J. Plant Biol. (2015) 58:391-401
DOI 10.1007/s12374-015-0379-z

ORIGINAL ARTICLE

Auxin Signaling is Involved in Iron Deficiency-induced Photosynthetic Inhibition and Shoot Growth Defect in Rice (*Oryza sativa* L.)

Kaidong Liu[1,†,*], Runqing Yue[2,†], Changchun Yuan[1], Jinxiang Liu[1], Lei Zhang[3], Tao Sun[4], Yanjun Yang[4], Shuanggui Tie[2] and Chenjia Shen[4,*]

[1]*Life Science and Technology School, Lingnan Normal University, Zhanjiang, PR China*
[2]*Henan Academy of Agricultural Sciences, Zhengzhou, PR China*
[3]*Department of Plant Pathology, Washington State University, Pullman, WA, USA*
[4]*College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, PR China*

Received: August 4, 2015 / Accepted: October 1, 2015

Abstract Iron deficiency is one of the most serious nutrient limiting factors that affect rice plant growth and photosynthesis. Several phytohormones, including auxin, participate in iron uptake and homeostasis. However, how auxin signaling is involved in iron deficiency-induced inhibition of shoot growth and photosynthetic efficiency is largely unknown. The Nipponbare (NIP) seedlings displayed typical chlorotic symptoms, biomass reduction and photosynthesis depression when subjected to iron deficiency. We measured the soluble Fe content in the shoots under different conditions. The soluble Fe content in the shoots under Fe deficiency was increased by 1-naphthoxyaceticacids (1-NOA) treatment and was decreased by 1-naphthaleneacetic acid (NAA) treatment. Blocking (1-NOA treatment) or enhancement (NAA treatment) of auxin signaling also affects photosynthetic parameters under Fe deficiency conditions. Furthermore, rice microarray data (GSE17245 and GSE39429) were used to analyze the relationship between iron deficiency responses and auxin signaling in shoots. Most iron deficiency response gene expression levels in the shoots increased under exogenous auxin treatment, and most auxin early response gene expression levels responded to iron deficiency. It suggested that there is a crosstalk between iron deficiency signaling and auxin signaling. Our results indicated that iron deciency-induced growth inhibition and photosynthesis depression were mediated by systemic auxin signaling.

Keywords: Auxin, Auxin-response *cis*-elements, Auxin signaling, Iron deficiency, Rice

[†]These authors contributed equally to this work

*Corresponding author; Kaidong Liu, Chenjia Shen
Tel : +86-759-3183086, +86-571-28865198
E-mail : liukaidong2001@126.com, shencj@hznu.edu.cn

Introduction

Iron (Fe) is an essential micronutrient for plant growth, metabolism and photosynthesis. Because of its low availability, iron deficiency is a widespread dietary problem affecting more than half of the world's population (Lee et al. 2009). Rice is an important crop in Asia. Rice plant often suffers from Fe deficiency and exhibits Fe-deficiency symptoms, such as chlorosis caused by chlorophyll synthesis deficiency, which leads to reduced yield and quality (Bashir et al. 2011). Thus, to elucidate the mechanisms underlying how plants sense and respond to Fe deficiency helps us to increase crop plant yields in an Fe-limited agricultural environment (Hindt and Guerinot 2012).

When plants are grown in an iron limited soil/environment, they develop several physiological and morphological responses to boost Fe uptake and homeostasis so that they can supply critical cellular processes (Grotz and Guerinot 2006; Kim and Guerinot 2007). In particular, Fe plays an important role in maintaining the structure and function of the photosynthetic electron transfer chain (Eberhard et al. 2008). Three major complexes of the photosynthetic apparatus: the light-harvesting photosystem I (PSI), photosystem II (PSII) and cytochrome *b6f,* have been reported to be Fe-containing protein complexes (Briat et al. 2014). Iron translocation, storage and utilization enhancement lead to large increases in the chlorophyll concentration and maximum quantum yield of photosynthesis in phytoplankton (Hiscock et al. 2008). Several photosynthetic parameters are also altered by Fe deficiency (Sarvari et al. 2010). The maximum quantum efficiency of PSII photochemistry (Fv/Fm), the potential photochemistry efficiency (Fv/F_0), the electron transfer rate (ETR) and photochemical quenching of fluorescence (qP) are gradually reduced by Fe deficiency whereas the initial fluorescence (F_0) rises (Bertamini

et al. 2001). However, the mechanism underlying Fe control of photosynthetic efficiency through the adjustment of cellular metabolism needs further investigation.

Many studies have explored Fe-deficiency responsive molecular mechanisms and gene regulation networks (Kong and Yang 2010; Schmidt and Buckhout 2011). Several phytohormones and small signaling molecules take part in Fe deficiency responses, such as auxin (Bacaicoa et al. 2009; Chen et al. 2010), abscisic acid (ABA) (Lei et al. 2014), brassinosteroids (BR) (Wang et al. 2012) and nitric oxide (NO) (Jin et al. 2011). Recently, it has been reported that the cellular indole-3-acetic acid (IAA) level was related to Fe status, i.e. a higher IAA concentration was detected in Fe-deficient roots than in Fe-sufficient roots (Jin et al. 2007). IAA is mainly synthesized in shoots and is then transported to the roots as a chemical signal during Fe-deficient conditions (Bacaicoa et al. 2011). Under Fe stress conditions, elevated IAA content can induce ferric-chelate reductase (FCR) activity, which has been proved to be the rate-limiting step for iron uptake and homeostasis (Connolly et al. 2003). Auxin is also strongly involved in root branching and root hair induction under iron deficient conditions (Benkova and Bielach 2010; Li and Schmidt 2010). It has been shown that there is a crosstalk between IAA and NO in root architecture change under iron deficiency (Chen et al. 2010). NO may act as a downstream target of IAA to control iron deficiency response and regulation of Fe acquisition genes in plants (Chen et al. 2010). Auxin response factors and auxin polar transporters have also been reported to be involved in iron accumulation and homeostasis in rice. OsARF12, a transcription activator for auxin response genes, affects iron accumulation (Qi et al. 2012), and OsABCB14, an auxin polar transporter, is involved in auxin transport and iron homeostasis (Xu et al. 2014). These previous studies have provided a novel insight into the relationship between auxin and Fe acquisition.

The genome-wide gene expression profile of Arabidopsis and rice significantly changed under iron-limited conditions (Zheng et al. 2009; Ogo et al. 2011; Schuler et al. 2011; Li et al. 2014). A series of Fe-decient responsive genes contain an ARF-binding motif (AuxRE) in their promoter regions, which suggests that auxin signaling is actively involved in the Fe-decient response (Romera et al. 2011; Li et al. 2014). The expression level of IRON-REGULATED TRANSPORTER 1 *(IRT1)* is regulated by exogenous IAA in tomato (Graziano and Lamattina 2007). In cucumber plants, IAA application also induced the expression of many Fe-stress response genes, including *CsFRO1*, *CsIRT1*, *CsHA1* and *CsHA2*. This suggests that IAA is able to activate the expression of iron deficiency response genes at the transcriptional level (Bacaicoa et al. 2009; Bacaicoa et al. 2011). Many studies have shown that auxin is involved in the regulation of the Fe deficiency response, but how auxin is involved in Fe deficiency response at the transcription level is largely unknown. In this study, we took advantage of a set of published microarray data to analyze how auxin is involved in the iron deficiency response in rice (*Oryza sativa* L.) shoots (Zheng et al. 2009). Furthermore, our experimental results revealed a crosstalk between auxin signaling and iron deficiency-induced photosynthetic inhibition and shoot growth defects in rice.

Result

Morphological Changes and Soluble Fe Concentration Measurement

To explore the possible effects of auxin signaling on the morphological responses to Fe deprivation, wild type Nipponbare (NIP) seeds were grown in nutrient solution under different treatments: +Fe, +Fe+1-naphthaleneacetic acid (NAA), +Fe+1-naphthoxyaceticacids (1-NOA), –Fe, –Fe+NAA or –Fe+1-NOA. Without any phytohormone application, the –Fe treatment caused chlorosis and inhibition of shoot growth (Fig. 1A, B). Furthermore, we measured the biomass and shoot length to analyze the differences in phenotype under various conditions. The growth parameters were showed in Fig. 1C and D. To reveal the affection of hormones on Fe deficiency responses clearly, we calculated the change ratio between –Fe treatment and +Fe treatment. The biomass change ratio between the –Fe treatment and +Fe treatment was 54% and the shoot growth inhibition ratio between the –Fe treatment and +Fe treatment was 74%. When NAA was applied, the biomass change ratio between the –Fe+NAA treatment and +Fe+NAA treatment was 42% and the shoot growth inhibition ratio between the –Fe+NAA treatment and +Fe+NAA treatment was 61%. NAA application led to more severe chlorosis and shoot growth inhibition. When 1-NOA was applied, the biomass change ratio between the –Fe+1-NOA treatment and +Fe+1-NOA treatment was 84% and the shoot growth inhibition ratio between the –Fe+1-NOA treatment and +Fe+1-NOA treatment was 89% (Fig. S1A, B). As an auxin analog, 1-NOA blocks auxin signaling by inhibiting auxin influx transport (Parry et al. 2001; Hoyerova et al. 2008), and 1-NOA application partially relieved the symptoms of Fe deficiency, such as chlorosis and shoot growth.

As an important metal element involved in photosynthesis, soluble Fe concentration decline is the major cause of leaf chlorosis. Therefore, we measured the soluble Fe concentrations under different conditions. The data showed that the soluble Fe concentrations almost similar under Fe supply conditions (+Fe, +Fe+NAA and +Fe+1-NOA). The soluble Fe concentration under –Fe+NAA condition was lower than that under –Fe condition and the soluble Fe concentration

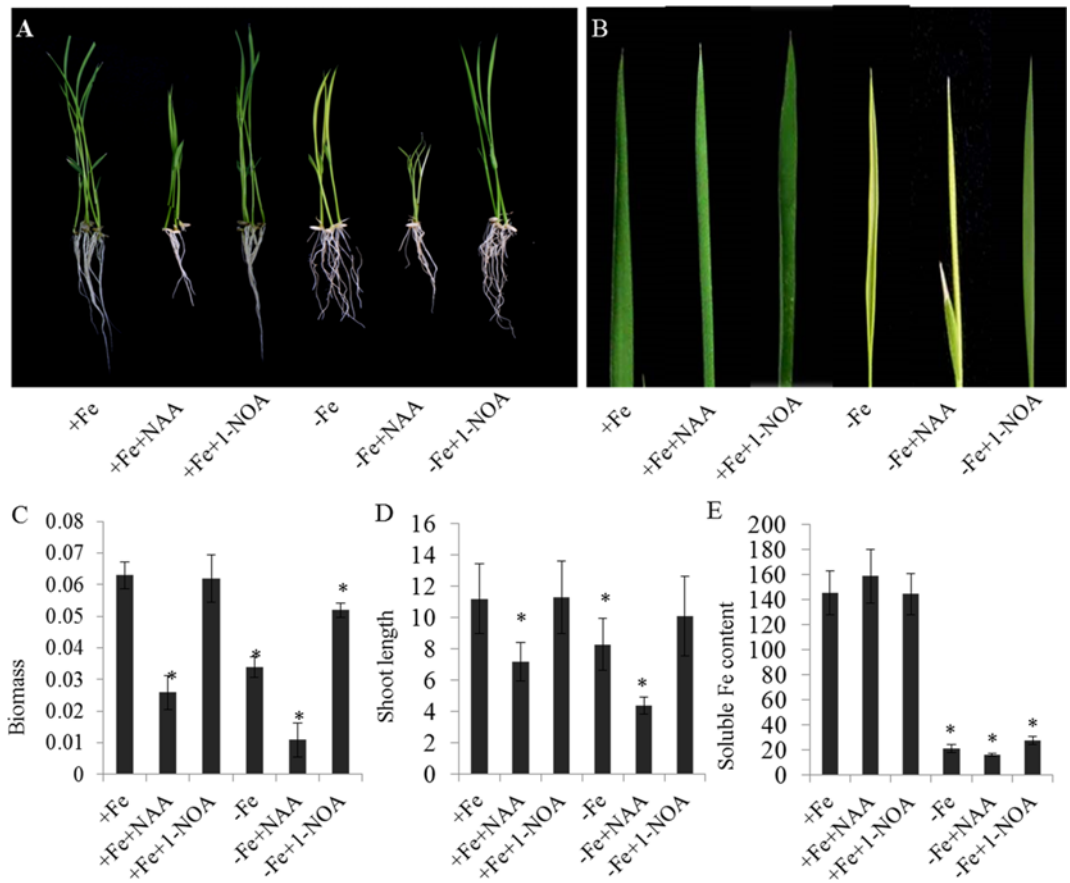

Fig. 1. Morphological responses and soluble iron content changes in rice plant under different conditions. Wild type NIP seedlings are grown in different conditions for 7 d. (A) Morphological responses of rice plants to different conditions, including +Fe, +Fe+NAA, +Fe+1-NOA, –Fe, –Fe+NAA and –Fe+1-NOA. (B) Photograph of shoot tips in rice plants grown in different conditions, including +Fe, +Fe+NAA, +Fe+1-NOA, –Fe, –Fe+NAA and –Fe+1-NOA. (C) Biomass under different hormone treatment conditions. (D) Shoot length under different hormone treatment conditions. (E) Soluble Fe concentrations under different hormone treatment conditions. Values are ± SD of five independent replicates. Signicant ($P < 0.05$) differences in biomass, shoot length and soluble Fe concentrations in the shoots of NIP seedlings under –Fe +NAA/+Fe+NAA and –Fe+1-NOA/+Fe+1-NOA conditions are indicated by an asterisk.

under –Fe+1-NOA condition was higher than that under –Fe condition (Fig. 1E). Then, we calculated the change ratio in the shoots between the –Fe treatment and +Fe treatment with different phytohormones. Our data showed that the soluble Fe concentration change ratio in the shoots significantly decreased from 0.146 (–Fe/+Fe condition) to 0.103 (–Fe+NAA/+Fe+NAA). The application of 1-NOA could relieve the rate of decline of soluble Fe concentration that caused by Fe deficiency (Fig. S1C).

Photosynthetic Parameters Analysis under Different Fe Deficiency Conditions

Fe deficiency causes a significant decrease in chlorophyll concentration. To investigate how auxin takes part in Fe deficiency inhibited photosynthesis, chlorophyll a, chlorophyll b and total chlorophyll concentrations were measured under +Fe, +Fe+NAA, +Fe+1-NOA, –Fe, –Fe+NAA or –Fe+1-NOA treatments. Our data showed that chlorophyll concentrations were similar for the Fe sufficient conditions (+Fe, +Fe+NAA and +Fe+1-NOA treatments), but were sharply reduced by Fe deficiency treatments (–Fe, –Fe +NAA and -Fe+1-NOA). The –Fe+NAA treatment caused a greater decline in chlorophyll concentrations than the –Fe treatment. In contrast to the NAA treatment, the chlorophyll concentrations under –Fe+1-NOA treatment were much higher than the –Fe treatment (Fig. 2A).

Photosynthetic parameters analysis revealed that Fe deficiency increased initial fluorescence (F_0) and simultaneously reduced the Fm' and Fv/Fm values. The Fv/Fm parameter, which assesses the maximum quantum efficiency of PSII photochemistry, under the –Fe treatment was lower than that under the +Fe treatment. All photosynthetic parameters were unchanged under the +Fe, +Fe+NAA and +Fe+1-NOA treatments, but were affected by Fe deficiency (Fig. 2B). Compared to the +Fe condition, –Fe deficiency increased the

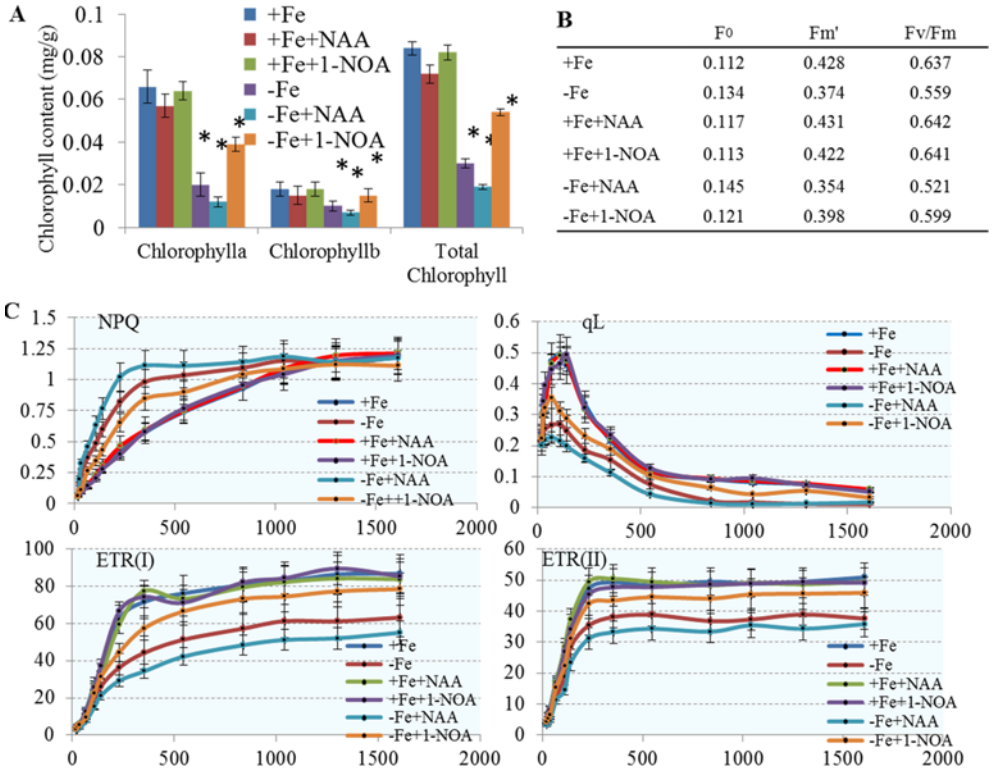

Fig. 2. Data analysis of chlorophyll concentrations and fluorescence parameters in NIP seedlings under different conditions. (A) Measurement of chlorophyll a, chlorophyll b and total chlorophyll concentrations in NIP seedlings under +Fe, +Fe+NAA, +Fe+1-NOA, –Fe, –Fe+NAA and –Fe+1-NOA treatments. (B) Determination of PSII quantum efficiency (Fv/Fm), initial (F_0) and the maximum (Fm) fluorescence yields in NIP seedlings under different conditions. (C) Assessment of PSI quantum yield, PSI electron transport rate (ETRI), PSII electron transport rate (ETRII), yield of energy dissipation by down-regulation (NPQ) and the fraction of PSII centers in an open state (qL) under different conditions. Data are shown as the mean ± SD (n = 5). Signicant ($P < 0.05$) differences in chlorophyll concentration measurements under –Fe, –Fe+NAA and –Fe+NOA treatments are indicated by an asterisk.

value of F_0 from 0.112 to 0.134, and reduced the values of Fm' (from 0.428 to 0.374) and Fv/Fm (from 0.637 to 0.559). Compared to +Fe+NAA condition, the –Fe+NAA treatment increased the value of F_0 (from 0.117 to 0.145) and reduced the values of Fm' (from 0.431 to 0.354) and Fv/Fm (from 0.642 to 0.521). Compared to the +Fe+1-NOA treatment, the –Fe+1-NOA treatment only slightly increased the value of F_0 (from 0.113 to 0.121) and reduced the values of Fm' (from 0.422 to 0.398) and Fv/Fm (from 0.641 to 0.599). These results indicated that NAA application enhanced photosynthetic parameter changes and 1-NOA attenuated changes caused by Fe deficiency. The other photosynthetic parameters, such as NPQ, qL, ETR(I) and ETR(II), also showed a similar change trend (Fig. 2C).

Quantitative RT–PCR Analysis of Several Fe Responsive Genes

In order to understand the mechanism behind the involvement of auxin in the Fe signaling system, qRT–PCR was performed to analyze the expression of several Fe responsive genes in the shoots under various treatments. In our study, a total of eight Fe responsive genes: two nicotianamine synthetase genes (*OsNAS1/2*) (Kobayashi and Nishizawa 2014), a (*OsYSL15*) (Aoyama et al. 2009), two Fe uptake strategy I genes (*OsIRT1/2*) (Lee and An 2009), a deoxymugineic acid synthase gene (*OsDMAS1*) (Bashir and Nishizawa 2006) and two ferric chelate reductase genes (*OsFRO1/2*) (Waters et al. 2002) were analyzed under +Fe, +Fe+NAA, +Fe+1-NOA, –Fe, –Fe+NAA and –Fe+1-NOA treatments. Our results showed that these Fe deficiency responsive genes were sharply induced by –Fe treatment. NAA application enhanced the induction and 1-NOA treatment attenuated the induction of the expression of these Fe deficiency responsive genes (Fig. 3).

Change ratio of Fe deficiency responsive genes was also calculated. The expression induction ratio of –Fe+NAA/+Fe+NAA increased compared to –Fe/+Fe. Meanwhile, the expression induction ratio of –Fe+1-NOA/+Fe+1-NOA decreased compared to –Fe/+Fe. Iron deficiency signaling was activated by exogenous auxin and was inhibited by 1-NOA application (Fig. S2).

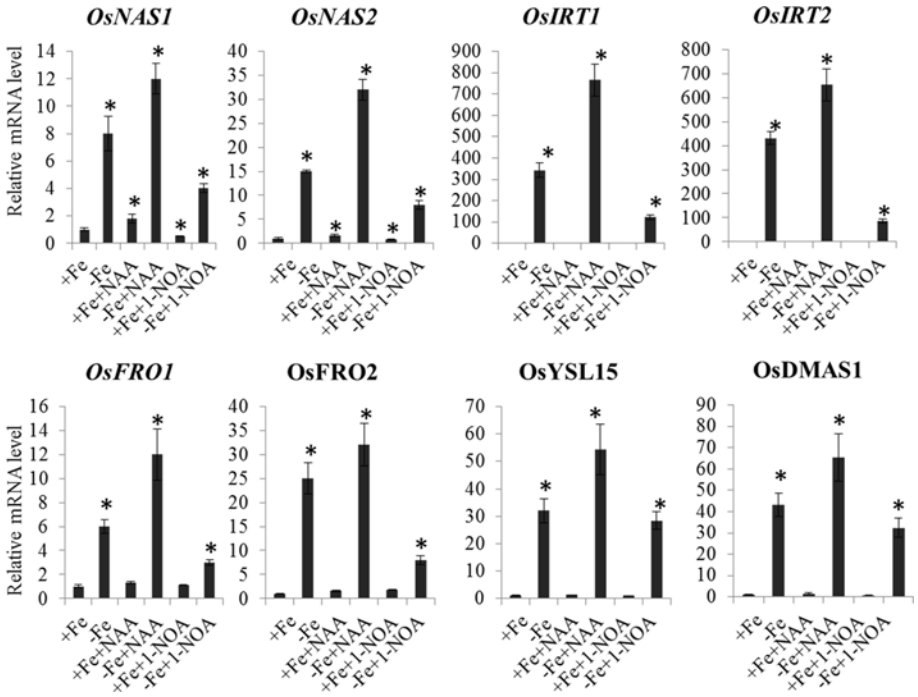

Fig. 3. Expression level analyses of eight Fe deficiency response genes in NIP seedlings. The expressions of *OsNAS1*, *OsNAS2*, *OsIRT1*, *OsIRT2*, *OsFRO1*, *OsFRO2, OsYSL15* and *OsDMAS1* in NIP under different conditions. All these data were based on the analysis of five independent biological repeats. Signicant ($P < 0.05$) differences in expression levels of Fe deficiency response genes under different conditions are indicated by an asterisk.

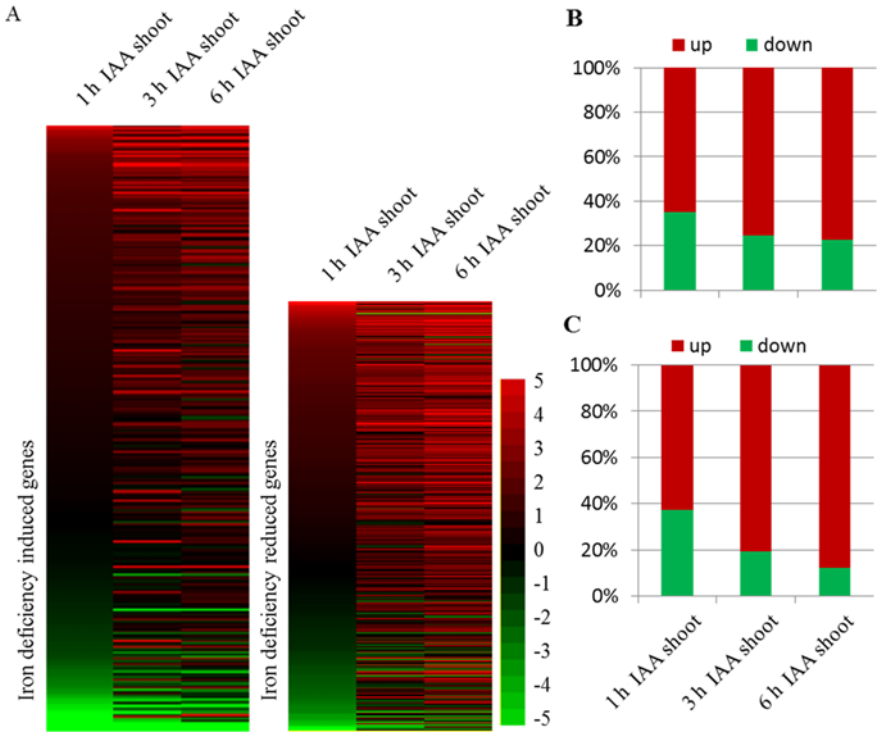

Fig. 4. Exogenous auxin was involved in the expression of iron deficiency response genes. (A) Heatmap for microarray profiles of iron deficiency genes response to IAA application. As Sato's published data (GSE39429), 8 microarray data were chosen for further analysis, and they were 0, 1, 3, 6 h IAA treatment (two repeats) in shoots (GSM968545, GSM968546, GSM968608, GSM968609, GSM968610, GSM968611, GSM968612, GSM968613). Totally 1324 iron deficiency induced genes (>5 folds) and 528 iron deficiency reduced genes (>5 folds) were chosen in this analysis. (B) Proportion of up-regulated to down-regulated iron deficiency induced genes in IAA treatment shoots. (C) Proportion of up-regulated to down-regulated iron deficiency reduced genes in IAA treatment shoots.

Auxin Signaling is Involved in the Iron Deficiency Response

Many studies have shown that auxin has specific roles during the iron deficiency response at the transcriptional level in plants (Bacaicoa et al. 2009; Chen et al. 2010). Eight microarray data sets were chosen for our analysis from Sato's (2012) published data (GSE39429), and these were 0, 1, 3, 6 h IAA treatments (two replicates per treatment) to the shoots (GSM968545, GSM968546, GSM968608, GSM968609, GSM968610, GSM968611, GSM968612, GSM968613) (Sato et al. 2013). Zheng's microarray data (GSE17245) identified 1324 iron deficiency induced genes (> fivefold) and 528 iron deficiency reduced genes (> fivefold). These genes were used to investigate how auxin is involved in the expression regulation of iron deficiency response genes in shoots (Zheng et al. 2009). Our data showed that most of the iron deficiency response genes were up-regulated by IAA treatment in the shoots (data shown in Table S2). Approximately, 65% of the iron deficiency induced genes were up-regulated in the shoots after 1 h of IAA treatment, and the proportion increased to nearly 80% after 6 h of IAA treatment (Fig. 4A, B). Approximately, 60% of the iron deficiency reduced genes were up-regulated after 1 h of IAA treatment, and the proportion increased to nearly 90% after 6 h of IAA treatment (Fig. 4A, C).

Analysis of Auxin-response Cis-element in the Promoters of Iron Deficiency Response Genes

Most auxin response gene promoters contain successive auxin-response elements (AuxRE) that are targeted by auxin-response factor (ARF) transcription factors (Sakamoto et al. 2013). In order to elucidate whether auxin signaling is involved in gene expression regulation during the iron deficiency response, we identified all promoters of the genes that were regulated by iron deficiency and had increased by 40-fold in the shoots, based on Zheng's rice microarray data (GSE17245). The GRE motif and MRE2 motif were present at a high frequency and occurred more than twice in each promoter (GRE: 2.28/promoter; MRE2: 3.25/promoter). TGA, AC, AUX1, AUX2 and MRE1 were also present in the iron deficiency response genes (TGA: 0.25/promoter; AC: 0.16/promoter; AUX1: 0.25/promoter; AUX2: 0.38/promoter; MRE1: 0.47/promoter) (Fig. 5).

Fe Deficiency Significantly Changes the Expression of Auxin Response Genes in Rice Shoots

The *OsIAA, OsGH3* and *OsSAUR* genes, the three major auxin response gene families (Abel and Theologis 1996), as well as *OsARF* genes, the most important auxin signaling

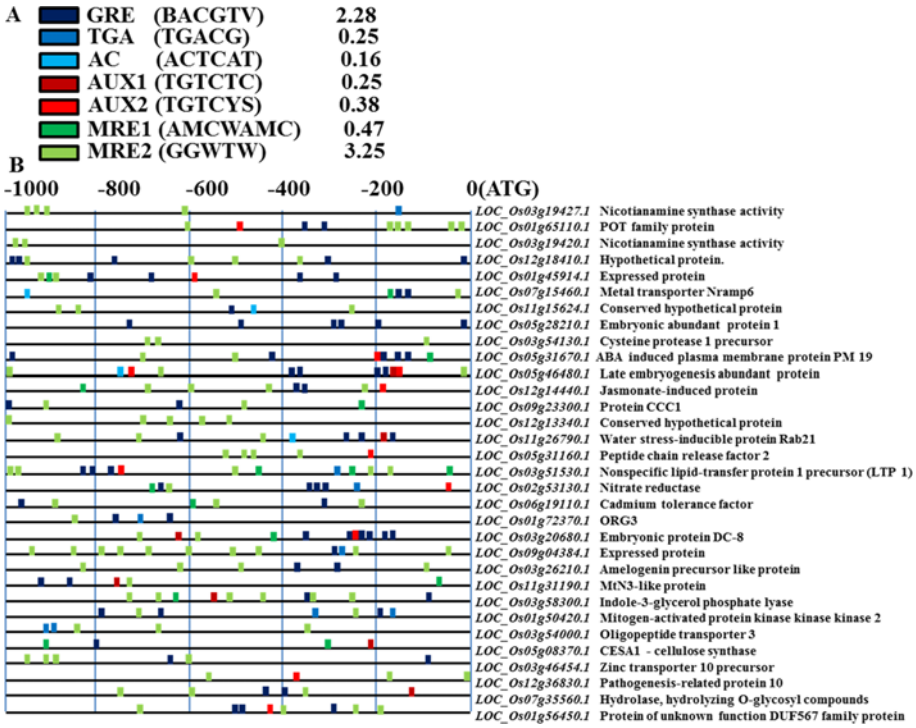

Fig. 5. Analysis of specific auxin-response *cis*-elements in iron responsive promoters in rice. (A) Different elements are given using the presented colour code. The promoters (-1000 to -1 bp) of iron deficiency response genes were scanned for AUX1 (TGTCTC core sequence), a less stringent variant called AUX2 (TGTVYS), three different ZREs (GRE, TGA and AC-motif), and two MREs (MRE1: AMCWAMC; MRE2: GGWTW). The average number of *cis*-elements per promoter is given next to the motif colour code. (B) The distribution of auxin-response *cis*-elements in iron responsive promoters.

pathway controllers, were analyzed in our study (Guilfoyle et al. 1998). To monitor the *OsARF*, *OsIAA,* *OsGH3* and *OsSAUR* gene expressions under Fe deficiency, we searched these auxin response genes in microarray data. In total, 20 *OsARF* genes, 27 *OsIAA* genes, 11 *OsGH3* genes and 30 *OsSAUR* genes were found in Zheng's microarray data (Table S3). The results showed that the expression of most auxin response genes in the shoots strongly responded to Fe deficiency treatment. Under Fe deficiency conditions, 35% of the *OsARF* genes were induced (> 1-fold) and another 35% were reduced (< –1-fold); in the *OsIAA* gene family, 29.6% of genes were induced and 48% were reduced; in the *OsGH3* gene family, 54.5% of genes were up-regulated and 45.5% were down-regulated; and in the *OsSAUR* gene family, 40% of genes were up-regulated and 46.7% were down-regulated. The expression of all the *GH3* family genes significantly changed under Fe deficient conditions (Fig. 6). Furthermore, several representative genes were chosen from different auxin response gene families to confirm the microarray data by qRT-PCR. There were *OsARF1*, *OsARF11* and *OsARF21* from the ARF family, *OsIAA1*, *OsIAA11* and *OsIAA21* from the IAA family, *OsGH3.1*, *OsGH3.6* and *OsGH3.12* from the GH3 family, and *OsSAUR11*, *OsSAUR21* and *OsSAUR31* from the SAUR family. The results obtained from qRT-PCR were similar to the microarray data (Fig. S3). The results indicated that auxin signaling was involved in the Fe deficiency response.

Discussion

How plants control their growth and development under Fe deficient conditions is an important question. Under Fe deficiency conditions, a specific morphological adaptive strategy, i.e. the inhibition of shoot growth and the induction of root hair elongation, has been developed by plants to response to Fe-limited environment (Sablowski et al. 1994). Auxin plays a pivotal role in these environmental response processes (Graziano and Lamattina 2007; Bacaicoa et al. 2009), including the developmental adaptations of root growth under Fe-deficient conditions (Giehl et al. 2012; Gruber et al. 2013). For example, Fe deficiency affects root architecture by regulating the AUX1-mediated auxin polar transport and auxin accumulation in the lateral root apices, which is a prerequisite for lateral root elongation (Giehl et al. 2012).

Iron deficiency regulates various morphological and physiological processes (Li et al. 2014). Several studies have shown that auxin plays a crucial role in altering root system architecture in response to limited Fe availability (Chen et al. 2010; Giehl et al. 2012). Iron is a limiting factor on biomass accumulation and growth in different crops (Takahashi et al. 2001; Jin et al. 2009; Jin et al. 2013). However, how auxin is involved in iron deficiency-induced inhibition of shoot growth and photosynthetic efficiency in rice plant remains largely unknown. The NIP seedlings displayed typical chlorotic symptoms and biomass reduction under Fe deficiency after 7 d

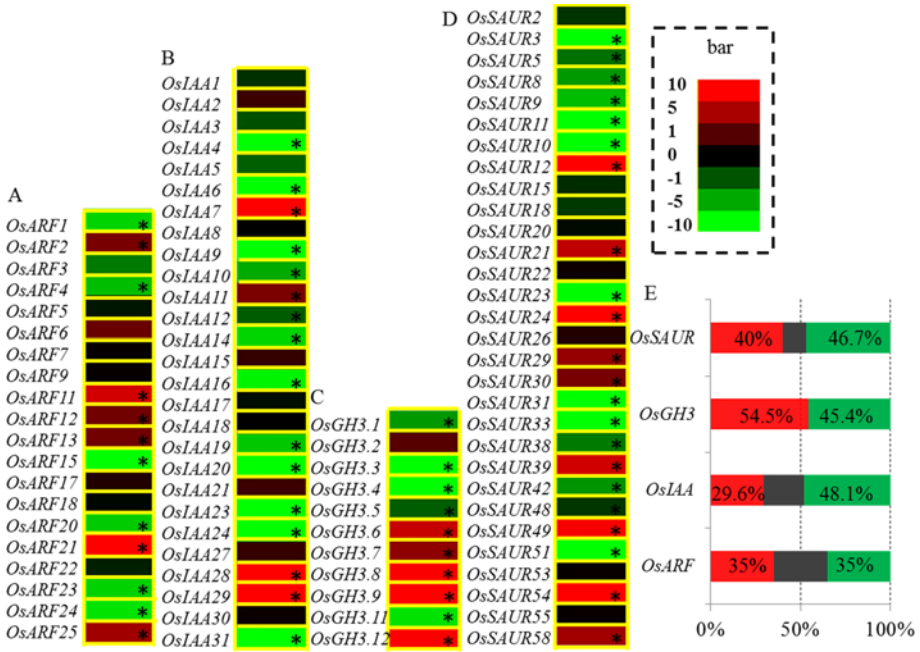

Fig. 6. Fe deficiency largely changed the auxin response gene expressions in rice. The expressions of *OsARF* (A), *OsIAA* (B), *OsGH3* (C) and *OsSAUR* (D) family genes were searched in microarray data (Accession Numbers GSE17245). Asterisks mark those ratios where auxin responsive gene expression in the Fe limited shoots was significantly different than in the Fe supplied shoots. (E) Proportion of up-regulated to down-regulated auxin early responsive genes in iron deficiency treatment in the rice shoots. The percentage shaded with red colour indicated up-regulated genes and the percentage shaded with green colour indicated up-regulated genes.

(Vigani et al. 2013). Our data confirmed that enhanced growth and soluble Fe accumulation under 1-NOA treatment in the Fe-deficient rice plants depended on auxin signal transmission during the iron deficiency response (Romera et al. 2011). The decrease in soluble Fe accumulation in the shoots was probably a main cause of the depression in chlorophyll synthesis, which explains the interveinal yellowing of leaves (Hiscock et al. 2008). When Fe is provided, both NAA and 1-NOA applications have no obvious effects on chlorophyll concentrations. However, significant differences in chlorophyll concentrations were observed among –Fe, –Fe +NAA and –Fe+1-NOA treatments (Fig. 2A). Previous studies have shown that a series of photosynthetic parameters were changed by Fe deficiency in a number of species (Sharma 2007; Msilini et al. 2013). The electron transfer system, including PSI and PSII, decreased in Fe-deficient maize and lettuce plants (Sharma 2007; Msilini et al. 2013). Our data showed that soluble Fe concentrations may be the key controller of several photosynthetic parameters, including NPQ, qL, ETR(I) and ETR(II) in rice. Furthermore, blocking (1-NOA treatment) or enhancement (NAA treatment) of auxin signaling also affects these photosynthetic parameters under Fe deficiency conditions.

A complicated molecular network maintains iron homeostasis (Lopez-Millan et al. 2013). Previous studies have demonstrated that iron deficiency was sensed in the underground parts of plants and then a signal was transmitted to the roots to regulate the expressions of iron uptake genes (Enomoto and Goto 2008; Garcia et al. 2011; Hindt and Guerinot 2012; Li et al. 2014). The hormone treatment results showed that interference or enhancement of auxin signaling could significantly affect the expression of these iron deficiency response genes in the shoots (Fig. 3). Similar results have also been recorded in *Malus. xiaojinensis* plants. The addition of IAA to decapitated shoots restored the iron deficiency physiological responses, whereas the auxin transport inhibitor, NPA, compromised iron deficiency responses (Wu et al. 2012). Our study showed that the changes in iron deficiency signaling coincided with morphological and physiological responses in rice plants. The most recent connection between auxin and Fe homeostasis in rice is the discovery that OsARF12, an auxin response factor, plays a role in the crosstalk between the auxin response and Fe signaling (Qi et al. 2012). Auxin signaling affects iron deficiency responses by interacting with iron deficiency signaling and by regulating the expression of several iron deficiency responsive genes (Hindt and Guerinot 2012; Wu et al. 2012). For example, Fe deficiency leads to increased auxin contents, which results in the enhanced expression of *FRO2* in Arabidopsis (Chen et al. 2010). In our study, two *AtFRO1* homologous genes and *OsFRO1/2*, were also found to be induced by NAA treatment and were inhibited by 1-NOA treatment under Fe deficient conditions. In the *osarf12* mutant, the auxin content was lower than in the WT, and the expression of *OsIRT1* was down-regulated (Qi et al. 2012). Many promoters of iron response genes contained a large number of auxin-response elements, which is the molecular basis for auxin regulated iron deficiency responses (Fig. 5).

Genome-wide transcriptome profiling in iron-starved plants provides information on genes that are involved in plant responses to iron deficiency (Zamboni et al. 2012). In our study, a set of published Fe deficiency and auxin response microarray data were used to analyze the expression patterns of Fe deficiency response genes under exogenous auxin treatment (Zheng et al. 2009; Sato et al. 2013). The data revealed that most of the iron deficiency response genes were up-regulated by exogenous IAA treatment in the shoots. In particular, the time course of the IAA treatments has a significant effect on expression regulation of iron deficiency response genes. It suggested that there is a crosstalk between iron deficiency signaling and auxin signaling.

Conclusions

Exogenous auxin application triggers and enhances the iron deficiency response, and blocking IAA transport prevents iron deficiency responses in the shoots. Our results strongly indicate that iron deciency-induced growth inhibition and photosynthesis depression are mediated by systemic auxin signaling. Further studies are needed to elucidate the mechanisms that govern auxin rice plant adaptation to important metal ion deficiencies.

Materials and Methods

Plant Materials and Growth Conditions

Rice plants *Nipponbare* (NIP) (*Oryza sativa* L.) were grown in standard culture solution in a glasshouse with a light: dark cycle of 16 : 8 h at 28 : 24 °C (day : night) and 60-70% humidity with a photoperiod of 16 h, the pH was adjusted to 5.8 with HCl. For hydroponics, seedlings were transferred to a plastic net floating on the nutrient solution containing either 100 μM EDTA–Fe (II) or 1 μM EDTA–Fe (II) for 7 d. The nutrient solution containing 100 μM EDTA–Fe (II) was used as Fe-sufficient treatment and nutrient solution containing 1 μM EDTA–Fe (II) was used as Fe-deficient treatment. The hydroponic experiments were performed in a standard rice culture solution containing 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 mM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.155 mM $CuSO_4$, 0.036 mM $FeCl_3$, 0.070 mM citric acid and 0.152 mM $ZnSO_4$. The phytohormone treatments were 10 μM of 1-naphthoxyacetic acid (1-NOA) and 1 μM of 1-naphthaleneacetic acid (NAA).

Soluble Fe Measurements

The shoot and root samples of 7-d-old seedlings were washed with oxalic acid three times and once with distilled water, then drained and

dried at 70 °C for 3 d. 25 mg aliquots of dried plant material were acid-digested for 6 h at 130 °C in 3 ml of ultrapure HNO

Quantitative RT-PCR (qRT-PCR) Analysis

Total RNA was isolated from the leaves of 7-d-old seedlings. The sequence of the related primers for qRT-PCR is listed in Table S1. The gene *OsACTIN* was used as an internal standard to calculate relative fold differences basing on the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) values. Briefly, 1 μL of a 1/20 dilution of cDNA in double distilled water was add to 5 μL of 2×UltraSYBR, 100 nM of each primer and water was then added to final volume 10 μL. The procedures for qRT-PCR were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 10 s, 58 °C for 10s, and 72 °C for 10s. Triplicate quantitative assays were performed on each cDNA sample. All the expression analysis was carried out for five biological repeats and the values shown in figures represent the average values of these five repeats.

Microarray Data Analysis

Microarray data sets published by Zheng (2009) and Sato (2012) were analyzed by software Expression Console of Affymetrix (http://www.affymetrix.com/), the graphic was made by TreeView 1.60 and ClustalW 2.20. The microarray data published by Zheng is downloaded from the supplemental data of the *Plant Physiology* paper (http://dx.doi.org/10.1104/pp.109). The genome-wide microarray data of rice published by Zheng were obtained from NCBI Gene Expression Omnibus (GEO) with Accession Numbers GSE17245. The genome-wide microarray data of rice published by Sato were obtained from NCBI Gene Expression Omnibus (GEO) with Accession Numbers GSE39429. For genes with more than one set of probes, the median values of expression were used. In our experiment, a specific fold change value (2x) in the expression levels is used to clarify the statistical analysis of significant differences among mock and the treatments.

Promoter Sequence Retrieval and Auxin-response Cis-element Analysis

The promoter sequences of corresponding iron deficiency response genes in the roots and shoots were used for analysis of specific ZRE, AuxRE and MRE *cis*-elements. The sequences of these promoters were obtained from website (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) and cleaned from promoters of alternative transcribed genes. The promoters (-1000 to -1 bp) of iron deficiency response genes were scanned for AUX1 (TGTCTC core sequence) and a less stringent variant called AUX2 (TGTVYS), and then the promoters were scanned for three different ZREs (GRE, TGA and AC-motif), and two MREs (MRE1: AMCWAMC; MRE2: GGWTW) (Berendzen et al. 2012).

Statistical Analysis of Data

For comparisons of biomass change ratio, shoot length change ratio and Fe content change ratio in different condition treatments, a two-sample *t*-test assuming unequal variances was performed with the values of –Fe+NAA/+Fe+NAA ratio or –Fe+NOA/+Fe+NOA ratio compared with values of –Fe/+Fe ratio. Significance was defined as $P \leq 0.01$.

Chlorophyll Fluorescence Parameter Measurements

Chlorophyll fluorescence parameters were recorded with the pulse-amplitude modulated fuorometer (Dual-PAM; Heinz Walz, Effeltrich, Germany). The plants were dark adapted for 1 h prior to determination of PSII quantum efficiency (Fv/Fm). The effect of short-term acute high-light stress was assessed by exposing leaves to a light intensity of 2000 mmol $m^{-2}s^{-1}$ in the Dual-PAM for 1 h, followed by determination of the three components of non-photochemical quenching (qE, qT and qI, with qI being a measure of PSII photo-inhibition) during a subsequent 15 min relaxation period in the dark. Recordings and calculations were carried out using Dual-PAM 1.7 data analyses. The saturation pulse method, originally developed for chlorophyll fluorescence quenching analysis, was used to assess PSI quantum yield. The electron transport rate of PSII (ETRII), yield of energy dissipation by down-regulation (NPQ) and the fraction of PSII centers in an open state (qL) were calculated as Kramer described (Kramer et la. 2004). Light-response curves for chlorophyll fluorescence and P700 assessment were measured simultaneously in detached, fully expanded new fronds with a Dual-PAM-100 measuring system (Walz-Effeltrich Germany). These were immediately dark adapted for 30 min. All the parameter analysis was carried out for five biological repeats and the values shown in figures represent the average values of these five repeats.

Measurement of Chlorophyll Content

Plant tissues were collected after centrifugation at 14,000 × *g* in a table-top centrifuge. The medium was removed by aspiration and the pellet was immediately frozen in liquid nitrogen and held at 80 °C. The xanthophyll cycle pigments and chlorophyll abundances were determined by HPLC after extraction in 100% acetone.

Seedling Biomass Measurement

The germinated seeds of NIP were grown in the iron contained culture solution (100 μM Fe-EDTA) or in the Fe deficient solution (1 μM Fe-EDTA) for 7 d. The shoot samples were washed five times in deionized water to clean. The plant shoot tissues were blotted dry with a paper towel and weighed using an electronic balance for biomass measurement. All the biomass analysis was carried out for five biological repeats and the values shown in figures represent the average values of these five repeats.

Acknowledgements

This work was supported by National Natural Science Foundation of China (31401935) and Key Laboratory of Zhanjiang Ttropical Characteristic Plant Resources Technology Development (grant no.2014A06008). We are grateful to Dr. Yanhua Qi (College of Life Sciences, Zhejiang University) and Dr. Dean Jiang for technical support.

Author's Contributions

KL and CS designed experiments; KL, RY, CY, JL and TS carried out experiments; YY and ST analyzed experimental results. LZ analyzed the data. CS and KL wrote the manuscript.

Supporting Information

Table S1. Primer sequences for gene expression related to iron signal.
Table S2. Auxin up-regulated genes response to iron deficiency.
Table S3. Expression levels of auxin response genes under Fe deficiency

condition.
Fig. S1. The Biomass ratios, shoot length ratios and soluble Fe content ratios between Fe sufficient and Fe deficient under different hormone treatment conditions.
Fig. S2. The expression change ratios of Fe deficiency responsive genes in NIP under different conditions
Fig. S3. The expression level of several representative genes from different auxin response gene families.

References

Abel S, Theologis A (1996) Early genes and auxin action. Plant Physiol 111:9–17

Aoyama T, Kobayashi T, Takahashi M, Nagasaka S, Usuda K, Kakei Y, Ishimaru Y, Nakanishi H, Mori S, Nishizawa NK (2009) OsYSL18 is a rice iron(III)-deoxymugineic acid transporter specifically expressed in reproductive organs and phloem of lamina joints. Plant Mol Biol 70:681–692

Bacaicoa E, Mora V, Zamarreno AM, Fuentes M, Casanova E, Garcia-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

Bacaicoa E, Zamarreño ÁM, Leménager D, Baigorri R, García-Mina JM (2009) Relationship between the Hormonal Balance and the Regulation of Iron Deficiency Stress Responses in Cucumber. J Amer Soc Hort Sci 134:589–601

Bashir K, Ishimaru Y, Nishizawa NK (2011) Identification and characterization of the major mitochondrial Fe transporter in rice. Plant Signal Behav 6:1591–1593

Bashir K, Nishizawa NK (2006) Deoxymugineic Acid synthase: a gene important for fe-acquisition and homeostasis. Plant Signal Behav 1:290–292

Benkova E, Bielach A (2010) Lateral root organogenesis - from cell to organ. Curr Opin Plant Biol 13:677–683

Berendzen KW, Weiste C, Wanke D, Kilian J, Harter K, Droge-Laser W (2012) Bioinformatic *cis*-element analyses performed in Arabidopsis and rice disclose bZIP- and MYB-related binding sites as potential AuxRE-coupling elements in auxin-mediated transcription. BMC Plant Biol 12:125

Bertamini M, Nedunchezhian N, Borghi B (2001) Effect of Iron Deficiency Induced Changes on Photosynthetic Pigments, Ribulose-1,5-Bisphosphate Carboxylase, and Photosystem Activities in Field Grown Grapevine (*Vitis Vinifera* L. cv. Pinot Noir) Leaves. Photosynthetica 39:59–65

Briat JF, Dubos C, Gaymard F (2014) Iron nutrition, biomass production, and plant product quality. Trends Plant Sci 20:33–40

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

Connolly EL, Campbell NH, Grotz N, Prichard CL, Guerinot ML (2003) Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. Plant Physiol 133:1102–1110

Eberhard S, Finazzi G, Wollman FA (2008) The dynamics of photosynthesis. Annu Rev Genet 42:463–515

Enomoto Y, Goto F (2008) Long-distance signaling of iron deficiency in plants. Plant Signal Behav 3:396–397

Garcia MJ, Suarez V, Romera FJ, Alcantara E, Perez-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

Giehl RF, Lima JE, von Wiren N (2012a) Localized iron supply triggers lateral root elongation in Arabidopsis by altering the AUX1-mediated auxin distribution. Plant Cell 24:33–49

Giehl RF, Lima JE, von Wiren N (2012b) Regulatory components involved in altering lateral root development in response to localized iron: evidence for natural genetic variation. Plant Signal Behav 7:711–713

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

Grotz N, Guerinot ML (2006) Molecular aspects of Cu, Fe and Zn homeostasis in plants. Biochim Biophys Acta 1763:595-608

Gruber BD, Giehl RF, Friedel S, von Wiren N (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. Plant Physiol 163:161–179

Guilfoyle TJ, Ulmasov T, Hagen G (1998) The ARF family of transcription factors and their role in plant hormone-responsive transcription. Cell Mol Life Sci 54:619–627

Hindt MN, Guerinot ML (2012) Getting a sense for signals: Regulation of the plant iron deficiency response. Biochim Biophys Acta 1823:1521–1530

Hiscock MR, Lance VP, Apprill AM, Bidigare RR, Johnson ZI, Mitchell BG, Smith WO Jr, Barber RT (2008) Photosynthetic maximum quantum yield increases are an essential component of the Southern Ocean phytoplankton response to iron. Proc Natl Acad Sci USA 105:4775–4780

Hoyerova K, Perry L, Hand P, Lanková M, Kocábek T, May S, Kottová J, Paces J, Napier R, Zazimalová E (2008) Functional characterization of PaLAX1, a putative auxin permease, in heterologous plant systems. Plant Physiol 146:1128–1141

Jin CW, Du ST, Chen WW, Li GX, Zhang YS, Zheng SJ (2009) Elevated carbon dioxide improves plant iron nutrition through enhancing the iron-deficiency-induced responses under iron-limited conditions in tomato. Plant Physiol 150:272–280

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 62:3875–3884

Jin CW, He XX, Zheng SJ (2007) The Iron-Deficiency Induced Phenolics Accumulation May Involve in Regulation of Fe(III) Chelate Reductase in Red Clover. Plant Signal Behav 2:327–332

Jin CW, Liu Y, Mao QQ, Wang Q, Du ST (2013) Mild Fe-deficiency improves biomass production and quality of hydroponic-cultivated spinach plants (*Spinacia oleracea* L.). Food Chem 138:2188–2194

Kim SA, Guerinot ML (2007) Mining iron: iron uptake and transport in plants. FEBS Lett 581:2273–2280

Kobayashi T, Nishizawa NK (2014) Iron sensors and signals in response to iron deficiency. Plant Sci 224:36–43

Kong WW, Yang ZM (2010) Identification of iron-deficiency responsive microRNA genes and cis-elements in *Arabidopsis*. Plant Physiol Biochem 48:153–159

Lee S, An G (2009) Over-expression of *OsIRT1* leads to increased iron and zinc accumulations in rice. Plant Cell Environ 32:408–416

Lei GJ, Zhu XF, Wang ZW, Dong F, Dong NY, Zheng SJ (2014) Abscisic acid alleviates iron deficiency by promoting root iron reutilization and transport from root to shoot in *Arabidopsis*. Plant Cell Environ 37:852–863

Li H, Wang L, Yang ZM (2014) Co-expression analysis reveals a group of genes potentially involved in regulation of plant response to iron-deficiency. Gene 554:16–24

Li W, Schmidt W (2010) A lysine-63-linked ubiquitin chain-forming conjugase, UBC13, promotes the developmental responses to iron deficiency in *Arabidopsis* roots. Plant J 62:330–343

Lopez-Millan AF, Grusak MA, Abadia A, Abadia J (2013) Iron deficiency in plants: an insight from proteomic approaches.

Front Plant Sci 4:254
Msilini N, Essemine J, Zaghdoudi M, Harnois J, Lachaâl M, Ouerghi Z, Carpentier R (2013) How does iron deficiency disrupt the electron flow in photosystem I of lettuce leaves? J Plant Physiol 170:1400–1406
Ogo Y, Itai RN, Kobayashi T, Aung MS, Nakanishi H, Nishizawa NK (2011) OsIRO2 is responsible for iron utilization in rice and improves growth and yield in calcareous soil. Plant Mol Biol 75:593–605
Parry G, Delbarre A, Marchant A, Swarup R, Napier R, Perrot-Rechenmann C, Bennett MJ (2001) Novel auxin transport inhibitors phenocopy the auxin influx carrier mutation *aux1*. Plant J 25:399–406
Qi Y, Wang S, Shen C, Zhang S, Chen Y, Xu Y, Liu Y, Wu Y, Jiang D (2012) OsARF12, a transcription activator on auxin response gene, regulates root elongation and affects iron accumulation in rice (*Oryza sativa*). New Phytol 193:109–120
Romera FJ, García MJ, Alcántara E, Pérez-Vicente R (2011) Latest findings about the interplay of auxin, ethylene and nitric oxide in the regulation of Fe deficiency responses by Strategy I plants. Plant Signal Behav 6:167–170
Sablowski RW, Moyano E, Culianez-Macia FA, Schuch W, Martin C, Bevan M (1994) A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J 13:128–137
Sakamoto T, Morinaka Y, Inukai Y, Kitano H, Fujioka S (2013) Auxin signal transcription factor regulates expression of the brassinosteroid receptor gene in rice. Plant J 73:676–688
Sarvari E, Gaspar L, Solti A, Meszaros I, Zaray G, Fodor F (2010) Cd-Fe interactions: comparison of the effects of iron deficiency and cadmium on growth and photosynthetic performance in poplar. Acta Biol Hung 61 Suppl:136–148
Sato Y, Takehisa H, Kamatsuki K, Minami H, Namiki N, Ikawa H, Ohyanagi H, Sugimoto K, Antonio BA, Nagamura Y (2013) RiceXPro version 3.0: expanding the informatics resource for rice transcriptome. Nucleic Acids Res 41:D1206–1213
Schmidt W, Buckhout TJ (2011) A hitchhiker's guide to the *Arabidopsis* ferrome. Plant Physiol Biochem 49:462–470
Schuler M, Keller A, Backes C, Philippar K, Lenhof HP, Bauer P (2011) Transcriptome analysis by GeneTrail revealed regulation of functional categories in response to alterations of iron homeostasis in *Arabidopsis thaliana*. BMC Plant Biol 11:87
Sharma S (2007) Adaptation of photosynthesis under iron deficiency in maize. J Plant Physiol 164:1261–1267
Takahashi M, Nakanishi H, Kawasaki S, Nishizawa NK, Mori S (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. Nat Biotechnol 19:466–469
Vigani G, Tarantino D, Murgia I (2013) Mitochondrial ferritin is a functional iron-storage protein in cucumber (*Cucumis sativus*) roots. Front Plant Sci 4:316
Wang B, Li Y, Zhang WH (2012) Brassinosteroids are involved in response of cucumber (*Cucumis sativus*) to iron deficiency. Ann Bot 110:681–688
Waters BM, Blevins DG, Eide DJ (2002) Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition. Plant Physiol 129:85–94
Wu T, Zhang HT, Wang Y, Jia WS, Xu XF, Zhang XZ, Han ZH (2012) 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
Xu Y, Zhang S, Guo H, Wang S, Xu L, Li C, Qian Q, Chen F, Geisler M, Qi Y, Jiang de A (2014) OsABCB14 functions in auxin transport and iron homeostasis in rice (*Oryza sativa* L.). Plant J 79:106–117
Zamboni A, Zanin L, Tomasi N, Pezzotti M, Pinton R, Varanini Z, Cesco S (2012) Genome-wide microarray analysis of tomato roots showed defined responses to iron deficiency. BMC Genomics 13:101
Zheng L, Wu J, Giraud E, He F, Cheng L, Wang F, Wu P, Whelan J, Shou H (2009) Physiological and transcriptome analysis of iron and phosphorus interaction in rice seedlings. Plant Physiol 151:262–274