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REGULAR ARTICLE

IAA producing *Bacillus altitudinis* alleviates iron stress in *Triticum aestivum* L. seedling by both bioleaching of iron and up-regulation of genes encoding ferritins

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Abstract
Background and Aims Many microbes are beneficial to plants and are termed as plant growth promoting bacteria (PGPB). This study explores the effect and mechanism of endophytic bacteria on wheat iron stress.
Methods Bacteria and wheat seedlings were hydroponically co-cultured under different concentrations of iron. Growth parameters were measured and transcriptions of ferritins as well as transporters were quantified by real-time quantitative PCR.
Results An endophytic *Bacillus altitudinis* WR10 was isolated from the root of *Triticum aestivum* L. The strain is resistant to 5 mM iron and it bioleaches more than 80% iron after 24 h of incubation. Meanwhile, WR10 produces as much as 35pM indole 3 acetic acid (IAA) during fermentation but there was no accumulation of cytokinin (zeatin to be precise). Inoculation of WR10 significantly improves the growth of the primary root and main sprout in wheat seedlings in a co-culture model under iron stress after two weeks hydroponic cultivation. The presence of WR10 up-regulates the expression of many genes encoding ferritins in wheat roots under iron stress.
Conclusions Besides its ability to bioleach iron, IAA producing *B. altitudinis* WR10 can alleviate iron stress in wheat by up-regulation of ferritin-encoded genes in roots, which is important for maintaining iron homeostasis.

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Keywords *Triticum aestivum* L. · *Bacillus altitudinis* · Iron toxicity · Indole 3 acetic acid · Ferritins

Abbreviations

B. altitudinis	*Bacillus altitudinis*
CTKs	Cytokinins
ELISA	Enzyme Linked Immuno-Sorbent Assay
IAA	Indole 3 Acetic Acid
PGPB	Plant Growth Promoting Bacteria
T. aestivum	*Triticum aestivum* L.

Introduction

Iron (Fe) is an essential micronutrient for both plants and animals. Though iron is widely found in soil and aquatic ecosystems, it is also known as a trace element due to its trace presence in the environment (Nagajyoti et al. 2010). As an essential element for plants, iron can be absorbed from both soil and water. In plants, iron performs biochemical and physiological functions in the processes of photosynthesis, chloroplast development and chlorophyll biosynthesis (Briat and Gaymard 2007; Briat et al. 2015). Furthermore, iron is a major constituent of cell redox systems (Schützendübel and Polle 2002). However, high concentrations of heavy metals are also widely regarded as environmental pollutants, and their toxicity is a problem of increasing significance for crop production and animal health (Becker and Asch 2005). In crops, on one hand, iron deficiency has been widely studied as a worldwide nutritional problem (Zuo and Zhang 2011). On the other hand, iron excess is a major threat in the humid tropical regions of Asia, Africa, and South America, where soils are frequently flooded or acidified (Khabaz-Saberi et al. 2010; Guo et al. 2010). Rice yields can be reduced by 12–100% depending on the intensity of iron stress and the tolerance of cultivars (Sahrawat 2004; Fofana 2009; Pereira et al. 2014).

Less significant but still prevalent, is that wheat grain yield is also influenced by iron stress and other factors in above areas, like phosphorus fertilizers and pesticides (Khabaz-Saberi et al. 2012). In hexaploid wheat, iron toxicity mainly occurs in acidic soils (Huang et al. 1995). Toxicity of iron in shoot tissues of wheat genotypes after water-logging of an acidic soil has been observed in Western Australia (Khabaz-Saberi et al. 2006, 2010). The toxicity in wheat was further verified using several acidic soils and two wheat varieties: a strong negative correlation ($r = -0.86$) was found between Fe concentration and dry weight of shoot after water-logging (http://www.aciar.gov.au/project/CIM/1996/025) (Setter et al. 2009). Furthermore, ammonium-based fertilizers are widely used for improving wheat production. However, repeated use of ammonium-based fertilizers leads to significant soil acidification (Johnson et al. 2010). Considering water-logging and the application of ammonium-based fertilizers are common practices in current agriculture, iron toxicity in wheat is increasingly drawing the attention of researchers and farmers.

Plants evolved to resist iron stress by down-regulation of iron transportation or up-regulation of ferritin which sequesters iron. Iron transportation is mediated by a series of transporters, including iron-regulated transporters 1 (IRT1), *NAC* transcription factor (NAM-B1), and so on. IRT1 is a member of the ZIP (Zink-regulated, Iron-regulated-like Proteins) metal transporter family and can transport multiple divalent metals (Kim and Guerinot 2007). NAM-B1 increases nutrient remobilization and regulates the translocation of iron from vegetative tissues to grain in wheat (Uauy et al. 2006; Waters et al. 2009). Ferritins are multimeric protein complexes that are composed of several different subunits. Ferritins can effectively control iron homeostasis since each molecule of ferritin sequesters up to 4500 atoms of iron (Briat 1996). Besides protection from iron stress, endophytes and rhizobacteria can have complex effects on plant iron resistance (Bar-Ness et al. 1992; Compant et al. 2010; Freitas et al. 2015). Many microorganisms are able to bioleach heavy metals that are widely present in waste-water, especially in industrial waste-water. This method of bioleaching heavy metals from waste-water, which is a major cause of soil contamination, is cheap and eco-friendly (Alluri et al. 2007; Jing et al. 2007). Therefore, several bacteria species, like *Bacillus* strains have been isolated and used for their inherent abilities to accumulate and absorb metal ions in water (Kim et al. 2007; Wen et al. 2013). *Bacillus* strains have also been widely isolated as endophytic plant growth promoting bacteria (PGPB), which improve soil fertility and produce plant hormones, such as indole 3 acetic acid (IAA) and cytokinins (CTKs) (Da Mota et al. 2008; Liu et al. 2013). However, using PGPB to alleviate iron stress and prevents iron toxicity in wheat has not been fully studied. Therefore, this study was designed to (1) characterize an endophytic isolate WR10, (2) alleviate the toxicity of excess iron in wheat seedlings using a co-culture model, and (3) explore the impact of endophytic *Bacillus altitudinis* WR10 on the expression of ferritins and transporters in wheat.

Materials and methods

Bacterial isolation

Bacterial strains were isolated from the roots of wheat with minor modifications of the protocol used by former researchers (Pereira and Castro 2014). Briefly, healthy

winter wheat, *Triticum aestivum* L. zhoumai 26 grown in the field in Zhoukou city in spring (N33°38′ E114°40′, around 180 days after seeding, namely during grain filling stage) was carefully collected and rinsed in water to clean any attachments. The primary roots, without lesions, were soaked in 70% ethanol for 5 min. Then, it was rinsed again in distilled water. The clean root tissue was cut into 0.5 cm segments. Segments of approximately 0.5 g were ground in 1 mL ddH_2O. The fluid was transported into a 1.5 mL tube, and then boiled in water at 100 °C for 5 min. After centrifugation at 2000RCF for 1 min, 0.2 mL of supernatant was removed and plated on the agar of *Bacillus megatherium* medium (HB8786, Hopebio Com. Ltd., Qingdao) and cultivated at 30 °C for 24 h. The last time rinsed water was collected for plating either Luria-Bertani (LB) or *Bacillus megatherium* agar. After incubation at 30 °C for 3 days, no visible colony on both control plates confirmed the effectiveness of root sterilization.

Identification of WR10 by staining and phylogenetic analysis

Staining was conducted with Gram staining solutions and Spore Staining Kit (Cat No. G1060 and G1132, Solarbio Com. Ltd., Beijing) according to the instructions and images were acquired with an optical microscope (MV5000, NOVEL) connected to a CCD camera (Mshot, Ningbo). Amplification of *16S rRNA* and *gyrB* gene of strain WR10 was performed with corresponding primers (supplementary Table S1, designed by GenScript Online PCR Primers Designs Tool) in 50 μl-reaction containing100nM dNTPs mix, 3 mM $MgCl_2$, 500 nM each primer in final concentration, 0.5 U of Taq DNA polymerase and 100 ng genomic DNA in 1 × Taq buffer. Genomic DNA was extracted with a TIANamp Bacteria DNA Kit (Tiangen, Cat No. DP302). DNA was quantified by reading Abs.260 nm with a spectrophotometer (Thermo Fisher, NanoDrop 1000). The thermo-cycling parameters are: initial de-naturation at 94 °C for 3 min; 35 amplification cycles of 94 °C de-naturation for 30 s, 55 °C annealing for 30 s, 72 °C elongation for 1.5 min; and a final polymerization step of 72 °C for 5 min with an Eppendorf master thermal cycler. The final PCR product was resolved in 1.0% agarose gel, excised and purified by a Universal DNA Purification Kit (Tiangen, Cat No. DP214). DNA sequencing was performed by ABI 3730 (Genescript, Nanjing) and the sequence was subjected to nucleotide BLAST analysis on the NCBI website (Zhang et al. 2000). The ClustalW software incorporated in the BioEdit was used with the progressive methods for multiple alignments. Phylogenetic trees were calculated by the BioEdit accessory application DNADist using the neighbor-joining method with representative sequences that were extracted from different species of *Bacillus* spp. (Hall 1999). Twenty *16S rRNA* sequences with more than 95% identity and fifteen *gyrB* sequences with more than 75% identity to WR10 were used for phylogenic trees construction, respectively (details in Table S2 and S3).

IAA and CTK assays

Glycerol stock of *B. altitudinis* WR10 was sub-cultured in LB broth as described above. Overnight cultivated WR10 was inoculated in 10 mL LB broth with an initial OD600 = 0.01. The tube was incubated at 30 °C, 150 rpm for 24 h. During incubation, samples were collected at different time points. Bacterial production of IAA and cytokinins was assayed by reading absorbance at 450 nm (Abs.450 nm) with a commercial Plant IAA ELISA Kit or Plant CTK ELISA Kit (Bluegene Co. Ltd., Shanghai) using a microplate reader (DNM-9602, CANY Tec, Shanghai).

Iron resistance and bioleaching ability

A single colony of WR10 was picked and propagated in LB broth shaking at 150 rpm, at 30 °C. Growth was monitored by reading OD600. Comparing to growth in LB broth, iron resistance was evaluated by relative growth under different concentrations of $FeSO_4$ (Khabaz-Saberi et al. 2010). The iron concentration in supernatants was determined based on a previous method with minor modification (Dawson and Lyle 1990). Briefly, iron concentration was measured by absorbance of Fe^{2+} complex at 425 nm at pH 6.0 using sulfosalicylic acid as a reducing agent.

Co-culture of *B. altitudinis* WR10 and *T. aestivum*

Co-culture was conducted in 100 mL half-strength Hoagland's medium (Orhan 2016) in a beaker for 2 weeks at room temperature (25 °C) in humid condition (80%) under dark or light for 12 h every day. As shown in supplementary Fig. S1, 12 beakers were assigned into

four groups (NC, Fe^{2+} 0 mM WR10 -; NC, Fe^{2+} 5 mM WR10 -; Treat, Fe^{2+} 0 mM WR10 +; Treat, Fe^{2+} 5 mM WR10 +). Briefly, each beaker in treated groups was inoculated with 1 mL pre-cultured *B. altitudinis* WR10 (about 10^8 cells), while NC groups were not inoculated with WR10. In Fe^{2+} 5 mM groups, 1 M $FeSO_4$ was added to each beaker to a final concentration of 5 mM. At last, three pieces of 3 mm filter paper (Cat No. 3030–861, Whatman) were placed on the surface of medium and seeded with 10 well pre-germinated *T. aestivum* grains. Wheat grains were sterilized by soaking in 0.1% $AgNO_3$ solution for 10 min and, then planted in Hoagland's medium for 3 days germination (Speakman and Krüger 1983). In this model, well-germinated grains of wheat were grown in half-strength Hoagland's medium containing 10^6cfu/mL WR10 that are alive but cannot propagate. To confirm bacterial cells are alive and not propagate, 1 mL suspension was removed at day 1, 7, 14, respectively for plating and counting colony-forming units. Analysis of count numbers showed that there is no significant decrease or increase of live cells during two weeks treatment. After the 2 weeks' co-cultivation, wheat seedlings were collected for the measuring of lengths of primary root and main sprout.

qPCR quantification of genes encoding ferritins and transporters in *T. aestivum*

After two weeks co-culture under either 0 or 5 mM iron, all shoots and roots in each group were collected separately for total RNA isolation. Total RNA was extracted using TRIzol (Cat No. 15596026, Invitrogen) and reverse transcribed using a SuperScript® III First Strand cDNA Synthesis Kit (Cat No. 18080051, Invitrogen). qPCR was conducted in 10 μL reaction in 8-tube strips with triplicates in a thermo cycler (CFX96, Biorad, USA) using Sybr Green as dye according to MIQE guidelines (Bustin et al. 2009). Parameters were designed according to a previous study (Finatto et al. 2015). Briefly, de-naturation at 94 °C for 30 s, annealing at 55 °C for 10 s, elongation at 72 °C for 30 s, and cycling for 40 cycles. Nine genes were amplified for real-time quantification, including 5 genes encoding ferritin or its subunits (labeled as G1 to G5: *TaFER1-A, TaFER1-B, TaFER1-C, TaFER2, TaFER2-B*, corresponding accession number is FJ225137, FJ225141, FJ225144, EU143671, and FJ225149), 3 genes encoding potential transporters (labeled as G6 to G8: *TaNAM, ZIP, UPP,* corresponding accession number is HM027575, AY864924, and HG670306), and one reference gene *GAPDH* (EU022331). To confirm *GAPDH* is a suitable reference gene, we compared the expression of *GAPDH* in both root and sprout under iron treatment in advance and its expression is relatively constant.

Data analysis

All data is mean +/− standard deviation of three experiments. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with Bonferroni post-test correction for multiple comparisons. The difference between means was compared by Tukey's test. A p-value <0.05 indicated statistical significance. *** $P < 0.01$, highly significant; * $P < 0.05$, significant.

Results

Isolation, staining, and identification of endophytic *B. altitudinis* WR10

Several *Bacillus* spp. were isolated from the roots of *T. aestivum* on a selective agar. Separate colonies with different morphology were randomly picked and propagated in LB broth. One isolate WR10 was used for gram-staining and spore-staining with respective reagents. As shown in Fig. 1a, WR10 cells are short, rod-like, and spores are small, round from a morphological aspect. Furthermore, WR10 seems are gram-positive as cells are stained to grey or blue (data unshown). Further *16S rRNA* alignment shows high identity of WR10 to a variety of *Bacillus* spp. (supplementary Table S2). In particular, the similarity of WR10 to *B. altitudinis* 41KF2b is 99% with the number of identical 1438 bp/total analyzed 1442 bp (only 4 gaps). Phylogenetic analysis based on both *gyrB* and *16S rRNA* suggests WR10 is closest to *B. altitudinis* 41KF2b (corresponding sequences are KJ_809604.1 and NR_042337.1 in Fig. 1b and c).

IAA and CTK production in *B. altitudinis*

Plant hormones play leading role in regulation of growth and productivity. It has been reported that many PGPB can produce different kinds of hormones, like IAA and CTK. In this study, production of IAA and CTK in WR10 was assessed using supernatant collected from different time points after inoculation, namely 0, 4, 8,

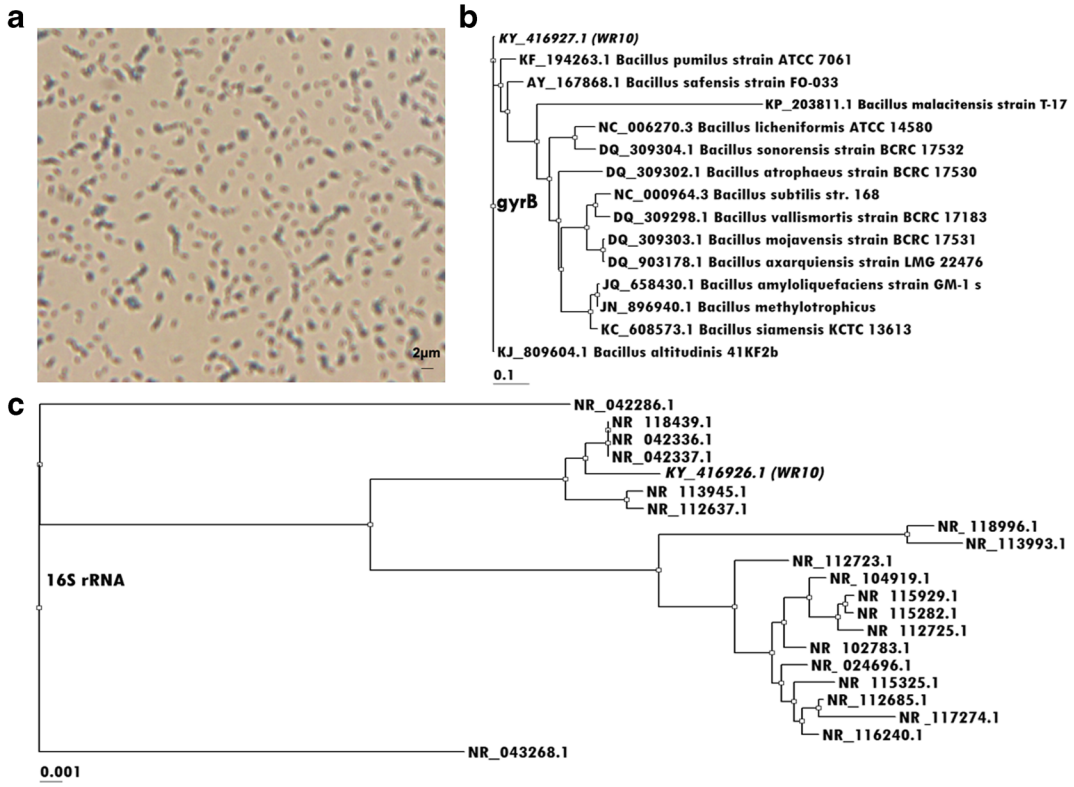

Fig. 1 Identification of WR10. (**a**) Staining of WR10 cells was conducted with a spore-staining kit according to instruction. The image was acquired by a light microscope using a 5.0 MP CMOS camera (MD50). The ClustalW software was used with the algorithm of progressive methods for multiple alignments. After alignment, all gaps were deleted. Phylogenetic trees were constructed by BioEdit using the Neighbor-Joining method for gene *gyrB* (**b**) and *16S rRNA* (**c**). There were 992 positions in *gyrB, and* 1409 positions in *16S rRNA* in the final dataset. The bootstraps value is 1000. Italic letters highlight genes from WR10. The detail bacterial names in Fig 1c can be found in supplementary Table S2

12, 16, and 24 h. Accumulation of IAA in the supernatants of WR10 shows similar pattern to growth curve (Fig. 2b v.s. Fig. 2a). According to a standard curve, WR10 produces as much as 35pM IAA perking at 12 h that is the late exponential growth phase. In contrast, there is low production of CTK as there is no obvious accumulation during growth (Fig. 2c).

Iron resistance and bioleaching ability

Resistance of WR10 to iron was evaluated by a growth inhibition experiment. As shown in Fig. 3a, 0.1-1 mM iron (supplemented as $FeSO_4$) has little impact on the growth of WR10, whereas 5 mM iron clearly inhibits the growth. After 24 h of incubation, WR10 can grow by around 70% compared to its counterpart without iron stress, even when as much as 5 mM iron is applied. To evaluate the iron bioleaching ability of WR10, the remaining iron in the cell free supernatants was assayed. At the beginning, 5 mM iron was added to the medium before inoculation (Fig. 3b, 0 h). After 12 h of incubation, more than 70% of the iron was absent from the medium. The value increased to more than 80% after 24 h of incubation indicating the strong iron bioleaching ability of WR10.

Co-culture of *B. altitudinis* WR10 and *T. aestivum*

A co-culture model was used for probing the potential effects of WR10 on *T. aestivum* growth under iron stress. As shown in Fig. 4, adding WR10 does not obviously affect wheat seedling growth in the absense of iron (Fig. 4a, Fe^{2+} 0 mM, WR10 +). While, in groups that were supplemented with 5 mM iron, adding WR10 improves growth (Fig. 4a, Fe^{2+} 5 mM, WR10 +). Furthermore, there is no significant difference in the mean number of lateral roots among groups co-cultivated with WR 10 and the NC group without iron stress. Co-culture

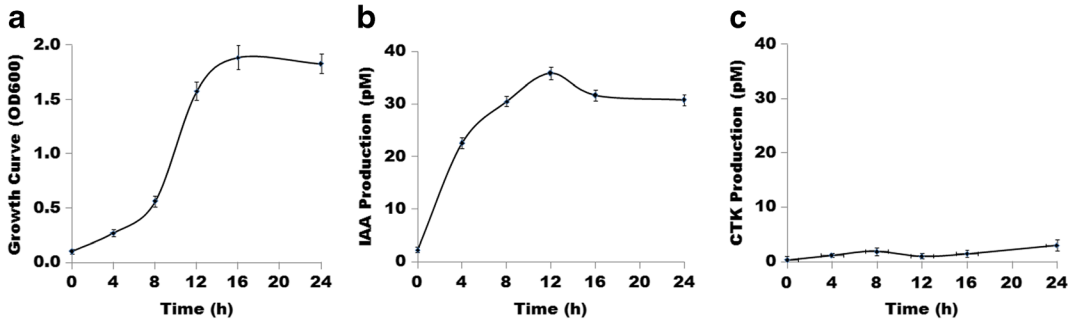

Fig. 2 Bacteria growth and hormone production. (**a**) Growth curve of WR10. Bacteria were grown in 5 mL LB broth shanking at 150 rpm, 30 °C incubator. **b** Production of IAA in WR10 by ELISA assay. **c** Production of CTK in WR10 by ELISA assay. A 0.5 mL culture fluid was collected at 0, 4, 8, 12, 16, and 24 h respectively, and supernantants were used for ELISA assay. Values are mean +/− standard deviation of three independent experiments with three technical replicates

with WR10 increases the mean number of lateral roots from roughly 1 to 4 under 5 mM iron that is detrimental to wheat seedlings (Fig. 4a and supplementary Table S4). Statistical analysis of the lengths of the primary root (Fig. 4b) and main sprout (Fig. 4c) of all seedlings in each group suggests adding WR10 can highly significantly decrease the toxicity of iron on rooting ($P < 0.01$), and significantly decrease the toxicity of iron on sprouting ($P < 0.05$).

qPCR quantification of genes encoding ferritins and transporters in *T. aestivum*

The expression of eight genes that encoding either ferritins (G1-G5) or transporters (G6-G8) in *T. aestivum* was quantified by qPCR analysis under different conditions. Firstly, without inoculation of WR10, iron has little impact on all tested genes, as their relative expressions are approximately 1–2 in roots (Fig. 5a, in blank columns, for detail data seen supplementary Table S5). In contrast, 5 mM iron up-regulates the expression of all tested genes encoded for ferritins and transporters in wheat sprouts in the absence of WR10 (Fig. 5b, in blank columns). Secondly, under 5 mM iron stress, WR10 up-regulates the expression of all genes encoding ferritins except G5 (*FER2-B*), with G3 (*FER1-C*) as high as 15.7-fold in roots. Impacts of WR10 on transporters in roots are limited, as only the expression of G7 (*ZIP*) gene was down-regulated more than 2-fold (Fig. 5a, in grey columns). In the sprouts, WR10 significantly down-regulates the expression of all genes (Fig. 5b, in grey columns). Totally, WR10 antagonizes the up-regulation effect of iron, which induces the expression of all tested genes in sprout. Furthermore, WR10 mainly enhances the expression of genes encoding ferritins in roots of wheat seedlings.

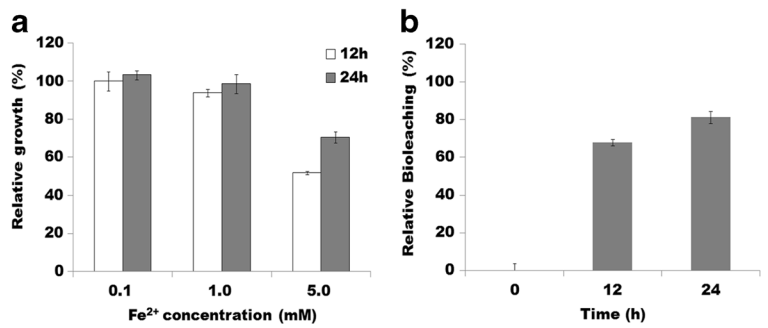

Fig. 3 Iron resistance and bioleaching ability. **a** Assay of iron resistance. Fresh pre-cultures of WR10 were inoculated in triplicate into 5 mL LB broth supplemented with 0, 0.1, 1, 5 mM (final concentration) $FeSO_4$. Growth was measured by reading OD600 at 12 h and 24 h, respectively. Data are mean of percentage relative to samples without adding $FeSO_4$. **b** Assay of bioleaching ability of WR10. Iron concentration in supernatants from tubes supplemented with 5 mM $FeSO_4$ was determined by reading Abs.425 nm using a spectrophotometer. Data are mean of percentage relative to samples without adding WR10

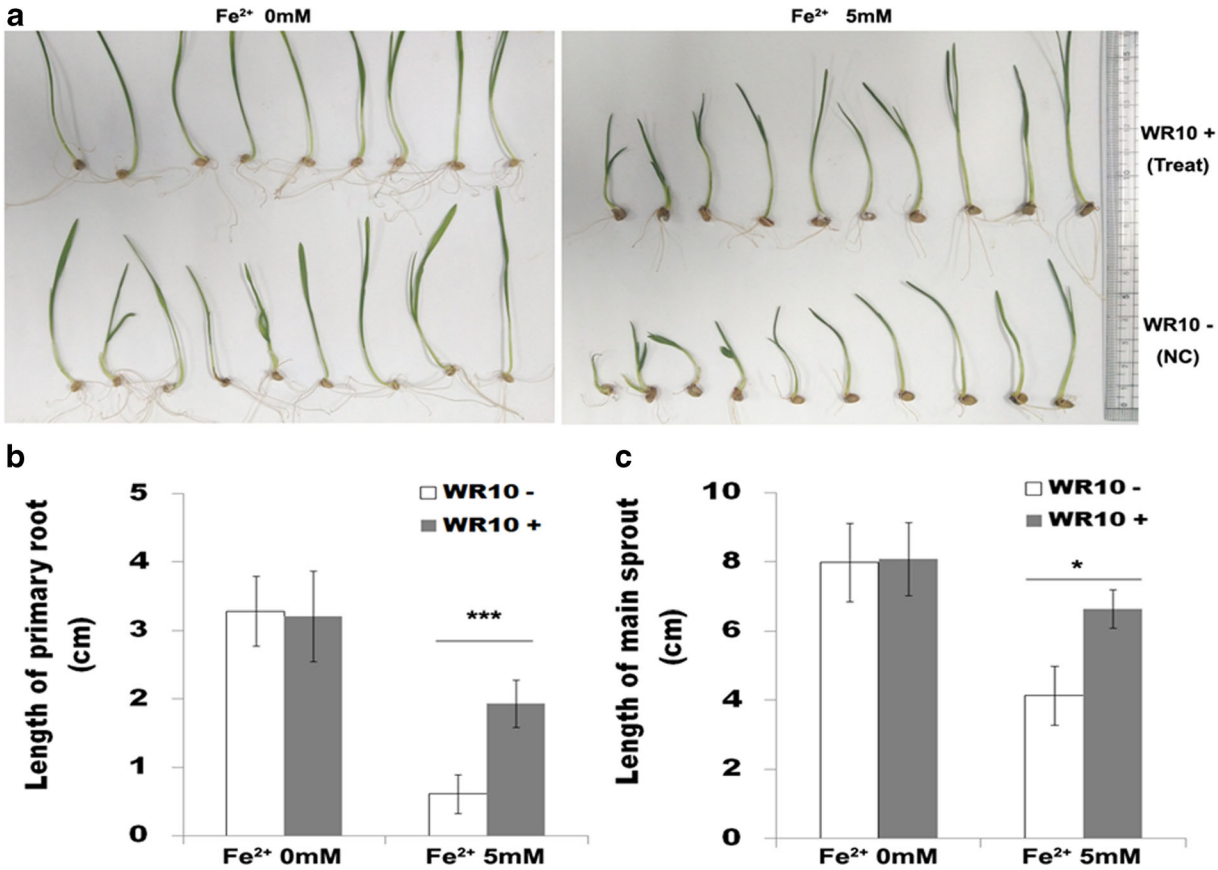

Fig. 4 WR10 alleviates iron toxicity to *T. aestivum* seedlings. Wheat seedlings were seeded on the surface of filter paper in 100 mL half-strength Hoagland's medium supplemented with 0 or 5 mM $FeSO_4$. Meanwhile, two groups were inoculated with 1 mL WR10 cells to a final cell density of 10^6cfu/mL. After two weeks, seedlings were collected and imaged (**a**). Lengths of the primary roots (**b**) and main sprouts (**c**) of wheat seedlings among different groups were compared. Data are mean +/− standard deviation of 10 seedlings. Fe^{2+} 0 mM, supplemented with 0 mM $FeSO_4$; Fe^{2+} 5 mM, supplemented with 5 mM $FeSO_4$; WR10 -, without adding WR10; Wr10 +, group inoculated with WR10. Statistical analysis was performed using two-way ANOVA with Bonferroni post-test correction for comparisons. *** Highly significant; * Significant

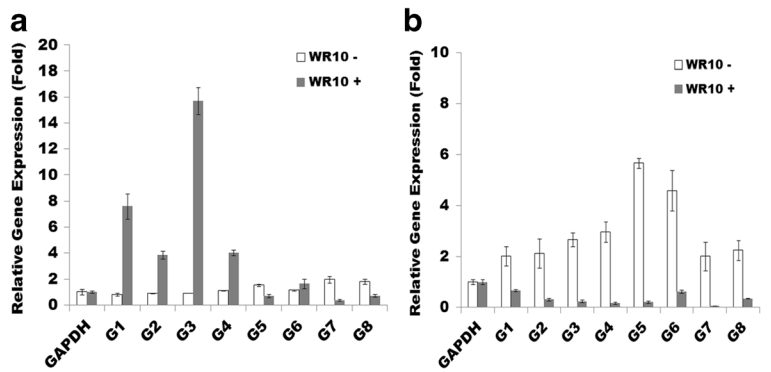

Fig. 5 Effects of WR10 on the expression of genes encoding ferritins and transporters in roots (**a**) and shoots (**b**) of wheat under 5 mM iron stress. WR10 -, groups without inoculation of WR10; WR10 +, groups inoculated with WR10. G1, *TaFER1-A*; G2, *TaFER1-B*; G3, *TaFER1-C*; G4, *TaFER2*; G5, *TaFER2-B*; G6, *TaNAM*; G7, *ZIP*; G8, *UPP*, unnamed protein product; *GAPDH*, glyceraldehydes-3-phosphate dehydogenase, which is used as a reference gene. Values are mean +/− standard deviation of three technical replicates and results of one representative of three independent experiments were shown

Discussion

Although iron is abundant in most mineral soils, iron toxicity symptoms only frequently occur under flooded conditions (Becker and Asch 2005). Iron toxicity in plants is related to high Fe^{2+} uptake by roots and its transportation to the leaves. Excess Fe^{2+} impairs cellular structure irreversibly and damages membranes, DNA and proteins (de Dorlodot et al. 2005). In tobacco and soybean, iron toxicity is accompanied by reduction of plant photosynthesis and yield (Sinha et al. 1997). In rice, which needs irrigation, iron toxicity is an important problem leading to significant reduction of yields and increasing sensitivity to pathogens (Finatto et al. 2015). In sandy regions, such as Western Australia and South China, water logging is common. In these regions, large portions of agricultural land is given over to seeding wheat, and water-logging often results in increased iron concentration in soil that affects the growth of the wheat (Khabaz-Saberi et al. 2010; Li et al. 2012). In this study, excessive iron toxicity on wheat was confirmed by hydroponic cultivation under different concentrations of $FeSO_4$.

PGPB are beneficial microbes that can enhance plant nutrition via improved nitrogen fixation, phosphate solubilization, or phytosiderophore production (Richardson et al. 2009). PGPB can also protect plants from pathogens, such as inhibition of phytoparasites, and/or induce systemic resistance (Couillerot et al. 2009; Lugtenberg and Kamilova 2009). Furthermore, some PGPB help plants withstand abiotic stresses including heavy metals and other pollutants; some are even able to increase the capacity of plants to sequester heavy metals (Jing et al. 2007; Vacheron et al. 2013). Therefore, utilizing PGPB is a promising approach to facilitate phytoremediation of contaminated soils (Zhuang et al. 2007; Shukla et al. 2011). As they are rarely influenced by environmental factors, such as the condition of soils and nutrients, endophytic isolates are sometimes preferred to rhizobacteria because they might have higher colonization ability and are already adapted to the inner tissues of plants (Franken 2012). Therefore, endophytic strains were isolated from the roots of wheat. Sequencing results of *16S rRNA* and *gyrB* genes verified the strain WR10 as *B. altitudinis*. Other strains in this species were also isolated recently from wheat rhizosphere by other researchers (Verma et al. 2016). The isolate WR10 characterized in this study possesses strong bioleaching ability to both copper (unpublished results) and iron (as shown in Fig. 3b). To precisely quantify the impact of PGPB on roots and the whole plant remains challenging (Compant et al. 2010). An in vitro co-culture model inoculated roots with PGPB and then monitored the resulting effects on the plant during cultivation. It has been shown that many PGPB increase the number and/or length of lateral roots (Combes-Meynet et al. 2011; Chamam et al. 2013), but may reduce the growth rate of the primary root (Dobbelaere et al. 1999). In line with previous reports, WR10 increases the number and length of lateral roots in the study (Fig. 4a). It is perhaps more interesting that WR10 increases the lengths of both primary root and main sprout under iron stress (Fig. 4b and c). This is in contrast to some previous reports, while it agrees with others that have same phenotypes in PGPB-inoculated plants growing in soil (El Zemrany et al. 2007; Veresoglou and Menexes 2010; Walker et al. 2012).

PGPB are able to produce a wide range of phytohormones, including auxins and/or cytokinins that can interfere with these hormonal pathways in plants. Indole-3-acetic acid (IAA) is the best-characterized auxin produced by many bacteria (Ali et al. 2009). Assay of IAA in the supernatants of WR10 shows that WR10 is a high auxin producer, contrasting with the widely studied PGPB *Phyllobacterium brassicacearum* STM196 and *B. subtilis* GB03 (Contesto et al. 2010; Zhang et al. 2007). It is believed that low concentrations of IAA can stimulate primary root elongation, whereas high levels of IAA stimulate the formation of lateral roots (Patten and Glick 2002; Remans et al. 2008). Cytokinin production, especially zeatin, has been documented in various PGPB as well, including *Bacillus* spp. (Hussain and Hasnain 2009). However, there is no obvious production of cytokinins in WR10.

Both ferritins and transporters are major regulators for iron homeostasis in plants (Kim and Guerinot 2007). The qPCR quantification of 8 genes encoding either ferritins (G1-G5) or transporters (G6-G8) shows up-regulation of the expression in the majority of genes encoding ferritins in roots under iron stress after inoculation of WR10 (Fig. 5a). More precisely, ferritins TaFER1-A (G1) TaFER1-C (G3) are leading responders for iron storage in roots, which attribute to iron resistance and act as metal detoxicants (Joshi et al. 1989). The expressions of *ZIP* (G7) and *UPP* (G8) are down-regulated in both root and sprout for decreased iron transportation. Results obtained in this study suggest a major effect of proteins ZIP, UPP, and TaNAM (G6) as

effluxes of iron; even UPP is an uncharacterized protein that is annotated as an iron transporter in the NCBI database (Waters et al. 2009; Barberon et al. 2011). Furthermore, the relative expression level of most of these genes is much lower in the shoot than in the root. In fact, many kinds of PGPB can enhance plant iron resistance through different kinds of mechanisms (Lin et al. 1983; Bar-Ness et al. 1992; Freitas et al. 2015). Furthermore, ferritins are expected to be used as a novel approach for solving iron problems in biology that has multiple applications (Theil et al. 2016).

In summary, we have isolated an endophytic growth promoting strain *B. altitudinis* WR10 from the root of wheat. Growth experiments showed that WR10 has strong resistance to iron stress. Therefore, we speculate the strain can alleviate iron toxicity in wheat. In the co-culture model, supplementation of WR10 improved lengths of both primary root and main sprout significantly suggesting significant effect on decreasing iron toxicity. To probe the mechanism behind this phenomenon, we tested the production of two kinds of hormones. ELISA results indicate the strain produces IAA that is an important regulator during plant growth. Furthermore, we conducted qPCR experiments to study whether ferritins and iron transporters in wheat can be regulated when co-cultivated with WR10. Interestingly, the expression of genes encoding ferritins is up-regulated in roots and down-regulated in shoots when co-cultivated with WR10 in the presence of high concentrations of iron. Up-regulated expression of ferritin-encoded genes in roots may contribute to improved iron resistance and down-regulated expression of ferritin-encoded genes in shoots may decrease iron toxicity. Considering the results obtained in the study, WR10 may benefit wheat growth in flooded regions by either bioleaching of iron or storage of excess iron by increased ferritins in roots, as well as the production of IAA.

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