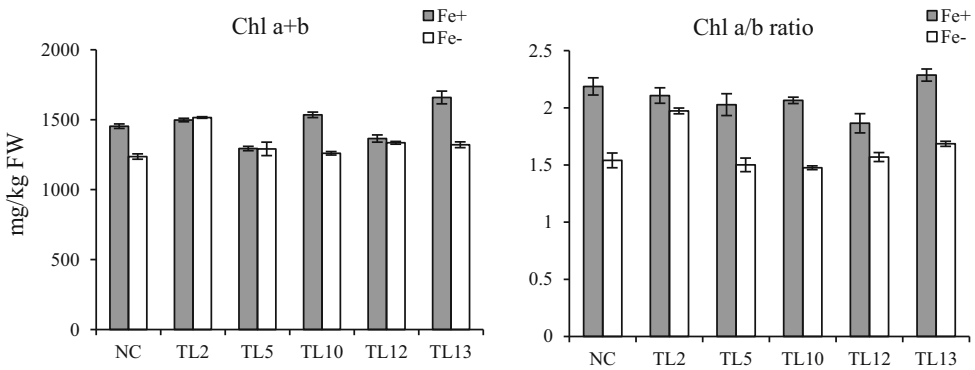


Fig. 7 Chlorophyll content and Chl a/b ratio in the control and *PtFIT*-transgenic poplar lines under iron-sufficient (Fe+) and iron-deficient (Fe−) conditions. *NC* the transgenic line with the empty vector (no target gene). Values are means of three replicates and standard errors are indicated as a *vertical line* on the top of each *bar*

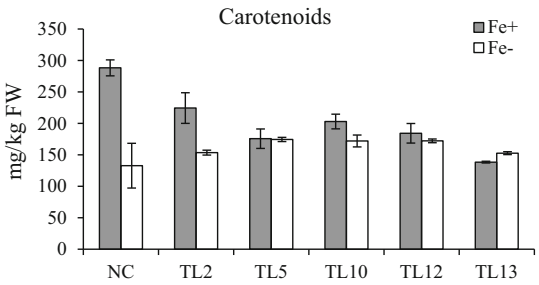


Fig. 8 The carotenoids content in the control and *PtFIT*-transgenic poplar lines under iron-sufficient (Fe+) and iron-deficient (Fe−) conditions. *NC* the transgenic line with the empty vector (no target gene). Values are means of three replicates and standard errors are indicated as a *vertical line* on the top of each *bar*

evaluate the possible function of *PtIRO* related to iron deficiency responses. This study also showed that the expression of *PtFIT* was higher in PtG than PtY at day 6 after the iron deficiency treatment. Research in *Arabidopsis* indicated that *AtIRT1* is a major downstream gene regulated by *AtFIT1* (Colangelo and Guerinot 2004; Jakoby et al. 2004; and Yuan et al. 2005). In this study, the correlation between *PtIRT1* and *PtFIT* responding to iron deficiency was strongly positive ($r = 0.94$) between *PtIRT1* and *PtFIT* in PtG (Fig. 10), but the correlation in PtY was weak ($r = 0.49$). This indicates that *FIT* in PtG functioned well in regulating *IRT1* to respond to iron deficiency as reported in *Arabidopsis* (Colangelo and Guerinot 2004; Jakoby et al. 2004; Yuan et al. 2005); however, in PtY, the regulation function of *FIT* might be inhibited or needs to be induced by other factors (Yuan et al. 2008) and lack of those factors or connections between those factors may explain such a weak correlation between *PtFIT* and *PtIRT*. In addition, the expression profile of these genes in response to iron deficiency and tissue specificity needs to be further compared in both iron deficiency tolerant and susceptible poplar trees.

To further characterize the gene function, *PtFIT* driven by a constitutive super promoter (a trimer of the octopine synthase transcriptional activating element affixed to the mannopine synthase 2′ transcriptional activating element plus minimal promoter) was transferred into another poplar species (*P. canescens* × *P. grandidentata* 'Cl6'). No enhanced constitutive expression of the *PtFIT* gene in

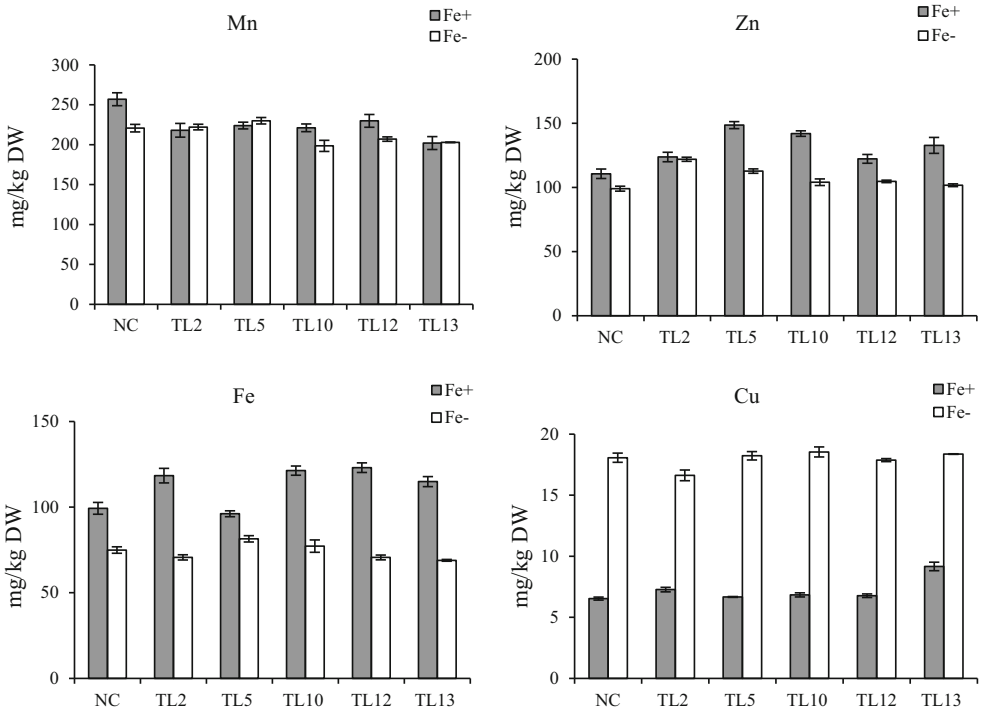


Fig. 9 The content of Mn, Zn, Fe, and Cu in the leaves of the control and *PtFIT*-transgenic poplar lines under iron-sufficient (Fe+) and iron-deficient (Fe−) conditions. *NC* the transgenic line with the empty vector (no target gene). Values are means of three replicates and standard errors are indicated as a *vertical line* on the top of each *bar*

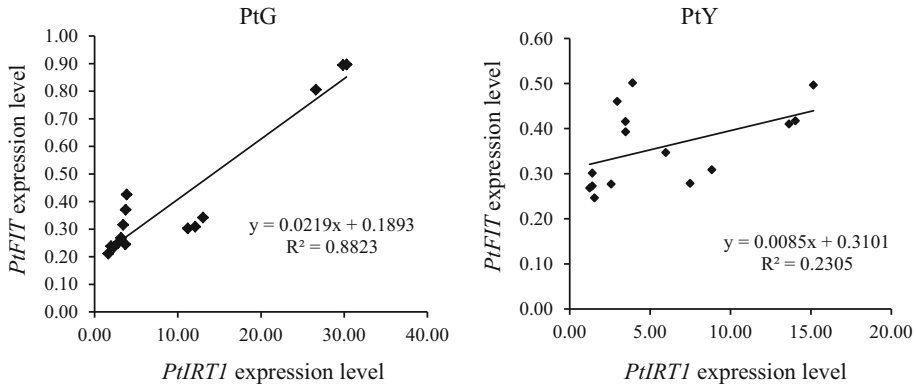


Fig. 10 Correlation between the expression level of *PtIRT1* and *PtFIT* responding to iron deficiency calculated by Pearson Correlation Coefficient Calculator. Expression levels are normalized to the ratio to *PtTIF5α* gene

transgenic lines was detected in this study. The transgenic plants had a relatively lower expression level of *PtFIT* under the iron-sufficient condition compared to the control, while the expression of *PtFIT* was induced by iron deficiency in most transgenic plants (Fig. 6). This indicates that there might be other factors regulating the expression of *PtFIT* and these factors can be activated by iron deficiency. Research showed that *FIT* also interacts with other elements (interactors) that regulate iron uptake. To date, many *AtFIT* interactors showed the positive regulation in response to iron deficiency; however, the negative regulation of *AtFIT* interactors might play an important role in preventing plants from metal toxicity caused by over-accumulation of iron or other metals (Brumbarova et al. 2014). The suppressed expression of *PtFIT* in transgenic plants under iron sufficiency might indicate the existence of such negative interactors of *PtFIT* in *Populus*. Similar interactors may also play roles in regulating the native promoter of the *FIT* gene; therefore, functional characterization of the gene promoter should be further investigated.

As expected, the expression of *PtIRT1* in *PtFIT*-transgenic plants was also inhibited by iron sufficiency and induced by iron deficiency (Fig. 6). Additionally, the fold change of the *PtIRT1* transcript was much greater in

transgenic lines than in the control (Supplementary Table 1). Previous research showed a similar result to which *AtIRT1* transcripts were not accumulated under the iron-sufficient condition, but increased under the iron-deficient condition in transgenic *Arabidopsis* plants over-expressing *AtFIT1* or *MxFIT*. This suggests that *PtFIT* might have the function to induce the expression of *PtIRT1* and the similar function was also found in *AtFIT1* and *MxFIT* (Jakoby et al. 2004; Yuan et al. 2008, 2014). We predict that *PtFIT* could induce the expression of *PtIRT1* and might play an important role in regulation of iron transport under iron deficiency. Under iron sufficiency, the *PtFIT*-transgenic poplar lines accumulated more Fe than the control plant.

In plants, the iron level significantly affects the chlorophyll formation and its structure by regulating the activities of heme enzymes in leaf tissues and the formation of thylakoid membrane in chloroplast. Decreased chlorophyll content was found as the consequence of iron deficiency (Marsh et al. 1963; Terry and Abadia 1986). Notably, one of the *PtFIT*-trangenic line (TL2) showed an increased chlorophyll (Chl a+b) content and Chl a/b ratio compared to the control under iron deficiency. This result is consistent with the findings in transgenic *Arabidopsis* in which overexpression of *MxFIT* increased chlorophyll content (Yuan et al., 2014). Interestingly, the TL2 line showed no enhanced Fe accumulation under iron deficiency, suggesting that TL2 might use iron more efficiently. These findings suggest genetic regulation of *PtFIT* has the potential to enhance the tolerance to iron deficiency in poplar trees.

In conclusion, two *bHLH* transcription factors (*PtFIT* and *PtIRO*) were cloned from *Populus tremula*. Functions of *PtFIT* in regulating iron deficiency were characterized. Results indicated that *PtFIT* might regulate *PtIRT1* that is directly involved in regulation of iron deficiency response in *Populus*. This study provides useful information for further understanding of the mechanisms of iron deficiency response in poplar trees and other woody species.

Author contribution statement DH and WD conceived and designed the study. The experiments were conducted by DH. WD supervised the research and guided data interpretation. DH and WD wrote the paper. All authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Brumbarova T, Bauer P (2005) Iron-mediated control of the basic helix-loop-helix protein FER, a regulator of iron uptake in tomato. Plant Physiol 137:1018–1026

Brumbarova T, Bauer P, Ivanov R (2014) Molecular mechanisms governing *Arabidopsis* iron uptake. Trends Plant Sci. doi:10.1016/j.tplants.2014.11.004

Buckhout TJ, Yang TJ, Schmidt W (2009) Early iron-deficiency-induced transcriptional changes in *Arabidopsis* roots as revealed by microarray analyses. BMC Genom 10:147

Carretero-Paulet L, Galstyan A, Roig-Villanova I, Martinez-Garcia JF, Bilbao-Castro JR, Robertson DL (2010) Genome-wide classification and evolutionary analysis of the *bHLH* family of transcription factors in *Arabidopsis*, poplar, rice, moss, and algae. Plant Physiol 153:1398–1412

Colangelo EP, Guerinot ML (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell 16:3400–3412

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL (2001) Maize yellow stripe 1 encodes a membrane protein directly involved in Fe(III) uptake. Nature 409:346–349

Dai W, Cheng ZM, Sargent W (2003) Plant regeneration and *Agrobacterium*-mediated transformation of two elite aspen hybrid clones from in vitro leaf tissues. In Vitro Cell Dev Biol Plant 39:6–11

Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. Science 320:942–945

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci USA 93:5624–5628

Giehl RF, Lima JE, von Wiren N (2012) Localized iron supply triggers lateral root elongation in *Arabidopsis* by altering the AUX1-mediated auxin distribution. Plant Cell 1:33–49

Guerinot ML, Yi Y (1994) Iron: nutritious, noxious, and not readily available. Plant Physiol 104:815–820

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC (2003) The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol 20:735–747

Hoagland DR, Arnon DI (1939) The water culture method for growing plants without soil. Calif Agr Expt Sta Circ 347

Inoue H, Kobayashi T, Nozoye T, Takahashi M, Kakei Y, Suzuki K, Nakazono M, Nakanishi H, Mori S, Nishizawa NK (2009) Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings. J Biol Chem 284:3470–3479

Ivanov R, Brumbarova T, Bauer P (2012) Fitting into the harsh reality: regulation of iron-deficiency responses in dicotyledonous plants. Mol Plant 5(1):27–42

Jakoby M, Wang HY, Reidt W, Weisshaar B, Bauer P (2004) FRU (HLH029) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. FEBS Lett 577:528–534

Jeong J, Connolly EL (2009) Iron uptake mechanisms in plants: functions of the *FRO* family of ferric reductases. Plant Sci 176:709–714

Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation and regulation in higher plants. Ann Rev Plant Biol 63:131–152

Larionov A, Krause A, Miller W (2005) A standard curve based method for relative real time PCR data processing. BMC Bioinform 6:62

Latchman DS (2007) Gene regulation: a eukaryotic perspective. 5th edition. Taylor & Franics Group e-Library, New York

Legay S, Guignard C, Ziebel J, Evers D (2012) Iron uptake and homeostasis related genes in potato cultivated in vitro under iron deficiency and overload. Plant Physiol Biochem 60:180–189

Li XX, Duan XP, Jiang HX, Sun YJ, Tang YP, Yuan Z, Guo JK, Liang WQ, Chen L, Yin JY, Ma H, Wang J, Zhang DB (2006) Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and *Arabidopsis*. Plant Physiol 141:1167–1184

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Meth Enzymol 148:350–382

Ling HQ, Bauer P, Bereczky Z, Keller B, Ganal M (2002) The tomato *fer* gene encoding a bHLH protein controls iron-uptake responses in roots. Proc Natl Acad Sci USA 99:13938–13943

Lingam S, Mohrbacher J, Brumbarova T, Potuschak T, Fink-Straube C, Blondet E, Genschik P, Bauer P (2011) Interaction between the bHLH transcription factor FIT and ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3-LIKE1 reveals molecular linkage between the regulation of iron acquisition and ethylene signaling in *Arabidopsis*. Plant Cell 23:1815–1829

Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN (2010) The bHLH transcription factor POPEYE regulates response to iron deficiency in Arabidopsis roots. Plant Cell 22:2219–2236

Marsh HV Jr, Evans HJ, Matrone G (1963) Investigation of the role of iron in chlorophyll metabolism. I. Effect of iron deficiency on chlorophyll and heme content and on the activities of certain enzymes in leaves. Plant Physiol 38:632–638

Massari ME, Murre C (2000) Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol Cell Biol 20:429–440

Maurer F, Mueller S, Bauer P (2011) Suppression of Fe deficiency gene expression by jasmonate. Plant Physiol Biochem 49:530–536

Maurer F, Naranjo Arcos MA, Bauer P (2014) Responses of a triple mutant defective in three iron deficiency-induced *BASIC HELIX-LOOP-HELIX* genes of the subgroup Ib(2) to iron deficiency and salicylic acid. PLoS ONE 9(6):e99234

Murata Y, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T (2006) A specific transporter for iron(III)-phytosiderophore in barley roots. Plant J 46:563–572

Ohashi-Ito K, Bergmann DC (2006) *Arabidopsis* FAMA controls the final proliferation/differentiation switch during stomatal development. Plant Cell 18:2493–2505

Pagnussat GC, Yu HJ, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie LF, Ye D, Sundaresan V (2005) Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. Development 132:603–614

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. Nature 397:694–697

Römheld V, Marschner H (1986) Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. Plant Physiol 80:175–180

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. Meth Enzymol 411:134–193

Schuler M, Keller A, Backes C, Philippar K, Lenhof H, Bauer P (2011) Transcriptome analysis by GeneTrail revealed regulation of functional categories in response to alterations of iron homeostasis in *Arabidopsis thaliana*. BMC Plant Biol 11:87

Seguela M, Briat JF, Vert G, Curie C (2008) Cytokinins negatively regulate the root iron uptake machinery in *Arabidopsis* through a growth-dependent pathway. Plant J 55:289–300

Simon R, Lam A, Li M, Ngan M, Menenzes S, Zhao Y (2007) Analysis of gene expression data using BRB-array tools. Cancer Inform 3:11–17

Sivitz AB, Hermand V, Curie C, Vert G (2012) Arabidopsis bHLH100 and bHLH101 control iron homeostasis via a FIT-independent pathway. PLoS ONE 7:e44843

Takagi S (1976) Naturally occurring iron-chelating compounds in oat- and rice-root washing. I. Activity measurement and preliminary characterization. Soil Sci Plant Nutr 22:423–433

Terry N, Abadia J (1986) Function of iron in chloroplasts. J Plant Nutr 9:609–646

Vert G, Briat JF, Curie C (2001) *Arabidopsis* IRT2 gene encodes a root-periphery iron transporter. Plant J 26:181–189

Wang HY, Klatte M, Jakoby M, Baumlein H, Weisshaar B, Bauer P (2007) Iron deficiency-mediated stress regulation of four subgroup Ib BHLH genes in *Arabidopsis thaliana*. Planta 226:897–908

Wang L, Cui Y, Liu Y, Fan H, Du J, Huang Z, Yuan Y, Wu H, Ling HQ (2013) Requirement and Functional Redundancy of Ib subgroup bHLH proteins for iron deficiency responses and uptake in *Arabidopsis thaliana*. Mol Plant 6:503–513

Waters BM, McInturf SA, Stein RJ (2012) Rosette iron deficiency transcript and microRNA profiling reveals links between copper and iron homeostasis in *Arabidopsis thaliana*. J Exp Bot 63:5903–5918

Xu HM, Wang Y, Chen F, Zhang XZ, Han ZH (2011) Isolation and characterization of the iron-regulated MxbHLH01 gene in *Malus xiaojinensis*. Plant Mol Biol Report 29:936–942

Yin L, Wang Y, Yuan M, Zhang X, Pan H, Xu X, Han Z (2013) Molecular cloning, polyclonal antibody preparation, and characterization of a functional iron-related transcription factor IRO2 from *Malus xiaojinensis*. Plant Physiol Biochem 67:63–70

Yin L, Wang Y, Yuan M, Zhang X, Xu X, Han Z (2014) Characterization of *MxFIT*, an iron deficiency induced transcriptional factor in *Malus xiaojinensis*. Plant Physiol Biochem 75:89–95

Yuan Y, Zhang J, Wang DW, Ling HQ (2005) AtbHLH29 of *Arabidopsis thaliana* is a functional ortholog of tomato FER involved in controlling iron acquisition in strategy I plants. Cell Res 15:613–621

Yuan Y, Wu H, Wang N, Li J, Zhao W, Du J, Wang D, Ling HQ (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in *Arabidopsis*. Cell Res 18:385–397