Tree Genetics & Genomes (2014) 10:573–584
DOI 10.1007/s11295-014-0705-5

ORIGINAL PAPER

Transcriptomic analysis demonstrates the early responses of local ethylene and redox signaling to low iron stress in *Malus xiaojinensis*

Shaojia Wang · Binbin Lu · Ting Wu · Xinzhong Zhang · Xuefeng Xu · Zhenhai Han · Yi Wang

Received: 14 August 2013 / Revised: 5 February 2014 / Accepted: 11 February 2014 / Published online: 23 March 2014

Abstract *Malus xiaojinensis* is an iron-efficient apple species, while the mechanism of its iron tolerance was not fully understood. This study was designed to obtain transcript sequence data and to examine gene expression in roots and leaves under iron deficiency based on RNA-Seq and bioinformatic analysis to provide a foundation for understanding the molecular mechanism of iron absorption after iron starvation. There were 74,839 transcripts with a mean length of 864 bp obtained from 454 and Illumina sequencing. The 21,037 transcripts were expressed differentially in root and leaf samples after iron starvation, involved in iron uptake, iron remobilization, and signal transduction based on GO biological process classification. Iron uptake was enhanced 12 h after iron deficiency treatment, while iron remobilization was reinforced after 2 days iron deficiency. Ethylene and reactive oxygen scavenger (ROS)-mediated signaling pathways were activated after 12 h of iron starvation, while the auxin-signaling pathway was inhibited at 12 h and activated on the 2nd day of iron starvation. Abscisic acid (ABA) and jasmonic acid (JA) signal pathway responded after 2 days of iron starvation. Therefore, in *M. xiaojinensis*, iron uptake was enhanced in the earlier period and iron remobilization was promoted in the later period of iron deficiency. Ethylene and ROS signaling pathway responded in the earlier period.

Communicated by D. Chagné

Electronic supplementary material The online version of this article (doi:10.1007/s11295-014-0705-5) contains supplementary material, which is available to authorized users.

S. Wang · B. Lu · T. Wu · X. Zhang · X. Xu · Z. Han · Y. Wang (✉)
Institute for Horticultural Plants, China Agricultural University, Yuanmingyuan West Road, Haidian District, Beijing 100193, People's Republic of China
e-mail: wangyi@cau.edu.cn

Keywords RNA-seq · Iron deficiency · Signal transduction · Ethylene · ROS · *Malus xiaojinensis*

Introduction

Iron is essential for the growth and development of all plants. Iron deficiency induces chlorosis, inhibit plant development, and reduce crop yield and quality. Some species are tolerant to iron starvation, such as *Malus xiaojinensis*, *Prunus cerasifera*, *Prunus cerasus*, *Citrus limonia*, and *Citrus volkameriana* (Han et al. 1994a, 1998; Tagliavini and Rombolà 2001). However, the mechanisms of their resistance to iron deficiency in woody plants are not fully understood.

Plants improve iron uptake when suffering from iron deficiency. Non-graminaceous plants excrete protons and phenolic compounds from their roots into the rhizosphere in low-iron conditions, which is thought to help increase the solubility of ferric ions or to support the reducing capacity of Fe on the root surface, and the related genes *IRT1*, *FRO2*, and *HAs* are upregulated significantly (Kobayashi and Nishizawa 2012).

Besides improving iron uptake, plants can also resist iron deficiency by enhancing iron remobilization. When suffering iron deficiency stress, expression of citric acid synthase (CS) and nicotianamine synthase (NAS) encoded genes were upregulated (Han et al. 2013; Higuchi et al. 2001). Compared with iron-sufficient conditions, the NA content became higher in roots and stems in *Arabidopsis* after iron starvation (Douchkov et al. 2005). Citrate is also accumulated in pea roots and leaves (Kabir et al. 2012). Another iron transporter, NRAMP3, which locates in the vacuolar membrane and can transfer iron from the vacuole to the cytoplasm was upregulated upon iron deficiency (Bereczky et al. 2003).

M. xiaojinensis is a Fe-efficient apple species (Han et al. 1994a) that shows no significant iron deficiency symptoms

when the iron concentration is 10 μM in the medium (Han et al. 1994b). Although the leaves become yellow after 9 days iron starvation (4 μM iron) in *M. xiaojinensis*, this phenomenon occurs later than in the Fe-inefficient species *Malus baccata*. In *M. xiaojinensis*, as a non-graminaceous plant, the rhizosphere pH decreases significantly and the activities of ferric reductase and iron transporters are upregulated to cope with iron deficiency, which indicates iron uptake is enhanced (Zhang et al. 2012; Wu et al. 2011). In addition, iron deficiency-induced upregulation of *MxNAS1* and *MxCS1* expression demonstrated that iron remobilization is also enhanced after iron starvation in *M. xiaojinensis* (Zhang et al. 2009; Han et al. 2013). However, whether the iron tolerance of *M. xiaojinensis* is due to the enhancement of iron uptake or remobilization is not clear.

Signal substances, such as plant hormones, nitric oxide (NO), and hydrogen peroxide (H_2O_2), are key regulators in abiotic stress responses. Among them, NO, auxin, and ethylene are the three most important signaling substances responsive to iron deficiency stress (García et al. 2011). Sodium nitroprusside (SNP), a NO donor, completely prevented maize leaf interveinal chlorosis, resulting in a 70 % increase in chlorophyll content compared with control plants (Graziano et al. 2002). In tomato, the application of the NO-scavenger cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] inhibited the expression of iron deficiency-induced genes *LeFER*, *LeFRO1*, and *LeIRT1* in roots (Graziano and Lamattina 2007). In *M. xiaojinensis*, the expression of *MxFIT*, *MxFRO2*, and *MxIRT1* is increased significantly with 50 μM SNP treatment in roots compared with control plants, while cPTIO completely represses *MxFIT*, *MxFRO2* and *MxIRT1* in roots after iron starvation compared with controls (unpublished data).

Auxin is one of the most important signal substances in iron deficiency. In *Arabidopsis*, Fe-deficiency-induced reductase activity is enhanced by 40 % after treatment with a-naphthaleneacetic acid (NAA) compared with Fe-sufficient plants, and is strongly inhibited by growing in 1-naphthylphthalamic acid (NPA) condition compared with Fe-deficient plants. In addition, there is a fourfold increase in indole-3-acetic acid (IAA) accumulation in the roots of Fe-deficient plants compared with those of Fe-sufficient plants (Chen et al. 2010). In *M. xiaojinensis*, IAA content in roots is increased by 32 and 67 % after 3 and 6 days, respectively, growing in iron-deficient conditions. In addition, *MxFRO2* is upregulated after supplying NAA in the medium for 3 or 6 days and inhibited by NPA treatment (Wu et al. 2010).

Ethylene is another signal substance in the iron deficiency response. Ferric reductase activity is enhanced by 2-, 2.7-, 2.2-, and 5.4-folds in pea (*Pisum sativum* L. "Sparkle"), sugar beet (*Beta vulgaris* L.), tomato (*Lycopersicon esculentum* Mill. T3238FER), and *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh "Columbia"] respectively, after 24 h growing in the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) supplying medium (Romera et al. 1999). The expression of the Fe-deficiency-induced genes *OsIRO2*, *OsNAS1*, and *OsIRT1* is increased by 3-, 28-, and 3-folds, respectively, in rice after ACC treatment for 5 days. In addition, ethylene accumulates in pumpkin and cucumber roots after 5 and 6 days iron starvation, respectively (Romera et al. 1999; Waters and Blevins 2000).

Ethylene, auxin, and NO can interact at different levels. Auxin can enhance ethylene production by affecting ACC synthase activity and promote NO accumulation in plant roots (Morgan and Hall 1962; Abeldeng et al. 1995; Du et al. 2008). On the other hand, ethylene can affect auxin accumulation and polar auxin transport (Swarup et al. 2002). ACC can also enhance NO emission in senescing pea leaves and NO production in the subapical regions of the roots of several Strategy I plants (García et al. 2011). NO induced upregulation of the expression of many genes involved in ethylene synthesis including *SAMS*, *ACS*, and *ACO* in *Arabidopsis* and cucumber (García et al. 2011). NO also can increase auxin content by reducing auxin degradation mediated by auxin oxidase (Xu et al. 2010).

Besides auxin, ethylene, and NO, other small molecular substances, such as abscisic acid (ABA), jasmonic acid (JA), cytokinin (CK), and H_2O_2, also participate in iron deficiency responses to some extent. JA and CK negatively regulate Fe-deficiency-induced genes in *Arabidopsis*. In both Fe-sufficient and Fe-deficient conditions, *AtFIT1*, *AtIRT1*, and *AtFRO2* are strongly repressed by growing in 100 μM methyl-jasmonate (MeJA) supplying medium for 6 h compared with control plants (Maurer et al. 2011). Ferric reductase activity decreases to 1/3 and the expression of *AtIRT1* and *AtFRO2* to 1/7 after growing in 1 μM N^6-Benzyladenine (6-BA) supplying medium for 2 days compared with control plants (Séguéla et al. 2008).

ABA and H_2O_2 might also respond to iron deficiency. ABA content is significantly decreased by growing in iron-deficient conditions (Bacaicoa et al. 2009). However, ferric reductase activity is strongly increased after growing in 0.5 μM ABA-supplying medium for 6 h (Bacaicoa et al. 2011). H_2O_2 content was increased 28 % in sunflower leaves by growing in iron-deficient conditions (Ranieri et al. 2001). In tomato, ascorbate peroxidase (APX), which participates in H_2O_2 catabolic processes, is upregulated after 9 days growing in iron-deficient conditions (Herbik et al. 1996).

It is not clear in which tissue signal substances respond firstly during iron deficiency. Auxin and ethylene accumulate in plant roots during iron deficiency, and NO emission is also enhanced; therefore, plant roots might be the first response tissue in iron deficiency stress (Bacaicoa et al. 2009; Wu et al. 2011; Romera et al. 1999; Graziano and Lamattina 2007). Reciprocal grafting experiments with the iron-efficient species *M. xiaojinensis* and the iron-inefficient species *M. baccata*

showed that iron deficiency induced upregulation of root proton extrusion, and ferric reductase activity only occurs when *M. xiaojinensis* was used as the stock (Wu et al. 2011).

Some researchers have indicated that signal substances are produced in leaves and then transmitted to roots to regulate downstream functional genes. For example, reciprocal grafting experiments showed that ferric reductase activity was higher when the Fe-hyperaccumulating mutant *dgl* was used as the shoot in pea (Grusak and Pezeshgi 1996). Split-root experiments showed that 3 days after transfer to culture medium, *IRT1* and *FRO2* messenger RNA (mRNA) accumulation was strongly decreased in the iron-deficient side and increased in the iron-sufficient side of the roots, which indicates signal substances derived from leaves (Vert et al. 2003).

Although previous researches show that NO, H_2O_2, and plant hormones are involved, to some extent, in iron deficiency stress responses, it is not clear in which order signal substances respond to iron deficiency in plants and whether all of these signal substances respond to iron starvation in *M. xiaojinensis*.

In this article, to obtain a general overview of Fe deficiency-induced changes in the transcriptome of *M. xiaojinensis*, 454 and Illumina RNA-seq were used to identify transcripts at different time points from 12 h to 9 days.

Materials and methods

Plant materials

M. xiaojinensis in vitro shoots were subcultured in MS medium (Murashige and Skoog 1962) containing 0.5 mg/l indole-3-butytric acid (IBA), 0.5 mg/l 6-benzyl aminopurine (6-BA), 3 % (w/v) sucrose, and 0.7 % (w/v) agar and propagated for 1 month. These strong and more than 4-cm-tall plants were transferred to 1/2 MS medium containing 0.5 mg/lIBA, 3 % (w/v) sucrose, and 0.7 % (w/v) agar to take root for another month. The tissue culture plantlets with roots extending nearly 2.0 cm were transplanted into a vessel containing a nutrient solution (1.9 g/l KNO_3, 1.65 g/l NH_4NO_3, 0.37 g/l $MgSO_4 \cdot 7H_2O$, 0.17 g/l K_2HPO_4, 0.0278 g/l $FeSO_4 \cdot 7H_2O$, 0.0373 g/l Na_2 ethylenediaminetetraacetic acid (EDTA) and the micronutrients HBO_3 1.54 mg/l, $MnCl_2 \cdot 4H_2O$ 0.912 mg/l, $ZnSO_4 \cdot 7H_2O$ 1.12 mg/l, $CuSO_4 \cdot 5H_2O$ 0.648 mg/l, and $[NH_4]_2MoO_4 \cdot 4H_2O$ 0.0408 mg/l, pH 6.3) for 4 weeks. The nutrient solution was replaced every week. Finally, uniform seedlings were selected for study. The seedlings were washed with distilled water twice, dried by absorbent paper and transferred into a new nutrient solution undergoing different treatments. (Supplementary Table S11 for 454 sequencing and Supplementary Table S12 for Illumina sequencing). At different time points, the white roots and the mature leaves (fully expanded) were sampled about 500 mg. All experiments were conducted in a growth chamber at 25±2 °C day/17±2 °C night with a 16 h photoperiod at a light intensity of 1,500 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

cDNA library construction and 454, Illumina sequencing

Total RNA of the samples for 454 sequencing was isolated according to the instructions of the TRIzol kit (Invitrogen, Shanghai, China) and then purified to exclude the transfer RNA (tRNA) and ribosomal RNA (rRNA) and to enrich the mRNA according to the instructions of the mRNA purification kit (Promega, Shanghai, China). mRNAs were reverse-transcribed by PowerScript™ Reverse Transcriptase (Takara, Dalian, China) with SMART IV™ Oligonucleotide (Takara) and CDS III/3′ PCR Primer (Beijing Genomics Institute). Long-distance PCR for double-strand complementary DNA (cDNA) amplification was performed with LA Taq Polymerase (Takara) for 15 cycles (96 °C for 1 min, 96 °C for 30 s, and 68 °C for 6 min) according to the SMART™ cDNA Library Construction Kit (Takara) User Manual. The poly-T stretch was broken, and the cDNA was randomly sheared in the condition of 30 psi nitrogen for 1 min, then cDNAs were purified using a DNA purification kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. After verified by 1.0 % agarose gel, the target fragments (500–800 bp) were collected using a Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, emPCR and 454 sequencing were performed according to GS FLX Titanium series kits (Roche Applied Science, Shanghai China).

Total RNA of the samples for Illumina sequencing was isolated using TruSeq Stranded mRNAs Sample Preparation kit (Illumina, Shanghai, China) according to the protocol. After that, mRNAs were purified using poly-T oligo-attached magnetic beads and fragmented, the first and second cDNA were synthesized and end-repaired. Adaptors were ligated after adenylation at 3′ end. After gel purification, cDNA templates were enriched by PCR. Finally, the short cDNA fragments were prepared for Illumina sequencing on an Hiseq 2000 (Illumina, San Diego, CA). Time points selecting in Illumina sequencing was based on our previous research, *MxVHA* was induced significantly at 12 h after iron starvation in root which indicated iron uptake was improved (Zhang et al. 2012), activity of ferric reductase improved, and pH value decreased began at 2 days after iron starvation in root, and the two values reached to peak in day 6 after iron starvation, while in day 9, the two values were back to normal levels (Wu et al. 2010). Therefore, roots and leaves samples were harvested at 0 h, 12 h, 2 days, 6 days, and 9 days after iron starvation.

Gene annotation and functional classification

All unigenes were blasted against the NCBI nonredundant nucleotide database (NT, by Nov. 3, 2010; *E* value

≤1e^{-10}), nonredundant protein database (NR, by Oct. 9, 2010; *E* value ≤1e^{-5}), and SWISS-PROT (downloaded from European Bioinformatics Institute by Oct. 28, 2010; *E* value ≤1e^{-10}). The BLAST result accessions against SwissProt were used to assign Gene Ontologies (GO) by WEGO.

Gene expression analysis

For gene expression analysis, the expression level of each gene in each treatment of Illumina sequencing was calculated by quantifying the number reads that mapped to each sequence using the Bowtie program. The raw gene expression counts were normalized using RPKM (reads per kilobits per million) method (Mortazavi et al. 2008). Compared with normal treatment, genes which expressed differentially in iron deficiency stress were identified by edgeR (Robinson et al. 2010) and required to have a twofold change and a *p* value ≤0.01. Heat maps illustrating expression patterns of various subgroups of differentially expressed genes were generated by TIBCO Spotfire software (https://tryspotfire.tibco.com/).

Quantitative PCR

Total RNA was extracted using CTAB method (Zhang et al. 2005). After DNase1 (Takara, Tokyo, Japan) treatment 30 min in 37 °C, purified RNA was identified by 1.0 % agarose gel, and the concentration was determined by using Nanodrop 2000 instrument (Thermo Scientific), the first strand cDNA for each sample was made from 1 μg RNA using M-MLV RT (Takara). Gene-specific primers were designed using Primer software (Supplementary Table S13). Samples and standards were run in triplicate on each plate using the 1×SYBR Master Mix (Taraka) on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. qPCR was performed in a 20 μl reaction containing 6.8 μl dd H_2O, 10 μl 1×SYBR Master Mix, 0.4 μl forward primer, 0.4 μl reverse primer, 0.4 ROX Reference DyeII, and 2 μl template cDNA. The PCR condition were 30 s of pre-denaturation, 40 cycles of 5 s at 95 °C and 30 s at 60 °C. The expression was calculated by "comparative C_T method" (Schmittgen and Livak 2008) using the C_T value of the *β-actin* (Kürkcüoglu et al. 2007), the primer was listed in Supplementary Table S13. The data were processed using Microsoft Excel 2007, and the differences were evaluated using two-sample *t* tests assuming unequal variances. Differences were defined as significant if *p* value ≤0.01 and fold change ≥2, and asterisks were labelled on the top of their columns.

Results

RNA-seq

A number of 1,200,975 short reads from 454 and Illumina sequencing were assembled into 80,380 contigs with a mean length of 680 bp. After filtering out homopolymers and short reads (<150 bp), 74,839 unigenes with a mean length of 864 bp were obtained. Illumina reads from each sample were mapped to 74,839 unigenes, 42.5–75.4 % reads were mapped to 74, 839 unigenes in different root and leaf samples, respectively (Supplementary Tables S1 and S2).

Approximately 66–91 % of the unigenes were annotated by BLASTx, with a threshold *E* value of 10^{-5}, from four public databases [i.e., the NCBI nonredundant (nr), Swiss-Prot protein, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups of proteins (COG) databases; Table 1]. Among the 74,839 *M. xiaojinensis* transcripts, 70,674 were significantly matched to 22,744 different "Golden Delicious" predicted proteins (BLASTx, *E* value threshold of 10^{-5}). Reciprocal BLAST searches, in which the Golden Delicious predicted sequences were queried against the *M. xiaojinensis* sequences (Velasco et al. 2010), revealed that 84,436 of the 95,232 proteins had significant similarity with *M. xiaojinensis* transcripts (tBLASTn, *E* value threshold of 10^{-5}), corresponding to 20,561 different *M. xiaojinensis* transcripts. A comparison of the best BLAST matches from these searches identified 13,704 unambiguous orthologs between *M. xiaojinensis* and Golden Delicious, based on significant reciprocal best matches (Fig. 1).

Expression profile in roots and leaves

To obtain a general overview of the *M. xiaojinensis* transcriptome during iron starvation, mature leaves and white roots were harvested after 12 h (12 h), 2 days (2d), 6 days (6d), and 9 days (9d) of iron deficiency. There were 3,482,026–17,118,764 reads obtained from the samples, respectively, and assembled into 67,844 unigenes.

Compared with root and leaf samples at 0 h of iron deficiency, a total of 21,037 unigenes were differentially expressed (>twofold change, *p* value ≤0.01) in root (R) and leaf (L) samples after 12-h, 2-day, 6-day, and 9-day iron-deficient treatment (Supplementary Table S3, Supplementary Table S4).

In root samples, the number of differentially expressed genes decreased gradually; 6,947 unigenes were expressed differentially in the R12h sample, then decreased to 2,743 in the R9d sample. However, there was no such tendency in leaf samples; 5,199, 4,776, 5,826, and 4,809 unigenes were expressed differentially in the L12h, L2d, L6d, and L9d samples, respectively (Supplementary Fig. S1).

Table 1 *Malus xiaojinensis* unigenes from 454 sequencing annotation to public databases BLASTx

Database	Annotated unigene numbers	Percentages	Annotated functional gene numbers	Database version	BLAST cutoff
NT	60707	81.11 %	23001	2010-11-03	$1e^{-10}$
NR	67964	90.81 %	29888	2010-10-09	$1e^{-5}$
KEGG	67441	90.11 %	26018	2010-09-01	$1e^{-5}$
SwissProt	49511	66.16 %	12842	2010-10-28	$1e^{-5}$
KOG	61113	81.65 %	13943	–	$1e^{-5}$

Expression profile of iron uptake and remobilization-related genes

M. xiaojinensis uses Strategy I to absorb iron. Many related genes were expressed differentially after iron starvation including iron uptake-related genes *MxIRT1*, *MxFRO2*, and *MxHAs* (Gao et al. 2011; Zhang et al. 2012), and iron remobilization-related genes *MxNAS1*, *MxCS1*, and *MxNRAMP1* (Zhang et al. 2009; Han et al. 2013). Expression profile showed that *MxHA2* and *MxFRO2* were upregulated significantly, by 11.35- and 8.72-folds, in the R12h sample, while at the same time point, the iron remobilization-related genes *MxNAS1*, *MxNRAMP1*, and *MxCS1* did not respond in leaf and root (Fig. 2, Supplementary Table S5). These results indicated that iron uptake in roots was already reinforced after 12 h of iron starvation, while iron remobilization-related genes had not responded.

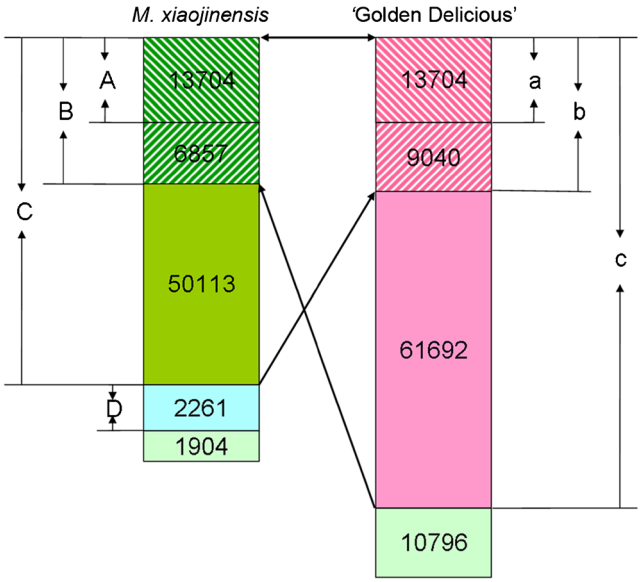

Fig. 1 Summary of the reciprocal comparisons between *Malus xiaojinensis* nucleotide sequences and "Golden Delicious" peptidic sequences. *A* Unambiguous orthologs between *M. xiaojinensis* transcripts and Golden Delicious peptidic sequences in *M. xiaojinensis* transcriptome; *a* unambiguous orthologs between *M. xiaojinensis* transcripts and Golden Delicious peptidic sequences in Golden Delicious genome. *B* Transcripts of *M. xiaojinensis* which are significantly matched by Golden Delicious peptidic sequences; *b* Golden Delicious peptidic sequences which are significantly matched by *M. xiaojinensis* transcripts. *C* Transcripts in *M. xiaojinensis* significantly match to the Golden Delicious peptidic sequences; *c* peptidic sequences in Golden Delicious genome which are significantly similarity with *M. xiaojinensis* transcripts. *D* Putative *M. xiaojinensis*-specific genes

In the R6d sample, although the expression of *MxFRO2* was increased 8.26-fold, *MxIRT1* was repressed significantly, which indicated that the enhancement of iron uptake had disappeared (Fig. 2, Supplementary Table S5). In contrast, the iron remobilization-related genes *MxNRAMP1* and *MxNAS1* were upregulated by 31.41-folds and more than 100-fold, respectively, which means that iron remobilization was reinforced in root samples (Fig. 2, Supplementary Table S5). Moreover, *MxNAS1* was also upregulated in the L6d sample, which showed that iron remobilization was also enhanced in leaves.

In the R9d sample, the iron remobilization-related genes *MxNAS1* and *MxCS1* were upregulated significantly, which indicated that iron transport in roots and iron remobilization in roots and leaves were reinforced.

In summary, iron uptake in roots was enhanced significantly from 12 h to 2 days, but expression of *IRT1* was downregulated in R6d sample, while iron remobilization was reinforced from days 6 to 9 in roots and leaves under iron starvation.

Expression profile of signal substances pathway

Ethylene-mediated signaling pathway

A number of genes in the ethylene-mediated signaling pathway were induced during iron starvation in *M. xiaojinensis*. Compared with the R0h sample, ethylene receptor *ERS1* was upregulated by 40-folds in the R12h sample (Fig. 3a, Supplementary Table S6). Moreover, the feedback inhibitor of ethylene-insensitive protein 3(EIN3) *EBF1* and three downstream transcription factors, *EIN3*, *ERF105*, and *RAP2.2*, were also upregulated significantly. The ethylene synthesis-related gene *ACO* was obviously repressed (Fig. 3a).

In the R2d sample, the expression of ethylene receptors *ETR* and *ETR2* were increased by 12.98- and 11.93-folds, respectively, and *EBF1* expression was increased 2.56-folds (Fig. 3a, Supplementary Table S6). The transcription factors *ERF4*, *ERF073*, and *RAP2.7* were also upregulated, while the ethylene receptor *ERS1*, which was upregulated in the R12h sample, was repressed significantly (Fig. 3a).

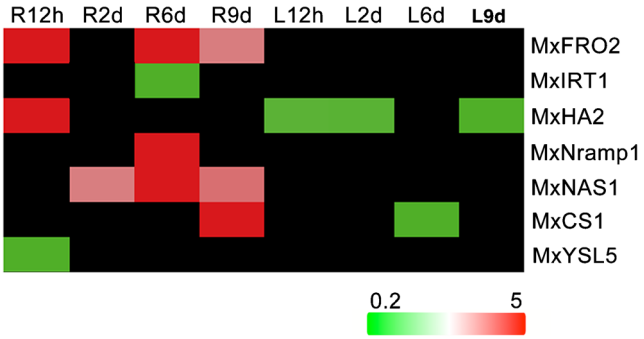

Fig. 2 Expression profile of genes related to iron uptake and transport. To be considered differentially expressed, the transcript must have a fold change ≥2 between iron deficiency stress and R0h sample, and a *p* value ≤0.01. While *red* cell means transcripts were induced due to iron starvation, *green* cell indicates transcripts were repressed after iron starvation and *black* cell means no significant fold change after iron starvation

In the R6d sample, the ethylene receptors *ETR* and *ETR2* (which were induced in the R2d sample) were downregulated significantly, and the transcription factors *ERF105* and *RAP2.2* were also clearly downregulated (Fig. 3a). In the R9d sample, genes in ethylene-mediated signaling pathway did not respond significantly, while the ethylene synthesis-related gene *ACO* was repressed, with expression of less than 1/100 compared with the R0h sample (Fig. 3a, Supplementary Table S6).

In leaf samples, *ERS1* was downregulated significantly at days 2 and 9 during iron starvation, while *ERF105* was repressed after 6 days iron starvation (Fig. 3a). The expression of other genes in the ethylene signal pathway showed no significant changes. Moreover, the ethylene synthesis-related gene *ACO* was upregulated in four leaf samples; its expression was increased by over 100 times in the L2d sample, and *ACS* was upregulated in the L2d and L9d samples (Fig. 3a, Supplementary Table S6).

Taken together, genes in the ethylene-mediated signaling pathways were enhanced in R12h and R2d samples after iron starvation and the enhancement were disappeared after 6 days, but they did not respond significantly in leaf samples. The ethylene synthesis genes *ACO* and *ACS* were only upregulated in leaves and downregulated in the R12h sample.

Auxin-mediated signaling pathway

The auxin-mediated signaling pathway was also activated. In the R12h sample, *IAA9* and *IAA13* repressors of auxin transcription factor ARF and auxin-repressed protein (ARP) were upregulated significantly; their expression levels were increased by 17.13-, 8.83-, and 10-folds compared with the R0h sample, respectively (Fig. 3b, Supplementary Table S7). A component of the SCF E3 ubiquitin ligase complex that can activate the auxin-mediated signaling pathway *SKP1* and the auxin influx transporter *LAX3* were also upregulated significantly (Fig. 3b).

In the R2d sample, although the ARF repressor *IAA13* was still induced, the fold changed from 8.83 to 2.64, compared with R0h sample, while another ARF repressor *IAA9* was not upregulated significantly (Fig. 3b, Supplementary Table S7). Moreover, the auxin receptor *TIR1*, another component of the SCF E3 ubiquitin ligase complex *CUL1*, some transcription factor *ARFs*, the auxin transporter *LAX1*, and Auxin-induced protein were all upregulated significantly (Fig. 3b, Supplementary Table S7).

In the R6d sample, auxin responsive genes were not upregulated, and the auxin transcription factors *ARF1*, *ARF15*, and *ARF9* were clearly repressed. In the R9d sample, only two genes responded significantly: *ARF1* was upregulated and *ARF4* was downregulated (Fig. 3b).

In the L12h sample, the auxin transcription factors *ARF1* and *ARF108* were repressed significantly, with relative expression values of only 0.07 and 0.01, respectively, compared with the R0h sample (Fig. 3b, Supplementary Table S7).

In the L2d sample, the ARF repressor *IAA9* was downregulated significantly, while the expression of auxin transporters *ABCB19* and *LAX3* were increased by 35.20- and 5.27-folds, respectively, compared with the L0h sample (Fig. 3b, Supplementary Table S7). The auxin transcription factor *ARF1* was upregulated, but *ARF108* was downregulated (Fig. 3b).

In the R6d sample, *ABCB19* and *LAX3* were still upregulated, with expression of *ABCB19* increased by over 100 times compared with the L0h sample (Fig. 3b, Supplementary Table S7). *ARF1* and Auxin-induced protein were also induced, and this tendency continued to 9 days of iron starvation (Fig. 3b, Supplementary Table S7).

In summary, the auxin-mediated signaling pathway responded in both roots and leaves during iron deficiency stress. In root samples, although auxin transport was enhanced at 12 h after iron starvation, the ARF repressors *IAA9* and *IAA13* were upregulated, which indicated the auxin-signaling pathway might be repressed. The auxin-signaling pathway was activated in R2d sample, accompanied by significant upregulation of *ARFs*. In leaf samples, auxin transport reinforced from days 2 to 6 during iron starvation.

ABA-mediated signaling pathway

A number of ABA-responsive genes were also expressed differentially during iron starvation. In the R12h sample, genes related to the ABA-mediated signaling pathway and ABA synthesis did not respond significantly (Fig. 3c). In the R2d sample, positive regulators of the ABA-signaling pathway were upregulated significantly, including the ABA receptor *PYL9*, the histidine protein kinase *ATHK1*, Mitogen-activated protein kinase3 and Syntaxin-61 (Fig. 3c). However, the transcription factor *ABI5* and the serine/threonine-protein kinase *SAPK1* were downregulated (Fig. 3c).

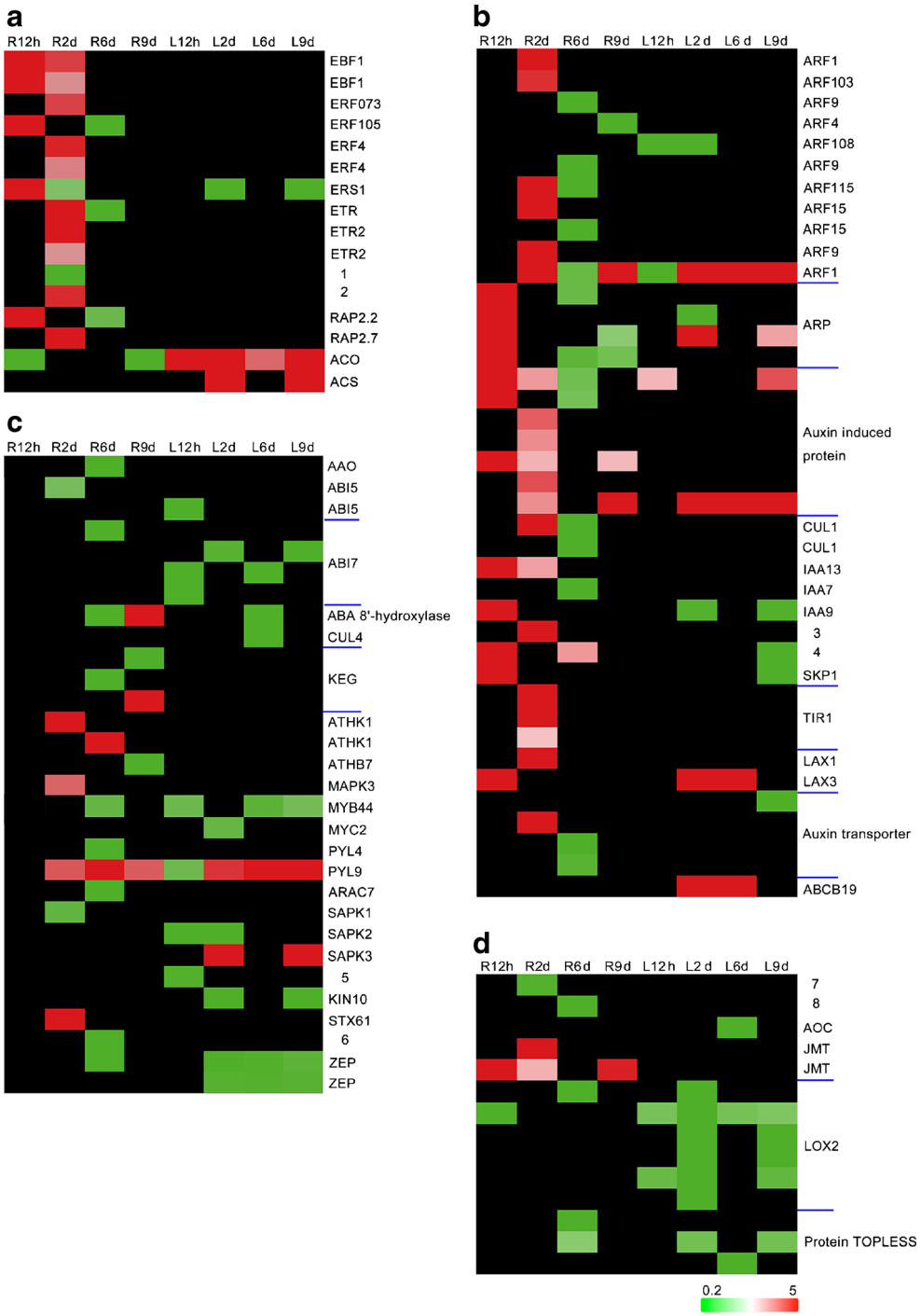

Fig. 3 Expression profile of genes related to plant hormone-related pathway. **a** Different expressed transcripts in ethylene biosynthetic process and ethylene-mediated signaling pathway. **b** Different expressed transcripts in auxin transport and auxin-mediated signaling pathway. **c** Different expressed transcripts in abscisic acid (*ABA*) biosynthetic process and ABA-mediated signaling pathway. **d** Different expressed transcripts in jasmonic acid (JA) biosynthetic, metabolic process, and JA-mediated signaling pathway. To be considered differentially expressed, the transcript must have a fold change ≥2 between iron deficiency stress and R0h sample, and a p value ≤0.01. While *red* cell means transcripts were induced due to iron starvation, *green* cell indicates transcripts were repressed after iron starvation and *black* cell means no significant fold change after iron starvation. Numbers from 1 to 8 in figures represent a gene name which is too long to write in the figure directly: *1* Ethylene-overproduction protein 1, *2* Protein XAP5 circadian timekeeper, *3* Protein transport inhibitor response 1, *4* Pyrophosphate-energized vacuolar membrane proton pump 1, *5* Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform, *6* Type I inositol-1,4,5-trisphosphate 5-phosphatase 1, *7* 4CL5-like, and *8* 6,7-dimethyl-8-ribityllumazine synthase

In the R6d sample, except for *PYL9* and *ATHK1*, which were upregulated, other genes related to abscisic acid signaling and synthesis were repressed significantly, including the ABA synthesis-related genes *AAO* and *ZEP*, the ABA degradation-related gene Abscisic acid 8′-hydroxylase, the ABA receptor *PYL4*, the transcription factors *ABI7*, *MYB44*, and *ARAC7*, and the negatively regulated ABA signal pathway gene E3 ubiquitin-protein ligase *KEG* (Fig. 3c).

In the R9d sample, Abscisic acid 8′-hydroxylase encoding gene was upregulated, with an expression value over 100 times greater than in the R0h sample (Fig. 3c, Supplementary Table S8). Although *PYL9* was upregulated, its expression was increased by only 3.6-fold, which was much less than in the R6d sample (Fig. 3c, Supplementary Table S8). *ATHB-7*, which can be upregulated by exogenous ABA, was repressed significantly.

In the L12h sample, genes related to the ABA-mediated signaling pathway were not upregulated significantly (Fig. 3c). The ABA receptor *PYL9*, ABA transcription factors *ABI5*, *ABI7*, and *MYB44*, and the serine/threonine-protein kinase *SAPK1* were all downregulated (Fig. 3c).

The ABA synthesis-related gene *ZEP* and the ABA transcription factor *ABI7* were repressed significantly from days 2 to 9 of iron starvation in leaf samples, while the ABA receptor *PYL9* was induced during this period (Fig. 3c).

To conclude, although the ABA-mediated signaling pathway positively regulated genes, *ATHK1*, *MAPK3*, and *PYL9* were upregulated in the R2d and R6d samples, ABA synthesis-related genes and ABA transcription factors were not, which indicated that the ABA-mediated signaling pathway was not activated after iron starvation. In leaf samples, only the ABA receptors *PYL9* and *SAPK3* were upregulated from days 2 to 9 during iron starvation, which showed that the ABA-signaling pathway was not activated in leaves.

JA-mediated signaling pathway

In root samples, the jasmonic acid synthesis-related genes *LOX2*- and 4CL5-like were downregulated significantly in the R12h and R2d samples, while the JA degradation-related gene Jasmonate O-methyltransferase (*JMT*) was upregulated. In the R6d sample, *LOX2* was still repressed, and JA-mediated signaling pathway-related genes were also downregulated (Fig. 3d). In the R9d sample, no genes were expressed differentially except *JMT*, which was induced upregulation. In leaf samples, JA synthesis and signaling pathway-related genes were downregulated at different times after iron starvation (Fig. 3d, Supplementary Table S9).

Therefore, JA synthesis was inhibited and degradation was enhanced in both root and leaf samples, while the JA-mediated signaling pathway might be repressed in root samples during iron starvation.

ROS genes

Reactive oxygen scavenger (ROS) scavenging protein-encoding genes were also expressed differentially in iron deficiency conditions. In the R12h sample, 24 genes from hydrogen peroxide catabolic process were expressed significantly differentially; 20 unigenes were upregulated including two *APX2*s, 14 *CAT*s, and four *POX*s, while the other four genes were downregulated including *APX3*, *APX5*, *CAT1*, and *POX10*. Based on GO cellular component classification, 16 of the upregulated genes were located in the cytoplasm and the extracellular region; among these, eight were induced significantly. In addition, 9 of 19 genes located in the peroxisome were induced upregulation (Fig. 4).

In the R2d sample, only four unigenes from hydrogen peroxide catabolic process were upregulated including

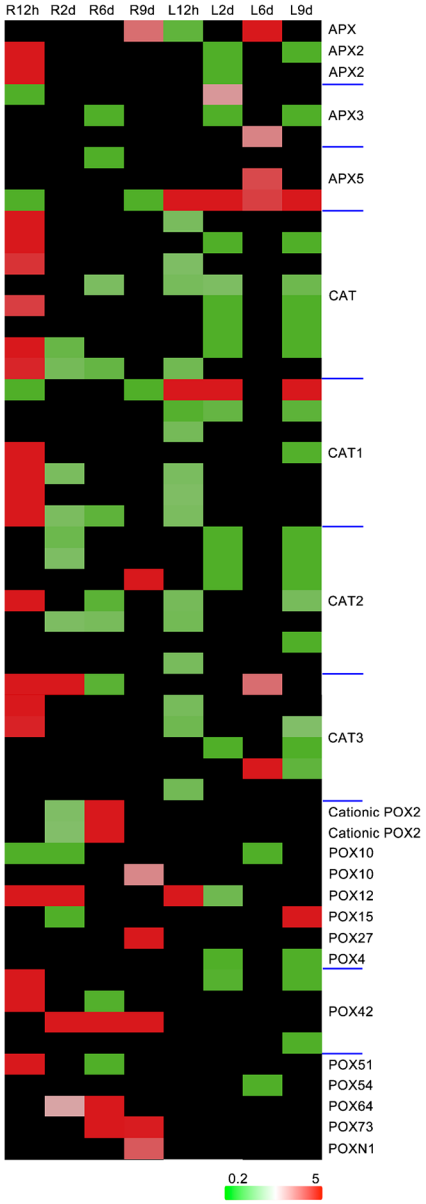

Fig. 4 Significantly different expressed genes in hydrogen peroxide catabolic process. To be considered differentially expressed, the transcript must have a fold change ≥2 between iron deficiency stress and R0h sample, and a p value ≤0.01. While *red* cell means transcripts were induced due to iron starvation, *green* cell indicates transcripts were repressed after iron starvation and *black* cell means no significant fold change after iron starvation

CAT3, *POX12*, *POX42*, and *POX64* (Fig. 4). There were 11 unigenes downregulated which comprised of two cationic *POX2*s, seven *CAT*s and two *POX*s (Fig. 4). There were also five unigenes expressed differentially in the extracellular region; cationic *POX2* and *CAT2* were repressed while only *POX42* was induced. *POX10* located in the cytoplasm and *POX15* located in the membrane were also repressed (Fig. 4).

In the R6d sample, among 15 differentially expressed genes from hydrogen peroxide catabolic process, only five were upregulated including cationic *POX2*, *POX42*, *POX64*,

and *POX73*; the expression of *POX2* and *POX73* were increased by over 100 times compared with the R0h sample. In addition, six *CAT*s were all significantly induced downregulation (Fig. 4, Supplementary Table S10).

In the R9d sample, only nine unigenes from hydrogen peroxide catabolic process were differentially expressed, among which *CAT1* and *APX5* were repressed significantly, while the other seven were upregulated strongly (Fig. 4).

In the L12h sample, among 19 differentially expressed genes from hydrogen peroxide catabolic process, only three were upregulated including *APX5*, *POX12*, and *CAT1*; the other 16 were repressed significantly including 15 *CAT*s and an *APX* (Fig. 4). Unigenes located in the cytoplasm, extracellular region, chloroplast envelope, and glyoxysome were repressed or did not respond (Fig. 4, Supplementary Table S10).

As in the L12h sample, there were also 19 unigenes from hydrogen peroxide catabolic process differentially expressed in the L2d sample, three of which were induced including *APX3/5* and *CAT1* (Fig. 4). The other 16 unigenes comprising ten *CAT*s, two *APX2*s, one *APX3*, and three *POX*s were downregulated. Unigenes located in the cytoplasm, extracellular region, chloroplast envelope, and glyoxysome were downregulated or did not respond (Fig. 4, Supplementary Table S10).

In the L6d sample, only eight unigenes from hydrogen peroxide catabolic process expressed differentially, six of which were induced while two were repressed significantly (Fig. 4).

In the L9d sample, 20 unigenes from hydrogen peroxide catabolic process were downregulated significantly, including two *CAT1*s, five *CAT2*s, three *CAT3*s, five other *CAT*s, and five encoding *APX2/3* and *POX4/42* (Fig. 4, Supplementary Table S10).

Taken together, the genes upregulated in the root samples were located in the cytoplasm, extracellular region, and peroxisome, which indicated that ROS scavenging had been activated. However, this activation disappeared in the R2d and R6d samples. While in the R9d sample, ROS scavenging was enhanced in the extracellular region and membrane, in leaf samples, ROS scavenging was only activated on day 6 of the iron starvation treatment.

qPCR verification

There were 11 unigenes chosen for quantitative polymerase chain reaction (qPCR) from the ethylene, auxin, ABA and JA synthesis, metabolism, and signaling pathways, and hydrogen peroxide catabolism. Their names and GO annotations were contig1137 (ERS1), contig247 (ERF105), contig2353 (IAA9), contig1260 (ARAC7), H_rep_c22375 (CAT1), H_rep_c2053 (TIR1), H_rep_c15522 (LOX2), 1254557 (ARF1), contig15484 (PYL9), contig16283 (ERF4), and contig12462 (Protein TOPLESS). Of all 88 relative expression time-points (11 genes×8 treatments), only seven differed from the RNA-seq data (Fig. 5).

Discussion

Iron uptake was enhanced in roots in the earlier period of iron starvation, while iron remobilization was reinforced in the later period. The iron uptake-related genes *FRO2*, *HA2*, and *IRT1* were upregulated at 12 h and 2 days of iron starvation. Although *FRO2* was still upregulated in the R6d and R9d samples, *HA2* and *IRT1* were not, and *IRT1* was even downregulated in the R6d sample. The iron remobilization-related genes *NRAMP1* and *NAS1* were upregulated in R6d, and *NAS1* was also upregulated in R9d sample.

In tomato, *LeIRT1* was upregulated after 6 h of iron starvation, and its expression reached a peak after 12 and 24 h, before decreasing a little at 48 h (Wang et al. 2002). *FIT* and *IRT* accumulated in *Arabidopsis* roots after 12 h of iron starvation and continued to accumulate to 5 days (Sivitz et al. 2011). These results indicate that Strategy I plants improve iron uptake in roots in the earlier period, while iron remobilization is enhanced in the later period. In *Arabidopsis* root samples, *AtNRAMP1* was upregulated on the 3rd day of iron deficiency treatment and its expression was further increased on the 5th day (Curie et al. 2000). In peanut, *NRAMP1* was upregulated in the roots after 4 days of iron deficiency, and the changes continued to 11 days (Xiong et al. 2012). Our previous studies showed that *MbNRAMP1* was upregulated on the 5th day of iron deficiency (Xiao et al. 2008). In *M. xiaojinensis*, another iron remobilization-related gene *NAS1* was also upregulated on the 5th day of iron deficiency and its expression was still elevated on the 15th day. Here, iron uptake was enhanced after 12 h and remained high after 2 days of iron deficiency in *M. xiaojinensis* roots, while iron remobilization was reinforced after 2 days.

Based on our expression profiling, the ethylene and ROS signal pathways might be more important in the low iron stress response than the other signal pathways. The ethylene-mediated signaling pathway was activated after 12 h of iron deficiency in roots, which was earlier than the other signal pathways. Ethylene accumulated in the roots of cucumber, pea, tomato, and pumpkin after iron deficiency, and in cucumber, the ethylene content increased in the first day of iron deficiency (Romera and Alcántara 2004; Bacaicoa et al. 2009). Moreover, the increased ethylene production by Fe-deficient roots paralleled the enhancement of ferric reductase activity and root acidification process (Romera et al. 1999; Waters and Blevins 2000). While in our results, ethylene synthesis genes *ACO* and *ACS* were not upregulated in roots might be because they did not expressed in roots or they were not induced in iron deficiency stress. In *Arabidopsis*, only four genes for ACC synthase *AtACS4*,

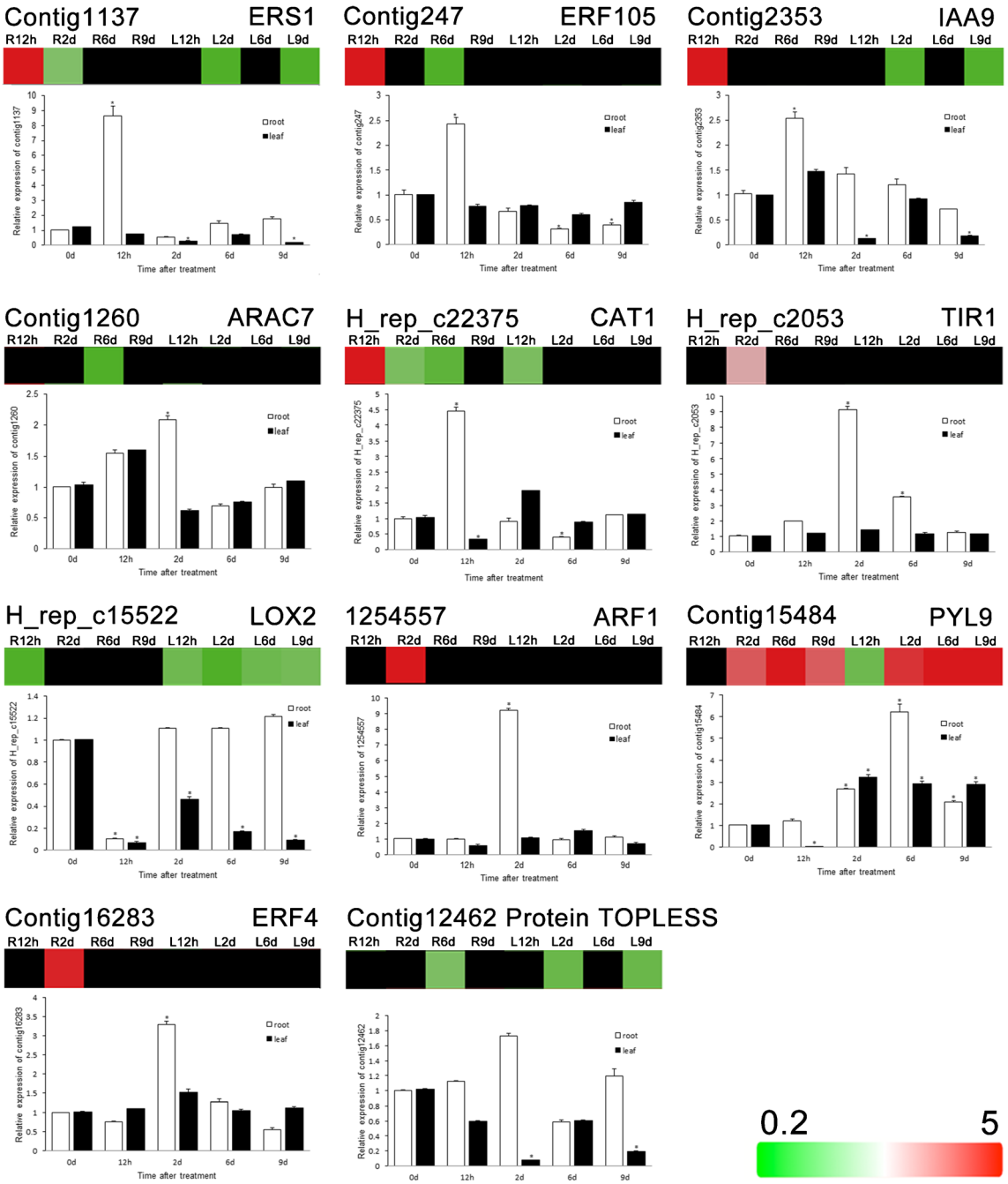

Fig. 5 Quantitative real time PCR confirmation of 11 significantly expressed genes in different signaling pathway. Histograms represent transcript abundance obtained for Contig1137, Contig247, Contig2353, Contig1260, H_rep_c22375, H_rep_c2053, H_rep_c15522, Contig12462, Contig15484, Contig16283, and 1254557. *Asterisks* were labeled on the top of the columns which indicate that the gene's expression had significant changes compared to R0 or L0 sample (fold change ≥2 and p value ≤0.01). Error bars on each column indicates SDs from three replicates

AtACS6, *AtACS9*, and *AtACS11*; and three for ACC oxidase, *AtACO1*, *AtACO2*, and *AtACOS3*, were examined in roots. And among all the genes, the expression of *AtACS11* and *AtACO3* were not increased. The other eight *AtACSs* and *AtACO4* were not examined in roots (García et al. 2010). Therefore, the downregulated *ACO* in root samples after iron deficiency might be homologous of *AtACO3* or *AtACO4*, no significant expression changes *ACS* in root samples after iron deficiency might be homologous of *AtACS11*. This evidence shows that in other plants, ethylene is also accumulated in roots after iron deficiency and is related to the improvement of iron uptake.

ROS scavenging was improved significantly after 12 h of iron starvation in *M. xiaojinensis* root and was activated on days 2 and 6, which indicated that the ROS signal pathway was activated at 12 h of iron deficiency and repressed on the

2nd day. In *Arabidopsis*, *POX* was also upregulated after 24 h iron deficiency and repressed after 3 days (Thimm et al. 2001).

The activation of the IAA-mediated signaling pathway occurred later than the enhancement of iron uptake. Expression profiling showed that iron uptake was upregulated strongly after 12 h of iron deficiency, at which point the ARF repressors *IAA9* and *IAA13* were upregulated, indicating the IAA signal pathway was suppressed. Many *ARFs* were upregulated after 2 days of iron deficiency, suggesting that the IAA-mediated signaling pathway was then activated.

In tomato, IAA accumulated significantly on the 6th day of iron starvation (Jin et al. 2011), but *LeIRT1* was upregulated in the 6th hour after iron starvation (Wang et al. 2002). Our previous research showed that IAA was accumulated in the *M. xiaojinensis* root after 3 days of iron deficiency, but did not accumulate before the second day (Wu et al. 2011).

On the other hand, NAA and IAA could enhance ferric reductase activity and iron uptake gene expression in iron-sufficient conditions in cucumber and *Arabidopsis*, and the IAA transport inhibitors NPA and TIBA repressed the enhancement of ferric reductase activity and iron uptake gene expression (Chen et al. 2010; Bacaicoa et al. 2011). In *M. xiaojinensis*, IAA also induced *MxFRO2* in iron-sufficient roots, while NPA repressed the upregulation of *MxFRO2* on the 3rd and 6th day of iron starvation (Wu et al. 2011).

Therefore, IAA was unrelated to the improvement of iron uptake after 12 h of iron starvation, but might be related to iron uptake enhancement at 2 days.

Conclusions

Based on expression profiling by RNA-seq, we found many transcripts that were expressed differentially in *M. xiaojinensis* during iron starvation including iron uptake and remobilization genes and many plant hormone pathway genes. The enhancement of iron uptake occurred earlier than the improvement of iron remobilization in roots. The expression profiles of plant hormone pathways showed that ethylene and ROS signaling responded earlier than the IAA, ABA, and JA signal pathways.

Acknowledgments This study was supported by the National Special Funds for Scientific Research on Public Causes (Agriculture) Project (201203075), the National High Technology Research and Development Program (2011AA001204), and the Modern Agricultural Industry Technology System (Apple). The study was also supported by the Key Laboratory of Beijing Municipality of Stress Physiology and Molecular Biology for Fruit Tree, the Key Laboratory of Biology, and the Genetic Improvement of Horticultural Crops (Nutrition and Physiology).

Data Archiving Statement Raw reads of the experiment are submitted to NCBI SRA database, the accession number of the project is SRP036137.

References

Abeldeng S, Nguyen MD, Chow W, Theologis A (1995) *ACS4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. J Biol Chem 270: 19093–19099

Bacaicoa E, Zamarreño ÁM, Leménager D, Baigorri R (2009) Relationship between the hormonal balance and the regulation of iron deficiency stress responses in cucumber. J Am Soc Hortic Sci 134:589–601

Bacaicoa E, Mora V, Zamarreño ÁM, Fuentes M, Casanova E, García-Mina JM (2011) Auxin: a major player in the shoot-to-root regulation of root Fe-stress physiological responses to Fe deficiency in cucumber plants. Plant Physiol Biochem 49:545–556

Bereczky Z, Wang HY, Schubert V, Ganal M, Bauer P (2003) Differential regulation of NRAMP and IRT metal transporter genes in wild type and iron uptake mutants of tomato. J Biol Chem 278:24697–24704

Chen WW, Yang JL, Qin C, Jin CW, Mo JH, Ye T, Zheng SJ (2010) Nitric oxide acts downstream of auxin to trigger root ferric-chelate reductase activity in response to iron deficiency in Arabidopsis. Plant Physiol 154:810–819

Curie C, Alonso JM, Le Jean M, Ecker JR, Briat JF (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. Biochem J 347:479–755

Douchkov D, Grycaka C, Stephan UW, Hell R, Bäumlein H (2005) Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. Plant Cell Environ 28:365–374

Du ST, Zhang YS, Lin XY, Wang Y, Tang CX (2008) Regulation of nitrate reductase by its partial nitric oxide in Chinese cabbage pakchoi (*Brassica chinensis* L. cv. *Baoda*). Plant Cell Environ 31: 195–204

Gao C, Wang Y, Xiao DS, Qiu CP, Han DG, Zhang XZ, Wu T, Han ZH (2011) Comparison of cadmium-induced iron-deficiency responses and genuine iron-deficiency responses in *Malus xiaojinensis*. Plant Sci 3:269–274

García MJ, Lucena C, Romera FJ, Alcántara E, Pérez-Vicente R (2010) Ethylene and nitric oxide involvement in the upregulation of key genes related to iron acquisition and homeostasis in *Arabidopsis*. J Exp Bot 61(14):3885–3899

García MJ, Suárez V, Romera FJ, Alcántara E, Pérez-Vicente R (2011) A new model involving ethylene, nitric oxide and Fe to explain the regulation of Fe acquisition genes in strategy I plants. Plant Physiol Biochem 49:537–544

Graziano M, Lamattina L (2007) Nitric oxide accumulation is required for molecular and physiological responses to iron deficiency in tomato roots. Plant J 52:949–960

Graziano M, Beligni MV, Lamattina L (2002) Nitric oxide improves internal iron availability in plants. Plant Physiol 130:1852–1859

Grusak MA, Pezeshgi S (1996) Shoot-to-root signal transmission regulates root Fe(III) reductase activity in the *dgl* mutant of pea. Plant Physiol 110:329–334

Han ZH, Wang Q, Shen T (1994a) Comparison of some physiological and biochemical characteristics between iron-efficient and iron-inefficient species in the genus *Malus*. J Plant Nutr 17:1257–1264

Han ZH, Shen T, Korcak RF, Baligar VC (1994b) Screening for iron-efficient species in the genus *Malus*. J Plant Nutr 17:579–592

Han ZH, Shen T, Korcak RF, Baligar VC (1998) Iron absorption by iron-efficient and -inefficient species of apples. J Plant Nutr 21:181–190

Han DG, Wang L, Wang Y, Yang GH, Gao C, Yu ZY, Li TY, Zhang XZ, Ma L, Xu XF, Han ZH (2013) Overexpression of *Malus xiaojinensis* CS1 gene in tobacco affects plant development and increases iron stress tolerance. Sci Hortic 150:65–72

Herbik A, Giritch A, Horstmann C, Becker R, Balzer HB, Stephan UW (1996) Iron and copper nutrition-dependent changes in protein

expression in a tomato wild type and the nicotianamine-free mutant chloronerva. Plant Physiol 111:533–540

Higuchi K, Watanabe S, Takahashi M, Kawasaki S, Nakanishi H, Nishizawa N, Mori S (2001) Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions. Plant J 25(2):159–167

Jin CW, Du ST, Shamsi IH, Luo BF, Lin XY (2011) NO synthase-generated NO acts downstream of auxin in regulating Fe-deficiency-induced root branching that enhances Fe-deficiency tolerance in tomato plants. J Exp Bot 62(11):3875–3884

Kabir AH, Paltridge NG, Adle AJ, Paull JG, Sangoulis JCR (2012) Natural variation for Fe-efficiency is associated with upregulation of strategy I mechanisms and enhanced citrate and ethylene synthesis in *Pisum sativum* L. Planta 235:1409–1419

Kobayashi T, Nishizawa NK (2012) Iron uptake, translocation, and regulation in higher plants. Annu Rev Plant Biol 63:16.1–16.22

Kürkcüoglu SK, Degenhardt J, Lensing J, Al-Masri AN, Gau AE (2007) I dentification of differentially expressed genes in *Malus domestica* after application of the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to the phyllosphere. J Exp Bot 58:733–741

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

Morgan PW, Hall WC (1962) Effect of 2,4-dichlorophenoxyacetic acid on the production of ethylene by cotton and grain sorghum. Physiol Plant 15:420–427

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5(7):621–628

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15:473–497

Ranieri A, Castagna A, Baldan B, Soldatini GF (2001) Iron deficiency differently affects peroxidase isoforms in sunflower. J Exp Bot 52: 25–35

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26(1):139–140

Romera FJ, Alcántara E (2004) Ethylene involvement in the regulation of Fe-deficiency stress responses by strategy I plants. Funct Plant Biol 31:315–328

Romera FJ, Alcántara E, De La Guardia MD (1999) Ethylene production by Fe-deficient roots and its involvement in the regulation of Fe-deficiency stress responses by strategy I plants. Ann Bot 83:51–55

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protocol 3:1101–1108

Séguéla 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

Sivitz A, Grinvalds C, Barberon M, Curie C, Vert G (2011) Proteasome-mediated turnover of the transcriptional activator FIT is required for plant iron-deficiency responses. Plant J 66:1044–1052

Swarup R, Parry G, Graham N, Allen T, Bennett M (2002) Auxin cross-talk: integration of signalling pathways to control plant development. Auxin Mol Biol 49:411

Tagliavini M, Rombolà AD (2001) Iron deficiency and chlorosis in orchard and vineyard ecosystems. Eur J Agron 15:71–92

Thimm O, Essigmann B, Kloska S, Altmann T, Buckhout TJ (2001) Response of *Arabidopsis* to iron deficiency stress as revealed by microarray analysis. Plant Physiol 127:1030–1043

Velasco R, Zharkikh A, Affourtit J et al (2010) The genome of the domesticated apple (*Malus*× *domestica* Borkh.). Nat Genet 42:833–839

Vert G, Briat JF, Curie C (2003) Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. Plant Physiol 132:796–804

Wang YH, David F, Garvin DF, Kochian LV (2002) Rapid induction of regulatory and transporter genes in response to phosphorus, potassium, and iron deficiencies in tomato roots. Evidence for cross talk and root/rhizosphere-mediated signals. Plant Physiol 130:1361–1370

Waters BM, Blevins DG (2000) Ethylene production, cluster root formation, and localization of iron(III) reducing capacity in Fe deficient squash roots. Plant Soil 225:21–31

Wu JJ, Wang C, Zheng LQ, Wang L, Chen YL, Whelan J, Shou HX (2010) Ethylene is involved in the regulation of iron homeostasis by regulating the expression of iron-acquisition-related genes in *Oryza sativa*. J Exp Bot 62:667–674

Wu T, Zhang HT, Wang Y, Jia WS, Xu XF, Zhang XZ, Han ZH (2011) Induction of root Fe(III) reductase activity and proton extrusion by iron deficiency is mediated by auxin-based systemic signalling in *Malus xiaojinensis*. J Exp Bot 63:859–870

Xiao HH, Yin LP, Xu XF, Li TZ, Han ZH (2008) The iron-regulated transporter, MbNRAMP1, isolated from *Malus baccata* is involved in Fe, Mn and Cd trafficking. Ann Bot 102:881–889

Xiong HC, Kobayashi T, Kakei Y, Senoura T, Nakazono M, Takahashi H, Nakanishi H, Shen HY, Duan PG, Guo XT, Nishizawa NK, Zuo YM (2012) *AhNRAMP1* iron transporter is involved in iron acquisition in peanut. J Exp Bot 63:4437–4446

Xu J, Wang W, Yin H, Liu X, Sun H, Mi Q (2010) Exogenous nitric oxide improves antioxidative capacity and reduces auxin degradation in roots of *Medicago truncatula* seedlings under cadmium stress. Plant Soil 326:321–330

Zhang YG, Cheng JH, Xu XF, Li TZ (2005) Comparison of methods for total RNA isolation from *Malus xiaojinensis* and cDNA LDPCR amplification. Biotechnol Bull 4:50–3

Zhang YG, Kong J, Wang Y, Xu XF, Liu LL, Li TZ, Zhu YJ, Han ZH (2009) Isolation and characterisation of a nicotianamine synthase gene *MxNas1* in *Malus xiaojinensis*. J Hortic Sci Biotechnol 84:47–52

Zhang Q, Wang Y, Zhang XZ, Yin LL, Wu T, Xu XF, Jia WS, Han ZH (2012) Cloning and characterization of *MxVHA-c*, a vacuolar H^+-ATPase subunit c gene related to Fe efficiency from *Malus xiaojinensis*. Plant Mol Biol Report 30:1149–1157