The Effect of Iron on the Primary Root Elongation of Arabidopsis during Phosphate Deficiency[1][W][OA]

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Root architecture differences have been linked to the survival of plants on phosphate (P)-deficient soils, as well as to the improved yields of P-efficient crop cultivars. To understand how these differences arise, we have studied the root architectures of P-deficient Arabidopsis (*Arabidopsis thaliana* Columbia-0) plants. A striking aspect of the root architecture of these plants is that their primary root elongation is inhibited when grown on P-deficient medium. Here, we present evidence suggesting that this inhibition is a result of iron (Fe) toxicity. When the Fe concentration in P-deficient medium is reduced, we observe elongation of the primary root without an increase in P availability or a corresponding change in the expression of P deficiency-regulated genes. Recovery of the primary root elongation is associated with larger plant weights, improved ability to take up P from the medium, and increased tissue P content. This suggests that manipulating Fe availability to a plant could be a valuable strategy for improving a plant's ability to tolerate P deficiency.

Phosphate (P) deficiency is a major constraint to crop production (Raghothama, 1999). Crop varieties that are adapted to P-deficient soils have been shown to have shallower, more highly branched root systems to maximize foraging in the relatively P-rich topsoil (Liao et al., 2001; Lynch and Brown, 2001). Further, it has been shown that uptake of P is greatly enhanced in barley (*Hordeum vulgare*) and peanut (*Arachis hypogaea*) cultivars that develop long root hairs compared to those that form short root hairs (Wissuwa and Ae, 2001; Gahoonia and Nielsen, 2004). Members of the Proteaceae and a few species in seven other families known for their P efficiency also develop cluster roots, a specialized root architecture consisting of many densely spaced rootlets (Lamont, 2003; Shane and Lambers, 2005). At present, little is known about how these root architecture differences arise during P deficiency.

One line of inquiry into this problem has been to study the root architecture changes that occur when the model plant Arabidopsis (*Arabidopsis thaliana*), specifically the Columbia (Col-0) accession, is subjected to P deficiency. It has been established that, under P deficiency, the elongation of the Arabidopsis primary root is inhibited (López-Bucio et al., 2002; Sánchez-Calderón et al., 2005; Jain et al., 2007). This response to P deficiency is rather unique to Arabidopsis accessions like Col-0. Most other plants allocate more resources to their roots during P deficiency and actually have longer roots. Maize (*Zea mays*) under P deficiency showed no reduction or slight enhancement in root elongation (Mollier and Pellerin, 1999). Similarly, elongation of the roots of rice (*Oryza sativa*) is observed during P deficiency (Shimizu et al., 2004). Further, a survey of 14 monocots and dicots in solution culture revealed that all the species tested had some degree of primary root elongation during P deficiency (Narayanan and Reddy, 1982). On the other hand, primary root inhibition is a well-documented response to the toxicity of a number of nutrients, including iron (Fe; Marschner, 1995).

Arabidopsis root architecture during P-deficiency conditions has not previously been thoroughly investigated in the context of how it is influenced by the specific and nonspecific interactions P has with other nutrients. Nutrient interactions have been shown to be important for the manifestation of deficiency and toxicity symptoms in many plants (Marschner, 1995). In some cases, deficiency and toxicity symptoms can be alleviated by increasing or decreasing another nutrient concentration, as in the case of adding extra potassium (K) to ameliorate Fe toxicity in rice (Li et al., 2001). There are a number of lines of evidence to suggest that nutrient interactions could be affecting the growth of Arabidopsis on P-depleted medium. For example, there is an increase in the expression of the ferritin Fe storage gene, *AtFER1*, and a decrease in the expression of the high-affinity Fe transporter, *AtIRT1*, observed in the P microarray analysis that correlates with the increase in tissue concentration of Fe in P-deficient plants (Mission et al., 2005).

Fe is always a concern for studies of P because it can interact with P in the soil/growth medium, at the root surface, and within the plant. About one-third of

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agricultural soils are P deficient because of the precipitation of P at least in part by Fe (Von Vexhall and Mutert, 1998). In nutrient solutions, P and Fe interact to form precipitates that result in reductions in the availability of both nutrients (Dalton et al., 1983). On the root surfaces, plaques of Fe form that act as a barrier to movement of P into the root (Zhang et al., 1999). Within the plant, P retention in the roots increases and P translocation to the shoot decreases in a concentration-dependent manner when Fe is absorbed at the same time as the P (Cumbus et al., 1977; Mathan and Amberger, 1977).

It has also been shown in plants that Fe is normally linked with P when it is bound in ferritin, which suggests that the ferritin-bound Fe is a potential plant sink for P (Waldo et al., 1995). It has been demonstrated that chlorosis can be induced in leaves by high P concentrations even when the levels of total Fe are comparable to those of healthy green leaves because P has made the Fe unavailable for the chloroplasts (DeKock et al., 1979). Besides these reports, the antagonistic interactions of P and Fe may manifest themselves in ways that have not been appreciated.

Here, we present evidence that the primary root growth inhibition of Arabidopsis (Col-0) during P deficiency is due to Fe toxicity in the root tip. When Fe is removed in P-deficient medium, the primary root continues to grow even though other tested aspects of the plant's response to P deficiency are unchanged. Further, when Fe is removed, there is overall improved growth of the plants on P-deficient medium. This suggests that manipulating the interactions P has with Fe within the plant root could be a novel way of improving the P nutrition of crops.

RESULTS

The Effect of the Interaction of P and Fe in the Nutrient Medium on the Growth of Arabidopsis

Several lines of evidence suggest that Fe could be interacting with P and influencing the growth of Arabidopsis (Col-0). To explore this, experiments were designed to examine the growth and root architecture of Arabidopsis on the medium with reduced concentrations of both P and Fe. The medium used in this study was based on the original Murashige and Skoog medium, as published by Murashige and Skoog (1962). Further, it was identical to that used by López-Bucio et al. (2002) for their work on the root architecture of Arabidopsis Col-0 ecotype during P deficiency. Four concentrations of added P were used: 1,000 μM (I), 100 μM (II), 10 μM (III), and 0 μM (IV); and two concentrations of added FeEDTA: 100 μM (a) and 10 μM (b). We observed that on medium containing 100 μM Fe, root elongation was directly correlated with the amount of P added to the medium. The shortest roots developed at the lowest P concentrations (Fig. 1, A and B). This observation was similar to that reported by López-Bucio et al. (2002), Sánchez-Calderón et al. (2005), and Jain et al. (2007).

In contrast, on medium containing only 10 μM Fe, the root architecture of Arabidopsis was markedly different and not correlated with the amount of P added to the medium (Fig. 1, A and B). There was no reduction in the primary root elongation when P was reduced in the medium. The marginally shortest primary roots were of plants grown on medium containing 1,000 μM P. These plants were chlorotic and likely Fe deficient because of the high P to Fe ratio in this medium.

The P content of tissues from plants grown with either 100 or 10 μM Fe was found to be proportional to the amount of P added to the medium (Fig. 1C). There were slight increases in the P content of plants grown on low Fe medium when compared to the P content of plants grown on normal Fe medium; however, even when plants with similar P content were compared, there was still much greater primary root growth when Fe was reduced in the medium. When root length is plotted against P content (Fig. 1D), it becomes evident that the root lengths of the plants grown on normal and low Fe medium lie on two separate nonparallel lines and that there is no correlation between primary root length and P content ($r^2 = 0.19$).

However, unlike P, root length is correlated with the plant's Fe content (Fig. 1, E and F). There is an increase in the Fe content of Arabidopsis when grown on medium with less added P, as previously reported in the microarray studies (Mission et al., 2005). Moreover, when the Fe content of plants is plotted against their root lengths, it is clear that root length is strongly correlated with tissue concentration of Fe ($r^2 = 0.99$).

Dry weights of the plants grown on normal and reduced Fe medium were found to be reduced for all the plants grown on medium with reduced concentrations of P. Dry weights were slightly larger for plants grown on low Fe medium than for normal Fe medium, except at the highest concentration of P. This suggests that, even though root elongation was independent of the P added to the medium, overall growth as measured by dry weight was not (Fig. 1G). When the total P taken up by the seedling is computed from the dry weight data and the tissue P concentration, it shows that the plants grown on low Fe medium were able to acquire at some concentrations more than twice as much P for their growth compared to plants grown on medium with normal levels of Fe (Fig. 1H).

In conjunction with the other experiments, attempts were made to model medium with either 1,000 μM (P+) or 0 μM (P−) P and either 100 μM (Fe+) or 10 μM (Fe−) Fe using the Visual Minteq chemical speciation and equilibrium computer program to determine whether the changes observed in the elemental composition of the plants were a consequence of the experimental design or mechanistic

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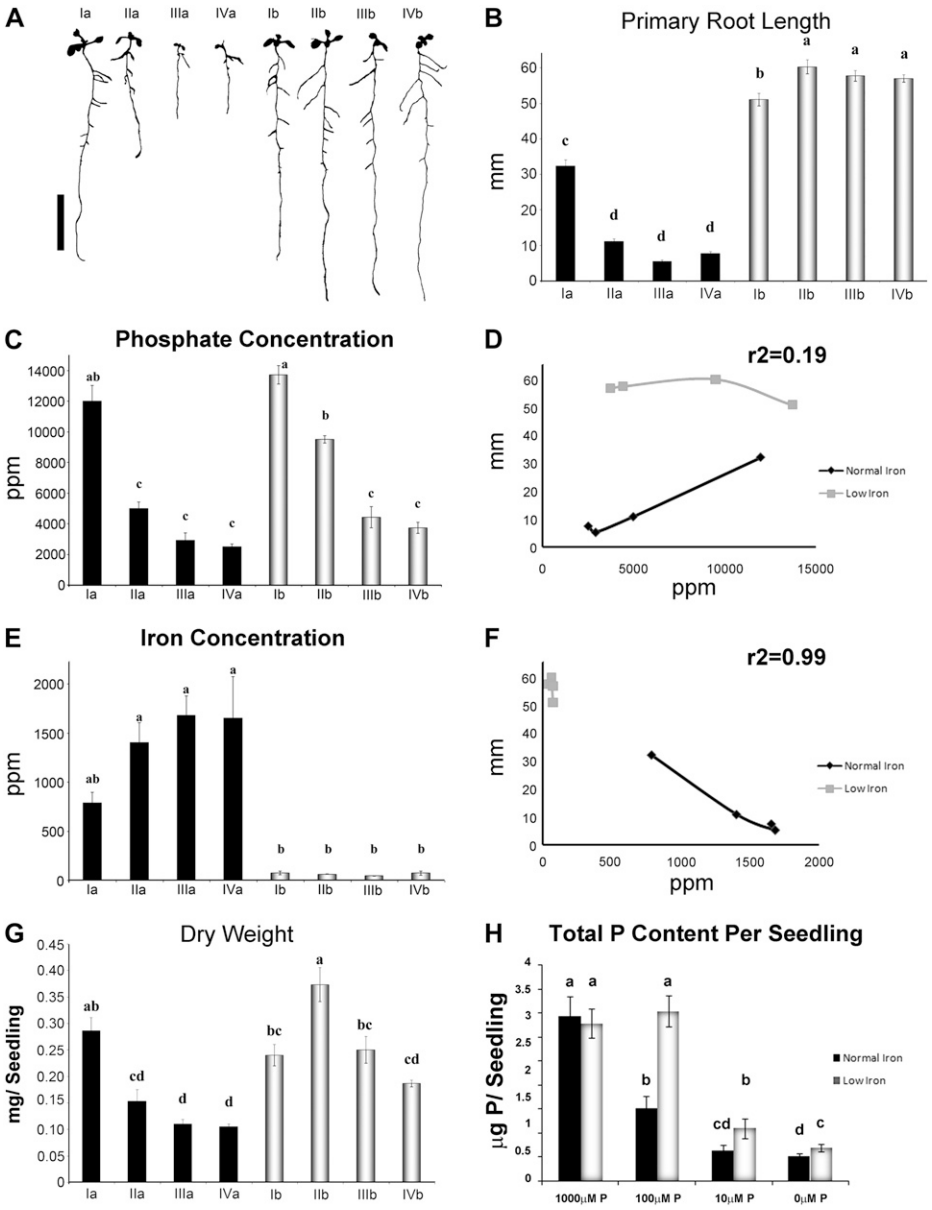

Figure 1. The growth and ion content of Col-0 accession of Arabidopsis seedlings grown on medium with different concentrations of P and Fe. Ia = 1,000 μM P, 100 μM FeEDTA; IIa = 100 μM P, 100 μM FeEDTA; IIIa = 10 μM P, 100 μM FeEDTA; IVa = 0 μM P, 100 μM FeEDTA; Ib = 1,000 μM P, 10 μM FeEDTA; IIb = 100 μM P, 10 μM FeEDTA; IIIb = 10 μM P, 10 μM FeEDTA; IVb = 0 μM P, 10 μM FeEDTA. A, Representative 9-d-old seedlings grown on medium with different concentrations of P and Fe. B, Primary root lengths as measured for 15 plants for each treatment using the ImageJ program. C, Tissue P concentrations as determined by ICP-MS for each treatment. D, Average tissue P concentrations for each treatment (C) plotted against the average primary root length for each treatment (B). E, Their tissue Fe concentrations as determined by ICP-MS for each treatment. F, Average tissue Fe concentrations for each treatment (E) plotted against the average primary root length for each treatment (B). G, Their dry weights as determined for three groups of 10 plants and these weights divided to determine individual seedling weights. H, Their total P content computed from the tissue concentration of P multiplied by their dry weight. Lowercase letters indicate significance by a two-factor ANOVA and Tukey's post-hoc analysis at a $P < 0.05$. r^2 indicates the correlation coefficient or amount of variation accounted for by the linear regression. Scale bar = 10 mm.

and associated with P deficiency (Allison et al., 1991; Parker et al., 1995). The predicted percent of the ions in their bioavailable form in the medium were calculated, with the assumption that, in most cases, the bioavailable forms were the ions in their free unassociated states. Verification of the computer predictions came in the form of their broad agreement with the elemental analysis of actual plants (Fig. 2).

When P was removed from medium with Fe, there was a 25% increase in the availability of free Fe, which supports our conclusion that reduced primary root elongation is due to Fe excess caused by an increase in Fe bioavailability. Furthermore, when Fe was reduced in the low P medium, the availability of free P was predicted to change by <2%, whereas the free Fe availability decreased by 60%. The predicted lack of a large increase in free P availability when Fe is reduced in the medium suggests that increased root elongation under these conditions is not due to an increase in free P availability as was recently suggested by Svistoonoff et al. (2007).

In addition to computer modeling, experiments with Dowex-purified agar were also done to show that neither a change in the availability of P nor impurities of P in medium were affecting the experiments with reduced P and Fe (Supplemental Fig. S1). These experiments again support the conclusion that it is not the absence of P, but the increased availability of Fe, that is responsible for the root inhibition that is normally observed when plants are grown on P-depleted medium.

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		H2PO4-	Fe++	K+	SO42-	Mg++	NH4+	NO3-	Mn++	Cu++	Ca++	Cl-	I-	Co++	Zn++	Na+	H3BO3	MoO42-	EDTA
Modified MS Medium	P+ Fe+	1mM	0.1mM	2.885mM	0.3801mM	0.15mM	2.06mM	3.94mM	0.1mM	0.1uM	0.3mM	0.6002mM	5μM	0.1μM	30μM	0.102mM	0.1mM	1μM	0.1mM
	P- Fe+	0μM	0.1mM	2.885mM	0.8801mM	0.15mM	2.06mM	3.94mM	0.1mM	0.1uM	0.3mM	0.6002mM	5μM	0.1μM	30μM	0.102mM	0.1mM	1μM	0.1mM
	P+ Fe-	1mM	0.01mM	2.885mM	0.3801mM	0.15mM	2.06mM	3.94mM	0.1mM	0.1uM	0.3mM	0.6002mM	5μM	0.1μM	30μM	0.102mM	0.1mM	1μM	0.1mM
	P- Fe-	0μM	0.01mM	2.885mM	0.8801mM	0.15mM	2.06mM	3.94mM	0.1mM	0.1uM	0.3mM	0.6002mM	5μM	0.1μM	30μM	0.102mM	0.1mM	1μM	0.1mM
Predicted Availabilty (%) by Visual Minteq	P+ Fe+	92.85	39.95	99.42	92.21	95.7	99.71	99.77	66.7	99.48	93.26	99.78	99.91	0.66	0.95	99.56	99.97	87.95	0
	P- Fe+	0	49.92	99.37	92.28	93.22	99.38	99.78	70.03	99.49	91	99.78	99.91	0.77	1.11	99.58	99.97	88.36	0
	P+ Fe-	93.92	68.79	99.42	92.17	95.65	99.71	99.77	91.89	0.62	93.22	99.78	99.91	55.46	62.52	99.56	99.97	87.86	0
	P- Fe-	0	90.75	99.37	92.33	93.16	99.37	99.77	92.89	0.62	90.94	99.78	99.91	54.64	62.04	99.57	99.97	88.27	0
Measured Tissue Content (PPM) by ICP-MS	P+ Fe+	11995+1038	788±112	38096±5065	nm	1579±142	nm	nm	470±39	5±0.3	2630±282	nm	nm	1±0.09	298±18	9913±282	nm	10+0.9	nm
	P- Fe+	2511±170	1655±423	13585±1831	nm	684±70	nm	nm	358±9	3±0.4	2008±95	nm	nm	1±0.09	353±31	4387±1051	nm	3±0.04	nm
	P+ Fe-	13719±601	76±17	48727± 2782	nm	2348 ±129	nm	nm	724± 24	11± 0.5	4317± 439	nm	nm	4± 0.7	2603± 263	10216±526	nm	44±3	nm
	P- Fe-	3731± 364	74±21	30292± 1655	nm	1408±61	nm	nm	436±13	10± 0.9	2538±54	nm	nm	2± 0.04	1037± 52	7880±603	nm	20±3	nm

Figure 2. Comparison of the concentration of ions added to medium as salts, with the predicted percentage of the ions in their bioavailable form in the medium, and with the tissue ion content of plants grown on the medium. P+Fe+ = 1,000 μM P, 100 μM; P+Fe− = 0 μM P, 100 μM FeEDTA; P+Fe− = 1,000 μM P, 10 μM FeEDTA; P−Fe− = 0 μM P, 10 μM FeEDTA. The free unassociated state of the ion in most cases is assumed to be the bioavailable form of the ion. The Visual Minteq predictions include the 20 μM of P and 10 μM of Fe introduced as impurities with the agar. However, by convention, they are not included as part of the basal nutrient solution recipe. The elemental analysis of 9-d-old seedlings was determined by ICP-MS.

Regulation of P Starvation-Responsive Genes in Arabidopsis Plants Grown on Low P and Low Fe Medium

To determine whether plants with elongated primary roots were P stressed at the molecular level, the expression of a number of P-responsive genes was assessed. RNA was isolated from the plants grown on the previously discussed medium. In addition, transgenic Arabidopsis plants containing a GUS reporter driven by the Pht1;4 promoter as a marker of P deficiency were grown under the same conditions and analyzed for their reporter gene expression (Karthikeyan et al., 2002). Six nutrient-responsive genes and an elongation factor (*EF1α*) were amplified with gene-specific primers. Four of these genes are known to be expressed at increased levels under P deficiency: *Pht1;4*, a high-affinity P transporter; *At4*, a riboregulator; *ACP5*, a purple acid phosphatase; and *PAP23*, a purple acid phosphatase. One of the genes is known to be down-regulated by P deficiency: *UBC24*, a ubiquitin-conjugating E2 ligase. The seventh is up-regulated by Fe deficiency: *IRT1*, the high-affinity Fe transporter.

As expected, plants grown on low P medium showed clear regulation of the genes known to respond to P deficiency (Fig. 3A; Supplemental Fig. S2). GUS activity was also found to be enhanced in plants grown on medium containing low P (Fig. 3B). Significantly, the Fe status of the medium had no effect on the response to low P as monitored by the expression of P-responsive genes and GUS activity. This confirms that primary root elongation is not correlated with P deficiency under our experimental conditions (Fig. 3A; Supplemental Fig. S2). Therefore, we conclude that primary root elongation is not a phenotypic surrogate for the molecular P-deficiency responses in Arabidopsis (Col-0).

The results with the transgenic plants highlight the likely reason that long-rooted seedlings acquire more P from P-deficient medium. They have increased capacity to explore the plate when compared to the short-rooted plants grown on low P, normal Fe medium. The ability of plants grown on medium containing reduced P and reduced Fe to take up P was confirmed using [^{33}P]. It was found that plants growing on medium containing low P and low Fe took up more than twice as much [^{33}P] as plants grown on medium with low P and normal amounts of Fe (Fig. 3C).

Effect of Additional Fe on Plants Grown on P-Sufficient Medium

The results of our previous experiments suggested that plants grown on medium containing sufficient P, but Fe added in excess of normal, would be short rooted but not P deficient. To test this, transgenic *pht1;4::gus* plants that had been grown on P-sufficient medium with double the concentration of Fe (1,000 μM P and 200 μM Fe) were examined. As expected, the seedlings had short roots (Fig. 4A). They had root lengths <10 mm and significant reductions in their dry weights (Fig. 4, B and C). However, contrary to predictions, the roots had elevated levels of expression of the *pht1;4* phosphate transporter, as indicated by GUS activity. Despite the abundance of P in the medium, these plants were P deficient (Fig. 4A).

Elemental analysis showed that plants grown on medium with elevated Fe had approximately 60% of the tissue P concentration as plants grown on P-sufficient medium with the normal concentration of Fe (Fig. 4D). Computer modeling predicted that the extra Fe added to the P-sufficient medium had little impact on the availability of P, just as it suggested that removal of the Fe from the basal medium would not severely impact P availability. This suggests that the reduction in P in the tissues of the plants grown on P-sufficient medium with additional Fe was likely a consequence of the short roots of these plants. This hypothesis is supported by the earlier evidence that showed that long-rooted plants took up more P from

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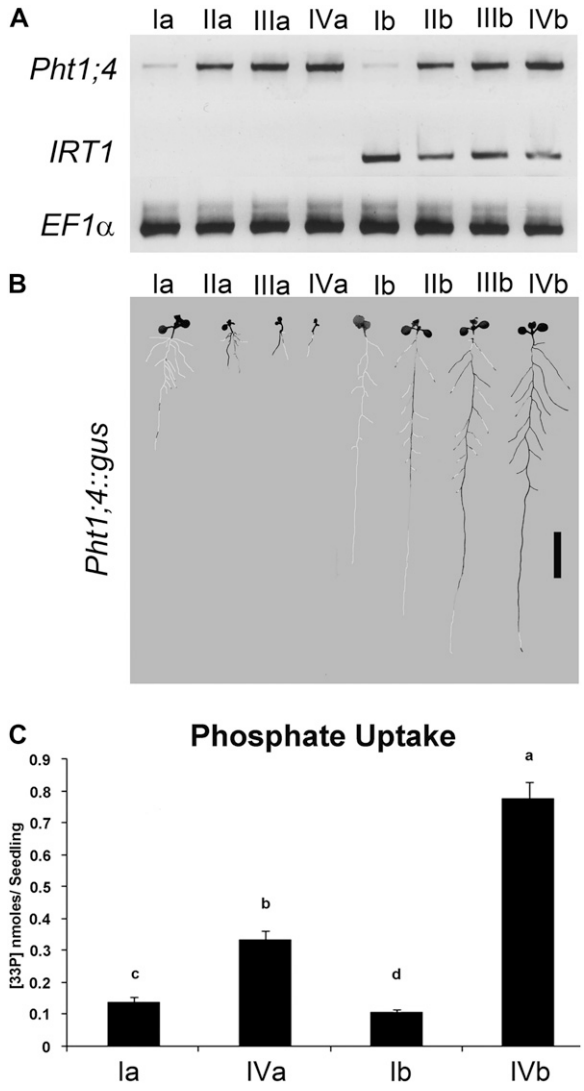

Figure 3. Gene expression changes and [^{33}P] uptake of plants grown on the same concentrations of P and Fe mentioned in Figure 1. Ia = 1,000 μM P, 100 μM FeEDTA; IIa = 100 μM P, 100 μM FeEDTA; IIIa = 10 μM P, 100 μM FeEDTA; IVa = 0 μM P, 100 μM FeEDTA; Ib = 1,000 μM P, 10 μM FeEDTA; IIb = 100 μM P, 10 μM FeEDTA; IIIb = 10 μM P, 10 μM FeEDTA; IVb = 0 μM P, 10 μM FeEDTA. A, RT-PCR of 9-d-old wild-type plants for *Pht1;4*, the high-affinity P transporter, and *irt1*, the high-affinity Fe transporter. B, Representative 9-d-old *Pht1;4*::*GUS* seedlings showing reporter gene expression. C, [^{33}P] uptake of seedlings grown on the four major media and then transferred for 2 h to identical uptake solutions containing 70 μM P and 0.15 μCi/mL [^{33}P]. Lowercase letters indicate significance by a two-factor ANOVA and Tukey's post-hoc analysis at a $P < 0.05$. Scale bar = 10 mm.

P-deficient medium than short-rooted plants (Figs. 1H and 3C). It is also supported by the similar changes in the tissue concentrations of nutrients, particularly the reductions in the concentrations of K and magnesium (Mg), of the short-rooted plants grown on P-sufficient medium with double the concentration of Fe compared to the short-rooted plants grown on P-deficient medium with the normal concentration of Fe (Figs. 2 and 4F).

Fe Is Inhibiting the Elongation of the Primary Root

Computer modeling and elemental analysis showed that the availability and tissue content of several other nutrients besides P and Fe were unintentionally altered in plants grown on medium with reduced P and Fe content (Fig. 2). These changes were investigated as potential causes or contributing factors in the primary root inhibition observed during P deficiency.

One source of these changes is that, in Murashige and Skoog-based nutrient solutions, Fe is solubilized by the addition of equal molar amounts of the chelator EDTA. EDTA balances the availability of Fe with the availability of the other metals in the medium because binding of metals is nonspecific and concentration dependent (Chaney, 1988). In accordance with this expectation, computer modeling predicted that the availability of several metals (manganese [Mn], cobalt [Co], and zinc [Zn]) were increased in addition to Fe in medium containing low P and normal levels of Fe (Fig. 2). However, computer modeling also predicted that the availability of these metals was increased in low P, low Fe medium (Fig. 2). Therefore, toxicity of these metals was ruled out as the cause of the primary root inhibition during P deficiency. This was further confirmed by the substitution of HBED, a chelator that only has limited interactions with the other metals (Supplemental Fig. S3).

Computer modeling predicted no major changes in the availability of K, Mg, and sodium (Na), but elemental analysis showed that there were large reductions in the tissue concentrations of these ions in seedlings grown on low P, normal Fe medium (Fig. 2). P deficiency was ruled out as the primary cause of the reduction of these nutrients because the severe reductions in these nutrients were observed only in short-rooted plants. However, it was still not known whether deficiencies of K and Mg were involved in, or the cause of, the primary root inhibition when P was removed from normal Fe medium. K is of particular interest because it has been previously implicated in cell expansion and root growth (Ashley et al., 2006). However, raising the medium and tissue concentrations of K and Mg did not rescue the root elongation as compared to that of the plants grown on low P and low Fe medium (Supplemental Fig. S4). This shows that reduced accumulations of K and Mg are not the causes of the inhibition of primary root elongation.

Primary Root Growth Inhibition Is a Response to the Locally High Concentrations of Fe within the Root

All of the evidence supports the conclusion that inhibition of the primary root growth of plants grown on medium containing reduced P is caused by Fe toxicity. However, it was not known whether the response was based on the whole plant Fe status or just on the Fe status of the root. To investigate this, a split-plate experiment was performed in which the top half of a petri dish contained either solidified 1,000 μM

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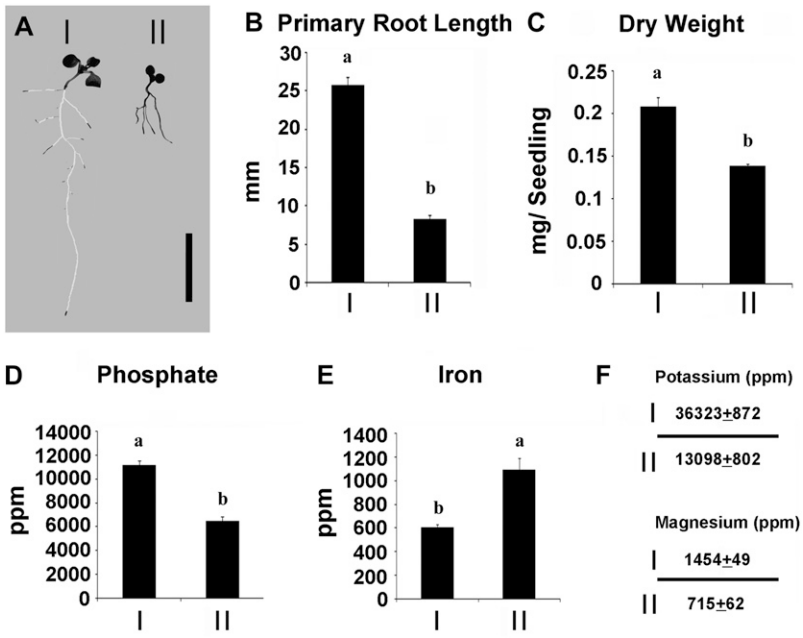

Figure 4. Growth and gene expression of *Pht1;4*:: GUS plants grown on P+ medium with twice the normal amount of Fe. I = 1,000 μM P, 100 μM FeEDTA; II = 1,000 μM P, 100 μM FeEDTA + 100 μM $FeSO_4$. A, Representative 9-d-old seedlings for each of the treatments. B, Primary root lengths as measured for 15 plants for each of the treatments using the ImageJ program. C, Dry weights as determined for three groups of 10 plants for each treatment with these weights divided to determine individual seedling weights. D, Their tissue P concentrations as determined by ICP-MS for each of the treatments. E, Tissue Fe concentrations as determined by ICP-MS for each of the treatments. F, Tables of the tissue K and Mg concentrations as determined by ICP-MS for each of the treatments. Lowercase letters indicate significance by a two-factor ANOVA and Tukey's post-hoc analysis at a $P < 0.05$. Scale bar = 10 mm.

P and 100 μM Fe (P+Fe+) or 0 μM P and 10 μM Fe (P−Fe−) medium, and the bottom half of the dish contained either solidified 1,000 μM P and 100 μM Fe (P+Fe+), 0 μM P and 100 μM Fe (P−Fe+), or 0 μM P and 10 μM Fe (P−Fe−) medium. Only when the bottom half of the plate contained low P, normal Fe medium did we observe cessation of the growth of the primary root (Fig. 5A). This strongly supports the conclusion that the primary root inhibition is a response to the Fe excess in the local root environment during P deficiency.

Effect on Root Growth in the Fe Hyperaccumulating Mutant *frd3* of Reducing P in the Medium

Based on the previous experiments, we predicted that the *frd3* Arabidopsis (Col-0) mutant, known to hyperaccumulate Fe in the roots (Rogers and Guerinot, 2002), should manifest hypersensitivity to root inhibition when grown on medium with reduced P. This mutant was found to have increasingly shorter roots compared to Col-0 wild type as Fe concentrations were raised from 10 μM to 100 μM in P-deficient medium (Fig. 5B). At 100 μM Fe, the primary root length of *frd3* was severely reduced compared to wild type. However, at 10 μM Fe, their primary roots were only slightly shorter than that of wild type (Fig. 5C).

Differences in the Response of the Llagostera-0 Accession to P Deficiency Are Because It Has Increased Tolerance to Fe

The Llagostera-0 (Ll-0) accession of Arabidopsis was previously identified as an accession that does not display the primary root inhibition normally observed for Col-0 of Arabidopsis when grown on medium containing reduced P (Chevalier et al., 2003). Based on the evidence presented here that the primary root inhibition is actually due to root Fe toxicity, we predicted that the Ll-0 accession is actually more resistant to root Fe toxicity.

Initially, on the basal medium used for this study, Ll-0 did not respond differently from the Col-0 accession. It was determined that the medium used by Chevalier et al. (2003), on which Ll-0 showed resistance to primary root inhibition, did not contain ammonia as the nitrogen source, unlike the basal medium used in our study. This led to the removal of the ammonia from all of the media used in the experiments with Ll-0. Ammonia is known to reduce the pH of the rhizosphere, which would be expected to increase Fe availability.

After removal of the ammonia from the medium, Ll-0 displayed the expected improvement in primary root elongation. At 50 μM Fe, the primary root of Ll-0 was more than twice that of Col-0 (Fig. 6, A and B). Elemental analysis of the Ll-0 and Col-0 plants established that the increased resistance to Fe toxicity-induced root inhibition was not due to Fe exclusion. The Ll-0 had approximately 60% higher tissue concentrations of Fe on medium concentrations of Fe (50 and 75 μM) in which there was increased elongation of the primary root of Ll-0 compared to Col-0 (Fig. 6, C and D).

DISCUSSION

In this study, the interactions of P and Fe were evaluated by assessing the growth of Arabidopsis Col-0 accession on medium in which the concentrations of P and Fe were varied simultaneously. Normally, when

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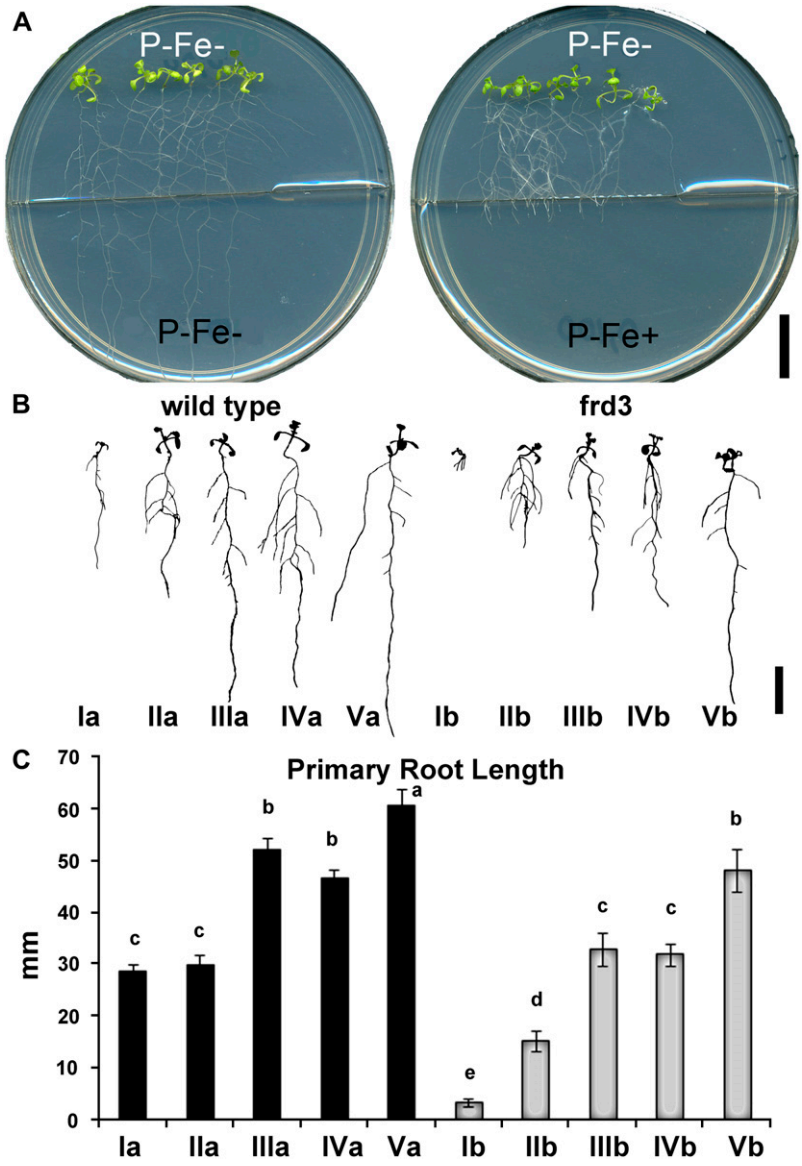

Figure 5. The response of the primary root to iron in the medium and within the plant. A, Split-plate experiment showing the growth of 14-d-old wild-type seedlings. The upper half of the plates contains 0 μM P, 10 μM FeEDTA (P−Fe−) medium. The lower half contains either 0 μM P, 10 μM FeEDTA (P−Fe−) medium or 0 μM P, 100 μM FeEDTA (P−Fe+) medium. B, Representative 9-d-old wild-type seedlings (a) compared to representative *frd3* mutant seedlings (b) grown on P− medium with different concentrations of Fe. *frd3* mutants have lower shoot Fe concentrations and elevated root Fe concentrations compared to wild-type plants. I = 0 μM P, 100 μM FeEDTA; II = 0 μM P, 75 μM FeEDTA; III = 0 μM P, 50 μM FeEDTA; IVa = 0 μM P, 25 μM Fe; V = 0 μM P, 10 μM FeEDTA. C, Primary root lengths of the wild-type and *frd3* seedlings grown under the different nutrient conditions. Lowercase letters indicate significance by a two-factor ANOVA and Tukey's post-hoc analysis at a $P < 0.05$. Scale bar = 10 mm.

P is removed from medium in which the Fe concentration is unaltered, the growth of the primary root of the Col-0 accession is inhibited (López-Bucio et al., 2002; Sánchez-Calderón et al., 2005; Jain et al., 2007). Further, when the available Fe is also reduced in low P medium, the primary root of the Col-0 ecotype continues to grow. It has been suggested that this is due to increase in the availability of P in the medium with reduced Fe (Svistoonoff et al., 2007). However, we conclude from the evidence presented here that the primary root elongation observed in Col-0 when grown on low P and Fe medium is, in fact, due to the elimination of Fe toxicity in the root.

Root lengths are found to show no correlation with the tissue concentrations of P or with the regulation of known P-responsive genes when plants grown on low Fe medium are considered. However, root lengths show a strong negative correlation with tissue concentrations of Fe, indicative of a response to Fe toxicity sensed by the roots in the absence of P. In support of this hypothesis, the *frd3* mutant, known to hyperaccumulate Fe in the roots, was found to be hypersensitive to inhibition of root elongation in low P medium. Furthermore, the Ll-0 accession of Arabidopsis, previously observed not to display the short root phenotype on medium containing low P, was found in this study to be more resistant to the inhibition of root elongation by Fe.

Many studies have shown that the primary root response of Arabidopsis during P deficiency is completely separate from the systemically controlled changes in gene expression (Linkohr et al., 2002; Ticconi et al., 2004; Jain et al., 2007). This study suggests that the two responses are separate because they are likely regulated by two different nutrients. Reduced primary root growth is due to Fe toxicity, whereas changes in

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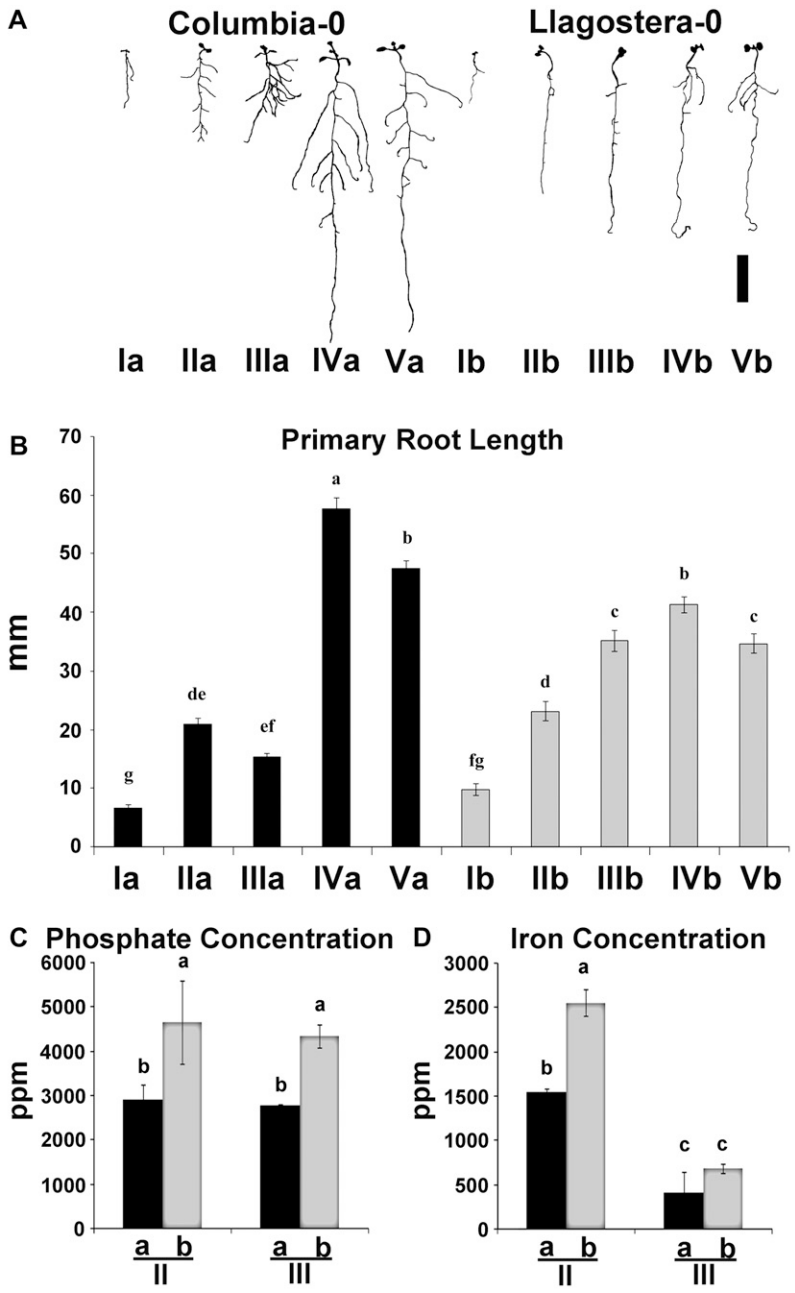

Figure 6. The primary root response of the Col-0 and Ll-0 accessions to increasing concentrations of Fe in P− medium without NH_4^+. I = 0 μM P, 100 μM FeEDTA; II = 0 μM P, 75 μM FeEDTA; III = 0 μM P, 50 μM FeEDTA; IVa = 0 μM P, 25 μM FeEDTA; V = 0 μM P, 10 μM FeEDTA. A, Representative 9-d-old seedlings of the Col-0 accession (a) compared to representative seedlings of the Ll-0 accession (b) from the different treatments. B, Primary root lengths of the Col-0 and Ll-0 seedlings as measured for 15 plants for each of the treatments using the ImageJ program. C, Tissue P concentrations as determined by ICP-MS of the short-rooted Col-0 plants compared to the long-rooted Ll-0 plants grown on medium II and medium III. D, Tissue Fe concentrations as determined by ICP-MS of the short-rooted Col-0 plants compared to the long-rooted Ll-0 plants grown on medium II and medium III. Lowercase letters indicate significance by a two-factor ANOVA and Tukey's post-hoc analysis at a $P < 0.05$. Scale bar = 10 mm.

the expression of genes involved in P nutrition are governed by P. However, this still leaves open the question of how the activity of P affects that of Fe beyond just limiting its availability in the medium. A recent report used x-ray probe microscopy to show that the localization and association of the Fe within the plant was different in P-deficient plants (Hirsch et al., 2006). In P-sufficient Arabidopsis (Col-0) plants, the Fe was localized to the vacuole where it was associated with P, K, calcium (Ca), Zn, and sometimes nickel (Ni). In P-deficient Arabidopsis (Col-0) plants, the Fe was found in the plastid likely bound to ferritin, and no Fe complexes with other elements were observed. The vacuoles under P-deficient conditions were largely free of Fe (Hirsch et al., 2006). The significance of this change in localization of the Fe remains to be investigated.

The split-plate results reported here are in agreement with the study by Svistoonoff et al. (2007), which showed that root tip contact with Fe in the absence of P is all that is necessary and sufficient for primary root inhibition. In their study, they reported that the absence of detectable expression of a multicopper oxidase (At1g23010) in the root tip in the Bay-0 accession of Arabidopsis allowed for the primary root elongation of this accession during P deficiency

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(Svistoonoff et al., 2007). Given that inhibition of root elongation in low P medium is, in fact, due to Fe toxicity, we would propose that alteration in the expression of this multicopper oxidase in Bay-0 is actually causing enhanced resistance to Fe toxicity. At least some classes of plant multicopper oxidases have been shown to have ferroxidase activity (Hoopes and Dean, 2004), and in yeast and algae multicopper oxidases have been linked to high-affinity Fe transport into the cell (Herbik et al., 2002). Whether the multicopper oxidase functions as a ferroxidase involved in Fe transport in the Arabidopsis root tip remains to be investigated.

Like Bay-0, the Ll-0 accession of Arabidopsis was also shown to have increased primary root elongation during P deficiency. This is likely due to the increased tolerance to high tissue concentrations of Fe and is not a consequence of Fe exclusion from the plant. A thorough understanding of the mechanisms by which these accessions tolerate Fe under P deficiency, and a thorough understanding of why other accessions are not able to stop the uptake of Fe when bioavailability increases, could have implications for developing plants with improved P efficiency on soils with low available P and high available Fe. It is clear from these studies that the longer rooted plants had higher P uptake rates, greater tissue P concentrations, greater total P content, larger dry weights, and, in general, appear better adapted to cope with P deficiency than the short-rooted Arabidopsis plants.

Conditions where P is limiting, and Fe is in excess, are abundant in agricultural situations. More than one-third of all croplands have severely acidic soils (Raghothama, 1999). The activity of Fe in acidic soils is generally associated with the fixing of P in the soil so that it is not available for uptake by the plant (Foth and Ellis, 1997). Aluminum (Al) toxicity is credited with the inhibition of root growth (Kochian et al., 2005). This study suggests that Fe toxicity could also be a factor in inhibiting root growth on these soils.

Fe toxicity is also a significant problem reducing yields in lowland rice cultivation on acidic waterlogged soils (Marschner, 1995) and, at least in some cases, Fe toxicity is associated with P deficiency (Sahrawat, 2004). This suggests that the insights gained from studying P deficiency in Arabidopsis might also be applicable for understanding Fe toxicity in other crops.

We conclude that Fe toxicity is the cause of the primary root inhibition observed in Arabidopsis Col-0 during P deficiency, and suggest that manipulating Fe may present a novel approach for improving the P nutrition of crops.

MATERIALS AND METHODS

Growth Medium

The P-sufficient, normal Fe control medium used for this study was a modified Murashige and Skoog medium that contained 2.06 mM ammonium nitrate, 1.88 mM potassium nitrate, 0.15 mM magnesium sulfate, 0.1 mM manganous sulfate, 0.03 mM zinc sulfate, 0.1 μM cupric sulfate, 0.3 mM calcium chloride, 5.0 μM potassium iodide, 0.1 μM cobalt chloride, 0.1 mM FeEDTA, 1 mM potassium phosphate, 0.1 mM boric acid, 1.0 μM sodium molybdate, 1.5% Suc, and 1.2% agar (Sigma A1296; lot 110K0195). The pH of the medium was adjusted to 5.7 before autoclaving.

Medium with lower concentrations of P were made by substituting potassium sulfate for the potassium phosphate so that the level of K in the medium remained at 2.885 mM. Concentrations of Fe were adjusted by manipulating the concentration of FeEDTA. Other reagents were added or substituted in the basal medium as noted in the text: EDTA (Mallinckrodt 4931–02); HBED [*N*,*N*′-di(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid monohydrochloride hydrate; Strem Chemicals reagent no. 07–0422]; or phytic acid sodium salt from corn (Sigma P8810).

The FeEDTA and FeHBED were added as stock solutions. FeEDTA was made by dissolving ferrous sulfate in a solution of sodium EDTA so that the Fe(II) and EDTA were in equal molar proportions. FeHBED was made by dissolving ferric sulfate in a solution of HBED so that the Fe(III) and HBED were in equal molar proportions.

An 8.35 mM stock concentration of phytic acid was made, adjusted to a pH of 5.7, and filter sterilized. This was added to autoclaved medium to the appropriate final concentration.

Plant Material and Growth Conditions

Seeds of the Col-0 and Ll-0 accessions of Arabidopsis (*Arabidopsis thaliana*), and those of the *frd3* mutant, were surface sterilized with 70% and then 50% household bleach solutions, and cold stratified for 2 d at 4°C. They were then directly sown on the medium on which their growth was being evaluated. These plates were placed vertically in a growth room under fluorescent lights with 16-h days and 8-h nights and a temperature of 25°C and left for 9 d (approximately 2 d to germinate and approximately 7 d of growth).

On day 9, the plates were scanned into Adobe Photoshop with an Epson Perfection 2400 scanner. After photo documentation, the seedlings were collected for root measurements, dry weight, inductively coupled plasma-mass spectrometry (ICP-MS) analysis, and other assays.

Purification of Agar

Agar (Sigma A1296; lot 110K0195) was rinsed several times and then left in distilled water. Dowex G55 (Sigma D3053) beads were loaded into dialysis bags and then circulated in the agar and water suspension overnight. The beads were removed and the agar rinsed several more times. The agar was drained over cheesecloth, squeezed of as much water as possible, and allowed to dry at room temperature. Purified and unpurified agars were analyzed by ICP-MS for their P and Fe content to demonstrate that the purification had been effective. The growth of Arabidopsis plants on washed and unwashed agars was also assessed on full nutrient medium to confirm that there were no unintended consequences of the purification.

Visual Minteq Analysis

Visual Minteq analysis was done according to Parker et al. (1995) using the ion concentrations of the modified Murashige and Skoog solution used in this study. Multiple analyses with different concentrations of ferric and ferrous Fe were considered. In all cases, the proportional increases in the availability of Fe in low P medium were the same. The results of the analysis when all of the Fe is in the ferrous form are presented. An extra 20 μM of P and an extra 10 μM of Fe were added to all the calculations to account for the impurities that were measured in the agar used in the medium preparations.

Primary Root Length Measurements

An Epson Perfection 2400 scanner was used to capture images of 15 to 20 seedlings with their roots spread out on agar plates. The image files were converted into the JPEG format and reopened in the ImageJ computer program. ImageJ is a free image analysis program that evolved from software developed at the National Institutes of Health, Bethesda, MD (Rasband, 1997–2006). The scale was set for the picture within the program. Then the drawing tools were used to trace over the primary roots. Based on the scale, the program automatically calculated the length of the root.

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Dry Weight Determination

Three groups of 10 9-d-old seedlings representing the different growth conditions were placed inside preweighed Eppendorf tubes. The plants were dried to constant weight at 65°C. The weights of the pools of seedlings were determined by subtracting the total weights by the weights of the tube. The individual weights of the seedlings were obtained by dividing the pooled weights by 10.

ICP-MS Elemental Profiling

Elemental profiling via ICP-MS was done on three groups of 10 seedlings that were 9 d old for each condition. Tissue concentrations were determined for lithium (Li), boron (B), Na, Mg, P, K, calcium (Ca), Fe, Co, copper (Cu), Zn, arsenic (As), selenium (Se), molybdenum (Mo), and cadmium (Cd). Plant tissue was placed into weighed Pyrex tubes and dried at 90°C overnight. The samples (0.5–1.0 mg) were digested with 0.25 mL of concentrated HNO_3 at 110°C for 2 h. Each sample was diluted to 2.0 mL with 18 MOhm water and analyzed on an Elan DRCe ICP-MS (Perkin-Elmer). Methane was used as a collision cell gas to measure Fe. Gallium and indium were used as internal standards, added to the digestion acid bottle to a concentration of 20 ppb. National Institute of Standards and Technology traceable single element ICP standards (Ultra Scientific) were used to make up the calibration standards.

[^{33}P] Uptake Studies

[^{33}P] uptake studies were done according to Mission et al. (2004) using 12 groups of 10 seedlings for each treatment. Briefly, 9-d-old seedlings were harvested from plates, rinsed at room temperature for 10 min in 5 mM MES, 0.1 mM $CaCl_2$ solution with a pH of 5.7, and transferred to the [^{33}P] uptake solution containing 5 mM MES, 0.1 mM $CaCl_2$, and 50 μM KH_2PO_4 at a pH of 5.7 with 0.15 μCi/mL to 0.2 μCi/mL of ^{33}P. The plants were allowed to take up P for 2 h at room temperature. Then the plants were desorbed of any remaining radioactivity for 30 min at 4°C in a solution of 5 mM MES, 0.1 mM $CaCl_2$, 1 mM KH_2PO_4, pH 5.7. Plants were transferred to preweighed scintillation vials, dried overnight at 65°C, and their weights determined the following day. Scintillation cocktail was added to the vials and the radioactivity counted in a Beckman LS3801 scintillation counter. The counts were normalized to the previously determined dry weights.

GUS Staining

GUS staining was done as described in Karthikeyan et al. (2002). Whole seedlings were incubated in GUS staining solution at 37°C overnight. The following day the stained seedlings were transferred to 70% (v/v) ethanol solution. Later the plants were spread on an agar plate and scanned into Adobe Photoshop with an Epson Perfection 2400 scanner. GUS staining solution contains 25 mg of 5-bromo-4-chloro-3-indoly-β-D-glucuronide in 50 mL of 100 mM sodium phosphate buffer with 0.1% (v/v) Triton X-100.

Reverse Transcription-PCR

Total RNA was extracted with phenol and guanidine thiocyanate. Reverse transcription (RT) and subsequent PCRs were done according the protocols supplied with the Superscript II RNase H− reverse transcriptase (Invitrogen; catalog no. 18064–014). PCR was done for 20 to 25 cycles depending on the gene. The genes and their primers are listed in Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. Effect of the purification of the agar on primary root elongation used to make medium without added P or Fe.

Supplemental Figure S2. RT-PCR of RNA isolated from 9-d-old wild-type seedlings for P-responsive genes in addition to the results presented in Figure 3.

Supplemental Figure S3. Growth of wild-type seedlings on P-deficient medium when the iron chelator is altered.

Supplemental Figure S4. Growth of wild-type seedlings on medium containing elevated levels of K and Mg.

Supplemental Table S1. List of primers used for RT-PCR of P- and Fe-responsive genes.

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