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A soil bacterium regulates plant acquisition of iron via deficiency-inducible mechanisms

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SUMMARY

Despite the abundance of iron in nature, it is the third most limiting nutrient for plants due to its minimal solubility in most soils. While certain soil microbes produce chelating agents that enhance the solubility of iron, the effectiveness of such siderophores in the assimilation of iron by plants is debated. With an increasing understanding that select soil microbes play a signaling role in activating growth and stress responses in plants, the question arises as to whether such symbionts regulate iron assimilation. Here we report a previously unidentified mechanism in which the growth-promoting bacterium *Bacillus subtilis* GB03 activates the plant's own iron acquisition machinery to increase assimilation of metal ions in Arabidopsis. Mechanistic studies reveal that GB03 transcriptionally up-regulates the Fe-deficiency-induced transcription factor 1 (*FIT1*), which is necessary for GB03-induction of ferric reductase *FRO2* and the iron transporter *IRT1*. In addition, GB03 causes acidification of the rhizosphere by enhancing root proton release and by direct bacterial acidification, thereby facilitating iron mobility. As a result, GB03-exposed plants have elevated endogenous iron levels as well as increased photosynthetic capacity compared with water-treated controls. In contrast, loss-of-function *fit1-2* mutants are compromised in terms of enhanced iron assimilation and photosynthetic efficiency triggered by GB03. In all studies reported herein, a physical partition separating roots from bacterial media precludes non-volatile microbial siderophores from contributing to GB03-stimulated iron acquisition. These results demonstrate the potential of microbes to control iron acquisition in plants and emphasize the sophisticated integration of microbial signaling in photosynthetic regulation.

Keywords: plant growth-promoting rhizobacteria, Fe-deficiency-induced transcription factor 1, ferric reductase, iron-regulated transporter 1, photosynthetic efficiency, volatile organic compounds.

INTRODUCTION

Iron is an essential micronutrient that participates in electron transfer reactions by reversible donation and acceptance of electrons. With the photosynthetic complex being one of the most iron-enriched cellular systems in nature with over 20 atoms per complex, photosynthetic activity is highly sensitive to the availability of iron (Spiller and Terry, 1980; Sandmann, 1985; Behrenfeld *et al.*, 1996). At a phenotypic level iron deficiency induces leaf chlorosis, due to reduced activity of the enzyme that catalyzes biosynthesis of the plant chlorophyll precursor protochlorophyllide, which requires metallic iron (Spiller *et al.*, 1982; Tottey *et al.*, 2003). Moreover, sustained iron shortages diminish photochemical capacity by reducing the number of photosynthetic units and perturbing the ultrastructure of chloroplasts (Stocking, 1975; Spiller and Terry, 1980; Terry, 1980). Iron is also a cofactor for several thylakoid-bound enzymes that protect chloroplasts from reactive oxygen species (Casano *et al.*, 2000; Murgia *et al.*, 2004).

A network of metabolic events is activated under iron deficiency to coordinate the mobilization of iron pools in the immediate vicinity of root epidermal cells and the uptake and distribution of iron within plants. In dicots and non-graminaceous monocots, a reduction-based iron uptake mechanism referred to as strategy 1 iron acquisition is induced (Marschner and Römheld, 1994). This strategy involves three steps for iron uptake: proton exudation to enhance iron mobility, reduction of Fe^{3+} to Fe^{2+} and import of Fe^{2+}. In the well-characterized Arabidopsis system proton

exudation is catalyzed by an ATPase belonging to the Arabidopsis H+-ATPase (AHA) gene family (Palmgren, 2001); however, the pump required for iron-deficiency-induced acidification has yet to be identified. Ferric reductase (FRO2) contains FAD and NADPH binding sites on the cytosolic face of the enzyme, while two heme groups are thought to reside in the membrane side, consistent with the transferring of electrons from cytosolic pyridine nucleotides to extracellular ferric chelates (Robinson *et al.*, 1999; Li *et al.*, 2004). Iron is subsequently taken up via the iron-regulated transporter 1 (IRT1) (Connolly *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002, 2003). Both IRT1 and FRO2 are subject to regulation by the Fe-deficiency-induced transcription factor (FIT1), which encodes a putative basic helix–loop–helix type protein that regulates iron uptake responses (Colangelo and Guerinot, 2004; Bauer *et al.*, 2007).

Low-molecular weight iron-binding molecules referred to as siderophores chelate $Fe^{3+}$and significantly increase the mobility of iron in the rhizosphere. In strategy II plants, phytosiderophore chelated Fe^{3+}can be directly shuttled into the roots without iron reduction by specific plant transporters (Curie and Briat, 2003). In addition to these phytosiderophores, soil microbes produce and release siderophores (Neilands and Leong, 1986; Briat, 1992) that are proposed to facilitate soil mobility and the uptake of iron by plants (Bar-Ness *et al.*, 1992; Glick, 1999). However, bacterially increased iron uptake for plants may not always correlate closely with microbial siderophores, since in some studies chelators are taken up by roots much less efficiently than synthetic or phytosiderophores (Crowley *et al.*, 1991; Marschner and Römheld, 1994). Moreover there is no precedent that soil bacteria directly regulate the acquisition of iron by plants.

Bacillus subtilis GB03 is a commercially available soil symbiont that emits a blend of volatile components that activate growth promotion in Arabidopsis (Ryu *et al.*, 2003; Paré *et al.*, 2005). Unlike several reported plant-growth-promoting rhizobacterial strains that activate plant responses by producing phytohormones, in GB03, a bouquet of over 30 bacterial volatile odors devoid of classical hormones is capable of triggering growth promotion without physical contact with the plant. These volatile organic compounds (VOCs) activate differential expression of approximately 600 transcripts related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation and iron homeostasis (Ryu *et al.*, 2003; Farag *et al.*, 2006; Zhang *et al.*, 2007). This blend of VOCs not only induces leaf cell expansion (Zhang *et al.*, 2007), but also augments plant photosynthetic activities, with increased synthesis of photosynthetic machinery (Zhang *et al.*, 2008). Given the importance of iron in photosynthesis, the potential role of GB03 VOCs in iron assimilation has been investigated. Herein is reported that GB03 VOCs stimulate iron uptake responses to elevate plant iron acquisition. GB03-triggered increases in photosynthetic capacity were examined in plants compromised in iron uptake and plants grown under different iron regimes to probe the role of iron in GB03-induced increases in photosynthesis.

RESULTS

GB03 directly and indirectly induces acidification of the rhizosphere

Since the solubility of iron improves with increasing soil acidity, strategy I plants effectively elevate the solubility of ferric iron by exuding protons into the rhizosphere (Palmgren, 2001). To examine whether GB03 affects the plant's capacity to acidify the rhizosphere, colorimetric acidification assays were performed by addition of the pH indicator bromocresol purple to the medium. Plants were initially grown in iron-sufficient medium with or without exposure to GB03 for 8 days, before transferring to pH indicator plates that turn yellow when the pH drops below 5.2. Within 24 h of transferring to the indicator medium, GB03-exposed plants (Figure 1a) exhibited greater acidification of the rhizosphere compared with water-treated controls (Figure 1b). Quantitative measurement of acidification of the medium revealed that root proton release capacity was increased three-fold with exposure to GB03 (Table 1). In contrast to partial yellowing of the medium (acidification) by GB03-exposed plants proximal to the root mass, complete yellowing of the medium occurred when GB03 was co-transferred with the plants (Figure 1c) suggesting that in addition to increasing root proton release, GB03 VOC emissions also directly acidify the plant growth medium. Indeed GB03 VOC emissions alone are sufficient to acidify the medium (Figure 1d, Table 1), demonstrating that GB03 effectively reduces the pH of the rhizosphere via direct acidification of the medium as well as induced root proton release.

GB03 activates root iron uptake transcriptionally and post-transcriptionally

In addition to acidification of the rhizosphere, strategy I plants coordinately regulate ferric reductase and ferrous iron transporter in response to iron demand (Connolly *et al.*, 2002, 2003). To determine whether GB03 regulates Fe^{3+}-chelate reductase and Fe^{2+} transporter *in planta*, transcript abundance of the Fe^{3+}-chelate reductase *FRO2* and the Fe^{2+} transporter *IRT1* were analyzed in Arabidopsis grown in medium supplemented with sufficient iron. Compared with control plants, *IRT1* gene expression in GB03-treated plants was up-regulated 2 and 4 days post-exposure, by approximately 20- and 10-fold, respectively (Table 2). Transcript abundance of *FRO2* in treated plants also increased within 2 days of GB03 exposure by slightly less than four-fold. Moreover, GB03-exposed plants exhibit higher ferric

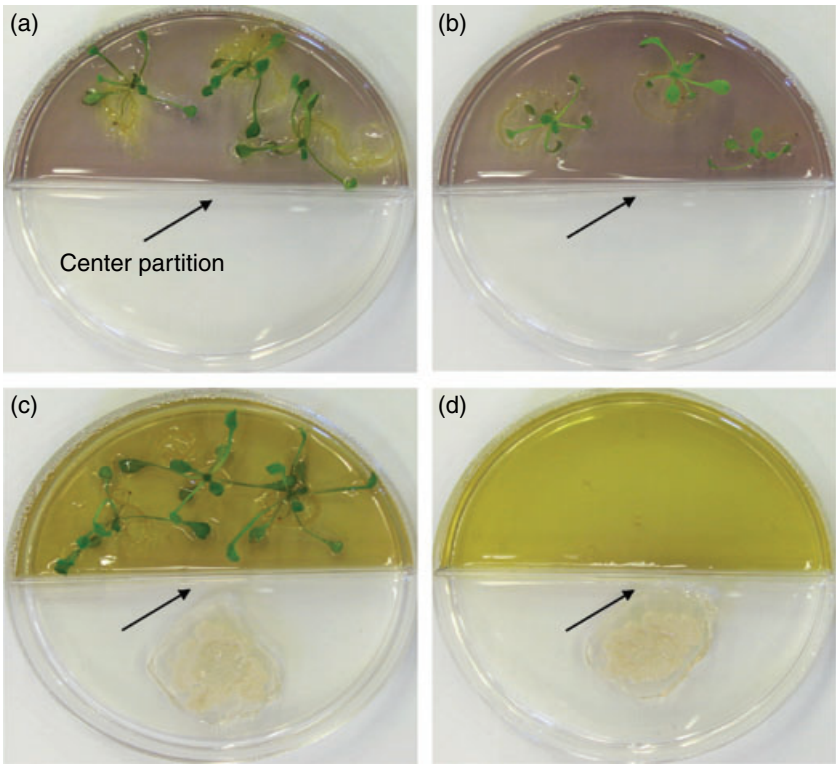

Figure 1. Plant or medium exposure to *Bacillus subtilis* GB03 volatile organic compound (VOCs) acidifies the medium.
Yellowing of the medium indicates a pH reduction to <5.2. Plants are grown with 50 μM Fe-EDTA and treated as indicated for 8 days and then transferred to bromocresol purple indicator plates that are devoid of exogenous iron for 24 h before photographing. GB03-treated plants (a), water-treated control plants (b), GB03-treated plants and bacterial introduction on the plate-half without the indicator medium (c), and no plant added and bacterial introduction on the plate-half without the indicator medium (d).

Table 1 Acidification of plant growth medium (n = 3, mean ± SD)

Treatment	$[H^+]$ release (nmol h^{-1})
Root acidification	
GB03-treated plants	0.90 ± 0.10 g^{-1} (FW)
H_2O-treated plants	0.28 ± 0.15 g^{-1} (FW)
Bacterial acidification	
GB03 VOCs	1.55 ± 0.13 per treatment

FW, fresh weight; VOC, volatile organic compound; GB03, *Bacillus subtilis* GB03.

Table 2 *Bacillus subtilis* GB03-induced gene expression in Arabidopsis (Col-0). Values detected by quantitative RT-PCR are relative to the lowest value for each gene (n = 3, mean ± SD)

	Day 2		Day 4	
Gene	GB03	H_2O	GB03	H_2O
IRT1	82.5 ± 9.8	4.3 ± 0.6	10.4 ± 1.0	1.0 ± 0.1
FRO2	6.0 ± 0.1	1.6 ± 0.2	2.5 ± 0.1	1.0 ± 0.1
FIT1	2.6 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.0 ± 0.1

reductase activity than controls. The GB03 induction of FRO2 transcript levels at day 2 was slightly greater than the increase in ferric chelate reductase activity of approximately three-fold after 1 week of exposure to GB03 (Figure 2). Transcript abundance and activity for iron acquisition along with medium acidification are elevated in GB03-treated plants, implicating GB03 in the activation of plant iron acquisition through regulation of strategy I iron-deficiency responses.

GB03 exposure increases plant iron content

Consistent with gene induction and medium acidification, exposure to GB03 increased whole-plant iron content (Figure 3a). Three days after treatment, iron content in GB03-treated plants increased over 30% compared with water-treated controls and increases in iron content in whole plant were more than two-fold at 5, 7 and 14 days post-GB03 exposure. Since lateral roots are more abundant in GB03-exposed plants compared with water controls (Zhang *et al.*, 2007), iron bound to apoplastic regions of the roots could account for the increased iron content at the whole plant level. However, iron content in GB03-exposed shoot were about two- and three-fold that of control plants, with 7 and 14 days of GB03 exposure, respectively (Figure 3b), confirming the elevation of iron acquisition with GB03 treatment.

FIT1 mediates GB03-enhanced plant iron uptake

Fe-deficiency Induced Transcription Factor 1 (*FIT1*) is a central component of iron homeostasis in Arabidopsis as it regulates iron-deficiency responses (Colangelo and Guerinot, 2004; Bauer *et al.*, 2007), though FIT1-independent

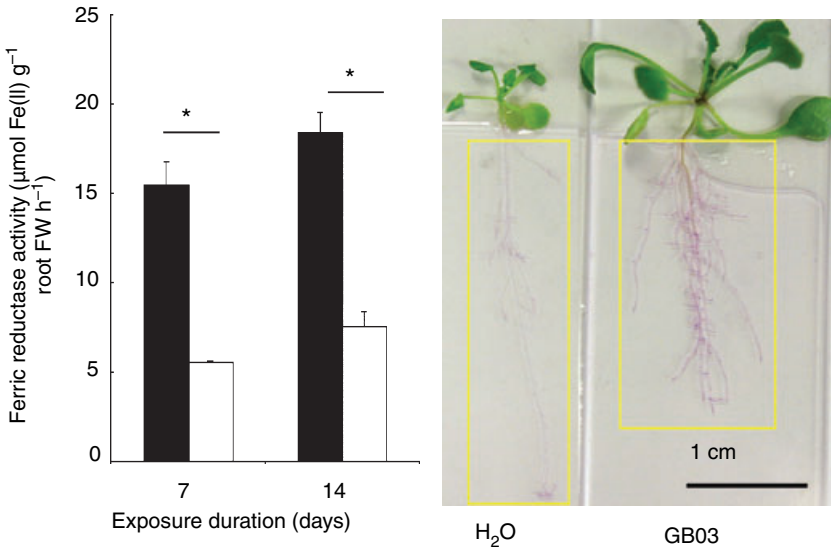

Figure 2. *Bacillus subtilis* GB03 increases root ferric reductase activity.
Plants were grown with 50 μM Fe-EDTA and treated with GB03 or water for 7 and 14 days. Root ferric reductase activities are quantified to the left of representative plants with greater ferric reductase activity (purple color) with GB03 treatment; yellow boxes demarcate the root region of plants with 14 days of indicated treatment. Black bars represent GB03 treatment, white bars water controls. FW represents fresh weight (n = 3 with error bars representing SD). An asterisk (*) indicates P-values ≤0.05 for GB03 treatment versus controls.

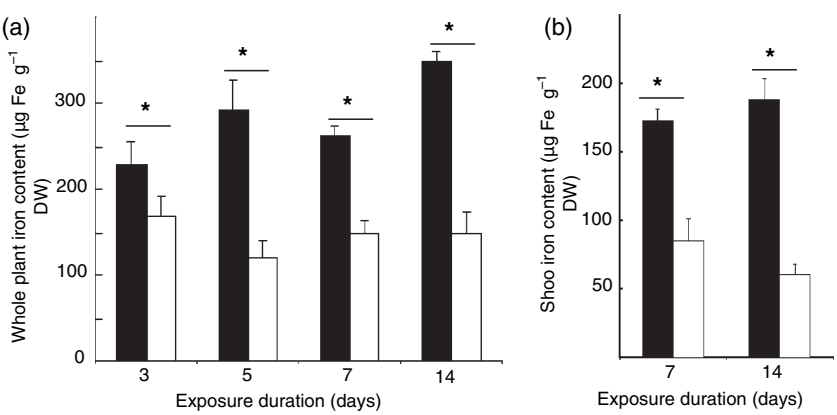

Figure 3. *Bacillus subtilis* GB03 enhances iron acquisition in plants.
Plants were grown with 50 μM Fe-EDTA and treated with GB03 or water for different periods of time as indicated. Elevated iron is detected in GB03-exposed plants as measured in whole-plants (a) or shoots (b). Black bars represent GB03 treatment, white bars water controls. DW represents dry weight ($n \geq 4$ with error bars representing SD). An asterisk (*) indicates P-values ≤0.05 for GB03 treatment versus controls.

regulation of iron uptake has been reported (Séguéla *et al.*, 2008). Parallel to the induced gene expression of *FRO2* and *IRT1*, plants exhibit increased transcript accumulation of *FIT1* with exposure to GB03 (Table 2), suggesting that GB03-regulated iron uptake responses may be mediated by FIT1 function. To test this hypothesis, the Arabidopsis *FIT1* loss-of-function mutant *fit1-2* was grown as wild-type plants under iron-sufficient conditions and exposed to GB03 or water. Unlike wild-type plants, GB03 did not trigger gene induction of either *FRO2* or *IRT1* in *fit1-2* plants, at 2 or 4 days post-treatment (Table 3). The *fit1-2* mutant also shows an unexpected reduction in *FRO2* transcript level at 2 days post-GB03 exposure (Table 3) suggesting that other GB03-regulated factors besides FIT1 can also induce or suppress *FRO2* gene expression. In fact, similar observations have been shown with iron deficiency in which genes such as *AHA7* are up-regulated in Col-0 and down-regulated in *fit1* plants (Colangelo and Guerinot, 2004). Nonetheless, these results demonstrated that FIT1 is required for GB03-triggered induction of *FRO2* and *IRT1* gene expression.

Table 3 *Bacillus subtilis* GB03-induced gene expression in Arabidopsis (*fit1-2*). Values detected by quantitative RT-PCR are relative to the lowest value for each gene (n = 3, mean ± SD)

	Day 2		Day 4	
Gene	GB03	H_2O	GB03	H_2O
IRT1	2.5 ± 0.2	2.3 ± 0.2	1.0 ± 0.04	1.6 ± 0.1
FRO2	1.0 ± 0.1	2.6 ± 0.2	1.5 ± 0.1	1.8 ± 0.1

Moreover, root ferric reductase activity in *fit1-2* plants was not altered with GB03 exposure (Figure 4a). Consistent with the loss of transcriptional and post-transcriptional induction, iron content did not increase in *fit1-2* plants with exposure to GB03 as in wild-type plants (Figure 4b). Iron deficiency increased the level of IRT1 protein while dysfunction of FIT1 abolished the accumulation of IRT1 protein independent of iron availability (Colangelo and Guerinot, 2004). Although IRT1 protein was not examined in this study, the fact that GB03 increases iron content in wild-type plants but not in *fit1*

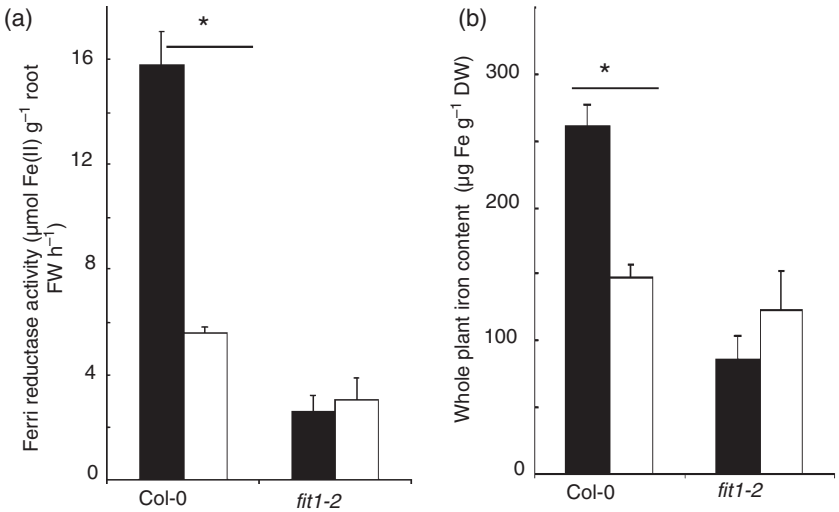

Figure 4. FIT1 mediates *Bacillus subtilis* GB03-stimulated plant iron uptake.
Unlike wild-type plants, Arabidopsis *fit1-2* mutant plants do not exhibit GB03-triggered increases either in root ferric reductase activity (a) or plant iron content (b). Plants were grown with 50 μM Fe-EDTA and treated with GB03 or water for 7 days. Black bars represent GB03 treatment, white bars water controls ($n \geq 3$, mean ± SD). An asterisk (*) indicates $P \leq 0.05$ for GB03 treatment versus controls.

mutant indicates that GB03-enhanced iron uptake in plants is dependent on FIT1 function.

GB03 augments photosynthesis via iron assimilation

Wild-type Arabidopsis exposed to GB03 exhibit increases in photosynthetic efficiency as well as chlorophyll content, compared with control plants (Zhang *et al.*, 2008). Since photosynthesis is highly sensitively to the availability of iron, the physiological significance of GB03-enhanced iron acquisition in plants was evaluated by studying iron uptake in GB03-triggered increases in photosynthetic capacity. Wild-type and *fit1-2* mutant plants were grown under iron-sufficient conditions and compared with or without GB03 exposure. Wild-type plants with 7 days' exposure to GB03, compared with control wild-type plants, accumulate a higher level of endogenous iron (Figure 4b) and appear greener (Figure 5a), showing 84 and 15% increases in chlorophyll content (Figure 5b) and photosynthetic efficiency (Figure 5c), respectively. In contrast, GB03 failed to increase either chlorophyll content (Figure 5b) or photosynthetic efficiency (Figure 5c) in *fit1-2* mutant plants, which did not exhibit GB03-triggered enhancement of iron content (Figure 4b). Therefore coordination of endogenous iron acquisition by GB03 via FIT1 appears to be necessary for increases in chlorophyll content and photosynthetic efficiency.

To further characterize the role of iron availability in GB03-induced increases in photosynthetic capacity, plants were supplemented with low (10 or 30 μM), sufficient (50 μM), or abundant (100 μM) iron (Connolly *et al.*, 2002). Endogenous iron almost doubled with GB03 exposure except under conditions of abundant iron where GB03 buffered iron uptake relative to controls (Figure 6a). With limited iron, GB03 increased photosynthetic efficiency and chlorophyll content; while the differences in photosynthetic apparatus

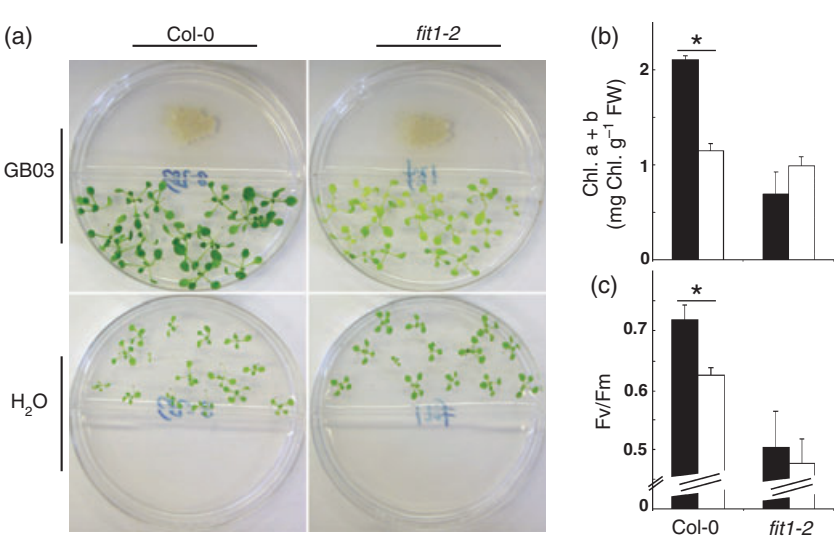

Figure 5. *Bacillus subtilis* GB03 enhances plant iron uptake and augments photosynthetic efficiency.
GB03 coordinately increases chlorophyll (*a* + *b*) content (b) and photosynthetic efficiency (c) in Col-0 but not in *fit1-2* plants. Plants were grown with 50 μM Fe-EDTA and treated with GB03 or water for 7 days. Black bars represent GB03 treatment, white bars water controls ($n = 4$, mean ± SD). An asterisk (*) indicates $P \leq 0.05$ for GB03 treatment versus controls.

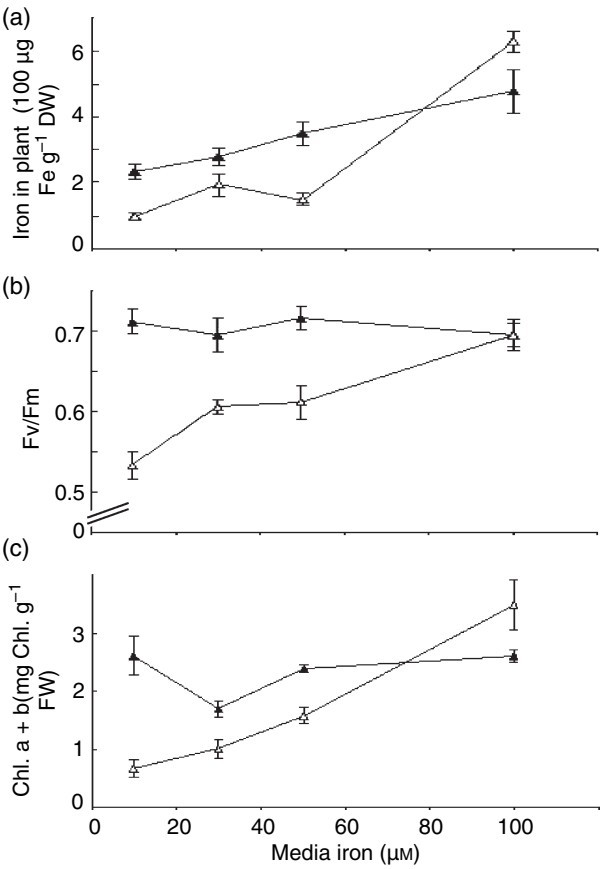

Figure 6. Increasing the availability of exogenous iron reduces *Bacillus subtilis* GB03-triggered increases in iron uptake (a), photosynthetic efficiency (b), and chlorophyll content (c) in Col-0.
Black and white triangles indicate GB03 treatment and water controls, respectively (n = 4, mean ± SD). Analysis is at 14 days post-treatment.

were minimized when exogenous iron was abundant (Figure 6b,c), indicating that endogenous iron is critical for GB03-increased chlorophyll content and photosynthetic efficiency.

DISCUSSION

Plant growth-promoting rhizobacteria (PGPR) form mutually beneficial associations with plants through a complex exchange of chemical metabolites within the rhizosphere. Microbial siderophores have been proposed to facilitate iron acquisition in plants. Herein we demonstrate that *B. subtilis* GB03 not only significantly acidifies the rhizosphere but also directly activates strategy I iron uptake responses, resulting in an elevated endogenous plant iron content. The partition separating bacteria from the plant (Figure 1) excludes the possibility that bacterial siderophores contribute to the increased iron uptake in GB03-exposed plants. GB03 VOCs also augment photosynthetic capacity via FIT1-dependent iron acquisition.

Microbial siderophores are observed to facilitate iron uptake by plants based on radiolabeling studies using Fe^{3+}-siderophores as the sole iron source (for review, see Bar-Ness *et al.*, 1992; Glick, 1999). However, in comparing siderophore uptake in microbe-rich and sterile conditions, an absence of microbes greatly reduces the rate at which siderophore-chelated iron is taken up by roots (Bar-Ness *et al.*, 1992). Therefore the close association of beneficial microbes seems to improve iron uptake by plants. In contrast, a recent study with purified microbial siderophores showed increased iron uptake (Vansuyt *et al.*, 2007). These combined observations suggest that iron chelation and bacterial signaling may participate in improved iron assimilation by plants. While it is generally assumed that bacterial activation of plant growth requires the presence of bacteria in the rhizosphere, our data clarify that without root colonization, GB03 VOCs induce iron-deficiency-like responses, resulting in elevated iron acquisition including acidification of the rhizosphere by GB03. Of the over 25 volatile metabolites that have been identified from the bacterial blend emitted from GB03, three components, glyoxylic acid, 3-methyl-butanoic acid and diethyl acetic acid, can be classified as organic acids (Farag *et al.*, 2006). Such acidification of the rhizosphere by bacterial metabolites, apart from its potential role in plant signaling to elevate iron assimilation by the plant, favors iron solubility in the rhizosphere. Indeed the solubility of macronutrients such as phosphate is also facilitated by such acidification of the rhizosphere (Sharma and Sahi, 2005). The bacterial volatiles thus far characterized chemically do not have known siderophore activity (Farag *et al.*, 2006) and the screening of individual bacterial VOCs for induction of iron uptake by plants has yet to be performed.

Although iron is the fourth most abundant element in the earth's crust, iron is mostly present as insoluble oxyhydroxide polymers that are not readily taken up by plants. As a result, iron can be a limiting micronutrient especially for plants grown in calcareous soils, which account for approximately one-third of the earth's soils (Guerinot and Yi, 1994). Traditionally iron deficiency occurs when iron drops below a critical tissue level that ranges from 50–100 µg g^{-1} plant dry weight (Guerinot and Yi, 1994); for Arabidopsis grown in half-strength MS medium containing 50 µM Fe-EDTA (Murashige and Skoog, 1962), basal iron content is well above this critical plant tissue iron level. While too much iron is toxic to plants, moderate increases in endogenous iron levels either by GB03-activated iron acquisition or increasing the availability of iron to the roots results in significant increases in the photosynthetic efficiency of plants.

The availability and redox potential of Fe^{2+}/Fe^{3+} enables its use in heme and iron–sulfur clusters that are essential for electron transfer reactions (Curie and Briat, 2003). The photosynthetic apparatus in plants is iron-enriched, containing two or three iron atoms per photosystme II (PSII);

twelve iron atoms per photosystem I; five per cytochrome b_6–*f* complex; and two per ferredoxin molecule (Varotto *et al.*, 2002); as a result, iron deficiency reduces reaction centers and electron carriers. For example, iron deficiency induces adaptive changes in the pool of PSII complexes that decrease the efficiency of plastoquinone-pool reoxidation resulting in higher levels of Q_A oxidation (Sharma, 2007). This loss in photosynthetic capacity renders the photosynthetic electron transport system susceptible to damage by light intensities normally encountered during growth, with PSII being especially sensitive (Sharma, 2007).

Treatment with GB03 stimulates iron acquisition and increases iron content in plants, which is consistent with its stimulatory effects on plant photosynthesis (Zhang *et al.*, 2008). Studies using *fit1* mutants demonstrate the significance of GB03-stimulated plant iron acquisition, as GB03 fails to enhance photosynthetic capacity in this mutant defective in iron uptake. In addition to mediating photosynthetic function, iron also plays an important role in plant stress responses. Upon pathogen attack, reactive Fe^{3+} is deposited at cell wall appositions where it accumulates and mediates the oxidative burst (Liu *et al.*, 2007). Iron homeostasis is also regulated by nitric oxide (NO), a cellular messenger in many physiological processes including hypersensitive responses and programmed cell death (Graziano and Lamattina, 2005). In addition the phytohormones ethylene and cytokinin have been shown to regulate plant responses to iron deficiency (Lucena *et al.*, 2006; Séguéla *et al.*, 2008). The participation of ethylene signaling in the regulation of plant iron uptake by GB03 was preliminarily examined by assaying an ethylene-insensitive mutant *etr1* with exposure to GB03 volatiles. Similarly to wild-type controls the GB03-treated *etr1* line exhibited higher shoot iron content, photosynthetic efficiency and chlorophyll content compared with control plants (HZ and PWP, unpublished data), suggesting that ethylene does not act in the GB03-regulated iron uptake signaling pathway or a GB03-derived inducer acts downstream of the ethylene receptor ETR1.

IRT1 is a high-affinity Fe^{2+} transporter that plays a key role in iron homeostasis (Varotto *et al.*, 2002; Vert *et al.*, 2002; Curie and Briat, 2003). In an aerobic rhizosphere, iron initially exists in the Fe^{3+} oxidation state before being reduced to Fe^{2+} for cellular functions. Expression of *FRO2* and *IRT1* is coordinately regulated in strategy I plants by iron starvation (Connolly *et al.*, 2003) and bacterial volatile stimulus as demonstrated in this study. FRO2-mediated ferric reduction is proposed to be the rate-limiting step in plant iron uptake under low-iron conditions (Connolly *et al.*, 2003). However in an Arabidopsis 35S-IRT1K146R,K171R double mutant exhibiting elevated iron accumulation an increase in ferric chelate reductase activity was not observed, suggesting that with sufficient iron, Fe^{3+} reduction may not be the rate-limiting step in iron uptake (Kerkeb *et al.*, 2008).

Given the importance of iron homeostasis in plant growth and development a highly orchestrated series of root-localized and shoot-borne signals have been characterized that tightly control the production of proteins involved in Fe^{3+} -chelate reductase activity and Fe^{2+} uptake, although initiation of these signals remains elusive (Romera *et al.*, 1992; Grusak, 1995; Schmidt and Schuck, 1996; Curie and Briat, 2003; Vert *et al.*, 2003). GB03-triggered induction of *IRT1* and *FRO2* gene is not observable in the *fit1* mutant, suggesting that FIT1 mediates regulation of *IRT1* and *FRO2* by GB03. Since *FIT1* itself is iron-regulated (Colangelo and Guerinot, 2004), an iron sensor upstream of FIT1 would be predicted. Characterization of activating signals in GB03 VOCs that stimulate acquisition of iron by plants may well reveal insights into early regulatory steps in plant iron uptake and homeostasis.

EXPERIMENTAL PROCEDURES

Plant materials and treatments

Arabidopsis thaliana seeds were surface sterilized and then planted on one side of specialized plastic Petri dishes (100 × 15 mm) that contain a center partition (I plates, Fisher Scientific, http://www.fishersci.com/); both sides contain half-strength MS solid medium with 0.8% (w/v) agar and 1.5% (w/v) sucrose. Growth medium containing 50 μM Fe-EDTA has been shown to be iron-sufficient for Arabidopsis growth (Connolly *et al.*, 2002; Vert *et al.*, 2002; Colangelo and Guerinot, 2004). Therefore, we refer to half-strength MS medium that contains 50 μM Fe-EDTA (Murashige and Skoog, 1962) as the iron-sufficient condition in this study. Media with iron concentrations different from the standard 50 μM were prepared according to Murashige and Skoog (1962), except that defined amounts of Fe-EDTA were added. Before being placed in the growth room, seeds were vernalized for 2 days at 4°C in the absence of light. Arabidopsis are grown under sterile conditions with a 16/8 h light/dark cycle at 200 μmol photons m^{-2} sec^{-1} light, 21 ± 4°C, and 40 ± 10% relative humidity.

One day before plant experiments, bacterial strains were streaked onto tryptic soy agar (TSA) plates and incubated at 28°C in the absence of light for 24 h. The PGPR cells were harvested from the TSA plates in double-distilled water (DDW) to yield 10^9 colony-forming units (CFU) ml^{-1}, as determined by optical density and serial dilutions with plate counts. Two-day-old Arabidopsis seedlings are then inoculated with 20 μl of bacterial suspension culture or DDW applied dropwise to the non-plant side of the Petri dish.

Proton concentration measurements in the rhizosphere

The pattern of pH change in the medium was determined by pH indicator visualization (Yi and Guerinot, 1996). For each I-plate, one half contains half-strength MS medium with 1.5% sucrose; the other half of the medium contains 0.2 mM $CaSO_4$ and the pH indicator bromocresol purple (0.006%), solidified with 0.7% agar. The pH of the medium was adjusted to 6.5 with NaOH. Arabidopsis seedlings were initially grown in half-strength MS medium and exposed to GB03 or DDW as a control for 8 days, then transferred to the medium with bromocresol purple. To quantify acidification of the plant growth medium, the experimental set-up is the same as described previously, except that agar was not added to the pH indicator

solution. Each plate contained equal amounts (10 ml) of pH indicator solution in each half of the plate. Before and after 24 h of acidification by roots or GB03 VOCs, the pH value of the pH indicator solution was determined by a pH meter (model 370 pH/ISE/mV/ORP/Temperature Meter, ATI Orion, http://www.thermo.com). Plate pH values without plant introduction or GB03 exposure were used as blank controls for calculation of the proton concentration. Root proton release (unit: mol g^{-1} fresh weight h^{-1}) was determined as $(10^{-\text{final pH}} - 10^{-\text{initial pH}}) \times 0.01$/fresh weight of roots/24 h.

Quantitative real-time PCR

The RNA was extracted following the Qiagen RNeasy protocol (Qiagen, http://www.qiagen.com/). Two-day-old seedlings were treated as described and whole plant RNA was extracted 2 and 4 days after treatments. The RNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech, http://www.nanodrop.com). The expression levels of genes in different RNA samples were detected by a Roche LightCycler 480 (http://www.roche.com/). The primers were designed by using the program PrimerQuest (Integrated DNA Technologies, http://www.idtdna.com/). 18S rRNA primers were used for RNA normalization. LightCycler RNA Master SYBR Green I (Roche) was used for one-step real-time RT-PCR. The PCR reactions were as follows: reverse transcription, 61°C for 20 min; denaturation, 95°C for 30 sec; amplification, 45 cycles of 95°C for 5 sec, 60°C for 20 sec and 72°C for 10 sec. The relative expression levels of all the samples were calculated and analyzed. The threshold cycle (*Ct* value) of the target genes and 18S RNA in different samples were obtained after quantitative real-time PCR reaction. In brief, the normalizer 18S RNA *Ct* value is subtracted from the *Ct* of the gene of interest (target gene) to produce the d*Ct* value of the sample. The d*Ct* value of the calibrator (the sample with the highest d*Ct* value) was subtracted from every other sample to produce the dd*Ct* value. Two to the power –dd*Ct* ($2^{-\text{dd}Ct}$) was taken for every sample as the relative expression levels. The following primers are used (5′ to 3′): [IRT1] ACCCGTGCGTCAACAAAGCTAAAG and TCCCGGAGGCGAAACACTTAATGA; [FRO2] TGTGGCTCTTCTTCTCTGGTGCTT and TGCCACAAAGATTCGTCATGTGCG; [FIT1] ACCTCTTCGACGAATTGCCTGACT and TTCATCTTCTTCACCACCGGCTCT; [18S RNA] AATTACCGCGGCTGCTGGCA and CGGCTACCACATCCAAGGAA.

Iron concentration measurements

Plant iron levels were determined as described (Lobreaux and Briat, 1991). Tissues (100–200 mg) were ground with liquid N2, mineralized according to Beinert (1978) and reduced with thioglycolic acid. The Fe^{2+}–*O*-phenanthroline complex was measured spectrophotometrically at 510 nm and iron concentration was reported on a tissue dry-weight basis using a wet-weight conversion factor determined by weighing the tissue aliquot before and after desiccation at 100°C.

Fe(III) reductase enzyme assay

Root-associated ferric reductase activity was assayed via spectrophotometric quantification of a purple-colored Fe(II)–FerroZine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine sulfonate] complex formed from the reduction of Fe(III) (Yi and Guerinot, 1996). The plant root system was submerged in the assay solution containing 0.1 mM Fe(III)-EDTA and 0.3 mM FerroZine (HACH Chemical Co., http://www.hach.com/). After a 20-min dark incubation, absorbance at 562 nm was recorded and the concentration of Fe(II)–FerroZine was calculated based on a molar extinction coefficient of 28.6 mM^{-1} cm^{-1} (Gibbs, 1976). An identical assay solution without root incubation was used as a blank. Assay solutions devoid of Fe(III) did not exhibit a difference in absorbance with and without root incubation, indicating that root-bound Fe(II) did not contribute to the measured ferric reductase activity.

Chlorophyll fluorescence measurements

Arabidopsis plants that have been exposed to GB03 VOCs or water for 2 weeks were used. Chlorophyll *a* fluorescence emission from attached leaves was measured with a pulse amplitude modulated fluorometer (PAM 101/103; Heinz Walz GmbH, http://www.walz.com/). Experimental protocol and nomenclature were based on descriptions by Maxwell and Johnson (2000). Plants were kept in Petri dishes during photosynthesis measurements. F_0 (minimal level of fluorescence in the dark-adapted state) measurements were performed on leaves previously kept in darkness for at least 30 min. After this, the maximal level of fluorescence in the dark-adapted state (F_m) was measured by means of a saturating flash. Saturating light pulses of 1-sec duration were provided by a KL 1500 light source (Schott, http://www.schott.com). The ratio F_v/F_m ($F_v = F_m - F_0$) was used to estimate the potential quantum yield of PSII photochemistry. Then the leaves were illuminated with actinic light (with an intensity as indicated in the figure legends) in order to activate photosynthetic reactions. Preliminary experiments have shown that 10-min illumination at room temperature is enough to obtain steady-state levels of fluorescence parameters. Once the leaves were adapted to the light, a saturating flash was applied to obtain the maximal level of fluorescence at light-adapted state (F_m'). Immediately after the flash, the actinic light was switched off and the measurement of F_0' was performed following the short-term application of low-intensity far-red light. The following parameters were calculated: coefficient of photochemical quenching [$qP = (F_m' - F)/(F_m' - F_0')$ where F is the level of fluorescence just before the pulse of saturating light was applied] reflecting the portion of PSII complexes with reaction centers in an 'open' state; quantum efficiency of electron transport through PSII complexes ($\Phi_{PSII} = (F_m' - F)/F_m'$); and quantum efficiency of non-photochemical dissipation in PSII complexes ($\Phi_N = F_0'/F_m'$) where Φ_{PSII} represents actual quantum yield of PSII photochemistry for light-acclimated samples under illumination while Φ_N reflects the non-photochemical dissipation in light-acclimated samples, which have all their PSII reaction centers in the 'open' state (Kornyeyev and Hendrickson, 2007).

The method known as chlorophyll fluorescence analysis is based on the detection of the fluorescence signal originating from PSII; data about the overall photosynthetic activity are also obtained with this method (Maxwell and Johnson, 2000). The level of PSII fluorescence depends on the reduction state of Q_A, the primary quinone acceptor. The effective electron transport results in a lower percentage of Q_A being reduced (lower *qP* levels) while reduction of electron transport downstream of Q_A including decreased photosystem I (PSI) activity or Calvin cycle inhibition leads to greater oxidation of the Q_A pool and consequently elevate the level of chlorophyll fluorescence. Actual (effective) quantum yield of PSII photochemistry (Φ_{PSII}) is shown to correlate well with the quantum efficiency of CO_2 fixation (Genty *et al.*, 1989; Edwards and Baker, 1993) as well as with the quantum efficiency of PSI (Harbinson *et al.*, 1989; Eichelmann and Laisk, 2000). Moreover, chlorophyll fluorescence analysis is a non-invasive and straightforward tool for probing functional activity of the photosynthetic apparatus.

Determination of chlorophyll contents

Fourteen days after treatments, weighed leaves were glass ground with aqueous acetone (80% v/v). Absorbance readings of the supernatant (centrifugation at 13 000 ***g*** for 5 min) were recorded at 470, 646.8 and 663.2 nm. Total chlorophyll content was calculated as $(7.15 \times A_{663.2} + 18.71 \times A_{646.8})/1000/(\text{fresh weight of leaves})$; calculated value were reported as mg chlorophyll g^{-1} fresh weight described by Lichtenthaler (1987).

Statistical analysis

Statistical analyses were performed with SAS software (SAS Institute, http://www.sas.com/). Significant difference between treatments was based on *P*-values ≤0.05.

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REFERENCES

Bar-Ness, E., Hadar, Y., Chen, Y., Shanzer, A. and Libman, J. (1992) Iron uptake by plants from microbial siderophores: a study with 7-nitrobenz-2 oxa-1,3-diazole-desferrioxamine as fluorescent ferrioxamine B analog. *Plant Physiol.* **99**, 1329–1335.

Bauer, P., Ling, H.Q. and Guerinot, M.L. (2007) *FIT1*, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in Arabidopsis. *Plant Physiol. Biochem.* **45**, 260–261.

Behrenfeld, M.J., Bale, A.J., Kolber, Z.S., Aiken, J. and Falkowski, P.G. (1996) Confirmation of iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean. *Nature*, **383**, 508–511.

Beinert, H. (1978) Micro methods for the quantitative determination of iron and copper in biological material. *Methods Enzymol.* **54**, 435–445.

Briat, J.F. (1992) Iron assimilation and storage in prokaryotes. *J. Gen. Microbiol.* **138**, 2475–2483.

Casano, L.M., Zapata, J.M., Martin, M. and Sabater, B. (2000) Chlororespiration and poising of cyclic electron transport. Plastoquinone as electron transporter between thylakoid NADH dehydrogenase and peroxidase. *J. Biol. Chem.* **275**, 942–948.

Colangelo, E.P. and Guerinot, M.L. (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell*, **16**, 3400–3412.

Connolly, E.L., Fett, J.P. and Guerinot, M.L. (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell*, **14**, 1347–1357.

Connolly, E.L., Campbell, N.H., Grotz, N., Prichard, C.L. and Guerinot, M.L. (2003) Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiol.* **133**, 1102–1110.

Crowley, D.E., Wang, Y.C., Reid, C.P.P. and Szaniszlo, P.J. (1991) Mechanisms of iron acquisition from siderophores by microorganisms and plants. *Plant Soil*, **130**, 179–198.

Curie, C. and Briat, J.F. (2003) Iron transport and signaling in plants. *Annu. Rev. Plant Biol.* **54**, 183–206.

Edwards, G.E. and Baker, N.R. (1993) Can CO2 assimilation in maize leaves be predicted accurately from chlorophyll fluorescence analysis? *Photosynth. Res.* **37**, 89–102.

Eichelmann, H. and Laisk, A. (2000) Cooperation of photosystem I and II in leaves as analysed by simultaneous measurements of chlorophyll fluorescence and transmittance at 800 nm. *Plant Cell Physiol.* **41**, 138–147.

Farag, M.A., Ryu, C.M., Sumner, L.W. and Paré, P.W. (2006) GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochem.* **67**, 2262–2268.

Genty, B., Briantais, J.M. and Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* **990**, 87–90.

Gibbs, C.R. (1976) Characterization and application of FerroZine iron reagent as a ferrous iron indicator. *Anal. Chem.* **48**, 1197–1201.

Glick, B.R., Pattern, C.L., Holguin, G. and Penrose, D.M. (1999) *Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria*. London: Imperial college Press, pp. 1–13.

Graziano, M. and Lamattina, L. (2005) Nitric oxide and iron in plants: an emerging and converging story. *Trends Plant Sci.* **10**, 4–8.

Grusak, M.A. (1995) Whole-root iron (III)-reductase activity throughout the life cycle of irongrown *Pisum sativum* L. (Fabaceae): relevance to the iron nutrition of developing seeds. *Planta*, **197**, 111–117.

Guerinot, M.L. and Yi, Y. (1994) Iron: nutritious, noxious, and not readily available. *Plant Physiol.* **104**, 815–820.

Harbinson, J., Genty, B. and Baker, N.R. (1989) The relationship between the quantum efficiencies of Photosystem I and II in pea leaves. *Plant Physiol.* **90**, 1029–1034.

Kerkeb, L., Mukherjee, I., Chatterjee, I., Lahner, B., Salt, D.E. and Connolly, E.L. (2008) Ironinduced turnover of the Arabidopsis IRON-REGULATED TRANSPORTER1 metal transporter requires lysine residues. *Plant Physiol.* **146**, 1964–1973.

Kornyeyev, D. and Hendrickson, L. (2007) Energy partitioning in photosystem II complexes subjected to photoinhibitory treatment. *Funct. Plant Biol.* **34**, 214–220.

Li, L., Cheng, X. and Ling, H.-Q. (2004) Isolation and characterization of Fe(III)-chelate reductase gene *LeFRO1* in tomato. *Plant Mol. Biol.* **54**, 125–136.

Lichtenthaler, H.K. (1987) Chlorophyll and carotenoids: Pigments of photosynthetic membranes. *Methods Enzymol.* **148**, 350–382.

Liu, G., Greenshields, D.L., Sammynaiken, R., Hirji, R.N., Selvaraj, G. and Wei, Y. (2007) Targeted alterations in iron homeostasis under lie plant defense responses. *J. Cell Sci.* **120**, 596–605.

Lobreaux, S. and Briat, J.F. (1991) Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. *Biochem. J.* **274**, 601–606.

Lucena, C., Waters, B.M., Romera, F.J., García, M.J., Morales, M., Alcántara, E. and Pérez-Vicente, R. (2006) Ethylene could influence ferric reductase, iron transporter, and H+-ATPase gene expression by affecting FER (or FER-like) gene activity. *J. Exp. Bot.* **57**, 4145–4154.

Marschner, H. and Römheld, V. (1994) Strategies of plants for acquisition of iron. *Plant Soil*, **165**, 261–274.

Maxwell, K. and Johnson, G.N. (2000) Chlorophyll fluorescence – a practical guide. *J. Exp. Bot.* **51**, 659–668.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, **15**, 473–497.

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S. and Soave, C. (2004) Arabidopsis thaliana plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *Plant J.* **38**, 940–953.

Neilands, J.B. and Leong, S.A. (1986) Siderophores in relation to plant growth and disease. *Ann. Rev. of Plant Physiol.* **37**, 187–208.

Palmgren, M.G. (2001) Plant plasma membrane H+-ATPases: powerhouses for nutrient uptake. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 817–845.

Paré, P.W., Farag, M.A., Krishnamachari, V., Zhang, H., Ryu, C.M. and Kloepper, J.W. (2005) Elicitors and priming agents initiate plant defense responses. *Photosynth. Res.* **85**, 149–159.

Robinson, N.J., Procter, C.M., Connolly, E.L. and Guerinot, M.L. (1999) A ferric-chelate reductase for iron uptake from soils. *Nature*, **397**, 694–697.

Romera, F.J., Alcantara, E., Diaz de, L.A. and Guardia, M. (1992) Role of roots and shoots in the regulation of the Fe efficiency responses in sunflower and cucumber. *Physiol. Plant*, **85**, 141–146.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Paré, P.W. and Kloepper, J.W. (2003) Bacterial volatiles promote growth in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **100**, 4927–4932.

Sandmann, G. (1985) Consequences of iron-deficiency on photosynthetic and respiratory electron-transport in blue-green-algae. *Photosynth. Res.* **6**, 261–271.

Schmidt, W. and Schuck, C. (1996) Pyridine nucleotide pool size changes in iron-deficient *Plantago lanceolata* roots during reduction of external oxidants. *Physiol. Plant*, **98**, 215–221.

Séguéla, M., Briat, J.F., Vert, G. and Curie, C. (2008) Cytokinins negatively regulate the root Iron uptake machinery in Arabidopsis through a growth-dependent pathway. *Plant J.* **55**, 289–300.

Sharma, S. (2007) Adaptation of photosynthesis under iron deficiency in maize. *J. Plant Physiol.* **164**, 1261–1267.

Sharma, N.C. and Sahi, S.V. (2005) Characterization of phosphate accumulation in *Lolium multiflorum* for remediation of phosphorus-enriched soils. *Environ. Sci. Technol.* **39**, 5475–5480.

Spiller, S. and Terry, N. (1980) Limiting factors in photosynthesis II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. *Plant Physiol.* **65**, 121–125.

Spiller, S., Castlefranco, A. and Castlefranco, P. (1982) Effects of iron and oxygen on chlorophyll biosynthesis. 1. In vivo observations on iron and oxygen-deficient plants. *Plant Physiol.* **69**, 107–111.

Stocking, C.R. (1975) Iron deficiency and structure and physiology of maize chloroplasts. *Plant Physiol.* **55**, 626–631.

Terry, N. (1980) Limiting factors in photosynthesis I. Use of iron stress to control photochemical capacity in vivo. *Plant Physiol.* **65**, 114–120.

Tottey, S., Block, M., Allen, M., Westergren, T., Albrieux, C., Scheller, H., Merchant, S. and Jensen, P. (2003) Arabidopsis CHL27, located in both envelope and thylakoid membranes, is required for the synthesis of protochlorophyllide. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.

Vansuyt, G., Robin, A., Briat, J.F., Curie, C. and Lemanceau, P. (2007) Iron acquisition from Fepyoverdine by *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* **20**, 441–447.

Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F. and Leister, D. (2002) The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. *Plant J.* **31**, 589–599.

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M.L., Briat, J.F. and Curie, C. (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. *Plant Cell*, **14**, 1223–1233.

Vert, G.A., Briat, J.F. and Curie, C. (2003) Dual regulation of the Arabidopsis high-affinity root iron uptake system by local and long-distance signals. *Plant Physiol.* **132**, 796–804.

Yi, Y. and Guerinot, M.L. (1996) Genetic evidence that induction of root Fe (III) chelate reductase activity is necessary for iron uptake under iron deficiency. *Plant J.* **10**, 835–844.

Zhang, H., Kim, M.S., Krishnamachari, V. *et al.* (2007) Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. *Planta*, **226**, 839–851.

Zhang, H., Xie, X., Kim, M.S., Kornyeyev, D.A., Holaday, S. and Paré, P.W. (2008) Soil bacteria augment Arabidopsis photosynthesis by decreasing glucose sensing and abscisic acid levels *in planta*. *Plant J.* **56**, 264–273.

