Putrescine Alleviates Iron Deficiency via NO-Dependent Reutilization of Root Cell-Wall Fe in Arabidopsis\(^1\)\(^{[\text{OPEN}]}\)

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Plants challenged with abiotic stress show enhanced polyamines levels. Here, we show that the polyamine putrescine (Put) plays an important role to alleviate Fe deficiency. The \(\text{alc2-1}\) mutant, which is defective in Put biosynthesis, was hypersensitive to Fe deficiency compared with wild type (Col-1 of Arabidopsis [\textit{Arabidopsis thaliana}]). Exogenous Put decreased the Fe bound to root cell wall, especially to hemicellulose, and increased root and shoot soluble Fe content, thus alleviating the Fe deficiency-induced chlorosis. Intriguingly, exogenous Put induced the accumulation of nitric oxide (NO) under both Fe-sufficient (+Fe) and Fe-deficient (-Fe) conditions, although the ferric-chelate reductase (FCR) activity and the expression of genes related to Fe uptake were induced only under -Fe treatment. The alleviation of Fe deficiency by Put was diminished in the hemicellulose-level decreased mutant-\(xth31\) and in the \(noa1\) and \(nia1nia2\) mutants, in which the endogenous NO levels are reduced, indicating that both NO and hemicellulose are involved in Put-mediated alleviation of Fe deficiency. However, the FCR activity and the expression of genes related to Fe uptake were still up-regulated under -Fe+Put treatment compared with -Fe treatment in \(xth31\), and Put-induced cell wall Fe remobilization was abolished in \(noa1\) and \(nia1nia2\), indicating that Put-regulated cell wall Fe reutilization is dependent on NO. From our results, we conclude that Put is involved in the remobilization of Fe from root cell wall hemicellulose in a process dependent on NO accumulation under Fe-deficient condition in Arabidopsis.

Iron is an essential element for plant growth and development, and iron deficiency is the most common micronutrient deficiency in the world. To cope with iron deficiency, plants have evolved two distinct mechanisms for Fe acquisition from the rhizosphere. Strategy I, found in all dicots and monocots with the exception of graminaceous species, is characterized by (1) release of protons to acidify the rhizosphere, which is mediated in Arabidopsis (\textit{Arabidopsis thaliana}) by the proton-translocating ATPase AHA2 (\textit{ARABIDOPSIS PLASMA MEMBRANE H\textsuperscript{+}-ATPASE ISOFORM 2}; Curie and Briat, 2003; Santi and Schmidt, 2009); (2) inducing ferric chelate reductase activity mediated by FRO2 (\textit{FERRIC REDUCTASE OXIDASE2}; Robinson et al., 1999); and (3) uptake of Fe\(^{2+}\) by the metal transporter IRT1 (\textit{IRON REGULATED TRANSPORTER1}; Eide et al., 1996; Vert et al., 2002). Strategy II, utilized by graminaceous monocots (Römheld and Marschner, 1986), is characterized by enhanced release of phytosiderophores that form chelates with Fe(III) (Curie and Briat, 2003). However, in addition to Fe acquisition, the mechanisms underlying the mobilization of Fe(III) also are a major challenge for us to understand.

Recently, accumulating evidence has shown that phenolic compounds are important for iron mobilization. Rodríguez-Celma et al. (2013) showed that secretion of phenolics is critical for Arabidopsis Fe acquisition from low bioavailability sources, and then Fourcroy et al. (2014) and Schmidt et al. (2014) demonstrated that coumarins are the active compounds in this process. Schmid et al. (2014) confirmed that secretion of coumarins is an essential aspect of Arabidopsis Fe acquisition and provided extensive information on metabolomic changes elicited by Fe deficiency. However, under certain conditions Fe is not readily available, and Fe is difficult to mobilize; thus, Fe stored in the plant needs to be reutilized. For example, phenolics are secreted to remobilize the root apoplastic Fe and improve Fe nutrition in red clover (\textit{Trifolium pratense}) and rice (\textit{Oryza sativa}) (Jin et al., 2007; Bashir et al., 2011). Moreover, Lei et al. (2014) reported that the cell wall can prove Fe nutrition in red clover (\textit{Trifolium pratense}) and rice (\textit{Oryza sativa}) (Jin et al., 2007; Bashir et al., 2011). Moreover, Lei et al. (2014) reported that the cell wall can be an important Fe source during periods of limited Fe supply. As the first barrier to encounter the soil environment, the cell wall is a pivotal site for most cationic ions in plants (Lozano-Rodríguez et al., 1997; Carrier et al., 2003). Hemicellulose contributes to the overall

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Al/Cd accumulation in the cell wall of Arabidopsis (Zhu et al., 2012, 2013) and also acts as a Fe pool (Lei et al., 2014). Over 75% of Fe in the root is retained in the cell wall (Bienfait et al., 1985), especially in the hemicellulose fraction (Lei et al., 2014). Thus, the cell wall is not only a site to immobilize an element and restrict its entrance into the cell, but also can serve as a pool to provide the nutrient when the supply from the growth medium is limited. However, the upstream mechanism of Fe reutilization through the cell wall, especially hemicellulose, is still far from clear.

The responses to Fe deficiency in plants involve numerous phytohormones and signaling molecules, including auxin (Römheld and Marschner, 1981; Chen et al., 2010), ethylene (García et al., 2010; Wu et al., 2011), and NO (Graziano and Lamattina, 2007; Chen et al., 2010). Polyamines share common substrates with nitric oxide (NO) (Shi and Chan, 2014), and polyamines like spermidine and spermine rapidly induce a burst of NO in various plant species, indicating that NO is a potential intermediate of polyamine-mediated signaling.

Polyamines, including putrescine (Put), spermidine, and spermine, are low M₀ natural compounds with nitrogen-containing aliphatic structure and influence basic physiological and developmental events, such as cell division and differentiation, rhizogenesis, leaf senescence, zygotic, somatic embryogenesis, and development of flowers and fruits (Feirer et al., 1984; Galston et al., 1995; Bouchereau et al., 1999; Kakkar et al., 2000; Tun et al., 2001; Shi and Chan, 2014). The metabolism of polyamines in plant tissues is subject to strict regulation, and polyamine levels in plant roots change upon exposure to abiotic stress such as salt, drought, low and high temperature, heavy metals (Cu, Cr, Fe, and Ni), and oxidative stresses (Liu et al., 2005; Cheng et al., 2009; Wimalasekera et al., 2011; Tavladoraki et al., 2012).

Ample evidence demonstrates the involvement of Put in responses to various types of abiotic stress, such as mineral deficiency in barley (Hordeum vulgare) leaves (Smith, 1973), high osmotic pressure in barley, corn, wheat, and wild oat leaves (Flores and Galston, 1982a), low pH in peeled oat (Avena sativa L. var Victory) leaf (Young and Galston, 1983), potassium deficiency in oat shoot and Arabidopsis thaliana (L.) Heynh (Young and Galston, 1984; Watson and Malmberg, 1996), and cadmium toxicity in oat and bean leaves (Weinstein et al., 1986). In animals, Put is produced either from Orn by Orn decarboxylase or from Arg by Arg decarboxylase (ADCA) (Hanfrey et al., 2001). As there is no detectable Orn decarboxylase activity in Arabidopsis, the ADC route is critical for Put biosynthesis. Although there are two genes responsible for ADC activity, Urano et al. (2004) reported that the expression of ADC2 correlates well with the increment of free Put, indicating ADC2 plays an important role in Put biosynthesis in Arabidopsis. However, the role of Put under Fe deficiency in plants remains unknown.

In this study, we found that Fe deficiency results in enhanced Put levels. Further, whereas exogenous Put alleviated Fe deficiency, the adc2-1 mutant, in which endogenous Put is decreased, exhibited a Fe deficiency-sensitive phenotype. We demonstrated that Put acts upstream of NO to decrease the Fe binding capacity of the cell wall, especially that of hemicellulose, thus resulting in greater Fe reutilization.

RESULTS

Fe Deficiency Induces Put Accumulation in Roots of Arabidopsis

To investigate the effect of Fe deficiency on Put metabolism, we exposed Arabidopsis plants to Fe-deficient (-Fe) nutrient solution for 7 d. Fe deficiency induced Put accumulation in the roots within 1 d (Fig. 1A). Put levels remained high through day 5 and then decreased to the same level as control by day 7 (Fig. 1A). These results demonstrate that Fe deficiency results in the rapid accumulation of Put in the roots of Arabidopsis.

Put Alleviates Fe Deficiency

To quantify the effects of exogenous Put application on the response of Arabidopsis to Fe deficiency, plants were treated with or without 0.1 mM Put under Fe deficiency. After 7-d treatments, Fe deprivation resulted in chlorosis in the newly expanded leaves (Fig. 1C). However, this typical Fe deficiency symptom was hardly observed in the -Fe+Put treatment, consistent with the increment in Soil and Plant Analyzer Development (SPAD), an indicator of the total chlorophyll content (Fig. 1D). The soluble Fe in the roots (Fig. 1E) and shoots (Fig. 1F) increased in -Fe+Put relative to -Fe, in good agreement with the phenotype observed (Fig. 1C).

To analyze the role of endogenous Put under Fe deficiency, we used the adc2-1 mutant, in which the free Put content is reduced to about 25% of that in wild-type Columbia ecotype (Fig. 1B; Urano et al., 2004). The SPAD values of wild type and adc2-1 were similar under Fe-sufficient (+Fe) or +Fe+Put treatment. Under -Fe treatment, the leaf chlorosis was more pronounced in adc2-1, but could be alleviated by exogenous Put (Fig. 1C). Furthermore, there was less shoot soluble Fe in adc2-1 under the Fe-deficient condition (Fig. 1F), although there was almost no difference in the root soluble Fe or root and shoot total Fe (Fig. 1E; Supplemental Figure S1, A and B), [ID]SUPFIG[1/ID] Put applied exogenously could increase the shoot soluble Fe content in adc2-1 back to the wild-type level under Fe-deficient conditions (Fig. 1F). Moreover, Put application also increased the root and shoot symplastic Fe in the wild-type and adc2-1 mutant, whereas less symplastic Fe was detected in the adc2-1 mutant root and shoot (Supplemental Figure S1, C and D). These results are in accordance with the increased root and shoot soluble Fe content in -Fe+Put versus -Fe treatment in wild type and the decreased shoot soluble Fe content in adc2-1 versus wild type (Fig. 1, C and D), further indicating that the increment of the Put levels under Fe-deficient conditions is beneficial to the Arabidopsis (Fig. 1).
Stimulation of the plasmalemma NADPH-ferric chelate reductase (FCR; Robinson et al., 1999) activity and extrusion of proton to the rhizosphere, which can be measured as changes in pH value of the growth solution (Jin et al., 2007, 2009), are hallmark responses to Fe deficiency in dicots (Robinson et al., 1999; Curie and Briat, 2003). Here, we found that the pH of the -Fe solution was decreased greatly after 1 d of Put treatment (Fig. 2, A and B), while the FCR activity was remarkably enhanced after 5 d of -Fe+Put treatment (Fig. 2, C and D). Moreover, the adc2-1 mutant showed less root FCR activity than wild-type plants and could be rescued by exogenous Put (Fig. 2D). These findings suggest that the increased Put content under Fe-deficient conditions was responsible for the increased FCR activity.

Interestingly, Put applied alone also leads to a decrease in the pH value (Fig. 2A). The solubility of Fe decreases up to 1000-fold for each unit increase in pH (Santi and Schmidt; 2009), but as there was no Fe in the nutrient solution, this reduction of the pH cannot explain the alleviation of Fe deficiency symptom (Fig. 1C). Furthermore, when the solution was buffered at pH 5.6 with 10 mM MES, the root and shoot soluble Fe content showed no significant difference from those in the nonbuffered solution (Supplemental Figure S2, A and B). Santi and Schmidt (2009) suggested that the rhizosphere acidification in response to Fe deficiency is chiefly mediated by AHA2. Thus, if lower pH increases the availability of Fe, there should be more soluble Fe in wild-type plants compared with the aha2 (Salk_022010) mutant under Fe-deficient conditions. However, we observed almost no difference in the root and shoot soluble Fe content between wild type and aha2, neither under -Fe nor under -Fe+Put treatment (Supplemental Figure S2, C and D). Therefore, the effect of Put observed here is not attributable to the effects of a lower pH in the media, but rather is caused by the synergetic action of Put and -Fe.

The FCR activity reached a maximum level at 7 d (Fig. 2D), as did the expression of FIT1, FRO2, and IRT1 (Fig. 2, E-G). We wondered whether exogenous Put would affect FCR activity under Fe-sufficient conditions. Neither the relative expression of FIT1, FRO2, and IRT1 nor the root FCR activity was affected by +Fe+Put treatment compared with +Fe treatment (Fig. 2), indicating that Put can trigger root FCR activity only under Fe deficient conditions.

Put Acts Upstream of NO Accumulation

It has been previously demonstrated that NO was involved in the induction of FCR activity (Graziano and Lamattina, 2007). To examine whether Put acts upstream or downstream of NO under Fe-deficient conditions, we first analyzed the effects of exogenous application of Put on NO accumulation in wild type. As shown in Figure 3, Put treatment significantly increased the NO accumulation under both Fe-sufficient and Fe-deficient conditions. However,
the increment of NO accumulation under Fe-sufficient conditions may not be efficient to induce the FCR activity compared with the increment of NO accumulation under Fe-deficient conditions (Fig. 3 and Fig. 2).

We further analyzed the effect of Put in noa1 and nia1nia2 mutants, both of which are defective in NO biosynthesis (Guo, 2006; Wilson et al., 2008; Chen et al., 2010). Put failed to alleviate the Fe deficiency symptoms in both mutants (Fig. 4A). Consistent...
with this, there was no increment of FCR activity (Fig. 4D), no change in the root and shoot soluble Fe content in either mutant (Fig. 4, B and C), and no induction of the relative expression of FIT1, FRO2, and IRT1 (Fig. 4, E-G). All of these results indicate that both NOA-dependent and NR-dependent production of NO acts downstream of Put under Fe-deficient conditions.

Figure 3. Effect of Put on NO accumulation in wild type. A, NO production reported as green fluorescence by using DAF-FM DA probes in representative roots. B, NO production expressed as relative fluorescence intensity. Fluorescence intensity in the +Fe-treated root was set to 100%, and intensity in the tested root was calculated relative to the corresponding control value. Error bars represent ± sd (n = 4). Different letters represent significant difference at P < 0.05.

Figure 4. Effect of exogenous Put on Fe deficiency symptoms in Arabidopsis wild type and noa1 and nia1nia2 mutants. Six-week-old seedlings were treated with or without 0.1 mM Put in the presence or absence of 50 μM Fe for 7 d, the Fe deficiency symptoms (A) were taken using a digital camera, and root and shoot samples were taken to measure the root soluble Fe content (B), shoot soluble Fe content (C), root FCR activity (D), the expression of genes related to Fe uptake (E, F, G), cell wall Fe content (H), cell wall hemicellulose content (I), and Fe content in the hemicellulose (J). Expression levels in the +Fe-treated sample for each gene were set to 100%, and levels in the tested sample were calculated relative to the corresponding control value. Error bars represent ± sd (n = 4). Different letters represent significant difference at P < 0.05.
Put Induces Fe Remobilization from the Root Cell Wall Hemicellulose

As there was no available Fe in the nutrient solution, the increased FCR activity could not be responsible for the increased root soluble Fe content under Fe-deficient conditions. This raised the question of the source of the soluble Fe. Since the cell wall plays pivotal roles in Fe reutilization in Arabidopsis during Fe deficiency (Lei et al., 2014), we wondered whether Put treatment led to a release of the cell wall-retained Fe. Indeed, we found that the cell wall fractions of wild-type and adc2-1 roots contained about 4.1 and 6.6 μg bound Fe/mg under Fe-deficient conditions (Fig. 5A), but only about 3 μg/mg in the -Fe+Put treatment (Fig. 5A). These results indicate that Put induces Fe remobilization from the root cell walls.

We previously demonstrated that hemicellulose contributes greatly to the metal binding in cell walls of rice and Arabidopsis (Yang et al., 2008, 2011; Zhu et al., 2012, 2013). Here, we found that -Fe+Put treatment significantly decreased hemicellulose content compared with -Fe treatment alone (Fig. 5B). In accordance with the changes in the cell wall components, the Fe content in the hemicellulose fraction was also much lower with -Fe+Put treatment than with -Fe treatment alone (Fig. 5C), suggesting that Put alleviated Fe deficiency through reutilization of Fe from root cell wall hemicellulose.

To further clarify the role of hemicellulose in the Put-mediated alleviation of Fe deficiency, we used the xth31 mutant, in which the endogenous hemicellulose content is decreased (Zhu et al., 2012). As shown in Figure 6, Put was unable to alleviate the Fe deficiency phenotype in the xth31 mutant (Fig. 6A), with no change in the root and shoot soluble Fe content (Fig. 6, B and C), although there was still an induction of the FCR activity and the expression of Fe uptake related genes: FIT1, FRO2, and IRT1 (Fig. 6, D-G). These results indicate that the ability of Put to alleviate Fe deficiency is dependent on hemicellulose.

The Hemicellulose-Mediated Function of Put in Fe Deficiency Is Dependent on NO Accumulation

In this study, we found that Put could not alleviate Fe deficiency in NO-related mutants (noa1 and nia1nia2) or in a hemicellulose content-reduced mutant (xth31) (Fig. 4 and 6). This prompted us to explore the relationship between NO and hemicellulose in Put alleviation of Fe deficiency. There was no difference in cell wall hemicellulose content or cell wall hemicellulose Fe content between noa1 and nia1nia2 mutants under -Fe+Put or -Fe treatment (Fig. 4, H-J). The fact that there was still an induction of FCR activity and the expression of Fe-related genes in the xth31 mutant (Fig. 6) indicates that the effect of Put is mediated through NO, as shown in the model proposed in Figure 7.

DISCUSSION

Put Alleviated Fe Deficiency

The experiments described here demonstrate the beneficial effect of Put on plants exposed to Fe-deficient conditions. The chlorosis induced by Fe deficiency was obviously alleviated upon treatment with exogenous Put (Fig. 1). Further, the Put level-reduced mutant adc2-1 showed more pronounced chlorosis than did wild type (Fig. 1). These results suggest that high levels of Put under Fe deficiency are causally related to the tolerance of Fe-deficient conditions by Arabidopsis.

Put Alleviates Fe Deficiency by Inducing Reutilization of Cell Wall Fe, Particularly from Hemicellulose

Accumulating evidence shows that the cell wall plays a significant role in regulating plant adaptive responses to Fe deficiency stress. Jin et al. (2007) provided the first evidence, to our knowledge, that Fe stored in the apoplast can be remobilized via Fe deficiency-induced secretion of phenolics in red clover and that the remobilized Fe can be found in the shoots; thus, the Fe nutrition of the shoots was improved. Lei et al. (2014) reported that the content of Fe bound to the cell wall decreases under Fe-deficient conditions in Arabidopsis. Exogenous application of ABA to Fe-deficient Arabidopsis significantly decreases cell wall Fe content, and as a result, more Fe is released and the plant becomes more Fe-deficiency resistant. Here, we found that the amount of root cell wall Fe was significantly increased in the Put level-reduced adc2-1 mutant and decreased in -Fe+Put treatment (Fig. 5A), indicating that Put promotes the remobilization of root cell wall Fe.

Figure 5. Effect of endogenous and exogenous Put on the cell wall Fe content (A), cell wall hemicellulose content (B), and cell wall hemicellulose Fe content (C). Six-week-old wild type and adc2-1 were placed in +Fe or -Fe medium with or without 0.1 mM Put for 7 d. Error bars represent ± SD (n = 4). Different letters represent significant differences at P < 0.05.
The cell wall is composed mainly of the matrix polysaccharides, including cellulose, hemicellulose, and pectin (Cosgrove, 2005). Pectin has been regarded as the main source of the negative charges of the cell wall and is generally attributed to the binding of cations, such as Al in tobacco (Chang et al., 1999), wheat (Zheng et al., 2004), maize (Eticha et al., 2005), rice (Yang et al., 2008), and Arabidopsis (Yang et al., 2011). However, recent evidence from Zhu et al. (2012) showed that hemicellulose make the greatest contribution to Al retention in the cell wall. Hemicellulose, which is tightly bound to the surface of cellulose to form a strong and resilient network, is synthesized in the Golgi and consists of a group of neutral or slightly acidic polysaccharides, such as xyloglucan, mannan, and arabinoxylan (Carpita and Gibeaut, 1993; Cosgrove, 2005; Zhu et al., 2012). In our previous study, we measured the Fe retained in different cell wall components and found that the largest pool of Fe was bound to hemicellulose (Lei et al., 2014). Upon treatment with exogenous Put, significantly less Fe was found that retained in hemicellulose (Fig. 5C). In fact, the hemicellulose content was significantly decreased by Put treatment (Fig. 5B), indicating that cell wall hemicellulose contributed greatly to the Put-mediated alleviation of Fe deficiency. Furthermore, we found that Put failed to alleviate Fe deficiency in the hemicellulose-reduced xth31 mutant (Fig. 6), implying that a certain hemicellulose level is necessary for Put to relieve Fe deficiency.

**Put Acts Upstream of NO under Fe Deficiency**

How does Put affect the hemicellulose content under Fe-deficient conditions? Put and NO share the common substrates, and NO has been demonstrated to function as a signal molecule in plant responses to low Fe conditions (Chen et al., 2010). For example, NO can induce many important genes regulating Fe acquisition, such as *FRO1*.

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**Figure 6.** Effect of exogenous Put treatment on the xth31 mutant with decreased hemicellulose content. Phenotypes of the plants (A), root soluble Fe content (B), shoot soluble Fe content (C), FCR activity (D), and the expression of genes related to Fe uptake (E, F, G). Six-week-old wild type and xth31 were placed in +Fe or -Fe medium with or without 0.1 mM Put for 7 d. Expression levels in the +Fe-treated sample for each gene were set to 100%, and levels in the tested sample were calculated relative to the corresponding control value. Error bars represent ± SD (n = 4). Different letters represent significant difference at P < 0.05.

**Figure 7.** Proposed model illustrating the link between Put and alleviation of Fe deficiency. Fe deficiency induces the increment of root Put level, which results in the pH reduction and NO accumulation. Moreover, cell wall hemicellulose acts downstream of NO in the Put-mediated Fe deficiency response. However, the reduced pH is not involved in the Fe remobilization induced by Put during Fe deficiency.
expression in tomato (Graziano and Lamattina, 2007) and FRO2 and GENERAL REGULATORY FACTOR11 (GRF11) expression in Arabidopsis (Chen et al., 2010; Yang et al., 2013). Both Put and NO content were increased in Fe-deficient roots (Fig. 1A and Fig. 3), does one event precede the other? In this study, we found that either Put applied exogenously or Fe deficiency could increase the root NO levels (Fig. 3) and that NO accumulation was further increased under -Fe+Put treatment (Fig. 3). Consistent with this, FCR activity was further induced under -Fe+Put compared with -Fe (Fig. 2D), in concert with the up-regulation of the expression of Fe uptake-related genes, FIT1, FRO2, and IRT1 (Fig. 2, E-G), which is in accordance with Chen et al. (2010). These findings indicate that Put may act upstream of NO to induce the expression of FRO2 and the activity of FCR. This conclusion was further demonstrated by the lack of FCR induction in both noa1 and nia1nia2 mutants upon Put treatment under Fe-deficient conditions (Fig. 4D). It is interesting that the increment of NO accumulation under +Fe+Put treatment failed to induce FCR activity compared with +Fe treatment (Fig. 2C and 3). The possibility that there is a tight threshold of NO accumulation that leads to the induction of the FCR activity awaits further study.

Put-Mediated Changes in Cell Wall Hemicellulose Content under Fe Deficiency Is Dependent on NO

As Put could influence both NO accumulation and hemicellulose content, we explored the relationship between NO and hemicellulose content. We monitored the hemicellulose content and Fe retention in the cell wall of noa1 and nia1nia2 mutants under -Fe and -Fe+Put treatment. Put influenced neither the hemicellulose content nor the cell wall Fe content in noa1 and nia1nia2 mutants; thus, root and shoot soluble Fe content remained unchanged (Fig. 4). This result further indicated that Put-mediated changes in cell wall hemicellulose contents under Fe deficiency depend on the NO level.

In conclusion, we propose a model to link the Fe deficiency-induced accumulation of Put level to Fe deficiency responses (Fig. 7). Previous reports showed that both NO accumulation and hemicellulose reduction are involved in response to Fe deficiency (Chen et al., 2010; Lei et al., 2014). Here, we further demonstrated that Fe deficiency induces Put accumulation in roots and that Put is involved in the remobilization of Fe from root cell wall hemicellulose through the accumulation of NO. As a result, there is more soluble Fe in the roots and leaves, thus alleviating the Fe deficiency-induced chlorosis. In other words, plants with less endogenous Put have less Fe reutilization from the root cell wall and thus are more sensitive to Fe deficiency.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Columbia ecotype (wild-type) of Arabidopsis (Arabidopsis thaliana) and the mutants generated in this background, including ada2-1 (Salk_026916C), ada2 (Salk_022010), xiih31 mutant (decreased hemicellulose levels), NOA-deficient mutant noa1, and the NR-null-deficient double mutant nia1nia2 were used in this study. Seedlings were germinated on a sponge in Murashige and Skoog nutrient solution for 3 weeks in an environmental controlled growth chamber. Subsequently, seedlings of similar rosette diameters were transferred to nutrient solution containing Murashige and Skoog salts for another 3 weeks. These 6-week-old plants were subjected to the following treatments: -Fe (the complete nutrient solution as control), -Fe+Put (complete nutrient solution plus 0.1 μM Put), -Fe (control solution without Fe), and +Fe+Put (Fe solution plus 0.1 μM Put). Put standard (Putrescine dihydrochloride, ref. P7505) was purchased from Sigma Chemical, according to Urano et al. (2004). For the pH buffered and nonbuffered solution, 10 mM MES was included or not in each of the above nutrient solutions (+Fe, +Fe+Put, -Fe, and -Fe+Put). All treatment solutions were renewed every 3 d. After 1 week, the plants were harvested and separated into roots and shoots and washed with deionized water. The nutrient medium consisted of the following macronutrients in mM: KNO3, 6.0; Ca(NO3)2, 4.0; MgSO4, 1; NH4H2PO4, 0.1, and the following micronutrients in μM: Fe(III)-EDTA, 50; H3BO3, 12.5; MnSO4, 1; CuSO4, 0.5; ZnSO4, 1; H2MoO4, 0.1; NiSO4, 0.1 according to Murashige and Skoog (1962). The final pH was adjusted to 5.6 with 1 μL KOH. The growth conditions for the seedlings were at a temperature of 24°C, a light intensity of 140 μmol m−2 s−1, and a 16-h/8-h day/night rhythm. The growth conditions were the same for all experiments unless otherwise specified.

Measurement of Chlorophyll Fluorescence

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

Put Measurement

Free polyamines were analyzed by HPLC according to Flores and Galston (1982b) and Morgan (1998) with some modifications. After treatment, roots were homogenized in 5% HClO4 in chilled mortars and pestles. After the homogenates were incubated in ice-cold water for 1 h, 10 μL benzoyl chloride and 2 mL saturated sodium chloride were added, and the samples were mixed vigorously to disperse the benzoyl chloride. Then the reaction was allowed to proceed at room temperature for 30 min. The polyamines were subsequently extracted with 2 mL 2 M NaOH and 2 mL diethyl ether and centrifuged briefly to separate the layers after vigorous vortexing for 30 s. The mixtures were separated into two phases, aqueous and organic. The organic phase, containing the polyamines, was completely dried using a rotary evaporator at 40°C, redissolved in 100 μL methanol, and assayed immediately. Aliquots of samples were diluted 5- to 20-fold before injection into the HPLC, with excitation at 350 nm and emission at 495 nm. Standards were treated in a similar way.

Solution pH Measurement

The pH in nutrient solution was measured every other day with a pH electrode (METROHM).

Gene Expression Analysis

Plants were grown in the same conditions used for inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis. Root tissues were collected 7 d after treatments began and were immediately frozen in liquid nitrogen before total RNA extraction. Total RNA was isolated from the root using TRIzol (Invitrogen). cDNA was prepared from 1 μg of total RNA using the PrimeScript RT reagent kit (Takara). For quantitative real-time PCR analysis, 1 μL 10-fold-diluted cDNA was used for quantitative analysis of gene expression performed with SYBR Premix Ex Taq (Takara) with the following pairs of gene-specific primers. FIT (forward: 5'-GGAGAAGGTGGTTTGCACATCTC-3'; reverse: 5'-GGTAGGCAAGTGAATCTCAGTC-3'); FRO2 (forward: 5'-CGTTCGCTGACGGATTCCTCG-3'; reverse: 5'-CGACTGTCGATGGCCGCTATG-3'); IRT1 (forward: 5'-CGTTGGACTCTCTAAATGC-3'; reverse: 5'-CGATAATCGACATTCCACCG-3'). Each cDNA sample was run in triplicate. Expression data were normalized to the expression of TUBULIN (forward: 5'-AAGTCTCGGAAATGTTT-3'; reverse: 5'-CTCCCAATGAGTGACAAA-3') (Chen et al., 2010).
Cell Wall Extraction and Fractionation

Extraction of crude cell wall materials and subsequent fractionation of cell wall components were carried out according to Zhong and Lauchli (1993) with minor modifications according to Yang et al. (2011). After treatment, roots were ground in liquid nitrogen with a mortar and pestle, homogenized by incubation in 75% ethanol for 20 min in an ice-cold water bath, and then centrifuged at 8,000 rpm for 10 min and the supernatant was removed. Then, the pellet was homogenized and washed with acetone, methanol:chloroform (1:1), and methanol for 20 min each, with the supernatant removed after centrifugation.

Cell wall samples (about 2 mg) were treated three times with 1 mL water at 100°C for 1 h, and the residue was further extracted twice with 1 mL 24% (v/v) KOH and 0.02% (v/v) KBH₄ at room temperature for 12 h. The two supernatants were combined in a 2-mL tube and centrifuged at 12,000 rpm for 10 min to recover the hemicellulose fraction.

Total Polysaccharide Measurement

The total polysaccharide contents in the hemicelluloses fractions were determined by the phenol sulfuric acid method (Dubois et al., 1956) and expressed as Glc equivalents. Briefly, 200-µL hemicellulose extracts were incubated with 1 mL 96% H₂SO₄, and 10 µL 80% phenol at room temperature for 15 min, and then the mixed solutions were incubated at 100°C for 15 min. After chilling, the absorbance was measured spectrophotometrically at 490 nm.

Measurements of Fe Content in Plants

Extraction of water-soluble Fe was performed according to Cassin et al. (2009). Briefly, after plants were treated as indicated, 0.5- to 1-g root and shoot samples were ground in liquid nitrogen with a mortar and pestle, homogenized by incubation in 75% ethanol for 20 min in an ice-cold water bath, and then centrifuged at 8,000 rpm for 10 min. The supernatants were combined in a 2-mL tube and centrifuged at 12,000 rpm for 10 min to recover the hemicellulose fraction.

Measurements of Cell Wall and Cell Wall Hemicellulose Fe Content in Plants

Fe in cell wall was extracted with 2 N HCl for 24 h with occasional shaking. Fe concentrations in these cell wall extracts and Fe content in the above extracted hemicellulose were determined by ICP-AES (IRIS/AP optical emission spectrometer).

Root FCR Activity Determination

FCR activity was determined according to Grusak (1995) and Lei et al. (2014). Briefly, whole excised roots were placed into a test tube filled with 5 mL assay solution consisting of 0.5 mM CaSO₄, 0.1 mM 4-morpholineethanesulfonic acid, 0.1 mM bathophenanthroline-disulfonic acid disodium salt hydrate, and 100 mM Fe-EDTA at pH 5.5 adjusted by 1 M NaOH. The tubes were placed in a dark room at 25°C for 1 h, with periodic hand swirling at 10-min intervals. The absorbance of the assay solutions was recorded by a spectrophotometer at 535 nm, and the concentration of Fe[II][bathophenanthroline-disulfonic acid disodium salt hydrate] was quantified using an extinction coefficient of 22.14 mM⁻¹ cm⁻¹.

Determination of NO Content in Roots

The endogenous levels of NO in roots were determined using 4-aminophenylmethylnitramino-2-7-difluorofluorescein diacetate (DAF-FM DA) probes and epifluorescence microscopy. The root tips were incubated with 5 µM DAF-FM DA in dark for 30 min, washed three times in PBS (pH 7.4), and analyzed microscopically. The fluorescent intensity was recorded using Photokhop 7.0 (Adobe Systems) according to Besson-Bard et al. (2009).

Statistical Analysis

Each experiment was repeated at least three times, and one set of data are shown in the Results. Data were analyzed by one-way ANOVA and the means were compared by Duncan’s multiple range test. Different letters and asterisks on the histograms indicate that the means were statistically different at the P < 0.05 level.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of endogenous and exogenous Put on Fe-deficiency symptoms in Arabidopsis.

Supplemental Figure S2. Effect of pH on the Put-mediated alleviation of Fe-deficiency in Arabidopsis.

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