Physiological and Transcriptome Analysis of Iron and Phosphorus Interaction in Rice Seedlings[1][C][W]

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The antagonistic interaction between iron (Fe) and phosphorus (P) has been noted in the area of plant nutrition. To understand the physiology and molecular mechanisms of this interaction, we studied the growth performance, nutrient concentration, and gene expression profiles of root and shoot segments derived from 10-d-old rice (*Oryza sativa*) seedlings under four different nutrient conditions: (1) full strength of Fe and P (+Fe+P); (2) full strength of P and no Fe (−Fe+P); (3) full strength of Fe and no P (+Fe−P); and (4) without both Fe and P (−Fe−P). While removal of Fe in the growth medium resulted in very low shoot and root Fe concentrations, the chlorotic symptoms and retarded seedling growth were only observed on seedlings grown in the presence of P. Microarray data showed that in roots, 7,628 transcripts were significantly changed in abundance in the absence of Fe alone. Interestingly, many of these changes were reversed if P was also absent (−Fe−P), with only approximately 15% overlapping with –Fe alone (–Fe+P). Analysis of the soluble Fe concentration in rice seedling shoots showed that P deficiency resulted in significantly increased Fe availability within the plants. The soluble Fe concentration under –Fe–P conditions was similar to that under +Fe+P conditions. These results provide evidence that the presence of P can affect Fe availability and in turn can influence the regulation of Fe-responsive genes.

Plants require a variety of nutrients for growth. Although iron (Fe) is quite abundant in the earth's crust, it is often a limiting resource for growth, due to low availability (Guerinot and Yi, 1994). Fe-deficient plants often develop interveinal chlorotic symptoms in young leaves and display a considerable decrease in leaf net photosynthetic rate (Briat et al., 1995). Severe deficiency leads to growth retardation, stasis, and ultimately death. Given the importance of Fe for plant growth, specific mechanisms have evolved in plants to optimize Fe acquisition.

In nongrass plants, Fe deficiency stimulates the expression of a ferric-chelate reductase (FRO) and ferrous transporters (IRTs; Curie and Briat, 2003), typically referred to as a strategy I approach (Marschner et al., 1986). H^+-ATPases acidify the rhizosphere and increase Fe solubility in the soil (Dell'Orto et al., 2000). Ferrous iron (Fe^{2+}), reduced by FRO from its ferric form (Fe^{3+}), can then be transported into root cells by the IRTs (Curie and Briat, 2003). In Arabidopsis, FRO2 and IRT1 are regulated by a basic helix-loop-helix (bHLH) family transcriptional factor, FIT1 (Colangelo and Guerinot, 2004). A different strategy, referred to as strategy II, is used in gramineous plants (Marschner et al., 1986). In these plants, an increase in the biosynthesis and secretion of high-affinity Fe^{3+} chelator(s) called phytosiderophores (PS) is observed under Fe-deficient conditions. PS chelates Fe^{3+} to form a PS-Fe^{3+} complex, which can then be transported by corresponding Yellow Stripe (YS) family transporters (Curie et al., 2001; Curie and Briat, 2003). The two strategies outlined above are not exclusive, and rice (*Oryza sativa*) employs both strategies (Marschner et al., 1986; Ishimaru et al., 2006; Cheng et al., 2007; Walker and Connolly, 2008).

Extensive studies on Fe uptake and its regulation in plants have identified transcription factors (TFs) and

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corresponding cis-acting regulatory elements in a variety of genes, including a variety of transporters associated with Fe uptake along with various signaling and regulatory factors for Fe homeostasis (Walker and Connolly, 2008). Such studies have led to the examination of various approaches to increase Fe uptake in plants. For example, rice does not have an inducible FRO; therefore, when rice was engineered to have such an activity, it resulted in enhanced Fe uptake (Ishimaru et al., 2007).

As may be expected, there is an interaction between the uptake of several nutrients in plants. For example, zinc uptake has been suggested to positively correlate with Fe uptake (Grotz and Guerinot, 2006). Also, phosphorus (P) deficiency has been shown to result in increased Fe concentration (Misson et al., 2005; Hirsch et al., 2006). Elevated Fe concentrations in the P-deficient plants were linked to increased Fe availability in the plant and in the medium in the absence of P (Waldo et al., 1995; Misson et al., 2005). However, removal of Fe in the P-deficient medium (−Fe−P) resulted in improved plant growth (Ward et al., 2008). Several marker genes involved in the maintenance of Fe homeostasis were found to be altered under P deficiency. P deficiency resulted in decreased expression of the Fe transporter *IRT1* in roots and increased the expression of *AtFER1* (a protein involved in Fe storage in the chloroplast) in leaves (Misson et al., 2005). Notably, the expression of genes changing in abundance under P-deficient conditions was not altered in response to Fe deficiency (Ward et al., 2008), indicating that the phenotype restoration in P-deficient plants was not due to improvements in the P status in these plants.

Several studies have carried out detailed molecular analyses of the effects of Fe or P deficiency alone. These studies reveal that the absence of either of these nutrients triggers large-scale molecular responses. In Arabidopsis (*Arabidopsis thaliana*), Fe deficiency appears to increase energy demand, increasing the capacity for oxidative phosphorylation, which is likely to be exacerbated by the lack of Fe (Thimm et al., 2001). In rice, the enzymes involved in 2′-deoxymugineic acid (DMA) synthesis and transporters for the uptake of Fe (III)-DMA are strongly induced under conditions of Fe deficiency (Kobayashi et al., 2005). In Arabidopsis, there is a genome-wide reprogramming of metabolism and regulatory networks in response to P deficiency (Morcuende et al., 2007). Notably, P deprivation leads to the accumulation of carbohydrates, organic acids, and amino acids (Morcuende et al., 2007). Similarly, in rice, P deficiency affects metabolism, increases carbon flux via glycolysis for the synthesis of organic acids, alters lipid metabolism, and leads to changes in the abundance of transcripts encoding proteins involved in aluminum, Fe, and zinc metabolism (Wasaki et al., 2003).

In this study, a genome-wide microarray approach was employed to investigate the rice transcriptome alteration, not only in response to one specific nutrient (Fe or P) but also to explore the context of interaction between Fe and P signaling in rice. Our results showed that the majority of transcript changes observed with −Fe growth conditions were repressed if P was also absent (−Fe−P). The results presented provide, to our knowledge, the first molecular evidence demonstrating that the Fe-deficiency response is controlled by the amount of available Fe instead of total Fe concentration.

RESULTS

Physiological Evidence for Fe and P Interaction in Rice Seedlings

To investigate the molecular and physiological responses to Fe and/or P deprivation, rice seeds were grown for 10 d after germination in a nutrient solution supplied with either Fe and P (+Fe+P), P and no Fe (−Fe+P), Fe and no P (+Fe−P), or without both P and Fe (−Fe−P). In the presence of P, Fe deprivation (−Fe+P) resulted in retarded seedling growth and induced chlorosis in the leaves (Fig. 1, A–C). The average leaf chlorophyll concentration (soil-plant analyzer development [SPAD] value) of seedlings in −Fe+P treatment was significantly lower than that in the +Fe+P treatment (Fig. 1B). The chlorotic phenotype was consistent with the low Fe concentrations in both roots and shoots (Fig. 1D). In contrast to the Fe concentration, inorganic phosphate (Pi) and total P concentrations in both roots and shoots of the −Fe+P-treated plants were significantly higher than those of the plants exposed to +Fe+P treatment (Fig. 1, E and F), indicating that Fe deficiency promoted P uptake. In contrast, Pi and total P concentrations in the seedlings grown under P-deprivation conditions were significantly lower than those of seedlings grown under +P conditions, confirming the P deprivation (Fig. 1, E and F). P deficiency in the young seedling stage resulted in a significant increase in root length and SPAD value ($P < 0.05$; Fig. 1, A–C). Under P-deficient conditions, Fe uptake was significantly promoted, evidenced by the finding that shoot and root Fe concentrations in the seedlings grown under +Fe−P conditions were 2.1 and 1.8 times as much as those for seedlings grown under +Fe+P conditions, respectively (Fig. 1D). Interestingly, under −Fe−P conditions, although both shoot and root Fe concentrations remained low (Fig. 1D), no chlorotic symptoms were observed, unlike what was observed with the −Fe+ P growth conditions (Fig. 1, A and B). Thus, both the growth retardation and chlorosis observed were not attributable to a lower total Fe concentration alone.

General Features of the Fe-Starvation Expression Profiles

Global mRNA profiling in response to Fe and P deprivation was performed using the Affymetrix Rice GeneChip. Root and shoot segments derived from rice plants 10 d after germination under +Fe+P, −Fe+P,

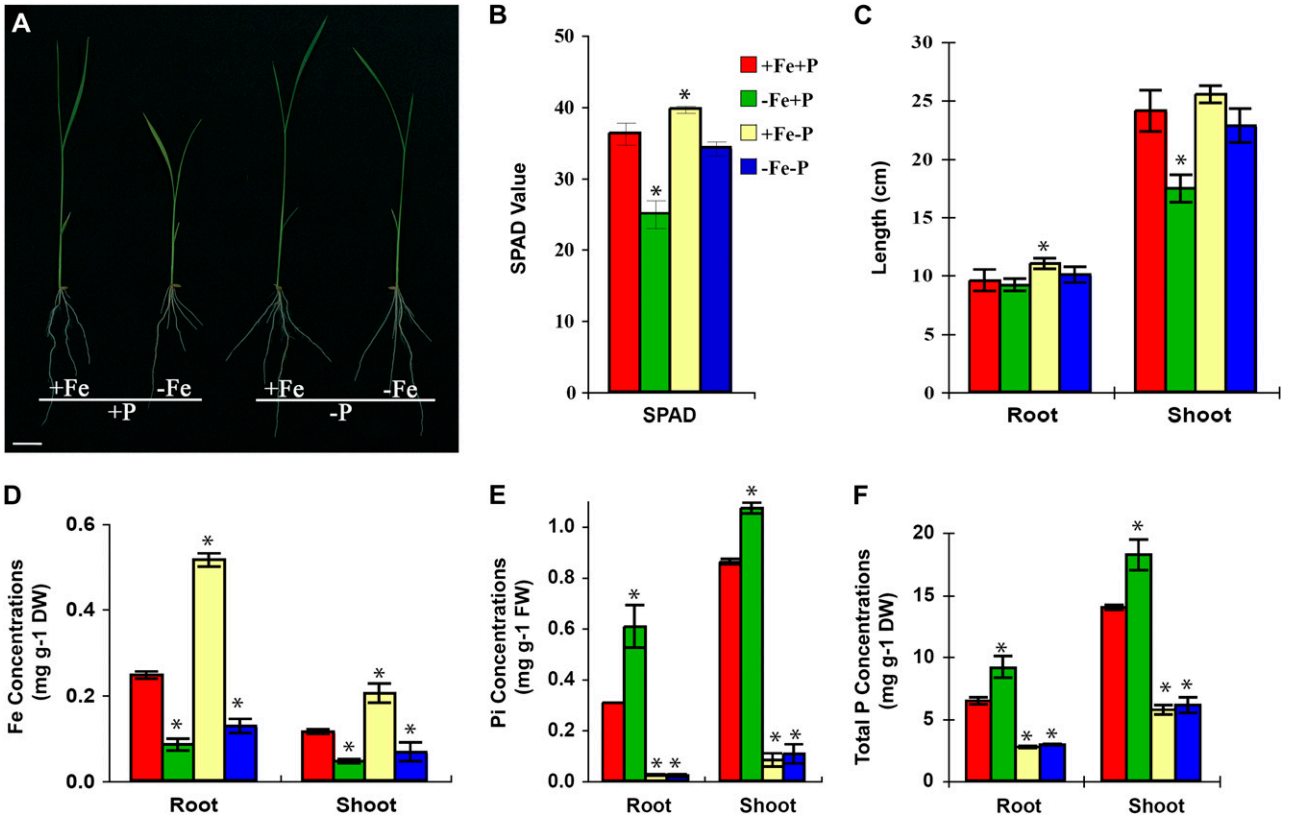

Figure 1. Growth and Fe and P analysis of rice plants grown under different nutrient regimes. Analysis was performed 10 d after germination under four different growth regimes (+Fe+P, −Fe+P, +Fe−P, −Fe−P). A, Photograph of rice plants grown in the four treatments. Bar = 2 cm. B, SPAD values of rice plants in the four treatments. C, Lengths of shoot and root of rice plants in the four treatments. D, Fe concentration of rice plants in the four treatments. E, Pi concentration of rice plants in the four treatments. F, Total P concentration of rice plants in the four treatments. Asterisks indicate significance using a two-sample *t* test assuming unequal variance. Significance was defined as $P \leq 0.05$. DW, Dry weight; FW, fresh weight.

+Fe−P, and −Fe−P conditions were analyzed. Principal component analysis of global microarray data from roots reveals that the −Fe+P treatment differs most from the other growth conditions (Fig. 2A), which is consistent with this treatment having the greatest number of significant changes in transcript abundance after false discovery rate correction, with 3,476 transcripts up-regulated and 4,152 down-regulated (Fig. 2B; Supplemental Table S1). In contrast, the −Fe−P and +Fe−P treatments are closer together on the principal component analysis plot, indicating that they share similarities in their global gene expression profiles and that many changes that occurred in the absence of Fe alone have been alleviated (Fig. 2A). This was also confirmed by the finding that the smallest number of genes changing in transcript abundance was observed when the treatments −Fe−P and +Fe−P were compared with each other, with 1,064 up-regulated and 989 down-regulated (Fig. 2B). This indicates that these two growth conditions elicit a very similar response, which differs greatly from the –Fe-alone conditions. Note also that the number of changes in transcript abundance observed during the absence of any one nutrient (i.e. −P or −Fe only) was greater than when both were absent, suggesting that an antagonistic effect exists between the two (Fig. 2B, first row and first column). This was most dramatic for the absence of Fe alone, which resulted in 7,628 changes (3,476 up and 4,152 down) compared with 4,242 changes (2,340 up and 1,902 down) when both Fe and P were absent in the growth medium.

A slightly different picture emerged when shoot tissue transcript profiles were analyzed. Here, the greatest difference was observed with −P alone (Fig. 2D), with 2,993 transcripts up-regulated and 2,699 down-regulated (Fig. 2E). Again, the lack of both Fe and P resulted in fewer changes than the sum of the individual changes (Fig. 2E), although it was notable that −Fe alone had the least effect on the number of altered transcripts compared with either −P alone or −Fe−P (Fig. 2E).

The number of genes that were significantly ($P < 0.05$) differentially expressed in roots and shoots by more than 2-fold in response to –Fe, –P, or –Fe–P are listed in Figure 2, C and F, respectively. Although each treatment produced a distinct response (e.g. for roots,

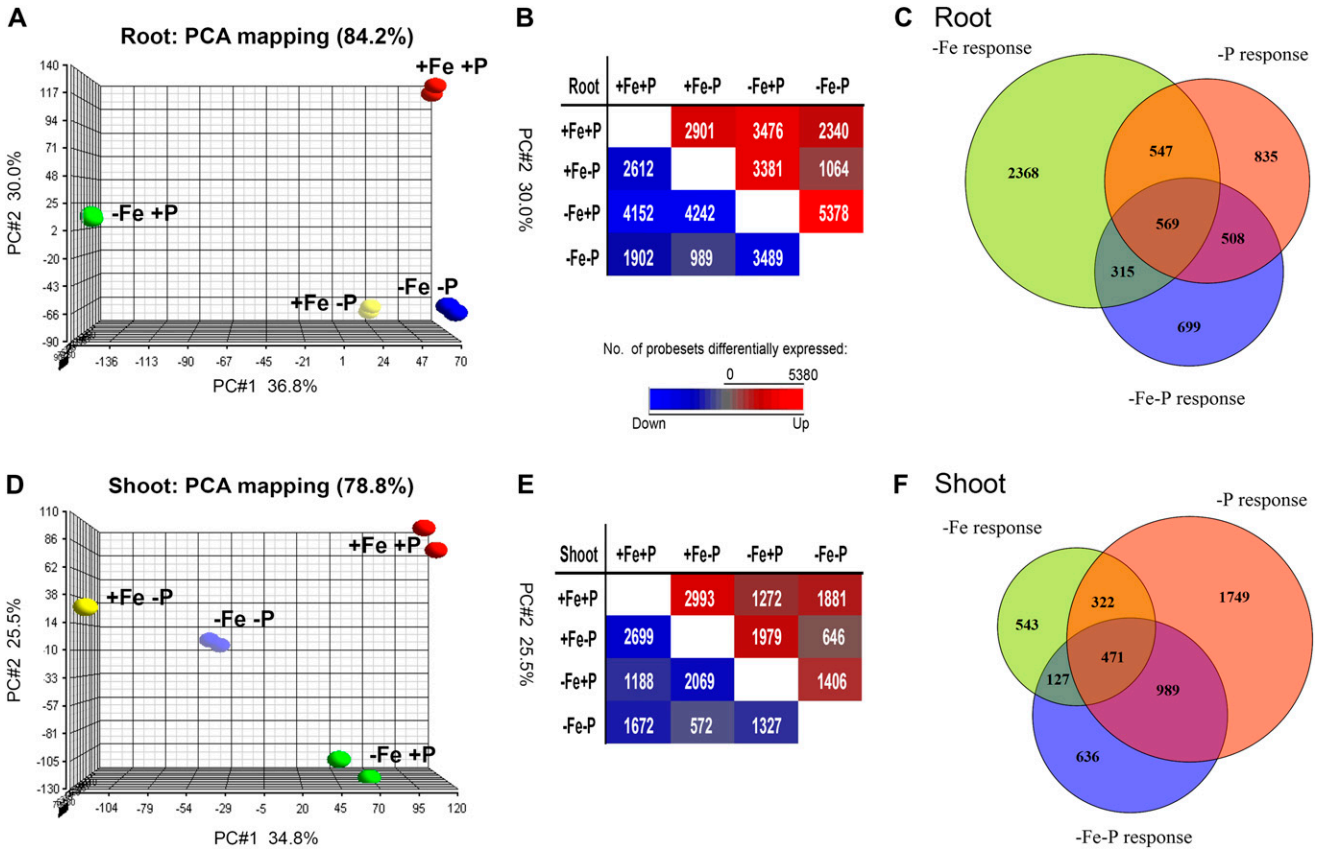

Figure 2. Overview of the changes in transcripts with different growth regimes. A and D, Principal component analysis (PCA) of the changes in transcript abundance in root (A) or shoot (D) under different growth regimes. B and E, The number of genes whose transcripts were significantly different in abundance (red = up-regulated, blue = down-regulated) in root (B) or shoot (E). C and F, Venn diagrams showing the number of probe sets increased or decreased ($P < 0.05$ and fold change > 2) in response to −Fe, −P, or a combination –Fe –P treatment in root (C) or shoot (F).

–Fe, 2,368; –P, 835; −Fe−P, 699), there was some overlap (e.g. for roots, –Fe and –P, 547; –Fe and −Fe−P, 315; –P and −Fe−P, 508; –Fe and –P and −Fe−P, 569; Fig. 2, C and F). It is clear that the Fe-deficiency response was much stronger in roots than in shoots at the level of transcript abundance.

Overall, it appeared that the absence of P could ameliorate the effects of the absence of Fe; this was particularly evident in the number of transcripts that were altered in abundance in root. At the molecular level, this effect is reflected in the growth phenotype, where an absence of both P and Fe results in plants that are not growth retarded compared with the absence of Fe alone (Fig. 1A). The changes in transcript abundance observed using microarray analysis were confirmed for a number of genes using quantitative reverse transcription (RT)-PCR (Table I). As roots are the site of uptake of Fe, most changes in transcript abundance with −Fe alone take place in the root, and a variety of genes encoding proteins involved in Fe metabolism (i.e. transporters and enzymes involved in strategy I or II Fe uptake) have been characterized in roots. Thus, we further investigated the effects of −Fe, −P, and −Fe−P in roots.

Genes Regulated in Response to Fe Deficiency in Roots

Under Fe-deficient conditions, a total of 3,799 probe sets (2,274 up and 1,525 down) significantly changed ($P < 0.05$, fold change > 2) in transcript abundance in roots (Fig. 2C) after false discovery rate correction. These genes were classified into three classes, according to their putative functions in Fe homeostasis: (1) Fe transport and mobilization (including DMA biosynthesis); (2) TFs; and (3) hormone-related and signal transduction proteins.

Genes involved in Fe acquisition and mobilization were greatly induced by Fe deficiency in roots. As rice plants take up both Fe^{3+}-phytosiderophore iron and Fe^{2+} (Ishimaru et al., 2006), the genes involved in both strategy II- and strategy I-like pathways were significantly changing in abundance under Fe-deficient conditions. As shown in Table II, most of the strategy II genes encoding enzymes in the Met cycle, DMA biosynthesis, and Fe(III)-DMA transporter gene-*OsYSL15* were up-regulated in both roots and shoots. Also, expression of *S*-adnosylmethionine synthetase gene (*OsSAM2*) and Rib 5-P isomerase family protein (*OsRPI*) was up-regulated; however, this was seen

Table I. *Validation of microarray results by RT-PCR*

Fold changes in transcript abundance as determined by microarray analysis (Chip) or by quantitative RT-PCR (qRT-PCR) are shown under the three different growth regimes. Positive numbers indicate increases in gene expression, whereas negative numbers indicate decreases in gene expression.

TIGR Locus Identifier	−Fe+P versus +Fe+P		+Fe−P versus +Fe+P	
	qRT-PCR	Chip	qRT-PCR	Chip
Genes in response to Fe				
Os07g48980	4.3	3.4	1.3	1.1
Os10g30770	3.8	3.8	4.3	3.5
Os01g03630	−50.0	−36.0	1.0	1.6
Os03g46470	33.6	190.6	−1.1	1.3
Genes in response to P				
Os12g01530	−1.3	−1.1	5.4	21.1
Os09g23300	−1.3	−1.6	3.4	3.1
Os03g05334	−1.2	−1.5	483.4	160.7
Os06g40120	−1.7	−1.4	11.5	4.7
Genes in response to Fe and P				
Os03g26210	3.2	5.6	−5.7	−17.2
Os06g29180	3.6	8.9	−2.5	−1.4
Os03g54000	5.7	13.5	−31.5	−4.5
Os03g19427	2.3	2.3	−395.0	−722.5

only in roots (Table II). Besides OsYSL15, the other YSL (for Yellow Stripe-Like) transporter, OsYSL2 (the nicotianamine-metal complex transporter), was also significantly induced under Fe-deficient conditions in both roots and shoots (Table II).

The Fe^{2+} transport genes *OsIRT1* and *OsIRT2* were increased in expression more than 190-fold in roots and 10- to 20-fold in shoots. One natural resistance-associated macrophage protein (NRAMP) family transporter gene, *OsNRAMP1* (Belouchi et al., 1997), was also highly induced, 8.7- and 301.5-fold in roots and shoots, respectively, under Fe-deficient conditions. Arabidopsis NRAMP family members, such as AtNRAMP1, AtNRAMP3, and AtNRAMP4, have been shown to function in Fe transport (Curie et al., 2000; Thomine et al., 2003). The induction of expression of the rice *OsNRAMP1* gene under Fe-deficient conditions indicates that this gene in rice is likely to have a similar function as its Arabidopsis homolog. A number of peptide transporters, including oligopeptide transporters, ATP-binding cassette (ABC) family transporters, and other peptide transporters, were also significantly induced under Fe-deficient conditions (Table I; Supplemental Table S2). Notably, the rice oligopeptide transporter *OsOPT7* (LOC_Os03g54000) was increased 50- to 60-fold in both roots and shoots under Fe-deficient conditions (Table II). Interestingly, its Arabidopsis homolog, oligopeptide transporter (*AtOPT3*), was recently reported to be involved in Fe homeostasis (Stacey et al., 2008), which suggests a similar role for this gene in rice. Another oligopeptide transporter family gene, designated *OsIROPT1* (LOC_Os01g65110), was induced greater than 1,000-fold under Fe-deficient conditions (Table II). This gene has not previously been associated with Fe uptake; however, its strong induction suggests that it could play a role in response to Fe deficiency. ABC family members have been reported to be involved in Fe transport across membranes (Briat et al., 2007). In this study, four putative ABC transporters were significantly induced by Fe deficiency (Table II). Several other peptide transporter genes were highly induced or suppressed by Fe deficiency, ranging from 15.7- to 73.1-fold increases and from 12.0- to 14.5-fold decreases in transcript abundance (Supplemental Table S2), suggesting function(s) in Fe homeostasis.

Changes in expression of a number of TFs were also evaluated. A total of 216 and 160 TFs showed more than 2-fold increases or decreases in root under Fe-deficient conditions (Supplemental Table S3). From these, only 22 and 24 TFs were also induced and repressed, respectively, in shoots. Several TFs that were previously reported to associate with Fe deficiency were significantly changed in abundance under −Fe conditions (Ogo et al., 2007). These TFs include the bHLH family protein IRO2 (Os01g72370) and its downstream targets OsNAC4 (Os01g60020) and the ethylene-responsive element-binding protein AP2 (Os03g64260). Two Fe deficiency-induced bHLH TF family proteins, OsIRbHLH1 (Os12g32400) and OsIRbHLH2 (Os03g26210), one unknown nucleus-localizing protein, and OsIRNLP1 (Os12g18410) and OsNAC1 (Os02g36880; Cheng et al., 2007) were all significantly ($P < 0.05$) up-regulated by more than 2.5-fold in roots (Table II). Apart from IRO2, the functions of these TFs have not been defined. These potentially represent novel regulators of the Fe-deficiency response.

Expression of some genes associated with the plant hormones, including auxin, abscisic acid (ABA), ethylene, and jasmonate metabolism, was also altered by Fe deficiency (Supplemental Table S2). Due to space limi-

Table II. *Selected genes showing 2-fold change in transcript abundance in response to Fe starvation with $P < 0.05$ (the full data set for the selected genes is shown in Supplemental Table S1)*

TIGR locus identifiers are given for each transcript at left. The –Fe ratio of these groups of genes are at least 2. Both the expression ratio in response to –Fe (−Fe+P versus +Fe+P), –P (+Fe−P versus +Fe+P), and –Fe–P (−Fe−P versus +Fe+P) of roots and shoots are shown. Positive numbers indicate increases in gene expression, whereas negative numbers indicate decreases in gene expression. The growth conditions, –Fe, –P, or −Fe−P treatment, and the microarray data analysis are detailed in "Materials and Methods." Genes was classified into six groups according to their putative functions. The description of annotation for each gene at right was according to TIGR general description and the RAP general description.

TIGR Locus Identifier	Fold Change in Root			Fold Change in Shoot			Description
	−Fe	−P	−Fe−P	−Fe	−P	−Fe−P	
DMA biosynthesis							
Os02g20360	5.1	−26.3	1.2	29.0	−3.6	−2.4	Nicotianamine aminotransferase 1 (OsNAAT1)
Os03g13390	8.3	−10.6	−1.5	10.8	−1.1	1.1	Deoxymugineic acid synthase (OsDMAS1)
Os03g19427	2.3	−722.5	1.1	1,807.8	1.0	1.0	Nicotianamine synthase 1 (OsNAS1)
Os03g19420	2.2	−893.4	−1.4	876.4	1.0	1.1	Nicotianamine synthase 2 (OsNAS2)
Os07g48980	3.4	−1.1	1.7	4.7	−1.9	1.3	Nicotianamine synthase 3 (OsNAS3)
Os12g39860	2.2	−1.8	1.1	2.6	1.1	1.1	Adenine phosphoribosyltransferase (OsAPT1)
Os06g02220	3.2	−4.9	−1.4	2.6	1.7	1.9	Methylthioadenosine/*S*-adenosyl homocysteine nucleosidase (OsMTAN)
Os04g57400	6.5	−1.0	1.4	2.9	1.4	1.8	Methylthioribose kinase (OsMTRK)
Os01g22010	4.4	−1.4	1.1	1.3	−1.7	−1.8	*S*-Adenosylmethionine synthetase (OsSAM2)
Os04g24140	5.3	−4.3	1.0	1.2	1.2	1.9	Ribose 5-phosphate isomerase family protein (OsRPI)
Os11g29370	6.4	−2.2	1.0	3.9	1.1	1.3	Dehydratase-enolase-phosphatase (DEP)
Os06g29180	8.9	−1.4	1.5	2.8	1.4	1.5	NAD-dependent formate dehydrogenase (FDH)
Os10g28350	4.8	−1.4	2.3	22.0	20.0	5.5	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase 1 (OsARD1)
Os03g06620	6.9	−2.0	1.5	2.1	1.4	1.3	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxygenase 2 (OsARD2)
Fe transport and mobilization							
Os07g15460	8.7	−10.3	3.8	301.5	−2.0	1.6	Putative NRAMP family metal transporter (OsNRAMP1)
Os02g43410	7.0	−71.0	1.1	27.7	−1.4	−1.3	Fe(III)-DMA transporter (OsYSL15)
Os02g43370	10.3	−1.0	1.0	5.4	1.0	1.0	Metal-nicotianamine transporter (OsYSL2)
Os03g46470	190.6	1.3	9.7	13.8	−1.1	1.2	Fe(II) transporter 1 (OsIRT1)
Os03g46454	192.6	−1.0	19.7	47.1	−1.0	−1.0	Fe(II) transporter 2 (OsIRT2)
Os03g54000	62.1	−1.0	1.0	58.1	−1.0	1.0	Oligopeptide transporter 3 (OsOPT7)
Os01g65110	45.4	−40.6	3.4	1,518.4	−1.0	2.2	OsIROPT1
Os01g42410	10.9	3.0	1.7	10.5	4.6	4.0	PDR5-like ABC transporter
Os04g11820	5.5	1.8	−1.6	3.9	4.4	3.0	ABC transporter family protein
Os08g30740	3.7	2.5	1.4	1.1	1.6	1.0	ABC transporter family protein
Os04g49890	3.6	1.6	1.1	−1.6	−2.6	−2.6	ABC transporter family protein
TF							
Os01g72370	34.5	−1.2	11.2	91.3	−1.1	−1.0	IRO2
Os03g64260	16.3	1.1	1.0	1.1	1.0	−1.0	Ethylene-responsive element-binding protein, putative, expressed (AP2)
Os01g60020	8.8	2.1	−1.7	−1.2	−3.8	−2.2	OsNAC4
Os12g32400	6.1	−6.3	1.6	1.0	1.0	1.0	OsIRbHLH1
Os03g26210	5.6	−17.2	2.0	71.5	−3.6	−1.1	OsIRbHLH2
Os12g18410	28.1	−22.9	1.8	682.9	−1.0	1.0	OsIRNLPl
Os02g36880	2.6	−1.6	−1.7	12.2	−2.0	−1.6	OsNAC1
Phytohormone related							
Os07g05940	10.2	1.1	1.1	−1.5	−1.7	−1.5	Viviparous-14
Os12g42280	11.4	−1.1	−1.1	1.0	1.0	1.0	Viviparous-14
Os05g28210	34.2	−1.0	−1.0	248.9	−1.0	1.0	Embryonic abundant protein 1
Os03g62060	39.2	−1.0	2.2	1.0	−1.2	1.0	IAA-amino acid hydrolase ILR1 precursor
Os06g44970	12.0	9.8	8.9	1.2	−1.0	1.0	Auxin efflux carrier component 2 (OsPIN1c)
Os09g37330	−15.6	1.1	−1.5	1.2	−1.0	1.0	Auxin-responsive SAUR gene family protein
Os01g56240	17.4	1.0	−1.0	1.0	1.0	1.0	Auxin-responsive SAUR gene family protein
Os08g39850	11.4	−1.1	1.0	5.3	−1.1	1.1	Lipoxygenase 8, chloroplast precursor
Os12g12720	13.9	−1.0	−1.1	1.0	1.0	1.0	Jasmonate-induced protein
Os03g52860	21.1	4.6	3.3	1.0	1.1	1.0	Lipoxygenase 2
Os03g55800	34.2	4.0	2.1	1.8	1.2	1.1	Cytochrome P450 74A1, chloroplast precursor
Os06g11290	76.0	2.6	2.6	1.2	2.8	1.0	12-Oxophytodienoate reductase 2
Os09g27820	13.9	10.1	7.2	2.4	1.5	1.3	1-Aminocyclopropane-1-carboxylate oxidase-1

tations, Table II only listed genes with a greater than 10-fold change (and $P < 0.05$). Among the greatly up-regulated genes, there were three ABA pathway genes (Os07g05940, Os12g42280, and Os05g28210) and five jasmonate metabolism-related genes (Os03g52860, Os08g39850, Os03g55800, Os06g11290, and Os12g12720), suggesting that the ABA and jasmonate signaling pathways may be involved in Fe deficiency-induced stress responses. One indole-3-acetic acid (IAA)-amino acid hydrolase (Os03g62060) has been proposed to cleave the IAA-amino acid conjugate to free IAA (LeClere et al., 2002). The auxin efflux carrier component encoding OsPIN1c (Os06g44970) and the auxin-responsive Small Auxin-Up RNA (SAUR) protein family gene (Os01g56240) were found to be induced by Fe deficiency (Table II). The gene encoding 1-aminocyclopropane-1-carboxylate oxidase-1 (Os09g27820), the rate-limiting enzyme in ethylene synthesis, was also seen to be up-regulated by 13.88-fold in –Fe-only conditions.

Effect of P Deficiency on Fe Deficiency-Regulated Genes

To investigate the effect of P supply on the expression of Fe response genes, we compared the −Fe response under +P and –P conditions. Out of the 3,476 transcripts significantly up-regulated in roots, under Fe-deficient and P-sufficient conditions (−Fe+P; Fig. 2B), 3,005 no longer responded to Fe deficiency when the P was removed from the medium (−Fe−P), representing 86.4% of the total number of Fe-responsive genes (Table III). Similarly, 90.3% of the 4,152 down-regulated genes under Fe-deficient and P-sufficient (−Fe+P) conditions showed no significant change in the –Fe-P conditions (Table III). This trend was also seen in shoots under Fe-deficient and P-sufficient (−Fe+P) conditions, where 90.0% and 91.8% of the 1,272 up-regulated and 1,188 of the down-regulated genes, respectively, were unresponsive under Fe- and P-deficient (−Fe−P) conditions (Table III). It is clear that genes found to be Fe responsive were also regulated by P starvation. It was seen that the regulation in response to Fe and P was antagonistic.

Genes Commonly Regulated by –Fe and –Fe–P

Although most of the Fe-deficient response genes could be ameliorated under –P conditions, there are still some genes altered in their transcript abundance in both treatments of –Fe and the combination of –Fe and –P. A total of 473 and 318 transcripts were up- and down-regulated in response to Fe deficiency regardless of +P or –P in the medium (Supplemental Table S4). A number of genes encoding proteins involved in cell wall-related metabolism showed similar induced expression patterns under the conditions of –Fe–P or –Fe+P (Table IV). These included genes encoding cellulose synthase, endoglucanase, expansin, and xyloglucan endotransglucosylase. Genes encoding proteins involved in growth and development were also overrepresented in –Fe under both –P and +P conditions, including two cyclin genes (Os04g47580 and Os01g59120), the putative chromosome condensation protein-encoding gene (Os05g41750), the origin recognition complex subunit 6 (Os07g43540), the mitotic chromosome and X chromosome-associated protein-encoding gene (Os01g67740), and two DNA replication licensing factor genes, *MCM2* (Os11g29380) and *MCM4* (Os01g36390; Supplemental Table S4). This suggests that Fe deficiency could lead to significant changes in growth and development regardless of P supply.

Table III. *The number of changes in transcripts for genes in response to Fe starvation under different P conditions*

Numbers of genes whose transcript change in response to Fe deficiency in P-sufficient and -deficient conditions were determined by comparing the levels of transcripts under −Fe+P versus +Fe+P or under −Fe−P versus +Fe−P. Differential expression analysis was performed with *P* value correction (Benjamini and Hochberg, 1995) at the 0.05 level.

Tissues	Nos. of Fe Response Genes					Percentage Genes[a]
	P Sufficient			P Deficient		
Roots	$P \leq 0.05$	Up	3,476	Up	191	86.4
				Down	280	
				No change	3,005	
		Down	4,152	Up	216	90.3
				Down	186	
				No change	3,750	
Shoots	$P \leq 0.05$	Up	1,272	Up	62	90.0
				Down	62	
				No change	1,145	
		Down	1,188	Up	56	91.8
				Down	41	
				No change	1,091	

[a]The percentage of genes that showed antagonistic responses to –Fe and –P.

Table IV. *Cell wall metabolism-related genes in response to Fe starvation with $P < 0.05$ (the full data set for the selected genes is shown in Supplemental Table S1)*

TIGR Locus Identifier	Fold Change in Root			Fold Change in Shoot			Description
	−Fe	−P	−Fe−P	−Fe	−P	−Fe−P	
Os08g02220	2.5	5.7	5.9	−1.0	−1.1	−1.0	Endoglucanase 1 precursor
Os01g21070	4.9	4.3	6.7	1.1	1.0	1.1	Endoglucanase 1 precursor
Os02g03120	6.4	3.2	10.6	1.0	−1.0	1.0	Endoglucanase 1 precursor
Os04g57860	40.3	20.3	9.0	1.0	1.0	1.0	Endoglucanase precursor
Os06g13830	7.5	2.2	3.9	1.2	−1.1	1.0	Endoglucanase precursor
Os05g08370	54.9	25.9	5.3	50.8	47.3	3.3	CESA1, cellulose synthase, expressed
Os07g24190	2.0	1.8	3.4	−1.1	1.3	−1.2	CESA3, cellulose synthase, expressed
Os07g36750	26.9	3.2	4.1	1.0	−1.0	−1.0	CSLF3, cellulose synthase-like family F
Os01g03710	11.4	1.9	2.3	−1.1	1.2	−1.3	Mannose-6-phosphate isomerase
Os05g29790	2.5	5.9	3.9	1.1	2.8	2.2	Papillar cell-specific pectin methylesterase-like protein
Os04g37650	3.9	3.1	3.4	1.5	1.1	1.1	Fucosyltransferase 7
Os04g51450	5.2	6.2	5.8	1.0	−1.0	1.0	Brassinosteroid-regulated protein BRU1 precursor
Os02g51040	7.8	2.5	8.3	1.3	−1.5	2.1	α-Expansin OsEXPA5
Os03g60720	2.4	2.7	3.5	−1.3	−3.1	−1.5	α-Expansin OsEXPA7
Os10g40700	4.1	1.1	2.7	−1.5	−7.5	−3.3	β-Expansin 1a precursor
Os03g44290	3.2	8.7	3.8	1.1	−1.6	−1.1	β-Expansin 4 precursor
Os04g44780	2.4	1.2	2.9	3.1	−1.3	1.0	β-Expansin 1 precursor
Os07g23660	6.1	8.2	20.6	−1.2	−1.6	−1.4	Vegetative cell wall protein gp1 precursor
Os03g05060	5.0	2.5	2.3	1.0	−1.0	−1.0	Xyloglucan galactosyltransferase KATAMARI 1
Os03g05110	2.1	1.7	2.2	−1.4	1.0	1.0	Xyloglucan galactosyltransferase KATAMARI 1
Os07g34580	7.5	3.8	5.5	3.3	1.2	1.6	Xyloglucan endotransglucosylase
Os06g48200	17.5	12.7	14.4	1.1	1.1	1.2	Xyloglucan endotransglucosylase
Os08g14200	9.3	3.1	5.0	−1.1	−1.1	−1.1	Xyloglucan endotransglucosylase
Os01g47400	3.3	2.2	2.5	−1.1	−1.2	−1.7	Hydrolase, hydrolyzing *O*-glycosyl compounds
Os06g10930	7.7	3.1	1.9	−1.0	−1.1	−1.0	Galactoside 2-α-L-fucosyltransferase
Os04g53950	4.4	4.3	6.4	5.4	−1.1	1.3	Glycosyl hydrolase family 16 protein
Os01g73730	5.0	5.1	4.8	1.0	−1.0	−1.0	Root cap family protein
Os10g31620	6.1	7.6	4.7	−1.1	1.0	−1.1	Glycine-rich cell wall structural protein 2 precursor

In order to determine the specific response to –Fe, we further investigated the genes that respond to –Fe (i.e. −Fe+P and −Fe−P). This selection resulted in 137 up-regulated and 66 down-regulated genes in roots (Supplemental Table S5). Among the 66 genes whose transcripts were down-regulated in abundance, 14 genes encoded TFs. Additionally, under –Fe or –Fe–P but not –P, two ferrous iron transporter genes, *OsIRT1* and *OsIRT2*, are still induced, although their induction decreased from more than 190-fold to about 10- to 20-fold (Table II). *OsNRAMP1*, another NRAMP family transporter gene (Os01g31870), and one ZRT/IRT-like protein 4-encoding gene (Os06g37010) are also included in the 137 up-regulated genes.

Genes Specifically Regulated by –Fe–P

In response to –Fe–P, 699 genes were significantly ($P < 0.05$) differentially expressed by a greater than 2-fold change (Fig. 2C; $P < 0.05$, fold change > 2). The transcripts with significant differential expression, defined as $P < 0.05$ with greater than 5-fold change, are listed in Table V. Three putative ubiquitin pathway genes were differentially expressed in response to the combination of –Fe–P. While RING-H2 finger protein ATL2M (Os01g53500) and Leu-rich repeat family protein (Os04g08390) were highly up-regulated, the F-box domain-containing unknown function DUF295 family protein (Os02g44990) was down-regulated. Among the two differentially expressed TFs, the bZIP TF (Os06g10880) increased in abundance while the MYB86 TF (Os02g02370) decreased in abundance. Interestingly, one gene encoding Argonaute4 (AGO4)-like protein (Os07g28850) was also down-regulated under conditions of –Fe–P. AGO has functions in both development and stress response by regulating gene expression at various levels, including chromatin remodeling and DNA methylation, translational repression, and RNA cleavage (Vaucheret, 2008). The AGO4-like protein reported here does not belong to the four plant AGO subgroups: AGO1, ZIPPY, AGO4, and MEL1 (Nonomura et al., 2007). In this study, a Ser/Thr-protein kinase WNK2 gene (Os07g39520) was found to be repressed by –Fe–P. WNKs have been shown to regulate ion transport in mammals (Gamba, 2005); whether the OsWNK2 acts similarly in ion transport in rice needs to be investigated. The down-regulation of metal ion transporter gene (Os08g03600) by –Fe–P may provide supportive evidence for the above hypothesis.

A large number of stress-related genes were significantly differentially expressed under –Fe–P conditions

Table V. *Selected genes showing a 5-fold change in root transcript abundance in response to −Fe−P treatment (the full data set for the selected genes is shown in Supplemental Table S1)*

TIGR Locus Identifier	Fold Change in Root			Fold Change in Shoot			Description
	−Fe −P	−Fe	−P	−Fe −P	−Fe	−P	
Regulated gene							
Os01g53500	9.8	1.1	1.6	−1.3	−2.3	−1.6	RING-H2 finger protein ATL2M
Os04g08390	5.6	1.5	1.5	1.5	3.9	2.2	Leucine-rich repeat family protein
Os02g44990	−5.9	−1.2	−1.8	−5.3	−1.8	−4.2	F-box domain-containing protein
Os06g10880	5.1	−1.1	1.4	−1.1	−1.0	−1.7	bZIP TF family protein
Os02g02370	−6.5	1.1	−2.0	1.1	1.1	1.1	TF MYB86
Os07g28850	−5.7	1.8	−1.3	−1.2	1.9	−1.2	Argonaute4 (AGO4)-like protein
Os07g39520	−7.1	−1.8	−2.0	−1.1	−1.2	−1.1	Serine/threonine-protein kinase WNK2
Transporter							
Os09g31130	−5.2	1.2	−1.1	−3.7	−2.1	−14.7	Tonoplast dicarboxylate transporter
Os08g03600	−5.5	−1.3	−1.6	−1.0	−1.1	−1.1	Metal ion transporter
Stress related							
Os02g09240	6.8	−1.4	−4.4	1.1	−1.1	1.0	Cytochrome P450 71D8
Os03g03720	10.8	−1.0	−1.3	1.6	1.4	1.0	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor
Os03g18200	6.4	−4.9	−1.2	1.3	1.0	−1.1	Heat shock protein-binding protein
Os06g39780	13.6	2.0	−1.0	1.6	2.3	−1.0	Cytochrome P450 76C1
Os07g33660	6.6	−1.7	2.0	1.9	1.2	1.3	Ankyrin repeat-containing protein
Os08g39860	7.5	−1.2	1.0	1.0	1.2	1.4	β-Glucosidase precursor
Os02g03570	6.1	−1.2	2.0	1.3	−1.0	1.6	18.3-kD class I heat shock protein
Os07g44920	17.1	−1.1	1.6	1.4	1.2	1.7	Similar to dirigent protein
Os01g47070	−5.4	−1.6	−1.1	−1.3	2.1	−3.4	Acidic chitinase OsChib3a precursor
Os04g45970	−5.0	1.8	−1.6	1.1	1.6	1.3	Glutamate dehydrogenase 2
Os05g40070	−12.2	3.2	−2.0	−1.0	1.0	1.0	Elicitor-induced DNA-binding protein
Others							
Os01g19740	9.2	−2.1	1.8	1.7	1.0	1.8	Chloroplast protein 12-1
Os01g43120	6.7	−1.1	2.0	9.5	2.9	5.8	ATP-dependent RNA helicase has1
Os04g48870	6.1	−1.2	1.9	1.1	−1.1	1.0	Nitrilase-associated protein
Os05g41420	5.2	−1.9	1.3	1.0	1.0	1.1	Nodulin-like protein
Os06g37610	5.5	1.2	1.2	1.9	1.6	1.2	Cyclopropane-fatty acyl-phospholipid synthase
Os07g35520	14.5	1.7	1.8	1.0	5.7	−1.2	Glucan endo-1,3-β-glucosidase
Os08g08960	6.9	−1.1	1.7	1.5	−2.2	2.2	Germin-like protein subfamily 1
Os08g10010	7.2	1.7	−1.1	1.0	1.1	1.3	Acyl-desaturase, chloroplast precursor
Os07g32710	26.8	−1.1	1.6	−1.2	2.5	1.4	Expressed protein
Os04g53710	−7.3	−1.8	−1.5	1.0	1.1	−1.0	Early noduin-like protein 1 precursor
Os01g36560	−8.4	1.7	−1.1	1.0	1.0	1.0	Nodulin-like protein
Os03g13050	−9.8	2.8	−1.4	−1.0	1.1	−1.0	Unknown function DUF607 family protein
Os04g55710	−5.5	−1.1	1.1	2.1	−7.2	−1.6	Similar to L-asparaginase
Os04g58280	−5.3	1.1	−1.3	−1.6	−1.1	1.0	Stem-specific protein TSJT1
Os03g12879	−5.8	1.1	−1.9	−3.4	−2.3	−3.4	Expressed protein
Os03g56500	−5.8	−1.5	−1.3	−1.3	1.0	−1.4	Expressed protein
Os04g06520	−5.1	−1.4	−1.1	−1.5	2.0	−2.4	Expressed protein
Os04g35750	−7.7	−1.2	−1.9	1.3	−1.0	1.2	Expressed protein
Os04g49370	−8.9	1.2	−1.6	−3.0	−2.5	−6.5	Expressed protein
Os12g40180	−5.9	1.4	−1.9	−1.1	−1.9	−2.2	Expressed protein

(Table V). Two cytochrome P450 genes (Os02g09240 and Os06g39780), two heat shock protein genes (Os02g03570 and Os03g18200), the glyceraldehyde-3-phosphate dehydrogenase B gene (Os03g03720), the ankyrin repeat-containing protein gene (Os07g33660), the noncyanogenic β-glucosidase precursor gene (Os08g39860), and the dirigent protein gene (Os07g44920) were induced in response to –Fe–P. Three stress-related genes, the acidic class III chitinase (OsChib3a precursor; Os01g47070), the Glu dehydrogenase 2 gene, and the elicitor-induced DNA-binding protein gene (Os05g40070), were repressed. These genes have been shown to be involved in common abiotic stress responses (Rizhsky et al., 2004; Misson et al., 2005). Thus, −Fe−P also appears to result in common abiotic stress responses.

Effect of P Deficiency on Fe Availability

To investigate whether P deficiency influences Fe availability, shoot soluble Fe concentrations of seedlings harvested from the different treatments were measured. Under Fe-sufficient conditions, the total and soluble Fe concentrations in shoots grown in

P-deficiency conditions were 1.8 and 1.4 times greater than those in P sufficiency (Figs. 1D and 3). The increased amounts of both Fe concentrations in shoots should mainly be attributed to the increase of Fe availability in hydroponic medium. The removal of Fe from the hydroponic medium resulted in very low levels of the total Fe concentrations in both –Fe+P and –Fe–P treatments compared with +Fe conditions, as expected (Fig. 1D). However, the amount of soluble Fe in shoots varied depending on the P supply (Fig. 3). The soluble Fe concentration of the plants grown in –Fe–P conditions was 1.27 μg g^{-1} fresh weight, which was double the amount of that in the –Fe+P treatment and similar to that in the +Fe+P treatment (Fig. 3). Thus, the absence of P increased the amount of soluble Fe to almost normal. Combining the results that the expression of Fe deficiency-responsive genes was antagonistically regulated by P deficiency with the finding that the presence of P can affect Fe availability, it is clear that that P can, in turn, regulate the Fe-responsive genes.

DISCUSSION

Many studies have shown that P can interact with Fe to form insoluble complexes in the growth medium or environments (Rediske and Biddulph, 1953; DeKock et al., 1979; Hirsch et al., 2006; Ward et al., 2008). In this study, although removal of Fe in growth medium resulted in very low shoot and root Fe concentrations regardless of the status of P supply (Fig. 1D), the chlorotic symptoms and retarded seedling growth were only observed on the seedlings grown in the presence of P (Fig. 1, A and B). Microarray data showed that the Fe deficiency-induced changes in the transcriptome were greatly reversed by the removal of P in the Fe-deficient medium (Fig. 2). Further analysis showed that the soluble Fe concentration in the seedlings grown in –Fe–P conditions was greatly higher than that in –Fe+P conditions (Fig. 3). We concluded that changes in the expression of Fe deficiency-responsive genes were dependent on the available Fe concentration within plants, which is not only influenced by the environmental Fe concentration but is also dependent on P concentration. Thus, the reason that Fe-responsive genes respond to P deficiency, as observed in previous studies (Wasaki et al., 2003; Misson et al., 2005), is due to the fact that the presence of P can affect the availability of Fe and, in turn, can regulate the Fe response genes.

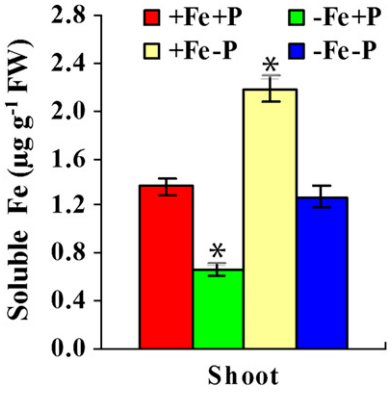

Figure 3. Shoot water-soluble Fe concentration of rice plants grown under different nutrient regimes. Asterisks indicate significance using a two-sample *t* test assuming unequal variance. Significance was defined as $P \leq 0.05$. FW, Fresh weight. [See online article for color version of this figure.]

It has been suggested that the alteration of root morphology by Fe deficiency is regulated by local Fe concentration, while many other –Fe physiological responses (i.e. ferric reductase activity) are controlled by shoot-derived Fe deficiency signals (Schikora and Schmidt, 2001). In our experimental conditions, removal of P improved the Fe deficiency situation in the shoots of –Fe-treated seedlings but did not change the Fe supplies in roots (Fig. 1, A and D). Thus, while most of the –Fe-responsive genes were offset by P deficiency, the genes that respond to the environmental Fe signal were not. For example, under –Fe conditions, the expression of many cell wall-related genes that are up-regulated in roots are still up-regulated in the conditions of –Fe–P, regardless of the difference in soluble Fe concentrations between –Fe+P and –Fe–P treatments.

P deficiency leads to high accumulation of Fe in Arabidopsis (Misson et al., 2005; Hirsch et al., 2006). Our study showed a similar increase of Fe concentration in P-deficient rice seedlings. In contrast to Arabidopsis, in which only the strategy I (IRT1) Fe transporter is used, rice also employs the strategy II pathway to take up Fe from the environment. Interestingly, under –P conditions, the transcripts encoding proteins in the strategy I Fe-uptake pathway showed no difference in expression, while the strategy II pathway (DMA synthesis and OsYSL15) was largely repressed. Further study can be carried out to determine whether the increased amount of Fe was simply attributed to the increased availability of Fe in medium or whether P deficiency triggers the expression of alternative Fe uptake transporter(s) or pathway(s), as has been suggested previously (Hirsch et al., 2006).

Besides the classic genes involved in Fe acquisition, this study also identified several other putative Fe transporter genes found to be up-regulated under Fe-deficient conditions, including *OsNRAMP1*, *OsOPT7*, *OsIROPT1*, and a number of ABC family transporter genes. *OsNRAMP1* was previously identified to be up-regulated in the *naat1* mutant (Cheng et al., 2007), suggesting that it has a role in the rice response to Fe deficiency. The oligopeptide transporter gene *OsOPT7* was induced by Fe deficiency. OsOPT7 was recently shown to transport the Fe^{3+}-nicotianamine complex (Vasconcelos et al., 2008). This gene shares high sequence similarity with Arabidopsis *AtOPT3*, which is also highly induced in Fe deficiency (Stacey et al.,

2008). In addition to those, *OsIROPT1* is a novel oligopeptide that was found to be induced by Fe deficiency in this study. Unlike the expression of *VIT1* in Arabidopsis, which is not responsive to Fe availability (Kim et al., 2006), *OsVIT1;1* is significantly ($P < 0.05$) down-regulated in shoots under Fe-deficient conditions, suggesting the existence of a vacuolar Fe transporter that functions in the regulation of intracellular Fe homeostasis in rice. ABC transporters had been reported to function in intracellular Fe homeostasis related to Fe-S cluster synthesis in mitochondria (Kushnir et al., 2001). The four induced ABC transporters (Table II) regulated by Fe status in this study might have similar functions in rice.

Under Fe-deficient conditions in Arabidopsis, it has been found that the energy demand increases, evidenced by the finding that glycolysis, the tricarboxylic acid cycle, the oxidative pentose phosphate pathway, and anaerobic respiration were all induced (Thimm et al., 2001). The increased expression of genes involved in energy supply observed in this study suggests that a similar response exists in rice. Furthermore, the synthesis of additional aerobic capacity may be limited, as evidenced by the induction of both an alternative NAD(P)H dehydrogenase and an alternative oxidase, which is further indication of increased energy requirements. Lipid synthesis genes induced in response to Fe depletion have been observed in yeast, where under Fe deficiency the activity of Fe-requiring proteins in lipid synthesis pathways was reduced, thereby limiting the levels of reaction products and resulting in decreased feedback inhibition and increased gene transcription (Puig et al., 2005). Consistent with that study, several lipid metabolism genes were also found to be highly up-regulated under –Fe conditions in this study.

Using the rice mutant *naat1*, five Fe-regulated TFs were found to be involved in Fe homeostasis (Cheng et al., 2007). We showed here that similar induction patterns are seen in the background of wild-type rice plants. In addition to these, a number of novel TFs, including bHLH, NAC, and MYB TFs, showed highly increased transcript abundance in response to –Fe, suggesting that there is still a lack of knowledge of the regulation of downstream responses to Fe deficiency or general abiotic stress. Further studies need to be carried out on this aspect through both forward and reverse genetic approaches.

In this study, the Fe- and P-deficiency treatments were conducted in the young rice seedlings. Although the seedlings may carry over some nutrient from rice grains and treatments only lasted for 10 d, the typical –Fe chlorosis symptom and –P symptom (longer roots) were observed (Fig. 1A). The phenotypes were consistent with their measured element concentrations. In addition, typical –Fe-responsive genes (*OsIRT1*, *OsNAS1*, and *OsYSL15*) and –P-induced genes (*OsIPS1* and *OsSPX1*) were up-regulated in our microarray and quantitative RT-PCR results. Thus, the treated seedlings were imposed with Fe- and/or P-deficient conditions as designed. During exposure of plants to –Fe and –P conditions within a relatively short period of time, the rice plants can still remobilize Fe and P from their stored form. It would be interesting to observe the response of rice plants to prolonged periods of –Fe and –P treatment. When the nutrition deprivation continues, to an extent that even the stored Fe and P are exhausted, rice plants would certainly exhibit altered growth phenotypes, and analysis of this response may be of interest in future studies.

MATERIALS AND METHODS

Plant Material and Cultivation Conditions

Rice (*Oryza sativa* 'Nipponbare') was adopted and grown in culture solution prepared as described (Yoshida et al., 1976). The solution contained 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 ZnSO4.

Rice seeds were germinated in distilled water for 2 d. After germination, 25 seedlings were transferred to a plastic net floating on the Yoshida nutrient solution (Yoshida et al., 1976). For –Fe+P treatment, $FeCl_3$ and citric acid were removed. For +Fe−P treatment, NaH_2PO_4 was removed. While for –Fe–P treatment, $FeCl_3$, citric acid, and NaH_2PO_4 were removed. After germination, the plants were grown on the described treatment medium for 10 d. Seedlings were grown in a growth chamber at 30°C/22°C day/night temperatures with a 12-h-light/12-h-dark regime (450 mmol photons m^{-2} s^{-1}).

Measurement of Chlorophyll Content

SPAD values (total chlorophyll content) were determined on the fully expanded youngest leaves of 10-d-old seedlings with a portable chlorophyll meter (SPAD-502; Minolta Sensing).

Measurement of the Total Fe and P Concentrations

To determine the concentrations of total Fe and P of the rice plants, the root and shoot samples were assayed separately. Shoot and root samples were ground to fine powders and digested with 5 mL of 11 M HNO_3 for 5 h at 150°C. Fe and P concentrations were measured using inductively coupled plasma mass spectrometry (Agilent 7500; Agilent Technologies).

Measurements of Pi and Soluble Fe Concentrations in Plants

Pi measurement followed the previously described method (Delhaize and Randall, 1995). Fifty micrograms of fresh sample was homogenized in 5 M H_2SO_4. The homogenate was then diluted 10 to 50 times with double distilled water. Pi concentration was analyzed 30 min after mixing with a malachite green reagent. The absorption values for the solution were determined at 650 nm wavelength. Pi concentration was calculated by normalization of fresh weight.

Extraction of water-soluble Fe was performed as described (Cassin et al., 2009). Briefly, approximately 0.5 to 1 g of shoots of 10-d-old seedlings was ground in liquid nitrogen and extracted by 5 volumes of deionized water at room temperature. After centrifugation, the supernatant was recovered. Fe concentration was measured by inductively coupled plasma mass spectrometry.

Affymetrix GeneChip Analysis

The GeneChip analysis experiment was designed as a two-factor experiment with four treatments: (1) +P+Fe; (2) –Fe+P; (3) +Fe–P; (4) –Fe–P. The experiment included two biological replicates. Each sample represented root and shoot material from five to 10 plants.

Total RNA was extracted using RNAzol (Campro Scientific) and glass beads according to the manufacturer's instructions. The integrity of each RNA sample obtained was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a Bioanalyzer 2100 (Agilent Technologies). The One-Cycle Target Labeling and Control Reagent kit (Affymetrix

900493) and the protocols optimized by Affymetrix were used to prepare biotinylated complementary RNA (cRNA; from 5 μg of total RNA) for microarray hybridization (n = 2 per group). The quality of intermediate products (i.e. biotin-labeled cRNA and fragmented cRNA) was again controlled using the RNA 6000 Nano Lab-on-a-chip and Bioanalyzer 2100. Microarray analysis was carried out using an Affymetrix technology platform and Affymetrix GeneChip rice genome array. Briefly, fragmented cRNA was mixed with spiked controls and applied to Affymetrix Test chips, and good quality samples were then used to hybridize with the rice GeneChip. The hybridization, probe array washing and staining, and washing procedures were executed as described in the Affymetrix protocols, and probe arrays were scanned with a Hewlett-Packard Gene Array Scanner (Leiden Genome Technology Center).

Microarray Data Analyses

Data analysis was carried out using Avadis 4.3 (Strand Genomics) and Partek Genomics suite software, version 6.3. Raw intensity data were initially normalized using the MAS5 algorithm, which allowed probe identifier present calls to be determined. Only those probe sets that were called present in at least two of two replicates under at least one treatment were included for further analysis. Ambiguous probe sets and bacterial controls were also removed, resulting in a final data set of 25,845 probe sets.

Using the 25,845 sets, probe intensities were analyzed using the GC-RMA algorithm and log transformed, and differential expression analysis was performed with P value correction (Benjamini and Hochberg, 1995) at the 0.05 level. This allowed the number of transcripts significantly changing to be calculated. For each of the 25,845 probe sets, in order to carry out hierarchical clustering, the maximum transcript abundance was assigned a value of 1 and all other expression values were made relative to this. Average linkage hierarchical clustering was carried out, and distinct clusters could be identified and were uniquely colored.

Functional Annotation and Statistical Analysis

For each probe set, the Gene Ontology annotations and transcript assignments were retrieved from Affymetrix. The National Science Foundation Rice microarray database was used to match each Affymetrix probe identifier to a National Science Foundation accession identifier and to a The Institute for Genomic Research (TIGR) locus identifier (LOC). These TIGR locus identifiers were then entered into the TIGR rice database, and the putative functions of the encoded proteins were derived (Yuan et al., 2005). In order to gather more functional information, all of the TIGR identifiers were matched to the respective Rice Annotation Project (RAP) identifier using the conversion file available (Tanaka et al., 2008). This enabled the extraction of the relevant functional information, including the RAP description and RAP Gene Ontology description. Lastly, in order to categorize the transcripts based on the FUNctional CATalogue (FUNCAT) of the encoded protein, the Australian National University genebins database was used for all 25,845 probe sets. Two FUNCATs were independently added: TFs, which were formed as a separate category based on the Database of Rice Transcription Factors (Gao et al., 2006), the Rice Transcription Factor Database (Riano-Pachon et al., 2007), and Caldana et al. (2007), and the FUNCATs of kinases, which were based on the rice kinase database (Dardick et al., 2007). In order to compare the differences between the percentile distribution of genes in a given FUNCAT within the 25,845 expressed probe sets and the percentage of genes in that FUNCAT in a given cluster, z score analysis was carried out. This determined the significance of the difference between the two proportions given that we know the sample sizes, frequency, and percentages for each set:

$$z = \frac{\hat{\pi}_1 - \hat{\pi}_2}{\sqrt{\hat{\pi}\left(1-\hat{\pi}\right)\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}}$$

The z scores were then matched to the cumulative standard normal table, and the P values were determined.

Quantitative Real-Time RT-PCR

Total RNA was extracted from plant samples using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. First-strand cDNAs were synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using the SYBR Premix Ex Taq (Perfect Real Time) Kit (TaKaRa Biomedicals) on a LightCycler480 machine (Roche Diagnostics) according to the manufacturer's instructions. The amplification program for SYBR Green I was performed at 94°C for 10 s, 58°C for 10 s, and 72°C for 10 s. Triplicate quantitative assays were performed on each cDNA sample. The housekeeping gene *ACTIN* was used as an internal control. The relative level of expression was calculated using the formula 2-Δ(ΔCp). All of the primers that were used for the RT-PCR are listed in Supplemental Table S4.

Statistical Analysis of Data

For comparisons of treatments in Figure 1, a two-sample t test assuming unequal variances was performed with all samples compared with wild-type plants grown in the presence of Fe and P. Significance was defined as $P \leq 0.05$.

All microarray data from this article can be found in the NCBI GEO data libraries under the accession GSE17245.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Full transcriptome data sets of the 25,845 expressed probe sets in this study.

Supplemental Table S2. Selected genes showing fold change > 2 in root transcript abundance in response to Fe starvation ($P < 0.05$).

Supplemental Table S3. TFs respond specifically to Fe deficiency (fold change > 2, $P < 0.05$).

Supplemental Table S4. Genes showing up- and down-regulation in response to Fe deficiency regardless of +P or −P in the medium (fold change > 2, $P < 0.05$).

Supplemental Table S5. Genes respond to −Fe and −Fe−P, but not −P alone (fold change > 2, $P < 0.05$).

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