Improved iron acquisition of Astragalus sinicus under low iron-availability conditions by soil-borne bacteria Burkholderia cepacia

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ABSTRACT
Soil bacteria can assist plant growth and increase uptake of nutrient elements, the question arises as to whether beneficial soil microbes confer augmented iron (Fe) content of host plants under Fe limited conditions. Herein, a novel strain of Burkholderia cepacia (strain JFW16) was isolated from rhizospheric soils of Astragalus sinicus grown under alkaline conditions. Inoculation of plants with B. cepacia JFW16 displayed increased endogenous Fe content compared with non-inoculated plants. Growth promotion and enhanced photosynthetic capacity were also observed for the inoculated plants. The inoculation with JFW16 significantly increased the rhizospheric acidification, and up-regulated the transcription of some Fe acquisition-associated genes in Astragalus sinicus. Moreover, the metabolic pathways of flavins were remarkably enhanced in the inoculated plants, showing the increased biosynthesis and release of flavins in roots. Collectively, these findings demonstrated the potential of B. cepacia JFW16 to improve Fe assimilation in agricultural crops.

KEYWORDS
Astragalus sinicus; Burkholderia cepacia; iron acquisition; alkaline soils; flavins

Introduction
Iron (Fe) is one of the most essential elements for plant growth and development because of its ability to change redox states, and it is required for some important physiological processes such as photosynthesis and respiration (Masse and Arguin 2005). Although Fe is highly abundant in the earth’s crust, it is exceedingly insoluble in alkaline soils. Fe often forms scarce solubility of Fe³⁺ oxyhydroxides that are not easily assimilated by most plant species (Lemanceau et al. 2009). Many crop plants such as barely and peanut often exhibit Fe deficiency-induced leaf chlorosis under alkaline conditions (Takahashi et al. 2001; Xiong et al. 2013). Calcareous soils account for about a third of the earth’s crust, Fe deficiency thus becomes one of major abiotic stresses that seriously affect plant growth and development, crop yield and quality (Takahashi et al. 2001; Xiong et al. 2013; Zhou et al. 2016). Fe deficiency is also one of the most wide-ranging nutrient deficiency in human dietary, which causes adverse impacts on approximate 2 billion people health, and even 0.8 million death annually (Murgia et al. 2012). For sustainable agriculture, it is an urgent need to improve the ability of plants to acquire Fe from low Fe-availability soils.

Hitherto, great efforts have been made to unravel the mechanisms of plant’s Fe acquisition. It is well known that plants have evolved two crucial strategies (strategy I and II) including Fe reduction and chelation to mine Fe from soils (Kim and Guerinot 2007). Strategy I plants including nongraminaceous monocots and dicots primarily rely on the reduction-based Fe acquisition mechanism by three processes: (I) root release of protons into rhizospheric soils for augmenting the solubility of Fe³⁺ oxyhydroxides, and this process is dependent on the activity of plasma membrane H⁺-ATPase (AHA2) (Santi and Schmidt 2009); (II) the conversion of Fe³⁺ to Fe²⁺ by the ferric-chelate reductase (FCR) (FRO2) (Connolly et al. 2003); and (III) the transportation of Fe²⁺ into the epidemic cells of roots via the plasma-localized iron-regulated transport (IRT1) protein (Vert et al. 2002). Moreover, these individual components of Fe acquisition machines in plants are finely controlled by Fe deficiency-induced transcription factors (FIT) such as FIT1, bHLH38 and bHLH39 (Wang et al. 2007; Yuan et al. 2008). However, plants equipped with the Fe reduction-based mechanism often display typical symptoms of Fe deficiency under alkaline conditions (Xiong et al. 2013; Zhou et al. 2016). The protons released by Fe-deficient plants are mostly buffered by high alkaline pH conditions, and the FCR activities are also seriously depressed (Ohwaki and Sugahara 1997), indicating that the Fe reduction-based mechanism is readily impeded in the strategy I plants grown in alkaline soils. Furthermore, many Fe-deficient strategy I plants also can release several secondary metabolites such as phenolics and flavins, which own redox and/or metal-complexing ability to enhance Fe acquisition (Jin et al. 2007; Rodriguez-Celma et al. 2011).

Graminaceous (strategy II) plants acquire Fe from external environments based on the Fe-chelation strategy (Curie et al. 2001). This mechanism is to synthesize and release the mugineic acid-family phytosiderophores (PS) for chelating Fe. Strategy II plants exhibit greater growth traits than strategy I plants under alkaline conditions. Since the PS-Fe³⁺ complexes are directly taken up by roots, without requirement for Fe-reduction of strategy I plants (Nozoye et al. 2011). Differing from the reduction-based Fe acquisition, the chelation-based system is much insensitive to alkaline conditions (Römheld and Marschner 1986). However, enough Fe cannot be acquired...
by either strategy I or II plants grown under high alkaline pH conditions. Importantly, sunflower and maize plants grown in sterilized soils display poor growth performance and Fe deficiency-induced chlorosis compared with the controls grown in non-sterilized soils (Masalha et al. 2000). Moreover, the growth of sorghum and rape was seriously repressed in sterilized soils, but recovered after the addition of Fe source into soils, indicating that certain soil microbes may facilitate plants to take up Fe from soils (Rocco et al. 2003).

Recently, many studies have indicated that plant roots can secrete several metabolites to attract colonization of diverse species of soil microbes in rhizosperic soils, and some of which can promote plant growth and enhance the tolerance of host plants to abiotic and biotic stresses (Dey et al. 2004; Mishra et al. 2014; Zhou et al. 2016; Zhou et al. 2017). These beneficial soil microbes are generally called as plant growth promoting rhizobacteria (PGPR). Importantly, some PGPR strains have great potential to improve Fe absorption in plants (Pii et al. 2015, 2016; Zhou et al. 2016). Bacillus subtilis GB03 activates the Fe acquisition machinery in Arabidopsis and cassava (Manihot esculenta) plants (Zhang et al. 2009; Freitas et al. 2015). Zhou et al. (2016) have reported that the inoculation with Paenibacillus polymyxa BFKC01 increases Fe content in Arabidopsis plants under alkaline conditions. Thus, application of soil beneficial bacteria will become a most promising path to enhance plant’s Fe acquisition under low Fe-availability conditions.

Astragalus sinicus is one of important leguminous forage crops and has been used as a pasture for animal in Asian countries (Chou et al. 2006). However, alkaline soils become the major constraints to the distribution and biomass of crop in agriculture (Sun et al. 2014; Jia et al. 2015). Recently, soil-borne bacteria Burkholderia cepacia has been attracted considerable attentions due to its diverse roles such as enhancement of nutrient use efficiency and control of fungal infection in host plants (Singh et al. 2013; Zhao et al. 2014; Ghosh et al. 2016). Here, we examined the activity of B. cepacia (strain JFW16) in plant Fe assimilation. The inoculation with JFW16 stimulated Fe deficiency responses and enhanced the release of flavins in Astragalus sinicus. Consequently, the inoculated plants displayed better growth traits than non-inoculated plants under alkaline conditions, implying that B. cepacia JFW16 can improve Fe nutritional status of agricultural crop effectively.

Materials and methods

Plant materials, growth conditions and bacterial treatments

Seeds of A. sinicus were surface-sterilized using 0.1% (w/v) HgCl₂ for 3 min followed by at least three rinses. Then, these seeds were cultured on 1/2 MS agar medium (pH = 6.2) containing 0.8% (w/v) agar and 1.5% (w/v) sucrose in plastic plots and were placed in a growth chamber at 25°C with a 14-h/10-h light/dark cycle (light intensity of 200 μmol m⁻² s⁻¹). After 10 days (d) of germination, the seedlings were transferred into soils at different pH values. Alkaline soils were generated by the addition of calcium oxide (CaO) into Pro-Mix (3:1:1, peat, vermiculite and perlite mix) (Premier Brands Inc., New Rochelle, NY, USA) according to the method reported by Kim et al. (2006). The pH of soils supplemented with 0.4% and 0.6% (w/w) CaO was about 7.8 and 8.2, respectively. Finally, the non-alkaline (pH 6.2) or alkaline soils were sterilized by autoclaving at 120°C for 1 h before its use.

B. cepacia (strain JFW16) was isolated from the rhizosphere of cultivated A. sinicus grown in alkaline soils and was confirmed by 16S rDNA sequencing (Genbank No. MF417470). Before bacterial inoculation, JFW16 was inoculated into Luria–Bertani (LB) liquid medium and incubated for 16 h in an orbital shaker (180 rpm) at 28°C. Then, this bacteria strain were collected by centrifugation at 6000 rpm at 4°C for 15 min, and the precipitate was washed and diluted to yield 10⁹ colony forming units (CFUs) mL⁻¹ in 0.1 M phosphate-buffered saline buffer (PBS, pH 7.2). Lastly, 1 mL of bacterial solution was inoculated into plant roots.

Measurement of photosynthetic parameters

To measure total chlorophyll content, about 500 mg of fresh leaf tissues was used to extract using aqueous acetone (80%, v/v), followed by centrifugation at 12,000 g at 4°C for 15 min. Finally, the absorbance of supernatant was detected at two wavelengths (645 and 663 nm), respectively. The amount of chlorophyll was calculated on the basis of the formulae: 8.02 × A663 + 20.21 × A645 as previously described by Porra (2002). Moreover, several chlorophyll fluorescence indexes including photosynthetic rate (Pn), ratio of the variable and maximum chlorophyll fluorescence (Fv/Fm) and effective quantum yield of PSII photochemistry (ΦPSII) were measured as reported by Du et al. (2015).

The content of two main types of reactive oxygen species (ROS) including O₂⁻ and H₂O₂ was quantified as recently by Liu and Pang (2010). The values of malondialdehyde (MDA) were determined by a thiobarbituric acid-based colorimetric method (Heath and Packer 1968). The values of electrolyte leakage (EL) were examined according to the method of Su et al. (2015). Activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) were measured according to the method of Mostofa et al. (2015).

Determination of Fe and IAA content

Approximate 500 mg of fresh shoot and root tissues was firstly separated and harvested from both the non-inoculated and inoculated plants. These samples were subjected to dehydrate and ground, and then digested with 12 mL nitric acid (HNO₃)/perhydrol (H₂O₂) (3:1, v/v) in a microwave system for 50 min. Subsequently, the digested solutions were centrifuged at 12,000 g for 20 min. The supernatant was used to measure Fe content by inductively coupled plasma-atomic emission spectroscopy according to the method reported by Lei et al. (2014). Additionally, the production of indole acetic acid (IAA) by bacteria in the culture medium was detected according to the method of Mei et al. (2014). The content of IAA in plants was examined by gas chromatography as reported by Ljung et al. (2005).

Analysis of CAS assays and rhizospheric pH values

The siderophore-producing strain of JFW16 was identified via the chrome azurol sulphonate (CAS) assay (Gaonkar et al. 2012). The pH values of plant rhizosphere were assayed by the pH
indicator of bromocresol purple (Orozco-Mosqueda et al. 2013). Modified MS agar medium containing different concentration of Fe source and bromocresol purple (0.006%) was adjusted to 8.0 by the addition of CaCO₃. The seedlings were firstly grown on modified MS agar medium for 10 d. Then, 5 μL of bacterial suspension culture (10⁶ CFU mL⁻¹ in a 0.1-M PBS buffer solution) was inoculated into plant roots and cultured for 16 h. Color observation of bromocresol purple was analyzed with a colorimetric scale at a pH range from 4.0 to 8.0.

**Measurement of FCR activity**

Root FCR activity was examined by quantifying the formation of Fe³⁺-FerroZine using a spectrometer as described recently by Yi and Guerinot (1996). Ten-day-old seedlings grown in 1/2 MS agar medium (pH 6.2, 7.8 or 8.2) were exposed to *B. cepacia JFW16* for 2 weeks. Then, these seedlings were incubated in a solution containing 0.2 mM Fe(III)-EDTA and 0.5 mM FerroZine at pH 6.2. After 1 h of dark incubation, the absorbance was detected at 562 nm, and a solution without root incubation was used as the control. Finally, the concentration of Fe³⁺-FerroZine was calculated according to the method described by Zhang et al. (2009).

**Gene expression analysis**

Total RNA was extracted from plant tissues using a TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions, and the residual DNA was digested by DNase I (Invitrogen, USA). The RNA was then reversely transcribed into first cDNA as templates of real-time quantitative polymerase chain reaction (qRT-PCR). The qRT-PCR reactions were performed in a 7500 real-time PCR machine (ABI, USA) according to the method described by Zhu et al. (2014). The housekeeping gene *ACTIN* was selected as internal reference to normalize targeted gene expression. Each experiment was carried out in three biological replicates. Primers used in this experiment were designed according to several homologous genes from other different plants species including *Medicago truncatula*, *Cicer arietinum* and *Arachis ipaensis* through the GeneFisher2 tool (http://bibiserv.techfak.Unibiele.feld.de/genefisher2/). Amplified product of PCR was further sequenced and verified by the NCB1 BLAST analysis. The primers were listed in supplementary Table S1.

**Observation of chloroplast ultrastructure**

Leaf samples were cut into 0.5 × 0.5 cm pieces, and immediately fixed in 2.5% glutaraldehyde for 4 h. After three rinses, these samples were fixed with 1.0% osmium tetroxide (OsO₄) for 1 h. Subsequently, the samples were subjected to successive treatments including gradient dehydration and embedding. Finally, the embedding samples were cut into sections (90 nm), and then observed using transmission electron microscopy (TEM) at 80 kV. At least 3 dependent samples and more than 10 chloroplasts were observed for each experimental group.

**Assays of flavins in roots and nutrient solutions**

Flavins in roots and nutrient solutions were measured according to the method described recently by Sisó-Terraza et al. (2016) with minor modifications. To analyze the accumulation of flavins in roots, 500 mg of root tissues were ground in 5 mL of methanol, and then centrifuged at 12,000 g for 10 min. Subsequently, the supernatant were vacuum concentrated and diluted to a final volume of 150 μL with a solution of 15% methanol and 0.1% formic acid. Moreover, nutrient solutions of plants grown under different pH conditions were passed through a Sep-Pak C18 cartridge (Waters Corp., Milford, MA, USA). Flavins were adsorbed in the cartridges and eluted using 50 mL of methanol. Then, the eluent was concentrated and diluted to a final volume of 200 μL. The extracted flavins was examined by high performance liquid chromatography (HPLC) coupled to electrospray ionization-mass spectrometry (time of flight) [ESI-MS (TOF)].

**Statistical analysis**

Each experiment was conducted three biological times. The bars indicated the standard deviation (SD) of the mean with an asterisk, showing significant differences between the control and bacteria-treated plants using two-way analysis of variance (ANOVA) followed by Tukey’s test at *P* < .05.

**Results**

**Effects of *B. cepacia JFW16* on plant growth**

To examine if *B. cepacia JFW16* enhanced plant’s Fe absorption under alkaline conditions, 10-day-old seedlings were transferred to alkaline soils and cultured for 4 weeks with or without exposure to JFW16, respectively. Although the non-inoculated (control) plants did not show visible chlorotic phenotypes when grown on normal soil (0.0% CaO, pH 6.2), the controls displayed severe yellowing leaves when grown in alkaline soils (0.4% CaO, pH 7.8; 0.6% CaO, pH 8.2), a growth condition which resulted in Fe deficiency due to low Fe solubility at high soil pH values (Figure 1A). Compared with the controls, the inoculated plants exhibited promoted plant growth and alleviated leaf chlorotic symptoms after 4 weeks of alkaline treatment (Figure 1A).

Furthermore, we measured several physiological indexes including total leaf area, fresh and dry weight in plants. Soil inoculation of plants with JFW16 significantly increased total leaf area under normal and alkaline conditions (Figure 1B). The inoculated plants also exhibited a marked increase in fresh and dry weight of plants compared with the controls (Figure 1C,D). Consistently, the inoculated plants exhibited stronger root growth phenotypes than the controls under normal and alkaline stress conditions (Figure 1A). In addition, IAA could be detected in the culture supernatant of JFW16 (Figure S1(a)), and the inoculated plants had higher IAA content than the controls after co-culture of 16 h (Figure S1(b)). Moreover, survival rates of plants were monitored until 8 weeks after alkaline treatments. The inoculation with JFW16 remarkably increased the survival rates of stress-treated plants. The controls grown in alkaline soils (pH 8.2) for 8 weeks could not survive, but the survival rates of inoculated plants remained approximate 80% (Figure 1E).

**Effects of *B. cepacia JFW16* on plant photosynthesis under alkaline stress**

Alkaline pH conditions often decrease the bioavailability of Fe in soils and further inhibit plant photosynthesis (Romera
and Alcántara 2004). In this study, the effects of JFW16 on photosynthetic capacity in plants were investigated. Ten-day-old seedlings were grown in soils containing 0.0%, 0.4% or 0.6% CaO for 4 weeks with or without exposure to JFW16. As shown in Figure 2A, the inoculation of plants with JFW16 exhibited a slight increase in total chlorophyll content under normal conditions compared with the controls. After 4 weeks of alkaline treatments, the amount of chlorophyll levels was remarkably decreased in the controls, whereas the chlorophyll levels were considerably higher in the inoculated plants than the controls. In accordance with this, the values of Pn were higher in the inoculated plants than the controls under alkaline conditions, but no significant differences under normal condition (Figure 2B). The values of Fv/Fm, an important indicator for the PSII photochemical efficiency, in the inoculated plants were also markedly higher than those in the controls under alkaline conditions (Figure 2C). A similarly changing tendency was observed for the values of $\Phi_{PSII}$ (Figure 2D). Thus, higher photosynthetic parameters in the stress-treated inoculated plants might indicate a great improvement of the photosynthesis and PSII activities.

**Effects of B. cepacia JFW16 on chloroplast development under alkaline stress**

The structure and functions of chloroplasts in plants are often seriously destroyed by Fe deficiency (Zhou et al. 2016). In this study, severe chlorotic symptoms were found in the controls grown under alkaline conditions, implying that alkaline-induced Fe deficiency interfered with normal chloroplast development. As shown in Figure 3A,D, the control and JFW16-inoculated plants exhibited full development of chloroplasts in mesophyll cells under non-stress conditions, whereas no significant difference in normal grana stacking and grana lamellae was found between the control and inoculated plants (supplementary Table S2). Moreover, less grana stacking and lamellae occurred in the plastids of the control plants grown in calcareous soils, exhibiting typical characteristics of Fe deficiency-induced thylakoid disorganization (Figure 3B,C). However, the inoculated plants displayed more grana lamellae than the controls under alkaline conditions (Figure 3E,F). For instance, more normal grana stacking obviously occurred in the inoculated plants grown in 0.4% CaO soils but hardly observed in the controls. The inoculated plants also had more grana lamellae than the controls when grown in 0.6% CaO soils.

**B. cepacia JFW16 alleviated alkaline-induced oxidative damage to plants**

To examine if the inoculated with JFW16 mitigated oxidative injury imposed by alkaline stress, the content of $H_2O_2$ and $O_2^-$ in leaves was measured. Under normal conditions, no marked difference in the levels of $H_2O_2$ and $O_2^-$ between the control and inoculated plants was observed (Figure 4A,B). After 4 weeks of alkaline treatments, the inoculated plants had lower $H_2O_2$ and $O_2^-$ levels than the controls (Figure 4A,B). Furthermore, we determined the values of EL and MDA, which are important indexes of membrane damage (Zhou et al. 2015). Consistently, alkaline treatments notably induced a great increase in MDA content in the control leaves (Figure 4C). However, the inoculated leaves displayed lower MDA levels than the
controls under alkaline conditions (Figure 4C). Moreover, the inoculation with JFW16 remarkably decreased the values of EL in leaves of the inoculated plants compared with the controls (Figure 4D).

In addition, several antioxidant enzymatic activities in plants were assayed. As shown in Figure 5, there was no marked difference in the antioxidant enzymatic activities between the control and inoculated plants under normal

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**Figure 2.** *B. cepacia* JFW16 increased the photosynthetic capacity of plants grown in alkaline soils. Ten-day-old seedlings were grown in normal or alkaline soils for 4 weeks with or without exposure to JFW16. The non-inoculated (NI) and JFW16-inoculated (I) plants were used to determine total chlorophyll content (A), Fv/Fm (B), ΦPSII (C) and Pn (D). Different letters above the bar represented significant differences using two-way ANOVA followed by Tukey’s test at $P < .05$.

**Figure 3.** TEM analysis of chloroplast development in mesophyll cells of both the non-inoculated (A-C) and JFW16-inoculated (D-F) plants. Ten-day-old seedlings were grown in normal or alkaline soils for 4 weeks with or without exposure to JFW16. Then, chloroplast structures in leaves from the non-inoculated and JFW16-inoculated plants were observed using TEM. (A, D), 0% CaO; (B, E), 0.4% CaO; (C, F), 0.6% CaO. Scale bar = 1 μm.
Figure 4. *B. cepacia* JFW16 decreased the levels of ROS, MDA and EL in plants under alkaline conditions. Ten-day-old seedlings were grown in normal or alkaline soils for 4 weeks with or without exposure to JFW16. The non-inoculated (NI) and JFW16-inoculated (I) plants were used to examine the levels of \( \text{O}_2^{-} \) (A), \( \text{H}_2\text{O}_2 \) (B), MDA (C) and EL (D). Different letters above the bar represented significant differences using two-way ANOVA followed by Tukey’s test at \( P < .05 \).

Figure 5. *B. cepacia* JFW16 enhanced antioxidant enzymatic activities in the alkaline-treated plants. Ten-day-old seedlings were grown in normal or alkaline soils for 4 weeks with or without exposure to JFW16. The non-inoculated (NI) and JFW16-inoculated (I) plants were used to measure the activities of antioxidant enzymes including SOD (A), CAT (B), APX (C), DHAR (D), MDHAR (E). Different letters above the bar represented significant differences using two-way ANOVA followed by Tukey’s test at \( P < .05 \).
conditions. However, the activities of SOD, converting of \(O_2^-\) to \(H_2O_2\), were strikingly increased in the inoculated plants compared with the controls under alkaline conditions, (Figure 5A). The activities of CAT, catalyzing \(H_2O_2\) to \(H_2O\), were remarkably higher in the inoculated plants than the controls (Figure 5B). The activities of APX, DHAR, MDHAR and GR, which are involved in the AsA-GSH cycle responsible for eliminating \(H_2O_2\), were observed greatly higher in the inoculated plants than the controls (Figure 5C-F).

**Effects of B. cepacia JFW16 on Fe accumulation in plants**

The solubility of Fe is extremely low under alkaline conditions, and thus Fe becomes an important factor that affects plant photosynthesis and chloroplast development (Masse and Arguin 2005). It has recently been indicated that the alkaline-tolerant plants own the strong systems to take up Fe from soils (Li et al. 2016). In this study, the inoculation with JFW16 ameliorated leaf chlorosis under alkaline stress, indicating a critical role of JFW16 in enhancing Fe assimilation. Moreover, shoot and root Fe concentrations were determined in both the control and inoculated plants. As shown in Figure 6, the inoculated plants accumulated 18% and 23% higher shoot and root Fe concentrations than the controls under normal conditions. When plants were grown in 0.4% CaO soils, shoot and root Fe concentrations were 32% and 39% higher in the inoculated plants than the controls, respectively. The inoculated plants had more shoot and root Fe content than the controls when grown in 0.6% CaO soils.

**B. cepacia JFW16 enhances Fe acquisition systems in plants**

In this work, the siderophore-producing ability of JFW16 was analyzed via the CAS assays. As shown in Figure S2, lots of yellow circles were produced around growth of JFW16 on CAS plates, implying that JFW16 released siderophores to chelate Fe from the plates. To further evaluate whether JFW16-induced rhizospheric acidification, the assays of colorimetric acidification were conducted by observing color alteration of the medium containing bromocresol purple. Plants were initially cultured on alkaline medium containing different concentrations of flavins and its derivatives such as 7-hydroxy-Rbfl (FC1), 7a-hydroxy-Rbfl (FC2) and 7-carboxy-Rbfl (FC3) are essential for Fe acquisition in plants under low Fe-availability conditions (Rodríguez-Celma et al. 2011, 2013). In this study, 10-day-old seedlings were transferred to different 1/2 MS liquid medium (pH = 6.2, 7.8 or 8.2) and grown for 2 weeks with or without exposure to JFW16. Then, the accumulation of flavins and its release by the control and inoculated plants were identified and quantified by HPLC-MS analyses. As shown in Table 1, the Rbfl concentrations of control roots were about 3.2 nmol g\(^{-1}\) FW, and the other forms of flavins were hardly detected under the condition of pH = 6.2. However, the inoculated plants had approximate 15.6 nmol g\(^{-1}\) FW, and the other forms of flavins were also evidently elevated. Similarly, the concentrations of flavins were strikingly higher in the inoculated plants than the controls under alkaline conditions. Furthermore, only traces of Rbfl (1.5 nM) were found in the

*Figure 6. B. cepacia JFW16 augmented shoot and root Fe content in plants. Ten-day-old seedlings were grown in normal or alkaline soils for 4 weeks with or without exposure to JFW16. The non-inoculated (NI) and JFW16-inoculated (I) plants were used to measure shoot and root Fe content. Different letters above the bar represented significant differences using two-way ANOVA followed by Tukey’s test at \(P < .05\).*
nutrient solution of control plants grown at pH = 6.2, whereas the inoculated plants displayed about threefold increase in flavins. Under alkaline conditions, the inoculation with B. cepacia JFW16 marked induced the release of flavins including riboflavin, FC1, FC2 and FC3 in plants. Furthermore, qRT-PCR analyses revealed that the expression levels of two Rbfl biosynthetic genes including \textit{AsGTPcII} and \textit{AsDMRLS} were significantly increased in the inoculated plants under alkaline conditions compared with the controls (Figure 9).

\textbf{Discussion}

In higher plants, several flexible mechanisms have been evolved to tolerate adverse environments during long-term evolution (Marschner and Römheld 1994; Li et al. 2016). It is one of striking strategies to recruit a wide range of beneficial bacteria around plant rhizosphere (Lebeis et al. 2015). The ability of plants to resist abiotic and biotic stresses can be enhanced by the intricately mutualistic interactions (Mishra et al. 2014; Sukweenadhi et al. 2015). Due to low Fe availability, Fe deficiency is increasingly becoming an

Table 1. Concentration of flavins accumulated in roots and released into nutrient solution by the non-inoculated and JFW16-inoculated plants responded to different pH conditions (pH 6.2, 7.8 or 8.2).

|                | Accumulated in roots | Exported to nutrient medium |
|----------------|----------------------|----------------------------|
|                | Rbf                  | FC1 | FC2 | FC3 |
| NI (pH 6.2)    | 3.2 ± 0.5e           | –   | –   | –   |
| I (pH 6.2)     | 15.6 ± 2.1d          | 1.3 ± 0.2d | 5.1 ± 0.8d | 6.2 ± 0.5e |
| NI (pH 7.8)    | 136.8 ± 12.5c        | 3.7 ± 0.9c | 38.7 ± 11.8c | 38.9 ± 6.3d |
| I (pH 7.8)     | 192.3 ± 22.1b        | 8.3 ± 1.7b | 51.9 ± 12.6b | 573 ± 11.5b |
| NI (pH 8.2)    | 185.9 ± 16.7b        | 4.2 ± 0.8c | 41.5 ± 9.3c | 47.2 ± 11.6c |
| I (pH 8.2)     | 237.9 ± 29.6a        | 11.5 ± 1.9a | 63.9 ± 13.6a | 78.2 ± 13.8a |

Note: Different letters above the bar represented significant differences among different treatments at \( P < .05 \).
under alkaline conditions. The increased antioxidant enzymes were significantly higher in the inoculated plants than the controls. Accordantly, the ROS-detoxifying enzymatic activities were over, alkaline stress remarkably induced a great increase in mesophyll cells of plants grown under alkaline stress. Moreover, alkaline stress considerably destroys the structures of chloroplasts and photosynthetic efficiency in plants (Li et al. 2016; Zhao et al. 2014; Ghosh et al. 2016). In this study, a novel role of *B. cepacia* JFW16 in regulating plant uptake of Fe was observed, which was positively associated with the activated plant’s Fe acquisition systems.

Plants often exhibit plant growth inhibition and yield loss, accompanying by a notable decrease in photosynthetic capacity under alkaline stress (Li et al. 2016; Zhou et al. 2016). In this study, the JFW16-inoculated plants displayed better growth performance with more shoot and root biomass compared with the controls. The chlorophyll levels and photosynthetic efficiency were also greatly higher in the inoculated plants compared with the controls. Along with the increased transcription of *AsFIT1*, the inoculated plants also displayed higher level of *AsIRT1*, *AsFRO2* and *AsAHA2* transcripts than the controls. However, this raised the question how does *B. cepacia* JFW16 stimulate Fe deficiency responses in plants? Some studies have shown that certain molecules such as auxin and siderophore can be secreted by soil microbes to regulate plant’s Fe acquisition mechanisms. Auxin has been shown to induce the nitric oxide (NO) synthesis in Fe-deficiency plants, thereby activating the expression of *FIT1*, *AHA2* and *FRO2*. Aznar et al. (2014) have reported that siderophore exposure can modulate cellular Fe distribution in plants, and further up-regulate the transcription of Fe uptake-related genes such as *IRT1* and *FRO2*. In this study, JFW16 also owned the ability to produce and secrete these signal molecules such as IAA and siderophore, indicating that the activated Fe deficiency of host plants was possibly dependent on several microbe-released signal molecules during plant-microbe interaction.

Soil bacteria also can acidify the rhizosphere for increasing the solubility of Fe directly by the secretion of organic acids such as glyoxylic acid, 3-methyl-butanolic acid and diethyl acetic acid (Farag et al. 2006). Thus, the JFW16-induced rhizospheric acidification increased Fe availability, and further alleviated adverse impacts imposed by alkaline pH conditions. Interestingly, Strategy I plants can produce and exude several secondary metabolites such as phenolics, flavonoids and flavins to increase Fe availability in the rhizosphere and in the root apoplast (Jin et al. 2007; Cesco et al. 2010; Rodriguez-Celma et al. 2011). Red clover (*Trifolium pratense*) increases the utilization of cell wall Fe pools under Fe-deficient conditions by the release of phenolics into root vicinity (Jin et al. 2007). Under Fe deficiency, the *Arabidopsis* plants secrete Fe-mobilizing phenolic compounds such as coumarins to acquire Fe by chelation and/or reduction of Fe³⁺ (Fourcroy et al. 2014).
Besides phenolics, some plant species such as sugar beet, spinach and alfalfa can synthesize and release abundant flavins that play redox and/or metal-complexing roles in augmenting Fe mobilization in the rhizosphere (Susín et al. 1993, 1994; Rodríguez-Celma et al. 2011). Importantly, some PGPR strains such as *Azospirillum brasilense* and *P. polymyxa* BFKCO1 can enhance Fe acquisition by increasing root-secreted phenolics (Pit et al. 2015; Zhou et al. 2016). Here, the inoculation with JFW16 markedly increased the synthesis and exudation of flavin compounds including Rbfl and Rbfl derivatives in the alkaline-treated plants. Along with the elevation of pH values from 6.2 to 8.2, the accumulation of flavins was greatly higher in the controls, while less Rbfl and Rbfl derivatives were release into the rhizosphere. In contrast, the inoculated plants exhibited the increased synthesis and root release of flavins under any alkaline pH conditions, indicating that JFW16-induced Rbfls exudation may facilitate plants to acquire Fe from low Fe-availability conditions. In addition, the H⁺-ATPase-mediated proton extrusion has been shown to enhance the release of flavins, in collaboration with the FCR activity (Rodríguez-Celma et al. 2011). Consistently, the JFW16-inoculated plants displayed up-regulation of the expression of *AsAHA2*, which may partially contribute to the increased release of flavins.

**Conclusion**

Based on the data shown in this study, the following model of *B. cepacia* JFW16-regulated Fe acquisition of host plants was proposed. As illustrated in Figure 10, the inoculation with JFW16 increased Fe assimilation in plants under alkaline conditions, which contributed to an integrated consequence of microbial metabolites (such as auxin and siderophore), induced rhizospheric acidity and activated Fe deficiency responses. This study provided important evidence that JFW16 could increase the bioavailability of Fe in alkaline soils and demonstrated a novel role of JFW16 in activating Fe acquisition systems in host plants.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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![Figure 10](image-url). A proposed model of *B. cepacia* JFW16-regulated Fe assimilation in plants. The release of certain signal molecules by JFW16 activates Fe deficiency responses by regulating the expression of *AsFIT1*, *AsAHA2*, *AsFRO2* and *AsIRT1*, and enhances the biosynthesis and export of flavins that effectively mobilize Fe from low Fe availability conditions.
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