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Involvement of endogenous salicylic acid in iron-deficiency responses in Arabidopsis

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Abstract

Several phytohormones have been demonstrated to be involved in iron (Fe) homeostasis. We took advantage of a salicylic acid (SA) biosynthesis defective mutant phytoalexin deficient 4 (pad4: T-DNA Salk_089936) to explore the possible effects of endogenous SA on the morphological and physiological responses to Fe deprivation. The morphological and physiological analysis was carried out between Col-0 and the pad4 mutant. Under an Fe-deficiency treatment, Col-0 showed more severe leaf chlorosis and root growth inhibition compared with the pad4 mutant. The soluble Fe concentrations were significantly higher in pad4 than in Col-0 under the Fe-deficiency treatment. Fe deficiency significantly induced SA accumulation in Col-0 and the loss-of-function of PAD4 blocked this process. The requirement of endogenous SA accumulation for Fe-deficiency responses was confirmed using a series of SA biosynthetic mutants and transgenic lines. Furthermore, a comparative RNA sequencing analysis of the whole seedling transcriptomes between Col-0 and the pad4 mutant was also performed. Based on the transcriptome data, the expression levels of many auxin- and ethylene-response genes were altered in pad4 compared with Col-0. Fe deficiency increases SA contents which elevates auxin and ethylene signalling, thereby activating Fe translocation via the bHLH38/39-mediated transcriptional regulation of downstream Fe genes.

Key words: Fe deficiency, hormones, nutrients, RNA sequencing, salicylic acid, transcriptome analysis.

Introduction

Iron (Fe) is an essential micronutrient for both plants and human beings. In plants, Fe is a ubiquitous cofactor in a series of cellular enzymatic reactions and developmental processes such as photosynthesis, respiration, hormone biosynthesis, and morphogenesis (Guerinot, 2001; Hansch and Mendel, 2009). Despite its abundance, Fe is predominantly present in its insoluble oxidized Fe (III) compound form in alkaline soil and is, therefore, unavailable to plants (Kim and Guerinot, 2007). To improve Fe bioavailability in crops and human diets, it is critical to reveal the mechanisms underlying the Fe-deficiency responses in plants (Zuo et al., 2012).

Two major strategies, a reduction-based strategy (Strategy I) in dicotyledonous plants and non-graminaceous monocots, and a chelation-based strategy (Strategy II) specific to graminaceous monocots, have been evolved in higher plants to maximize iron uptake and utilization under Fe-deficient
conditions (Walker and Connolly, 2008). In the Strategy I plant, the acidification of the local rhizosphere is mainly regulated by H^+\text{-}ATPase-mediated proton extrusion which results in the solubilization of Fe (Santi and Schmidt, 2009). The next steps in the Fe-acquisition process are the ferric-chelate reductase (FCR) activity of FERRIC REDUCTASE OXIDASE2 (FRO2) and the uptake of Fe^{2+} by the metal transporter IRON REGULATED TRANSPORTER1 (IRT1) (Curie and Briat, 2003). Meanwhile, changes in root architecture, such as enhanced root branching and subapical root hair development, are also required for Fe efficient absorption (Jin et al., 2008; Santi and Schmidt, 2009).

Due to the unavailability of Fe in Fe-limited soils, plants have evolved a series of physiological and morphological responses to maintain Fe homeostasis (Shen et al., 2015). Two transcriptional regulatory mechanisms, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR (FIT) network and the POPEYE (PYE) network, were identified to modulate Fe deficiency responses in Arabidopsis (Ivanov et al., 2012). The iron content in the FIT knockout mutant is severely reduced and nearly half of the Fe deficiency-inducible genes are down-regulated in the Fe-deficient roots of the mutant (Colangelo and Guerinot, 2004). The expression levels of four lb subgroup bHLH genes, bHLH38, bHLH39, bHLH100, and bHLH101, are dramatically induced by Fe deficiencies and inhibited by Fe re-supplementation (Sivitz et al., 2012; Wang et al., 2013). Evidence suggests that FIT regulates the expression of its target genes by interacting with these four lb subgroup bHLHs (Wang et al., 2007; Yuan et al., 2008). The second regulatory network for Fe-deficiency responses is controlled by the PYE bHLH protein which is also induced in Fe-deficient roots. The pye-1 mutant shows Fe-deficiency-sensitive phenotypes and its direct target genes, including NICOTIANAMINE SYNTHASE4, FRO3, and ZINC-INDUCED FACILITATOR1, are up-regulated under Fe-deficiency conditions in pye-1 mutants (Long et al., 2010; Li et al., 2015). Similar to FIT, PYE can also interact with bHLH transcription factors, such as IAA-LEUCINE RESISTANT3, and regulates responses to iron deficiency and auxin conjugation (Rampey et al., 2006; Long et al., 2010).

It is becoming increasingly obvious that phytohormones and other small molecules such as nitric oxide (NO) play important roles in responses to Fe deficiency (Hindt and Guerinot, 2012). Several hormones, including auxin, ethylene, and NO, are involved in Fe-deficiency responses as positive regulators (Romera et al., 2011). Auxin has important roles in the morphological changes of the root system in response to Fe availabilities (Schmidt and Schikora, 2001). However, Fe deficiency leads to elevated auxin synthesis and a high auxin level enhances the expression of FIT and FRO2 (Chen et al., 2010; Wu et al., 2012). In Arabidopsis, the AUX1-mediated auxin distribution is required for Fe-deficiency-dependent lateral root elongation (Giehl et al., 2012a, b). In rice, several Fe-deficient symptoms can be partially restored in the osarf16 mutant (Shen et al., 2015). Moreover, the osarbcl4 mutant also shows a lack of sensitivity to Fe deficiency (Xu et al., 2014). These new findings indicate an important cross-talk between auxin and Fe homeostasis. Similar to auxin, ethylene is also over-produced by Fe deficiency (Chen et al., 2010; Garcia et al., 2010). In Strategy I plants, the transcriptional regulation of a series of Fe-acquisition genes is controlled by ethylene (Lucena et al., 2006; Garcia et al., 2010). Recent observation showed that ethylene signalling pathway transcription factors ETHYLENE INSENSITIVE 3 and ETHYLENE INSENSITIVE 3-LIKE 1 (EIN3/EIL1) could directly interact with FIT and may function to reduce the photo-oxidative damage in Fe-deficient conditions (Lingam et al., 2011). The small signalling molecule NO has been involved in a huge array of physiological responses related to abiotic and biotic stresses, including the Fe-deficiency response (Wendehenne et al., 2004; Graziano and Lamattina, 2005). In maize, the severe symptoms of chlorosis in 15-d-old iron-deficient plants could be partially restored by the application of NO (Graziano et al., 2002). In tomato, Fe deficiency causes NO accumulation which suggested that NO production is an early response of roots to iron deprivation (Graziano and Lamattina, 2007). Recently, NO has been implicated in FIT protein stabilization. Blocking NO signalling causes a decrease in FIT protein levels and FIT activity (Meiser et al., 2011). Abscisic acid (ABA) is another important phytohormone that regulates senescence and stress responses in plants (Jaradat et al., 2013). Exogenous ABA treatment alleviated the chlorosis caused by Fe-deficiency by promoting the transport and redistribution of Fe between the roots and shoots (Lei et al., 2014). A bHLH transcription factor, CmbHLH1, has been cloned in chrysanthemum. CmbHLH1 enhanced Fe uptake by elevating H^+\text{-}ATPase-mediated acidification of the rhizosphere. The expression of CmbHLH1 was inducible through exogenous ABA application, suggesting that ABA may be positively involved in the process (Zhao et al., 2014).

Three other hormones, cytokinin (CK), jasmonate, and brassinosteroids (BRs), act as negative regulators of the Fe-deficiency responses (Hindt and Guerinot, 2012; Wang et al., 2012). The expressions of several Fe-acquisition-related genes, such as IRT1, FRO2, and FIT, are inhibited by both exogenous CK and methyl-jasmonate treatments (Seguela et al., 2008; Maurer et al., 2011). It is important to note that the negative regulator of Fe uptake, jasmonate, may be a potential antagonist of ethylene and NO signalling (Orozco-Cardenas and Ryan, 2002; Adams and Turner, 2010). BRs, which are steroid hormones, modulate numerous physiological processes in plants. In rice, a new study highlights the vital role of BRs in regulating the responses to Fe deficiency: a BR biosynthesis defective mutant, D2-I, displayed a greater tolerance compared with the wild type (Wang et al., 2015). Clearly, the Fe-deficiency response is regulated by a series of complex interactions among several different phytohormones (Hindt and Guerinot, 2012).

SA is an essential hormone in plant immunity and defence responses (Du et al., 2009). The lipase-like protein PAD4 is well known to function together with its interacting partner protein EDS1 to promote SA biosynthesis and signalling (Zhou et al., 1998; Jirage et al., 1999). The effects of aspirin (acetylsalicylic acid) on the expression of iron transport and storage genes have been reported in BV-2 microglial cells.
In plants, the expression levels of \textit{bHLH38} and \textit{bHLH39}, two important Fe deficiency regulators, are dramatically induced after an application of SA by the SA-inducible Dof transcription factor (Kang \textit{et al.}, 2003). It indicated that there is a link between SA and the up-regulation of the Fe-deficiency response (Maurer \textit{et al.}, 2014). However, no genetic evidence is available on the involvement of SA in plant nutrient-deficiency responses. In the present study, we investigated the role of SA in the response to Fe deficiency and explored the possible relationship between SA and Fe homeostasis.

**Materials and methods**

**Plant materials and growth conditions**

\textit{A. thaliana Col-0} (Columbia ecotype), \textit{phytoalexin deficient 4} (pad4) (T-DNA insert line: Salk\_089936), \textit{enhanced disease susceptibility 1} (eds1) (T-DNA insert line: Salk\_017710C), and \textit{isochorismate synthase 1} (ics1) (T-DNA insert line: SALK\_088254), were used in the present study. All the seeds were from ABRC. Seeds were surface-sterilized and germinated on plates containing 0.75\% agar-solidified Murashige and Skoog (MS) nutrient medium, adjusted to pH 5.8 with 1M NaOH. The seedlings were grown in an environmentally controlled growth chamber at 24 °C, a light intensity of 140 \textmu mol photons m\textsuperscript{-2} s\textsuperscript{-1} and a 16/8 h day/night rhythm. The seedlings were then subjected to two treatments, +Fe (the complete nutrient solid medium as a control) and –Fe (the complete nutrient solid medium, but without Fe), for 7 d.

**Gene cloning, plasmid construction, and transgenic plants**

\textit{PAD4} gene-specific primers (5′-GGTACCATGGACGATTGTCGATTCC-3′; 5′-GGATCCCTAAGTCTCCATTGCGTC-3′) were used to amplify (LA-Taq polymerase; Takara, Dalian, China) the full-length cDNA using RT-PCR which was subsequently cloned into the pDL28-Flag vector to create 35S::PAD4. Total RNA was extracted from leaves using RNAeasy plant mini kits (Qiagen) according to the manufacturer’s protocol. RNA quality was assessed by A260–A280 ratios (>2.0) using an ND-1000 UV Nanodrop spectrophotometer (Thermo Scientific, Shanghai, China) and by RNA integrity (>7.0) determined with a Bioanalyzer (Agilent, Santa Clara, CA, United States). For total RNA, DNA contamination was removed by DNase I. The qRT-PCR experiment was performed as described previously by Yang \textit{et al.} (2015). Each cDNA sample was performed in triplicate and data were normalized to the expression level of \textit{ACTIN} (AT1G01750).

**GUS staining**

GUS staining of the seedlings was carried out using a 100 \textmu M sodium phosphate buffer (pH 7.0) containing 0.1\% v/v Triton X-100 and 2 \textmu M X-Gluc (Biobasic Inc., Sangon, Shanghai, China) at 37 °C overnight. The tissues were observed using a Carl Zeiss LSM510 laser scanning system (http://www.zeiss.com/).

**Transcriptome analysis**

Total RNA from each shoot sample of Arabidopsis was obtained from seedlings using the total RNA purification kit (LC Sciences, Houston, TX, USA) and was further purified by TruSeq RNA LT Sample Prep Kit v2 (Illumina, CA, USA) according to the manufacturer’s protocol. Oligo-dT was used to yield poly(A+) mRNA from a total RNA pool consisting of equal quantities of total RNA from four sample types: +Fe/WT, +Fe/pad4, -Fe/WT, and -Fe/pad4. The purified mRNAs were fragmented using divalent cations under elevated temperatures and were then converted to dsDNA by two rounds of cDNA synthesis using reverse transcriptase and DNA polymerase I. After an end repair step, most dsDNA fragments were ligated with adapter oligos (Pruit \textit{et al.}, 2007). These cDNA libraries were sequenced using an Illumina 2000/2500 sequence platform at the LC Sciences Company (Hangzhou, China).

Based on the Illumina paired-end RNA-seq approach, we sequenced the transcriptomes of \textit{Col} and \textit{pad4} mutant shoots under +Fe and –Fe conditions, respectively. Prior to assembly, the low quality reads, such as reads containing sequences adaptors, reads containing sequencing primer and nucleotide with a quality score lower than 20, were eliminated. The raw sequence data have been submitted to the NCBI Short Read Archive with accession number GSE74515.
Differential expression analysis

The samples from each type were harvested in three different batches. The expression abundance of each assembled transcript was determined though reads per kilobase per million mapped reads (RPKM) values. All of the reads were mapped onto the non-redundant set of transcripts to calculate the abundance of assembled transcripts. The expressions of each gene between sample pairs (+Fe/WT versus +Fe/pad4, –Fe/WT versus –Fe/pad4, +Fe/WT versus –Fe/WT, and +Fe/pad4 versus –Fe/pad4) were measured using the numbers of reads having a specific match. Among the four samples, a minimum of a 2-fold difference in log2 expression was considered as a significant difference.

Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

GO enrichment analysis of differential expressed genes (DEGs) was performed using GOseq R packages based on Wallenius non-central hyper-geometric distribution. Based on the DEG analysis results, the significantly enriched GO terms for each cluster were filtered using hyper geometric test with a P-value of ≤0.01. A KEGG database resource (http://www.genome.jp/kegg/) was used for exploring high-level functions and utilities of the biological system, especially large-scale molecular datasets. In our experiment, the statistical enrichment of DEGs in the KEGG pathway was calculated by KOBAS 2.0 software (http://kobas.cbi.pku.edu.cn/download.do).

Results

Morphological and physiological evidence associated with the involvement of PAD4 in Fe-deficiency responses in Arabidopsis seedlings

To explore the possible effects of endogenous SA on the morphological and physiological responses to Fe deprivation, a pad4 mutant, which lacks a key player in SA biosynthesis, was used in our study (Vogelmünn et al., 2012). The seedlings of both Col-0 and pad4 were grown on under +Fe and –Fe treatments for one week. The results showed that pad4 was less sensitive to Fe deficiency compared with Col-0 (Fig. 1a, b). The pad4 mutant seedlings grew as well as Col-0 in the +Fe nutrient medium, but the –Fe treatment caused more severe leaf chlorosis and root growth inhibition in Col-0 than in the pad4 mutant. The chlorophyll contents in Col-0 and pad4 were similar under the +Fe treatment; however, the changes in the chlorophyll contents under Fe-deficiency conditions in pad4 were different from Col-0. The total chlorophyll contents in pad4 was 2-fold higher than in Col-0 under the –Fe treatment (Fig. 1c). After 7 d of –Fe treatment, the average biomass of Col-0 seedlings fell significantly from 0.023 g Fresh Weight per 8 Plants (FW/P) to 0.011 g FW/P. Compared with the +Fe treatment, the average biomass of pad4 only changed from 0.024 g FW/P to 0.017 g FW/P (Fig. 1d). There were no significant changes in the root lengths of Col-0 and pad4 under both the +Fe and –Fe treatments (Fig. 1e).

FCR activation and proton extrusion are the classical responses of Fe deficiency in Arabidopsis (Robinson et al., 1999; Curie and Briat, 2003). The FCR activities in roots and the pH values of the root bathing solution were measured to compare the differences between pad4 and Col-0. It was noted that pad4 also induced FCR activity and proton release but the induction level was lower compared with Col-0 (Fig. 2a). The release of protons was also decreased in pad4 compared with Col-0 under the –Fe treatment (Fig. 2b). There were no significant changes in FCR activity and proton extrusion under the +Fe treatment in both Col-0 and pad4.

PAD4 regulates soluble Fe concentrations under Fe-deficiency conditions

Fe is the most important metal ion associated with chlorophyll biosynthesis and soluble Fe deficiency is the major cause of leaf chlorosis (Zheng et al., 2009). Therefore, we measured the soluble Fe contents in Col-0 and the pad4 mutant under +Fe and –Fe treatments. The data showed that the soluble Fe concentrations in the shoots and roots of pad4 were the same as in Col-0 under +Fe conditions. However, the soluble Fe concentrations were significantly higher in the shoots and roots of pad4 than in Col-0 under the –Fe treatment which is in accordance with the phenotype observed (Fig. 2c, d). It is suggested that mutation in the PAD4 gene may alleviate the Fe-deficiency-induced symptoms by increasing the soluble Fe concentrations in the shoots and roots.

Major Fe pools exist in root apoplasts in plants (Jin et al., 2007b; Lei et al., 2014). The concentration of apoplastic Fe in the Col-0 roots was two times higher than that in the pad4 roots under the –Fe treatment (Fig. 2e). The increase in the soluble Fe may be due to the enhanced reutilization of Fe stored in the apoplastic pools. We also measured the total Fe contents in Col-0 and the pad4 mutant under +Fe and –Fe treatments but there were no significant differences in total Fe content between Col-0 and the pad4 mutant (Fig. 2f, g).

Complementation experiments to confirm the involvement of PAD4 in the Fe-deficiency response

To verify the involvement of PAD4 in Fe-deficiency responses, we produced transgenic lines expressing the full-length cDNA of the PAD4 gene under the control of the 35S promoter (over-expression line: pad4/OX) or under the control of the PAD4 promoter (complementation line: pad4/C) using pad4 mutants. The expression level of the PAD4 gene in different plant materials is shown in Supplementary Fig. S1a at JXB online. The pad4/OX and pad4/C lines were tested along with Col-0 for their growth phenotypes. The phenotypes of Col-0, pad4/OX, and pad4/C were approximately the same under the +Fe and –Fe treatments (Supplementary Fig. S1b–e). We also measured the chlorophyll contents, biomass, and root lengths of these materials, and no significant differences were observed (Supplementary Fig. S1f–h). The results confirmed that the Fe-deficiency partial insensitive phenotype was caused by the loss-of-function of PAD4.

The expression of the PAD4 gene was induced by Fe deficiency

The GUS reporter gene was used to evaluate the PAD4 expression pattern in the shoots and roots under different
Involvement of SA in iron-deficiency responses

Under the +Fe treatment, *PAD4* was slightly expressed in the leaf veins and pericycles. The expression level of the *PAD4* gene was largely increased by the Fe-deficiency treatment (Fig. 3a–h). The GUS activity determinations confirmed these changes in the *PAD4* expression pattern (Fig. 3i).

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**Fig. 1.** Morphological evidence that *PAD4* is involved in Fe deficiency responses. (a) The seedlings of both Col-0 and *pad4* were grown on under the +Fe treatment for one week. (b) The seedlings of both Col-0 and *pad4* were grown on under the –Fe treatment for one week. The bars in (a) and (b) equal 1 cm. (c) The chlorophyll contents in Col-0 and *pad4* under +Fe and –Fe treatments. (d) The biomass of Col-0 and *pad4* under +Fe and –Fe treatments. (e) The root lengths of Col-0 and *pad4* under +Fe and –Fe treatments. Significant (*P*<0.05) differences between Col and the *pad4* mutant are indicated by an asterisk.

**Fig. 2.** Physiological evidence that *PAD4* is involved in Fe deficiency responses. (a) The activity of FCR in the roots of Col-0 and *pad4* under +Fe and –Fe treatments for one week. (b) The pH of root bathing solutions of Col-0 and *pad4* under +Fe and –Fe treatments for one week. (c) The soluble Fe contents in shoots of Col-0 and *pad4* under +Fe and –Fe treatments. (d) The soluble Fe contents in roots of Col-0 and *pad4* under +Fe and –Fe treatments. (e) The root apoplast Fe contents in Col-0 and *pad4* under +Fe and –Fe treatments. Significant (*P*<0.05) differences between Col and the *pad4* mutant are indicated by an asterisk.
Fe deficiency enhances SA accumulation in both the shoots and roots

In this study, we investigated how Fe deficiency affects endogenous SA biosynthesis and metabolism using the PRI promoter:GUS construct as an SA signalling marker (Beilmann et al., 1992). Under +Fe conditions, the PRI gene was only expressed in leaf veins and leaf tips. After 7 d of the –Fe treatment, expression of the PRI gene had spread to the entire leaf and was strongly induced in the root pericycle in Col-0. However, the expression pattern of the PRI gene was different in pad4 compared with Col-0. The expression of the PRI gene was only slightly induced in the leaf tips in pad4 under the –Fe treatment. No transcript abundance of the PRI gene was detectable in the roots of pad4 (Fig. 4a–d).

Furthermore, we quantified the SA contents in Col-0 and the pad4 mutant under different Fe conditions. Free SA levels were approximate 5-fold and 9-fold higher under –Fe treatments in the shoots and roots of Col-0 than the pad4 mutant, respectively. The data showed that the Fe deficiency significantly induced SA accumulation in the roots and shoots of Col-0, and the loss-of-function of PAD4 slightly blocked this process (Fig. 4e, f).

To examine whether SA accumulation is required in the Fe-deficiency responses, we used two other mutants, ics1 and eds1, and an NahG transgenic line, all of which are defective in SA biosynthesis or signalling (van Wees and Glazebrook, 2003; Morse et al., 2007). We confirmed that all of these mutants and transgenic line were less sensitive to Fe deficiency compared with Col-0 (Fig. 5a, b). The chlorophyll contents (Fig. 5c) and average biomass (Fig. 5d) had decreased more severely in Col-0 than in the eds1 and ics1 mutants and in the NahG lines under the –Fe treatment. We also determined the SA contents in these mutants and the transgenic line. No significant induction of free SA was detected in either the ics1 and eds1 mutants or in the NahG transgenic line in both roots and shoots (Fig. 5e, f). Furthermore, the content of soluble Fe in the eds1 and ics1 mutants and the NahG line was much higher in both roots and shoots under the –Fe treatment than in Col-0. Meanwhile, the root apoplastic Fe contents of the eds1 and ics1 mutants and the NahG line were much lower in both the roots and shoots under the –Fe treatment than in Col-0. There were no significant differences in total Fe contents between Col-0 and the mutants and the transgenic line (Supplementary Fig. S2).

RNA sequencing analysis of seedling transcriptomes under different Fe conditions

To elucidate the molecular and metabolic mechanisms by which SA affects plants’ responses to Fe deficiency, we performed a comparative RNA sequencing analysis of the whole seedling transcriptomes. Complementary DNA libraries were constructed using total RNA extracted from the 7-d-old seedlings of Col-0 and the pad4 mutant growing under +Fe or –Fe treatment conditions. The pad4 mutant was used as an SA deletion mutant to investigate the effects of endogenous SA on the Fe-deficiency responses (Zhou et al., 1998). For each combination of genotype and Fe condition, there were three independent biological replicates. Using an Illumina Hiseq2000 sequencer, 552,900,240 valid data reads were obtained from the 12 cDNA libraries. The raw data of Illumina reads are available at the National Center for Biotechnology Information Sequence Read Archive browser (http://ncbi.nlm.nih.gov/sra; accession no. SRP74515). For each sample, ~85% of reads could be mapped to The Arabidopsis Information Resource (TAIR) reference genome TAIR10 and most of them aligned with unique genes without ambiguity. The global comparisons of the gene expression profiles of Col-0 and pad4 under different Fe conditions are shown in Supplementary Fig. S3. The differences in the expression levels between samples are log2-transformed ratios.

We first evaluated the differences in the transcriptomes of Col-0 seedlings between +Fe and –Fe treatments. Six well-known Fe-starvation-induced marker genes, including
| bHLH38, bHLH39, FRO2, IRT1, NAS4, and MYB72 were used in this study (Zhang et al., 2015). The induction of these six Fe-deficiency-induced marker genes in the –Fe Col-0 samples indicated that the treatment conditions for the RNA-seq experiments were appropriate and the data were trustworthy (Supplementary Table S1). We then compared the transcriptomes of the –Fe treatments to the transcriptomes of the +Fe treatments. In Col-0 seedlings, the expression was induced for 2,022 genes and was repressed for 2,323 genes under Fe-deficient conditions. In the pad4 seedlings, the expression was induced for 2,261 genes and was repressed for 2,405 genes by Fe-deficient conditions. Among these genes, 1,231 genes were up-regulated and 1,435 genes were down-regulated in both Col-0 and pad4 (Fig. 6a; Supplementary Table S2).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and Gene Ontology (GO) functional annotation of the differentially expressed genes

Under the –Fe treatment, the phenotype of pad4 is largely different compared with that of Col-0. Therefore, we then clustered the transcriptomes of Col-0 and the pad4 mutant under the –Fe treatment using the Hierarchical Clustering method. The differentially expressed transcripts between Col-0 and pad4 under the –Fe treatment were classed into five classes (from 1 to 5) (Fig. 6b). Within these five classes, genes related to several KEGG pathways were particularly enriched. In total, 11 KEGG pathways showed significant differences between Col-0 and the pad4 mutant (P value <0.01) (Fig. 6c). The first KEGG (ko00400) contained genes that are related to the 3-dehydroquinate synthase pathway. Among the 33 genes, 11 genes showed significant differences between Col-0 and the pad4 mutant (P value <0.01) (Supplementary Fig. S4 and Table S3). The second KEGG (ko04075) contained 239 serine/threonine-protein kinase SRK2 pathway-related genes. Among these genes, 42 genes showed significant expression differences between Col-0 and the pad4 mutant (P value <0.01) (Supplementary Fig. S5 and Table S4). The third KEGG (ko05204) contained 18 genes and seven of these S-(hydroxymethyl) glutathione dehydrogenase pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S6 and Table S5). The fourth KEGG (ko00196) contained 19 genes and seven of these light-harvesting complex I chlorophyll a/b binding protein 2 pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S7 and Table S6). The fifth KEGG (ko00941) contained 15 genes and six of these flavonol synthase pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S8 and Table S7). The sixth KEGG (ko00860) contained 31 genes and nine of these oxygen-independent coproporphyrinogen III oxidase pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S9 and Table S8). The seventh KEGG (ko04712) contained 21 genes and seven of these pseudo-response regulator 3 pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S10 and Table S9). The eighth KEGG (ko00270) contained 49 genes and 12 of these 1-aminocyclopropane-1-carboxylate synthase pathway-related genes showed significant expression differences (P value <0.01) (Supplementary Fig. S11 and Table S10). The

Fig. 4. Fe deficiency enhances SA accumulation in both the shoots and roots. The PR1 promoter:GUS was used as an SA signalling marker. (a) The expression of the PR1 gene in the shoots of Col-0 under different Fe conditions. (b) The expression of the PR1 gene in the roots of Col-0 under different Fe conditions. (c) The expression of the PR1 gene in the shoots of pad4 under different Fe conditions. (d) The expression of the PR1 gene in the roots of pad4 under different Fe conditions. (e) The free SA contents in the shoots of Col-0 and pad4. (f) The free SA contents in the roots of Col-0 and pad4. Significant (P <0.05) differences between Col and the pad4 mutant are indicated by an asterisk.
Shen et al.

The ninth KEGG (ko00380) contained 12 genes and five of these indole-3-pyruvate monooxygenase pathway-related genes showed significant expression differences ($P$ value <0.01) (Supplementary Fig.S12 and Table S11). The tenth KEGG (ko00940) contained 102 genes and 20 of these peroxidase pathway-related genes showed significant expression differences ($P$ value <0.01) (Supplementary Fig.S13 and Table S12). The eleventh KEGG (ko00592) contained 23 genes and 7 of these hydroperoxide dehydratase pathway-related genes showed significant expression differences ($P$ value <0.01) (Supplementary Fig.S14 and Table S13).

Upon carrying out an overrepresentation analysis of GO functional categories, the differentially expressed transcripts between Col-0 and pad4 under the –Fe treatment were found to be enriched in environmental stress-response functions (Supplementary Table S14). In total, 16 of the top 20 GOs which showed the most significant differences ($P <0.00001$) in expression change between Col-0 and pad4 are involved in the environmental stress response (Fig. 7). Thus, overall, the loss-of-function in pad4 results in an alteration of the expression pattern for genes that participated in stress responses. In these significant differential GOs, 468 genes were up-regulated and 542 genes were down-regulated in the pad4 mutant compared with Col-0 (Supplementary Table S15).

To examine further the role of endogenous SA in Fe-deficiency responses, the expression of several hormone responses and photosynthesis-related genes was analysed. Several phytohormones have been reported to be involved in Fe-deficiency responses (Garcia et al., 2011; Maurer et al., 2011; Lei et al., 2014). Based on the GO analysis, GO:0009737 (response to abscisic acid), GO:0009753 [response to jasmonic acid (JA)], and GO:0009723 (response to ethylene) showed significant differences between pad4 and Col-0 ($P <0.001$) (Supplementary Tables S16–S18). Furthermore, two photosynthesis-related GOs (GO:0009507 and GO:0015979) are shown in Supplementary Tables S19 and S20. Compared with Col-0, 24 photosynthesis-related genes were up-regulated and only two genes were down-regulated in pad4 under Fe deficiency condition. The data suggest that phytohormone responses and photosynthesis-related genes are regulated by the SA level under Fe-deficiency conditions.

Furthermore, a complex interplay of hormone pathways has been analysed using transcriptome data. GO functional
Involvement of SA in iron-deficiency responses

The expression of genes responding to Fe deficiency

To examine the effect of *PAD4* deletion on the regulation of genes related to Fe deficiency, a series of representative genes were selected from our transcriptome data for analysis in both Col-0 and the pad4 mutant under the +Fe and –Fe treatments. The expression of *bHLH104* and *ILR3* did not show significant induction by the –Fe treatment, while *bHLH34* and *bHLH115* showed a 1.5-fold induction upon the –Fe treatment in both Col-0 and the pad4 mutant. The data indicated that the level of *PAD4* did not influence the expression of the IVc subgroup *bHLH* genes. The expression of *IRT1* and *IRT2* (Ivanov et al., 2014), were also found to be inhibited in pad4 compared with Col-0 under the –Fe treatment. In addition, the expression of Fe translocation-related genes, including *YSL1*, *NAS4*, *ZIF1*, and *FRD3* (Green and Rogers, 2004; Haydon and Cobbett, 2007; Klatte et al., 2009), showed no significant differences between Col-0 and the pad4 mutant under both Fe-sufficient and Fe-insufficient conditions. In the selected Fe homeostasis regulation-related genes, *MTB10* was down-regulated in the pad4 mutant under the –Fe treatment (Supplementary Table S21).

Discussion

Plants have evolved an innate immune system that affords protection against infection by potential pathogens in challenging environments (Du et al., 2009; Carstens et al., 2014).
SA signalling is a major branch of the defence networks in various eukaryotes (Jones and Dangl, 2006). In Arabidopsis, PAD4 and its interacting protein EDS1, both of which show homology to acyl lipases, are key signalling components in the SA-signalling pathway (Feys et al., 2001). However, how SA signalling is involved in mineral nutrition deficiency responses is largely unknown.

It is becoming increasingly explicit that a complicated regulatory network is involved in modulating Fe homeostasis and deficiency responses (Zhang et al., 2015). Here, we find that PAD4 plays an integral role in the Fe-deficiency response. Fe deficiency leads to a severe reduction in biomass, a decrease in the chlorophyll content, and an inhibition of root elongation (Zheng et al., 2009; Shen et al., 2015). Under Fe-deficient conditions, pad4 mutant seedlings grew much better than the Col-0 seedlings and the chlorotic symptoms shown by the pad4 seedlings were also alleviated (Fig. 1). These results indicated that the mutation of PAD4 had a global impact on the plant responses to Fe deficiency. Generally, in Strategy I plants, Fe-deficiency responsive FCR is mainly localized in the root apex and subapical root regions (Römheld and Marschner, 1981). Hormones and other small molecules, such as auxin, NO, ABA, and ethylene, have been implicated in regulating the induction of FCR activity in root system responses to Fe deficiency (Chen et al., 2010; Jin et al., 2011; Lei et al., 2014). The pad4 mutant confers significantly decreased levels of FCR activity after 7 d of the –Fe treatment (Fig. 2a), implying that a certain level of SA signalling is necessary for the Fe-deficiency-based induction of root FCR activity.

Reutilization of Fe stored in the roots is a strategy used by plants to survive in Fe-limiting soils. Therefore, the Fe content in the root is always much higher than in the shoot (Lei et al., 2014). Under Fe-deficiency conditions, root apoplastic Fe can be partially reutilized to alleviate chlorosis (Jin et al., 2007a). Our data showed that the soluble Fe contents in pad4 are higher than in Col-0 both in the roots and shoots under the –Fe treatment. However, root apoplastic Fe content in pad4 is lower than in Col-0, indicating that pad4 promotes the reutilization of root apoplastic Fe. Changes in Fe availability could enhance signals to regulate the expression of genes involved in Fe transport and acquisition (Thimm et al., 2001; Zhang et al., 2015). Based on the expression analysis of Fe-deficiency-related genes, it appears that the PAD4-controlled SA content and signalling pathway was partially involved in the transcriptional regulatory network of the Fe-deficiency response. On the other hand, accumulating evidence has found that aspirin-induced iron loss might be involved in an anticancer mechanism (Mascitelli et al., 2010). SA co-ordination with Fe is well established in human cells (Xu et al., 2015). An application of aspirin in a spectrophotometric determination of trace Fe levels also indicated the co-ordination between SA and Fe (Andres et al., 1987). The increased soluble Fe content observed in the pad4 mutant might be a result of both the altered movement of Fe–SA coordinates and SA signalling.

A series of SA-deficient mutants and the transgenic line were used to confirm the roles of SA in Fe deficiency (van Wees and Glazebrook, 2003; Morse et al., 2007). All of these SA-deficient mutants and the transgenic line display
Involvement of SA in iron-deficiency responses

In Arabidopsis, endogenous SA promotes soluble Fe content by mobilizing Fe from the root apoplast, thereby alleviating the Fe-deficiency-induced chlorosis. Fe is so important for plants. However, PAD4 is well known to function, together with its interacting partner protein EDS1, to promote plant immunity and defence responses (Du et al., 2009). In natural environments, any reduction in the plant immune system is extremely dangerous, so evolution has not removed the functional PAD4 from plants.

We were particularly interested in how the endogenous SA affected metabolism and expression regulation under Fe deficiency. Data showed that, under Fe-deficient conditions, the mutation of the PAD4 gene caused many genes associated with different biological processes to be up- or down-regulated. Some of these genes are directly involved in the maintenance of chloroplasts and in photosynthesis (GO:0009507 and GO:0015979). The altered expression of these genes, therefore, is consistent with the observed phenotypic differences in chlorosis between pad4 and Col-0 under Fe deficiency. The evident changes in expression of a series of photosynthesis-related genes (24 genes were up-regulated and 2 genes were down-regulated) in pad4 seems especially significant (Supplementary Table S3). The induction of photosynthetic gene expression in pad4 under Fe-deficient conditions suggests an activation of Fe-dependent photosynthesis (Rodriguez-Celma et al., 2013; Shen et al., 2015). This is in agreement with the higher accumulation of soluble Fe which is required for chlorophyll biosynthesis in pad4 than in Col-0 under Fe-deficient conditions (Zheng et al., 2009).

Interestingly, the expression of many hormone-related genes showed significant differences between –Fe and +Fe conditions.

**Fig. 8.** Analysis of the complex interplay of the hormone pathways in the Fe-deficiency response in Arabidopsis. (a) The expression changes of various hormone response genes in pad4 compared with Col-0 under +Fe conditions. (b) The numbers of up- or down-regulated hormone response genes in the Col-0/pad4 comparison. (c) Significant P values of hormone-related GOs between –Fe and +Fe treatments in both Col-0 and pad4. (d) Schematic model of the involvement of SA in the hormone regulatory hierarchy of Fe deficiency responses.
treatments in both Col-0 and the pad4 mutant (Fig. 8c). The expression of ethylene-response (GO:0009723), ethylene-binding (GO:0051740), and ethylene-activated signalling pathway genes (GO:0009873) was found to be significantly changed by –Fe treatment in Col-0 ($P<0.01$). However, no significant differences in the expression of ethylene-response genes were observed between +Fe and –Fe treatments in the pad4 mutant (Fig. 8c). It suggested that the responses of many ethylene-related genes to Fe deficiency may depend on the full function of the PAD4 gene. Recently, a direct molecular link between ethylene signalling and the basic helix-loop-helix protein FIT was revealed. Ethylene, through the activation of the FIT, bHLH38 and bHLH39, regulates the expression of several Fe genes (Lucena et al., 2015). Two ethylene-regulated transcription factors, EIN3 and EIL1, were identified as protein interaction partners of the Fe-deficiency response regulator FIT (Lingam et al., 2011). Earlier evidence indicated that SALICYLIC ACID INDUCTION DEFICIENT2, which encodes the isochorismate synthase required for SA biosynthesis, is a downstream target of EIN3 and EIL1 (Chen et al., 2009). Compared with the WT, the expression of several photo-oxidative stress-response genes were largely changed in the ein3eil1 double mutant. Ethylene signalling might contribute to declining oxidative stress in response to Fe deficiency (Bauer and Blondet, 2011). In the pad4 mutant, the expression of oxidative stress-response genes was significantly changed ($P=1.8E^{-07}$) (Supplementary Table S5). SA, together with ethylene, may trigger Fe-deficiency responses by affecting the oxidation reaction at the transcriptional level.

Since previous studies have indicated that auxin plays a critical role in the regulation of Fe responses (Chen et al., 2010; Bacaicoa et al., 2011), it is necessary to clarify whether SA has an association with auxin in the regulation of the Fe-deficiency responses. In our study, the expression of auxin-related genes was also found to be significantly changed by the –Fe treatment in Col-0 ($P<0.01$) rather than the pad4 mutant (Fig. 8c). Under –Fe conditions, PAD4 is involved in the expression regulation of a large number of auxin-related genes, including auxin response (GO:0009733), auxin signalling pathway (GO:0009734), auxin homeostasis (GO:0010252), and auxin transport (GO:0010540) genes, suggesting a close relationship between SA and the auxin signalling pathway in the regulation of the Fe-deficiency response.

Furthermore, the expression of two subgroups of lb bHLH genes, bHLH38 and bHLH39 (Yuan et al., 2008), was analysed under different conditions. The expression of bHLH38 and bHLH39 was induced by –Fe treatments, but the levels of increase were much lower in pad4 than those in the Col-0 seedlings (Fig. 9; Supplementary Table S21). By combining the results of this study and previous studies, we propose the following model: Fe deficiency increases SA in seedlings, which elevates auxin and ethylene signalling and the subsequent induction of NO accumulation, thereby activating reduction-based Fe uptake via the bHLH38/39-mediated transcriptional regulation of downstream Fe genes (Fig. 8d).

In summary, through molecular and genetic studies combined with an RNA-seq analysis, we have uncovered a new function of SA in Fe-deficiency responses. In Arabidopsis, Fe deficiency enhances SA accumulation, and an elevated endogenous SA level is related to the Fe-deficiency response. Soluble Fe contents are induced in the pad4 mutant which alleviates the symptoms of chlorosis under the Fe-deficient condition. Based on the RNA-seq analysis, the expression of many hormone-response genes was changed in pad4 compared with Col-0. Hence, Fe acquisition might be under the control of a complex interplay of SA-auxin/ethylene signalling pathways.

### Supplementary data

Supplementary data can be found at JXB online.

Table S1. The induction of six Fe-deficiency-induced marker genes (RPKM) in the –Fe samples.

Table S2. The expression changes in both Col-0 and the pad4 mutant by Fe deficiency treatment.

Table S3. The expression changes of the KEGG (ko00400) genes in both Col-0 and the pad4 mutant.

Table S4. The expression changes of the KEGG (ko04075) genes in both Col-0 and the pad4 mutant.

Table S5. The expression changes of the KEGG (ko05204) genes in both Col-0 and the pad4 mutant.

Table S6. The expression changes of the KEGG (ko00196) genes in both Col-0 and the pad4 mutant.

Table S7. The expression changes of the KEGG (ko00941) genes in both Col-0 and the pad4 mutant.
Involvement of SA in iron-deficiency responses

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