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ORIGINAL ARTICLE

# *FreB* is involved in the ferric metabolism and multiple pathogenicity-related traits of *Verticillium dahliae*

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## Abstract

Ferric reductases are integral membrane proteins involved in the reduction of environmental ferric iron into the biologically available ferrous iron. In the most overwhelming phytopathogenic fungus, *Verticillium dahliae*, these ferric reductase are not studied in details. In this study we explored the role of *FreB* gene (VDAG_06616) in the ferric reduction and virulence of *V. dahliae* by generating the knockout mutants (*ΔFreB*) and complementary strains (*ΔFreB-C*) using protoplast transformation. When cultured on media supplemented with $FeSO_4$, $FeCl_3$ and no iron, *ΔFreB* exhibited significantly reduced growth and spore production especially on media with no iron. Transmembrane ferric reductase activity of *ΔFreB* was decreased up to 50% than wild type strains (Vd-wt). The activity was fully restored in *ΔFreB-C*. Meanwhile, the expression levels of other related genes (*Frect-4, Frect-5, Frect-6* and *Met*) were obviously increased in *ΔFreB*. Compared with the Vd-wt and *ΔFreB-C, ΔFreB-1* and *ΔFreB-2* were impaired in colony diameter and spore number on different carbon sources (starch, sucrose, galactose and xylose). *ΔFreB-1* and *ΔFreB-2* were also highly sensitive to oxidative stress as revealed by the plate diffusion assay when 100 μM $H_2O_2$ was applied to the fungal culture. When *Nicotiana benthamiana* plants were inoculated, *ΔFreB* exhibited less disease symptoms than Vd-wt and *ΔFreB-C*. In conclusion, the present findings not only indicate that *FreB* mediates the ferric metabolism and is required for the full virulence in *V. dahliae*, but would also accelerate future investigation to uncover the pathogenic mechanism of this fungus.

**Keywords** Ferric reductases · *Verticillium dahliae* · Oxidative stress · Carbon utilization · Virulence

## Introduction

Pathogenic fungi are the most dangerous threat to global food security (Pennisi 2010; Fisher et al. 2012) and the major causal agents affecting agricultural crops (Oerke 2006). *Verticillium dahliae* Kleb., a highly destructive soil-borne fungus belonging to the phylum Ascomycota, causes Verticillium wilt in more than 400 plants species, including a wide range of important ornamental, horticultural, agronomical and woody plants (Pegg and Brady 2002; Fradin and Thomma 2006). Once the plant is infected, fungicides cannot work effectively since *V. dahliae* resides in the vascular system, moreover, nearly 250–310 million US dollar losses are reported annually by the fungus (Fradin and Thomma 2006; Wang et al. 2016). As from the release of genomic sequences of this fungus by Broad Institute of MIT (http://http://www.broadinstitute.org/annotation/genome/verticillium_dahliae), multiple genes have been studied for their roles in the development and virulence of *V. dahliae*, which facilitate the molecular research on pathogenetic mechanism for eventual controlling (Klimes and Dobinson 2006; Hoppenau et al. 2014; Xiong et al. 2015; Qi et al. 2016, Su et al. 2017).

Iron is an essential element for almost all organisms as an electron donor and acceptor, as well a crucial cofactor for a variety of enzymes involved in different biological pathways (Weber et al. 2006). Despite its abundance, the bioavailability of iron is exceptionally limited (Philpott

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2006) due to the issue that iron rapidly oxidizes to the ferric state ($Fe^{3+}$) in the presence of oxygen and at neutral or higher pH values. This state is extremely insoluble in water and forms precipitates in the form of different salts (Guerinot and Yi 1994) which further limits its biological accessibility. Additionally, the ferrous form of iron can react with oxygen to produce hydroxyl radicals that are extremely toxic to the cells and causing damage to cellular macromolecules like DNA, membrane lipids and proteins (Imlay 2003). Therefore, mechanisms in reduction of iron as well its uptake and storage are tactfully controlled by organisms under different circumstances.

Ferric reduction is a vital step during the cellular iron uptake. Ferric reductases are integral membrane proteins involved in the reduction of the environmental ferric iron into the biological available ferrous iron (Dancis et al. 1990). In *Saccharomyces cerevisiae*, for the siderophore mediated iron uptake mechanism, the iron bound to siderophores is reduced by the cell-surface reductases, mainly *Fre1* and *Fre2*, then taken up by the ferrous transporters (Yun et al. 2001). This uptake is regulated in a similar way in *Candida albicans* (Morrissey et al. 1996). While studying the role of *CFL1* gene in iron acquisition in *C. albicans*, it was found that a compensatory mechanism exists for iron reduction (Xu et al. 2014), which the deletion of this gene resulted in increased expression levels of *FRP1, CFL2* and *FRE10*, the alternative ferric reductases.

Besides the role in iron reduction, the ferric reductase genes also have been associated with the tolerance to oxidative stress and virulence of a particular organism (Xu et al. 2014). The deletion of *Fre4* gene in *Cryptococcus neoformans* compromised the production of virulence factor melanin and increased the sensitivity to azole antifungal drugs in the mutant study (Saikia et al. 2014). In *Aspergillus fumigatus*, the disruption of *FreB* in combination with the inactivation of siderophore system, diminished the growth of the fungus, surface ferric reductase activity and oxidative stress tolerance (Blatzer et al. 2011). The *CFL1* gene deletion mutants of *C. albicans* exhibited hypersensitivity when exposed to higher concentration of hydrogen peroxide and menadione, and highly attenuated virulence indicating the role of this gene in the tolerance to oxidative stress and virulence (Xu et al. 2014).

Although ferric metabolism in other species has been intensively surveyed, FreB functions have not been reported yet in *V. dahliae*. To explore the role of *FreB* gene, we generated the *FreB* deletion and complementation strains using the protoplast transformation. Ferric reductase activity and iron uptake were evaluated in the *ΔFreB* and *ΔFreB-C* strains. Furthermore, we investigated the *FreB* relationship with carbon utilization, oxidative stress tolerance and virulence.

## Materials and methods

### Fungal growth and spore collection

*V. dahliae* (V991), highly toxic and defoliating wild type pathogenic strain provided by Prof. Guiliang Jian of Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), was cultured on PDA (potato dextrose agar) medium at 25 °C for 7–10 days. For spore collection, sterile distilled water was added to the plates and the surface of each plate was gently scraped using a sterile loop. The resulting suspension was filtered through a sterile 40 µm Nylon filter (Falcon, REF352340) and centrifuged at 4000 rpm for 5 min. The final spore concentration was adjusted to $1.5 \times 10^7$/ml.

### Protoplast isolation

Protoplast was isolated from freshly collected mycelia of *V. dahliae* as described in our previously published article (Rehman et al. 2016). Briefly, 2 ml *V. dahliae* spores ($1.5 \times 10^7$/ml) were cultured in 100 ml CM (Complete Medium) for 20 h at 28 °C and 150 rpm. After filtration, harvested mycelia were aseptically transferred into 10 ml enzyme mixture respectively and incubated at 33 °C for 2.5 h at 60 rpm. The mixture was filtered using sterile 40 µm Nylon filter to remove any hyphal fragments and the protoplast was centrifuged at 2800 rpm for 5 min. The supernatant was discarded and the pellet was washed 2–3 times with STC buffer (20% sucrose, 10 mM Tris–HCl, pH 8.0, and 50 mM $CaCl_2$). Finally the concentration of protoplast was adjusted to $10^6$/ml using STC buffer.

### Phylogenetic analysis

The whole sequence of *FreB* in *V. dahliae* was obtained from Verticillium genomic database (http://www.broadinstitute.org). The homologous amino acid sequences corresponding to FreB in other species were obtained using blastp search program of NCBI. Phylogenetic tree was constructed using MEGA7 software (Kumar et al. 2016).

### Targeted gene knockout and complementation

Flanking regions, about 1 kb upstream and downstream, of *FreB* gene were amplified with the primers FreB-5F/5R and FreB-3F/3R, respectively (Table 1). These fragments were fused with the neomycin resistance cassette (*Neo*) amplified with Neo-F/R primers by overlapping PCR to produce knockout gene fragment (Table 1; Fig. 1a). The resulting knockout gene fragment was introduced into the

**Table 1** Primers used in this study

| Primers | Sequence (5′–3′) |
|---|---|
| FreB-5F | GGAACCAGGTGCAAGGATAGC |
| FreB-5R | CAAGACAGCCCGCAAACAAGGCAGATGGGGGAAAGA |
| FreB-3F | CCCAGAATGCACAGGTAAGACGACACGGGCGGGTTAC |
| FreB-3R | TCGGAAGAGGAGCAGGAGGAA |
| Neo-F | GTTTGCGGGCTGTCTTGACG |
| Neo-R | TACCTGTGCATTCTGGGTAA |
| FreB-MF | CTTATGGCACGGTCATGTTG |
| FreB-MR | TGAGAACACCAGGTCCACAA |
| GFP-CF | aga***tacgta***CTTTCGACACTGAAATACGTCG |
| GFP-CR | ggg***tctaga***GCATCAGAGCAGATTGTACTGAGAG |
| Hyg-CF | ggg***tctaga***TTGAAGGAGCATTTTTGGGC |
| Hyg-CR | aga***aagctt***TTATCTTTGCGAACCCAGGG |
| GFP-F1 | AGCTGGACGGCGACGTAAAC |
| GFP-R1 | GATGGGGGTGTTCTGCTGGT |
| FreB-InF | GCAAAGATAATCTAGAATGGCATGAGCGAAGGTTAC |
| FreB-InR | CACAAACGGTGATACGTATTCTGTATTGCCCCCTGAAG |
| FreB-CF | CAATGCATCCCTCAGCAACT |
| FreB-CR | ACCAACGGTGGAGAAGATTG |
| Frect-4F | TGCTTCCCGTCTTGAGCAAT |
| Frect-4R | CACAGTGACCTCGACGTCAA |
| Frect-5F | AAGCGGAACAACTCTCAGGG |
| Frect-5R | TCCCCGAATTCAAAGAGGCC |
| Frect-6F | CGATTTTGACGACACCTCGC |
| Frect-6R | TTTCCCGACGGATGAGGAAC |
| Met-F | AGCCCCTGAAACATCTGACG |
| Met-R | CGTCAGGTACAGCAGTTCGT |
| Vta2-F | CTCGTCTGCTGCCCACTACTCTTCG |
| Vta2-R | GTGCGAGTCAACACTTACCTGTGCTTG |
| Glu-F | CAACCCGACCATCCCTCCCTCCAT |
| Glu-R | ACGTACTCGTCCGTGACGGCCTCCT |
| VdNoxB-F | TGCGTGGCAAGCATAAGACATAC |
| VdNoxB-R | GACAGCACGAGTGAAATCACCAAC |
| Vd-ActF | GGCTTCCTCAAGGTCGGCTATG |
| Vd-ActR | GCTGCATGTCATCCCACTTCTTC |
| Vd-ITSF | CCGCCGGTCCATCAGTCTCTCTGTTTATAC |
| Vd-ITSR | CGCCTGCGGGACTCCGATGCGAGCTGTAAC |
| Nb-ActF | GGACCTTTATGGAAACATTGTGCTCAGT |
| Nb-ActR | CCAAGATAGAACCTCCAATCCAGACAC |

Restriction sites are represented in bold italic letters

protoplasts isolated from *V. dahliae* by PEG-mediated transformation (Rehman et al. 2016). Transformants were selected based on neomycin resistance and the gene knockout mutants (*ΔFreB-1, ΔFreB-2*) were confirmed by PCR using primers FreB-MF/MR. To construct GFP tagged mutant (*ΔFreB-GFP*), pBI121 plasmid and *GFP* expression fragment (amplified with GFP-CF/CR primers) were digested with SnaBI and XbaI, and the digested fragments were ligated. Hygromycin resistance cassette (hyg, amplified with Hyg-CF/CR primers) was introduced into pBI121 via XbaI and HindIII restriction enzyme sites. The resultant plasmid was designated as pBI121-Hyg-GFP (Fig. 1b) and the protoplast obtained from gene knockout mutants were transformed with this plasmid. GFP transformants (*ΔFreB-GFP*) were selected based on GFP fluorescence and PCR with GFP-F1/R1 primers (Table 1). For complementary strain generation, *FreB* gene along with 2 kb upstream region for its own promoter was amplified with infusion primers FreB-InF/InR and the GFP expression cassette was replaced in pBI121-Hyg-GFP via infusion

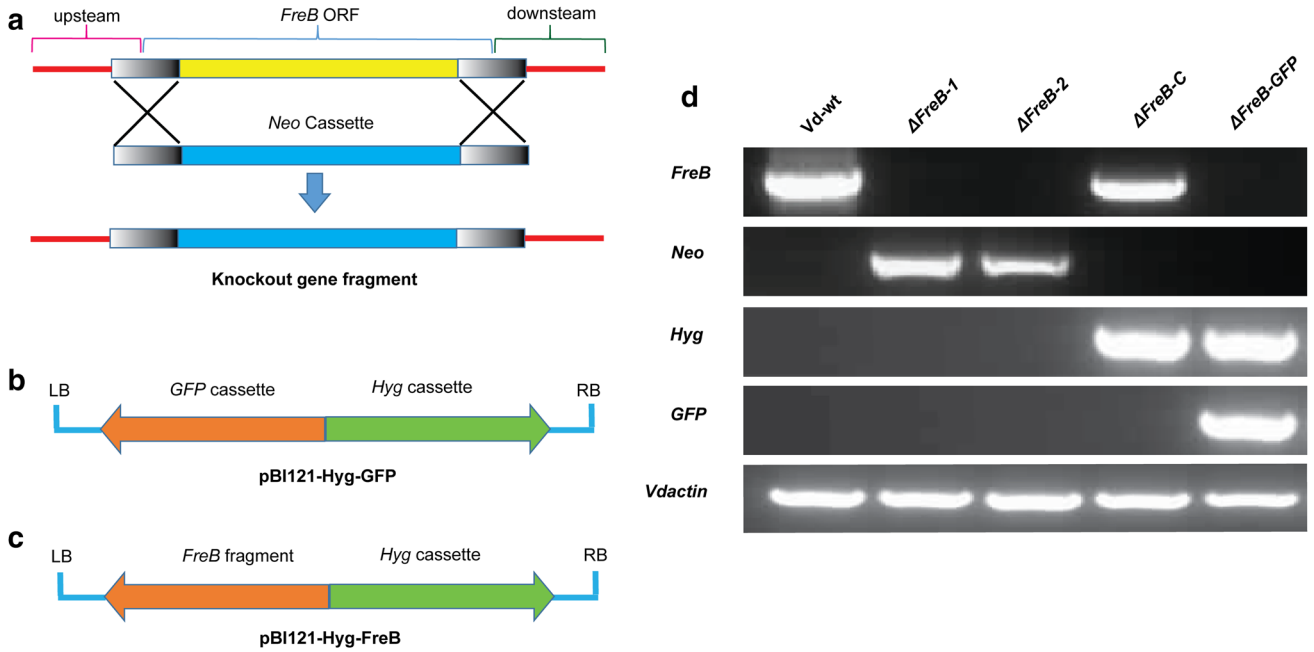


**Fig. 1** Generation of gene knockout, GFP tagged and complementary strains. **a** Gene knockout fragment was constructed by fusing about 1 kb upstream and downstream of *FreB* gene with neomycin resistance cassette via overlapping PCR and the resultant fragment was used for generating knockout gene mutant by transformation into Vd-wt protoplasts. **b** For GFP tagged mutants, *GFP* gene was introduced into pBI121 via SnaBI and XbaI digestion followed by the introduction of hygromycin B phosphotransferase (hpt) through XbaI and HindIII restriction digestion. The resultant plasmid (pBI121-Hyg-GFP) was used to transform the protoplast obtained from gene knockout mutants. **c** For complementary strain, *GFP* expression cassette in pBI121-Hyg-GFP was replaced with *FreB* gene along with 2 kb upstream region and 0.8 kb downstream region. The resultant plasmid pBI121-Hyg-FreB was introduced into the gene knockout protoplasts

cloning. The resultant plasmid was named as pBI121-Hyg-FreB (Fig. 1c) and introduced into the gene knockout protoplasts. The resultant complementary strains were selected by hygromycin resistance and further confirmed by PCR using FreB-CF/CR primers (Table 1).

## Observing the effect of iron source on the growth of mutants

To compare the growth of gene deletion mutants, wild type and complementary strains on media supplemented with different iron sources, Czapek Dox agar was either supplemented with 1 mM $FeSO_4$ or 1 mM $FeCl_3$. For no iron conditions, 150 µM BPS (as iron chelator) was added while $FeSO_4$ and $FeCl_3$ were omitted. Spore suspensions (10 µl, $10^6$/ml) from the respective strains (Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*) were placed in the center of each plate separately. The plates were incubated at 25 °C. The colony diameter and the number of spores produced by each strain were recorded after 12 days and photographed. Two repeats were performed for this experiment independently and the results were presented as means ± standard deviations.

## Ferric reductase assay

Ferric reductase assay was performed as described previously with some modifications (Nyhus et al. 1997; Xu et al. 2014). Spores were collected from iron depleted media and washed with assay buffer (50 mM citrate buffer, 5% glucose; pH 6.5) 2–3 times. The spores (10 µl, $10^6$/ml) from the relevant strains were incubated independently in the assay buffer in the presence of 1 mM $FeCl_3$ and 1 mM BPS at 25 °C. Cultures were assayed every 30 min for 6 h by removing the spores through centrifugation and measuring the optical density of the supernatant at 520 nm. A standard curve was generated by measuring the absorbance at 520 nm using known concentration of $FeSO_4$. The standard curve was used to determine the amount of iron reduced by the spores of the respective strains used in the ferric reductase assay. The assay was repeated for all the strains two times independently and the results were presented as mean ± SD.

## Iron uptake assay

Spores ($1 \times 10^7$/ml) of the respective strains (Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*) were cultured in Czapek Dox broth supplemented with 100 µM $FeCl_3 \cdot 6H_2O$

for 72 h. The iron content was measured every 24 h using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7900, CA, USA) (Huang and Lin 2001). Samples without any spores were used as negative control. Briefly, the cultures were centrifuged at 10,000 rpm for 3 min., the supernatant was transferred into a new clean tube and the mycelia were discarded. Samples (0.2–0.9 g) were taken; 7 ml nitric acid (standard reagent) and 3 ml hydrogen peroxide (standard reagent) were added and heated at 120–180 °C for 5–10 min in microwave digestion system. Samples were taken out of the digestion tank and heated on electric hot plate at 140–160 °C to adjust the volume to 1.0 ml. The samples were cooled down, and diluted with $H_2O$ (Ultrapure) to 25 ml. The iron contents were then measured using ICP-MS. Prior to analytical run, the ICP-MS was calibrated with the certified standard iron solution and its dilutions (Pepper et al. 2010). Experiment was repeated twice for each strain. The results were expressed as the iron contents of the supernatant (ppm) with means ± SD. The following formula was used to calculate the iron concentration in samples:

$$X = (\rho - \rho_0) \times V/m.$$

$X$ is the iron content in the samples, $\rho$ is the iron mass concentration in the samples, $\rho_0$ is the iron mass concentration in the blank, $V$ is the final volume of sample (25 ml) and $m$ is the weight of the samples.

## Expression analysis of related genes

In order to exploit the impact of *FreB* deletion on the regulation of other genes related to ferric reduction or metabolism, we analyzed the expression of *ferric reductase transmembrane component 4* (VDAG_06992, *Frect-4*), *ferric reductase transmembrane component 5* (VDAG_09294, *Frect-5*), *ferric reductase transmembrane component 6* (VDAG_09283, *Frect-6*) and *metalloreductase* (VDAG_07239, *Met*). Spores of the respective strains (Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*) collected from PDA plates were cultured in CM broth for 3 days. Mycelia were collected and RNA was extracted using RNA extraction kit (YPHBio, Tianjin, China) as per manufacturer's instructions. First strand cDNA was synthesized using Transcript® one-step gDNA removal and cDNA synthesis kit (Transgen Biotech, Beijing, China) as per manufacturer's instructions. Expression of the respective genes was analyzed by qRT-PCR in 7500 Real Time PCR System (ABI, Massachusetts, USA) using Frect-4F/4R, Frect-5F/5R, Frect-6F/6R and Met-F/R primers respectively. *Vd-actin* gene was used as a housekeeping gene and detected using Vd-ActF/R primers. The primers used are listed in Table 1.

## Growth of mutants on different carbon sources

The growth of *ΔFreB* mutants was compared on different carbon sources. For this purpose, Czapek Dox agar (without sucrose) was amended with starch (10 g/L), sucrose (30 g/L), galactose (10 g/L) and xylose (10 g/L), respectively. Spores (10 µl, $10^6$/ml) from the respective strains (Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*) were placed in the center of each plate separately. The plates were incubated at 25 °C. The colony diameter was measured at 3-day intervals for 12 days. The number of spores was counted after 12 days and the plates were photographed. The experiment was independently repeated two times for each set and the results were presented as means ± standard deviations.

## Oxidative stress assay and expression of oxidative stress response genes

For oxidative stress assay, 500 µl spores ($10^6$/ml) of the respective strains were cultured on Czapek dox agar plates separately. A hole was made in the center of each plate using a cork borer. 100 µl $H_2O_2$ (100 mM) solution was poured onto the hole of each plate. The plates were incubated at 25 °C for 1 week. The zone of inhibition was measured for each strain and photographs were taken. To observe the impact of oxidative stress on the expression of oxidative stress response genes, *Vta2* (HE972123.1), *Glutathione reductase* (VDAG_07524) and *VdNoxB* (VDAG_09930), spores from the respective strain were collected after exposing to oxidative stress and cultured for RNA extraction. qRT-PCR was conducted using gene specific primers (Vta2-F/R, Glu-F/R, VdNoxB-F/R, Table 1) as described previously. The experiment was repeated for each set two times independently and the results were presented as means ± standard deviations.

## Infection assay and quantification of fungal biomass in *Nicotiana benthamiana*

*Nicotiana benthamiana* plants were grown in growth chamber at 23–25 °C under a 16-h light/8-h dark photoperiod with 60–70% relative humidity. Spores were collected from 7 days old culture of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*. The spores were resuspended in a final concentration of $1 \times 10^7$/ml. *N. benthamiana* seedlings with six to seven true leaves were dug out of the pots, the roots were dipped in the spore suspension of either Vd-wt, *ΔFreB-1, ΔFreB-2* or *ΔFreB-C* for 2 min and immediately planted back into new pots. A total of ten plants were used for each strain and the experiment was repeated twice. Disease index was evaluated at 10, 11 and 12 days post inoculation (dpi). For estimating fungal biomass, total genomic DNA was isolated from roots, stems and leaves of the infected plants at 12 dpi using DNAsecure

plant kit (Tiangen, Beijing, China) and qRT-PCR was conducted using SYBR® Fast qPCR kit (KAPA Biosystems, Boston, MA, USA). Vd-ITSF and Vd-ITSR were used to amplify ITS1 and ITS2 regions of ribosomal RNA genes for quantifying fungal biomass in each sample (Tzima et al. 2012). *Nbactin* gene was used as internal control (Lee et al. 2013).

To observe the infection process, *N. benthamiana* plants were inoculated with Vd-GFP (wild type GFP strain) and *ΔFreB-GFP*, respectively. The roots of the infected plants were observed at 7 dpi using confocal microscope (LSM 700, Carl Zeiss, Jena, Germany) (Su et al. 2017).

### Disease index

To evaluate the disease severity on the plants, a five-grade (0–4) scale was used based on the previous studies with modifications (Wang et al. 2008, Su et al. 2017): 0 grade, no disease symptoms; 1 grade, wilting of less than two leaves; 2 grade, wilting of three to five leaves; 3 grade, wilting of more than five leaves and 4 grade, death of plants or near death. Each inoculation experiment comprised of ten plants and was conducted in triplicates. The disease symptoms were observed at 10, 11 and 12 days post-inoculation (dpi). The disease index was calculated using the following formula at the mentioned dpi and represented in percentage.

$$\mathrm{DI} = \left[\sum(\text{number} \times \text{grade})/(10 \times 4)\right] \times 100 \text{ (Su et al. 2017).}$$

## Results

### Phylogenetic analysis

The open reading frame (ORF) of *FreB* contains 1749 nucleotides coding for 582 amino acids (XP_009657615.1). The phylogenetic tree was constructed with MEGA7 using the neighbor-joining tree method and 1000 bootstrap replicates (Fig. S1). The phylogenetic analysis revealed that the orthologs of FreB are present in a number of fungal species including *V. longisporum, V. alfalfa, Fusarium* species, etc., however, in yeast and plants, no orthologs were found (Fig. S1). The similarity of *FreB* gene of *V. dahliae* with other fungal species is ranged from 59 to 99%. As indicated by the phylogenetic tree, *FreB* gene of *V. dahliae* is more closely related to that of *V. longisporum* (99%) and *V. alfalfa* (86%).

### Generation of *FreB* gene deletion and complementation mutants

To produce *FreB* gene deletion mutants, *FreB* gene was replaced in *V. dahliae* (V991) with neomycin resistance cassette (*Neo*). Gene deletion mutants were selected on the basis of neomycin resistance and a total of 20 neomycin resistant colonies were analyzed by PCR in which 5 colonies showed deletion of the *FreB* gene by homologous recombination. *ΔFreB-1* and *ΔFreB-2*, two independent gene deletion mutants, were selected for further analysis. For complementation, *FreB* gene along with 2 kb upstream region was introduced into one of the two gene deletion mutants via PEG-mediated protoplast transformation. Complementary mutants were selected on the basis of hygromycin resistance and finally verified by PCR (Fig. 1d). As expected, the target genes were detected in relevant strains. FreB-GFP mutants were initially screened on the basis of GFP fluorescence and finally a single colony was selected on media supplemented with hygromycin which was further confirmed by PCR using gene specific primers, GFP-F1/R1 (Fig. 1d; Table 1).

### *FreB* contributes to fungal growth in different iron sources

To determine the growth of gene deletion mutants on media with no iron and on media supplemented with different iron sources, $FeSO_4$ and $FeCl_3$, respectively, spores of the respective strains (Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C*) were cultured in the center of plates. The colony diameter was observed after 10 days. We found that the deletion of *FreB* gene had a significant influence on the utilization of iron sources. As shown in Fig. 2a, about 80% decrease in the mycelial growth of gene deletion mutants was found on media with no iron when compared with Vd-wt and *ΔFreB-C*. The growth of gene deletion mutants was found better on media using $FeSO_4$ as an iron source (2.75 and 2.7 cm colony diameter for *ΔFreB-1* and *ΔFreB-2*, respectively) in comparison with $FeCl_3$ (1.9 and 1.97 cm for *ΔFreB-1* and *ΔFreB-2*, respectively). No significant difference in the mycelial growth between Vd-wt and *ΔFreB-C* observed on media with either $FeSO_4$ (3.57 and 3.5 cm, respectively) or $FeCl_3$ (3.32 and 3.37 cm, respectively) as shown in Figs. 2b and S2. Collectively, our results indicate that the deletion of *FreB* gene had a significant effect on the fungus in utilizing $Fe^{3+}$ as an iron source. Moreover, this gene is also involved in adapting the fungus to iron starvation.

### Deletion of *FreB* gene impaired the surface ferric reductase activity and iron uptake

Ferric reductase assay relies on the formation of a red BPS-$Fe^{2+}$ complex. The activity is measured from the contribution of *FreB* gene in reducing $Fe^{3+}$ form ($FeCl_3$) to $Fe^{2+}$ form. The ferric reduction was assayed every 30 min for 6 h. The activity of ferric reductase increased for all the strains to a certain point and then declined onwards. The

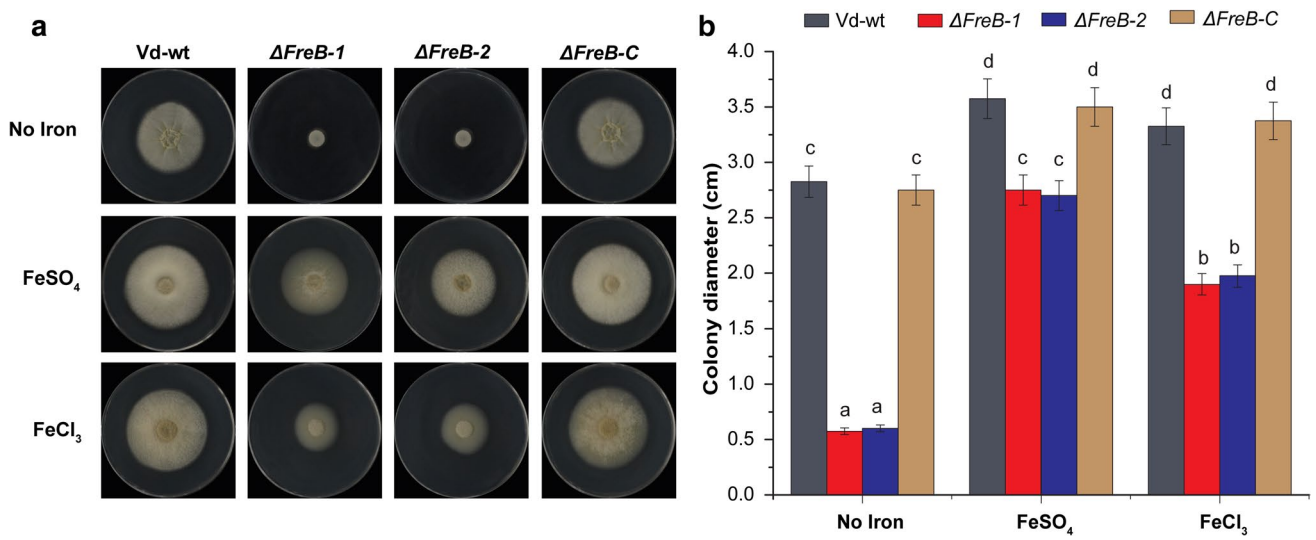


**Fig. 2** Iron utilization assay. The growth of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* was compared on Czapek Dox agar supplemented either with $FeSO_4$, $FeCl_3$ or both of them were omitted in case of limited iron conditions. Spore suspensions (10 µl, $10^6$/ml) from the respective strains were cultured on plates separately for 12 days. **a** Phenotypes of the growth of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* observed at 12 days and **b** Colony diameter. The experiment was repeated for each set two times independently and the results were presented as means ± standard deviations

maximum activity of Vd-wt and *ΔFreB-C* was found to be 176 and 161 µg $Fe^{2+}/10^7$ spores/h, respectively while almost 50% decrease was observed for gene deletion mutants in this activity, which accounts for 99 and 104 µg $Fe^{2+}/10^7$ spores/h for *ΔFreB-1* and *ΔFreB-2*, respectively, as shown in Fig. 3a. These results suggest that *FreB* gene is involved in the cell-surface reduction of $Fe^{3+}$ to $Fe^{2+}$. The detection of ferric reductase activity in the gene deletion mutants indicates the presence of a compensatory mechanism for ferric reduction.

To further characterize the *FreB* function in iron uptake, determination of the iron content was carried in broth after 24-, 48- and 72-h incubation (Fig. 3b). The iron content was decreased in Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* following the incubation. In *ΔFreB* mutants, the iron uptake was evidently attenuated as compared to Vd-wt and *ΔFreB-C*. Taken together, iron requisition was reduced after *FreB* disruption, which indicates *FreB* gene is important for iron uptake.

## Deletion of *FreB* gene resulted in increased expression level of related genes

The deletion of *FreB* gene resulted in elevated expression levels of other related genes. About 3-fold increase in the expression level of *Frect-4* (Fig. 4a) was observed in *ΔFreB-1* and *ΔFreB-2* than Vd-wt and *ΔFreB-C*. Similarly, 1.8, 3.5 and 1.4-fold increase in the expression level of *Frect-5, Frect-6* and *Met* was observed in the gene deletion mutants from the wild type and complementary strains (Fig. 4b–d). The increase in expression levels of these genes indicate that these genes somehow compensate for the ferric reductase activity in the mutants.

## *FreB* is involved in carbon utilization

To determine the role of *FreB* gene in the mycelial growth and sporulation of *V. dahlliae*, Czapek dox agar was supplemented with different carbon sources. The growth of *FreB* knockout mutants (*ΔFreB-1* and *ΔFreB-2*) was compared with that of Vd-wt and *ΔFreB-C*. The colony diameter of *ΔFreB* mutants was significantly less than that of the Vd-wt on all carbon sources. The observed colony diameter for *ΔFreB* mutants (*ΔFreB-1* and *ΔFreB-2*) on different carbon sources (starch, sucrose, galactose and xylose) was 3.8 and 3.7 cm, 2.6 and 2.5 cm, 3.3 and 2.9 cm and 3.3 and 3.4 cm, respectively, as compared to 4.7 and 4.5 cm, 4.7 and 4.6 cm, 4.2 and 4.1 cm and 3.9 and 3.8 cm of Vd-wt and *ΔFreB-C*, respectively (Fig. 5a, b). Similarly, the number of spores produced by the gene knockout mutants was distinctly less on all the carbon sources than the wild type and complementary strains (Fig. S3).

## Disruption of *FreB* gene resulted in increased susceptibility to oxidative stress and significant reduction in the expression level of oxidative stress response genes

Mutants that are defective in iron acquisition are more sensitive to the oxidative stress (Meneghini 1997; Achard et al. 2013; Xu et al. 2014). With this concept, we checked

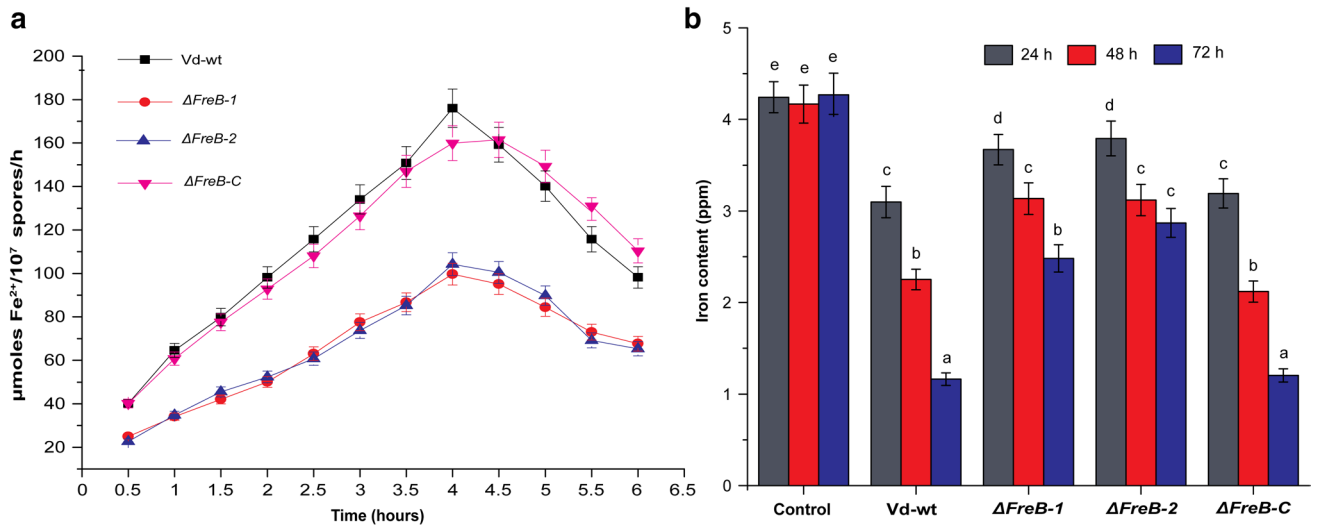


**Fig. 3** Ferric reductase and iron uptake assay. **a** Ferric reductase assay. Spores of the respective strains were collected from iron depleted media and washed with assay buffer (50 mM citrate buffer, 5% glucose; pH 6.5) 2–3 times and incubated in the assay buffer in the presence of 1 mM $FeCl_3$ and 1 mM BPS at 25 °C. Cultures were assayed after each 30 min for 6 h by removing the spores through centrifugation and measuring the optical density of the supernatant at 520 nm. A standard curve was generated by measuring the absorption at 520 nm using known concentration of $FeSO_4$. The standard curve was used to determine the amount of iron reduced by the spores of the respective strains used in the ferric reductase assay. **b** For iron uptake assay. Spores ($1 \times 10^7$/ml) of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* were cultured in Czapek Dox broth supplemented with 100 µM $FeCl_3 \cdot 6H_2O$ for 72 h. The iron content was measured after each 24 h using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7900, CA, USA). Samples with no spores were used as control. The cultures at each time point were centrifuged at 10,000 rpm for 3 min, the supernatant was transfer into a new clean tube and the mycelia were discarded. The iron content was measured in the supernatant of each culture. Experiment was repeated for each strain twice. The results were expressed as the iron contents of the supernatant (ppm) with means ± SD represented by the error bars

the sensitivity of gene deletion mutants to oxidative stress ($H_2O_2$) by measuring the zone of inhibition when $H_2O_2$ was added in the center of the plates after culturing the respective strains. The gene deletion (*ΔFreB-1* and *ΔFreB-2*) mutants were found highly sensitive to the oxidative stress than Vd-wt and *ΔFreB-C* (Fig. 6a), indicating the role of *FreB* in adaptation of the fungus to oxidative stress. The zone of inhibition for *ΔFreB-1* and *ΔFreB-2* was 2.7 and 2.78 cm, respectively, where 1.93 and 1.9 cm for Vd-wt and *ΔFreB-C* (Fig. 6b). When exposed to oxidative stress, a significant reduction in the expression level of oxidative stress response genes, *Vta2* (HE972123.1), *Glutathione reductase* (VDAG_07524) and *VdNoxB* (VDAG_09930), was observed in *ΔFreB-1* and *ΔFreB-2* as compared to Vd-wt and *ΔFreB-C* (Fig. 6c–e).

## *FreB* is required for full virulence in *V. dahliae*

To evaluate the role of *FreB* gene in the virulence of *V. dahliae, N. benthamiana* plants with 4–5 true leaves were inoculated with either *ΔFreB-1, ΔFreB-2*, Vd-wt or *ΔFreB-C*. Plants infected with *ΔFreB-1* and *ΔFreB-2* did not display clear disease symptoms as found in the plants inoculated with Vd-wt. The Vd-wt caused severe wilting symptoms; plants were nearly exanimate at 12 dpi as shown in Fig. 7a. Tobacco plants inoculated with *ΔFreB-1* and *ΔFreB-2* exhibited mild wilting at 12 dpi. The disease index for plants inoculated with Vd-wt was significantly higher (100%) at 12 dpi when compared with those inoculated with the *ΔFreB-1* and *ΔFreB-2* (46 and 44%) as shown in Fig. 7b. The virulence was fully restored in the complementary mutants (*ΔFreB-C*) after introducing *FreB* gene. The virulence caused by *ΔFreB-C* at 12 dpi was almost similar (95%) to that of the wild type fungus, Vd-wt (Fig. 7a, b).

To analyze the fungal biomass in various tissues (roots, stems and leaves) of the plants inoculated with gene deletion mutants, wild type and complementary strains, qRT-PCR was conducted. Regardless of the inoculated fungal strain, fungal biomass was found higher in roots followed by stem and leaves of the plants. Fungal biomass of the mutants (*ΔFreB-1* and *ΔFreB-2*) was significantly lower in all the tissues when compared with that of Vd-wt and *ΔFreB-C* as shown in Fig. 7c. The results of fungal biomass were consistent with the data obtained from disease index and the phenotypic observations. To observe the infection process, roots of the plants inoculated with Vd-GFP and *ΔFreB-GFP* were examined under confocal microscopy at 7 dpi. Multiple spore germination was observed in Vd-GF than in *ΔFreB-GFP* (Fig. 8). Taken together, our results suggest a close association of *FreB* gene with the virulence of *V. dahliae*.

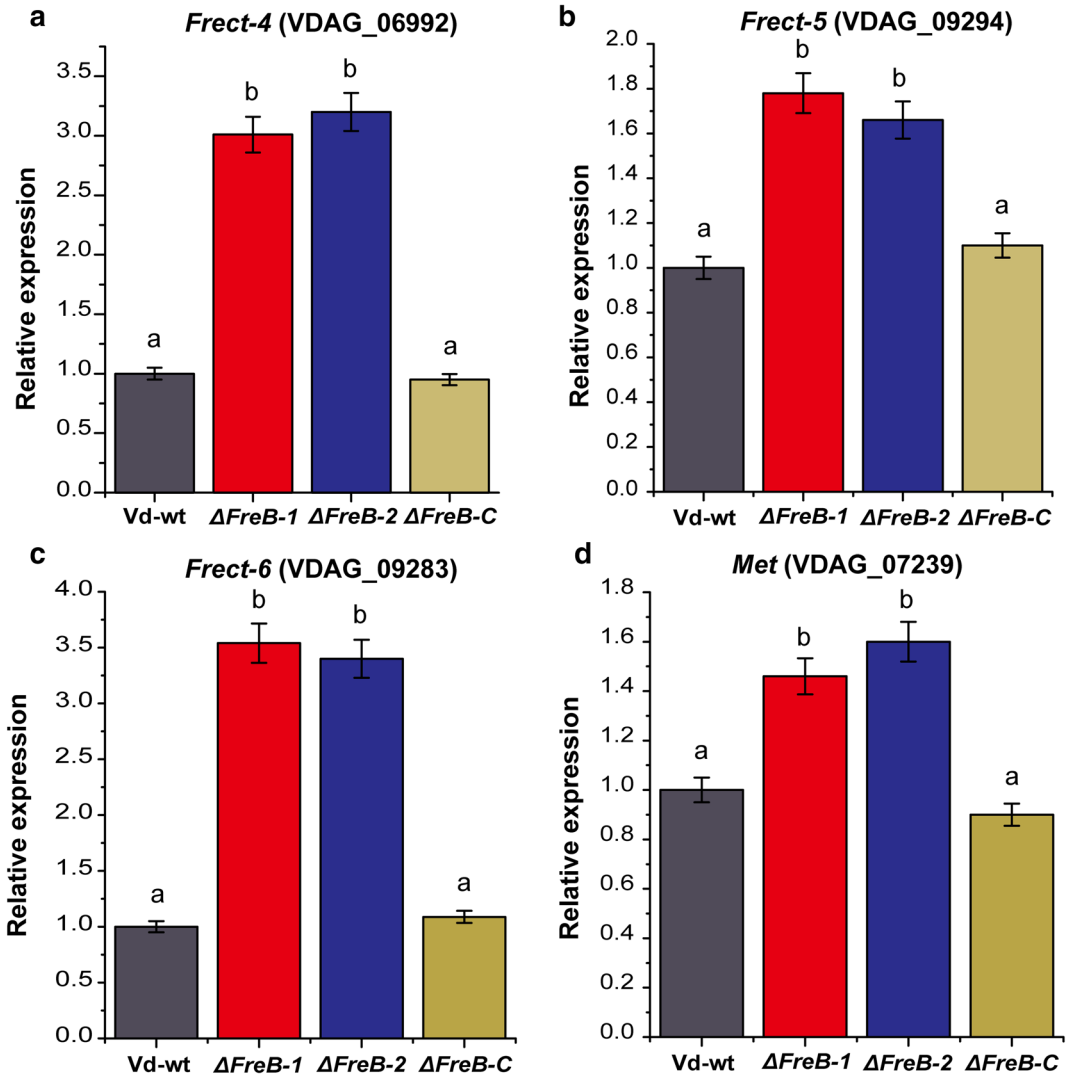


**Fig. 4** Expression levels of other related genes. Conidia from the respective strains were cultured in CM broth. Mycelia were collected for RNA extraction and the cDNA was synthesized. The relative expression of **a** *ferric reductase transmembrane component 4* (VDAG_06992, *Frect-4*), **b** *ferric reductase transmembrane component 5* (VDAG_09294, *Frect-5*), **c** *ferric reductase transmembrane component 6* (VDAG_09283, *Frect-6*) and **d** *metalloreductase* (VDAG_07239, *Met*) were analyzed in Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* were determined by qRT-PCR. *Vd-actin* gene was used as a housekeeping gene. Significant differences ($p < 0.05$) are indicated by different letters

## Discussion

Although many genes have been associated with pathogenetic mechanism in *V. dahliae* (Chen et al. 2016; Xiong et al. 2016; Zhang et al. 2017), few genes about ferric metabolism have been reported. In our study, we characterize the function of ferric reductase transmembrane component three precursor (*FreB*) in the cell-surface ferric reduction ($Fe^{3+}$ to $Fe^{2+}$) and iron uptake in *V. dahliae*. Meanwhile, multiple pathogenicity-related traits were mediated through *FreB* impact on ferric metabolism.

In previous studies, mutants that are deficient in iron uptake showed significant reduction in growth on media specially in limited iron condition, e.g., in *A. fumigatus*, combination of *FreB* gene disruption with the inactivation of siderophore system resulted in decreased growth rate under iron deficient conditions (Blatzer et al. 2011). Similar in iron limited conditions, reduced growth was found in the *sef1* deletion mutants of *C. albicans* (Homann et al. 2009; Chen and Noble 2012). Our results were in accordance with the previous studies, *ΔFreB* mutants showed significantly reduced growth on media with limited iron. In observation of the growth on media supplemented with $FeSO_4$ and $FeCl_3$ as iron sources, *ΔFreB* mutants exhibited better growth on $FeSO_4$ supplemented media while no significant difference in the growth of Vd-wt and

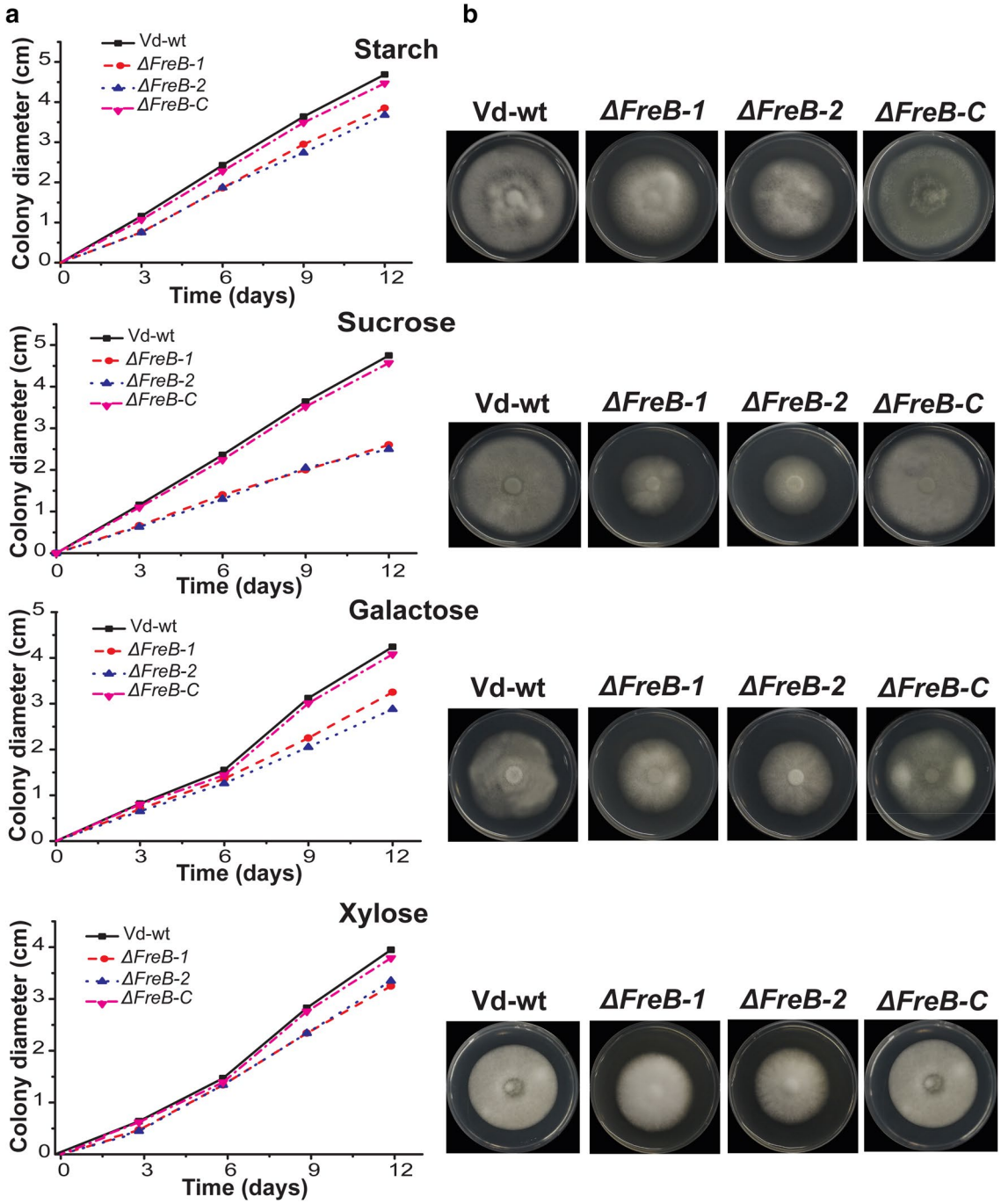


**Fig. 5** Carbon source utilization assay. **a** Colony diameter of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* on media supplemented with different carbon sources. Spores (10 µl, $10^6$/ml) from the respective strains were placed in the center of each plate separately and the colony diameter was measured at 3-day intervals for 12 days. **b** Phenotype of the growth of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* on media after 12 days. The experiment was repeated for each set two times independently and the results were presented as means ± standard deviations

*ΔFreB-C* was found on either media. These results suggest the role of *FreB* gene in adapting the fungus to iron starvation and iron reduction. The deletion of important genes can compromise the ability of the mutants to grow on different carbon sources (Liu et al. 2013; Zhang et al. 2015, 2016). In our study, the growth of mutants (*ΔFreB*) on media supplemented with different carbon sources showed significant differences with an overall trend in better growth for Vd-wt and *ΔFreB-C* on all the media, probably because iron is crucial for a number of enzymes

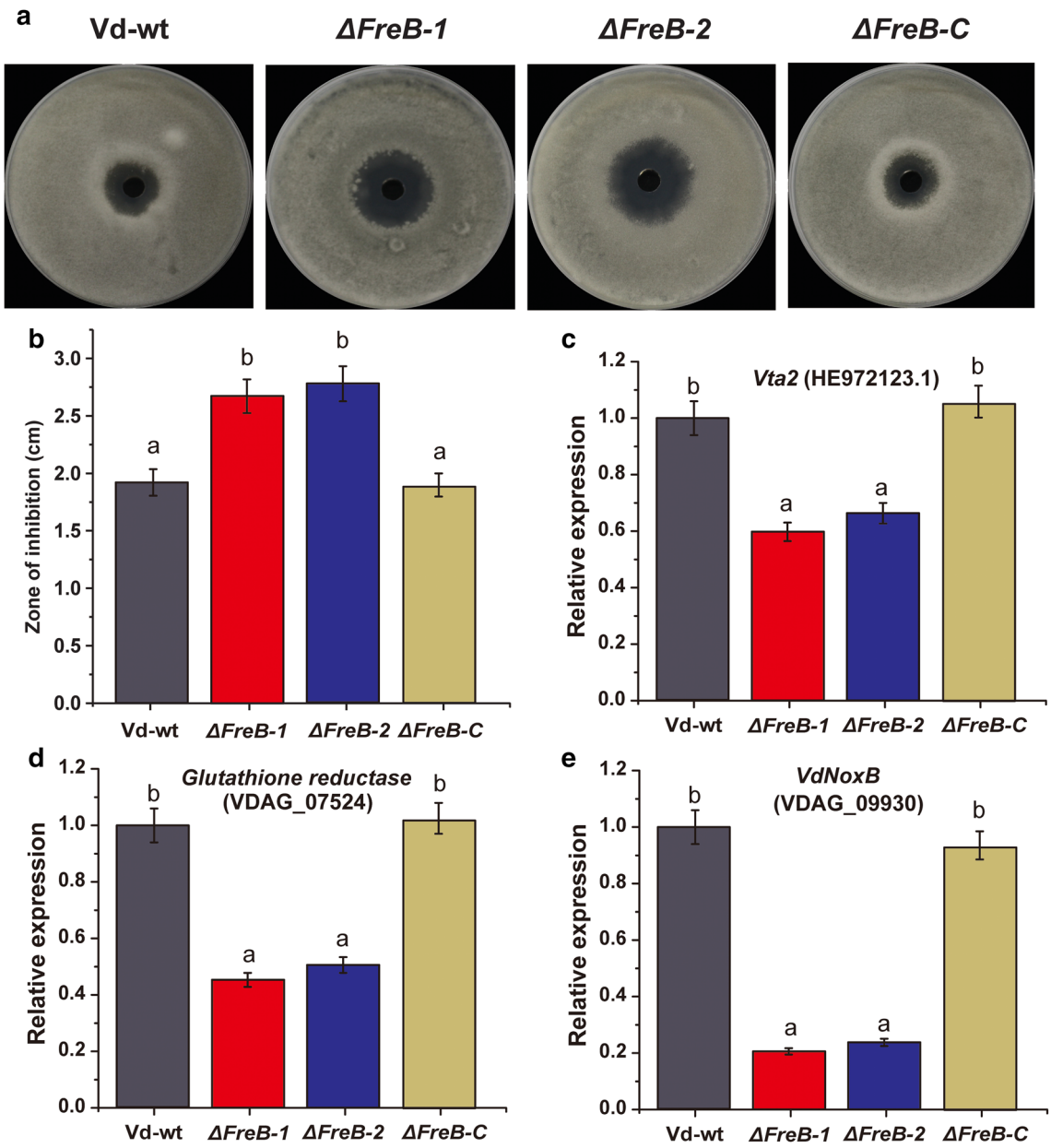


**Fig. 6** Oxidative stress assay expression level of oxidative stress response genes. For this assay, 500 µl spores ($10^6$/ml) of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* were spread on Czapek dox agar plates, respectively. A hole was made in the center of each plates using a cork borer. 100 µl $H_2O_2$ (100 µl, 100 mM) solution was poured onto the hole of each plate. **a** The zone of inhibition was observed after a week **b** measurement of zone of inhibition after a week. For expression analysis of oxidative stress response genes, spores were collected from the plates after exposing to oxidative stress and cultured in CM for RNA extraction. qRT-PCR was carried out using gene specific primers. Expression level of **c** *Vta2* (HE972123.1), **d** *Glutathione reductase* (VDAG_07524) and **e** *VdNoxB* (VDAG_09930). The experiment was repeated for each set two times independently and the results were presented as means ± standard deviations

in different metabolic pathways and the mutants are defective in iron reduction and uptake.

Deletion of *FreB* gene cause significant reduction in the surface ferric reductase activity. The reduction of iron ($FeCl_3$) increased with the increasing incubation time up to 4 h and then reduction in the ferric reductase activity was observed for both mutants and the wild type strains. However the ferric reductase activity in gene deletion mutants was significantly lower than the wild type and the complementary strains suggesting the involvement of *FreB*. We did not observe the complete loss of the activity in the gene deletion mutants, which indicate the existence

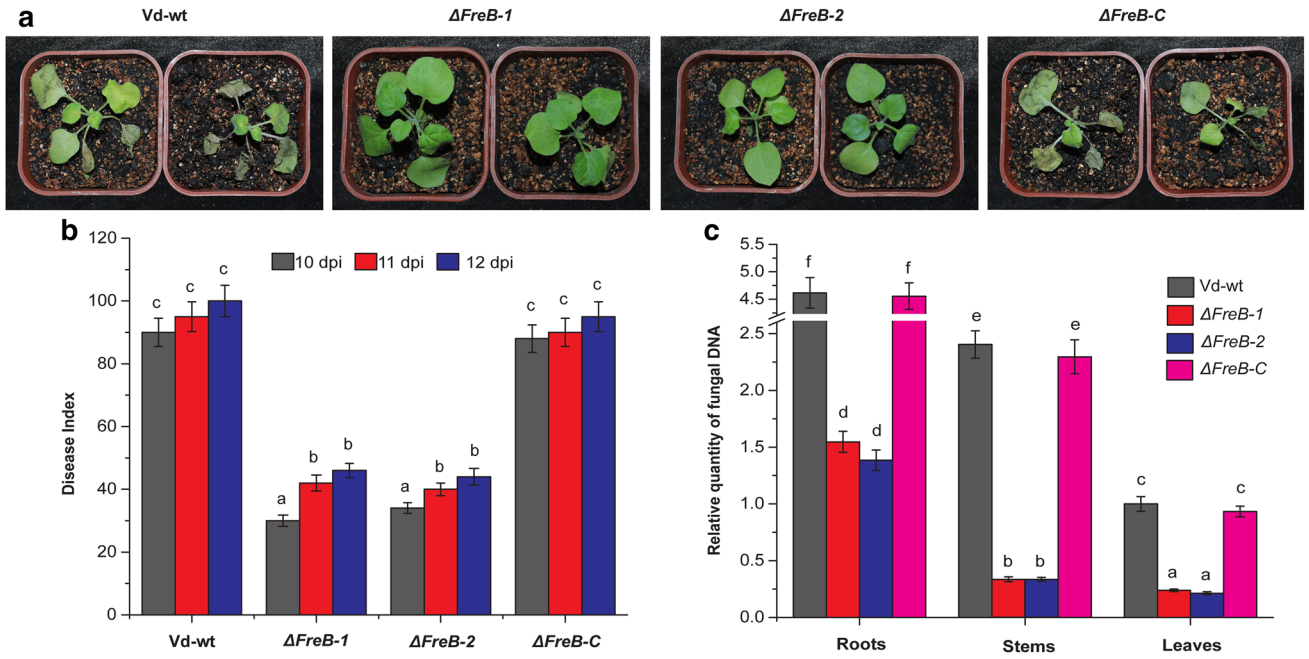


**Fig. 7** Pathogenicity assay. *Nicotiana benthamiana* seedlings, with 6–8 true leaves, were inoculated with the spores ($10^7$/ml) of Vd-wt, *ΔFreB-1, ΔFreB-2* and *ΔFreB-C* for 2 min by root-dip method. **a** Observation of the disease phenotypes for seedlings inoculated with different strains at 12 days post-inoculation (dpi). **b** Disease index measured for seedlings at 10, 11 and 12 dpi. **c** Fungal biomass measured as relative quantity of fungal DNA by qRT-PCR in different tissues of the infected seedlings at 12 dpi by amplifying ITS1 and ITS2 of rDNA

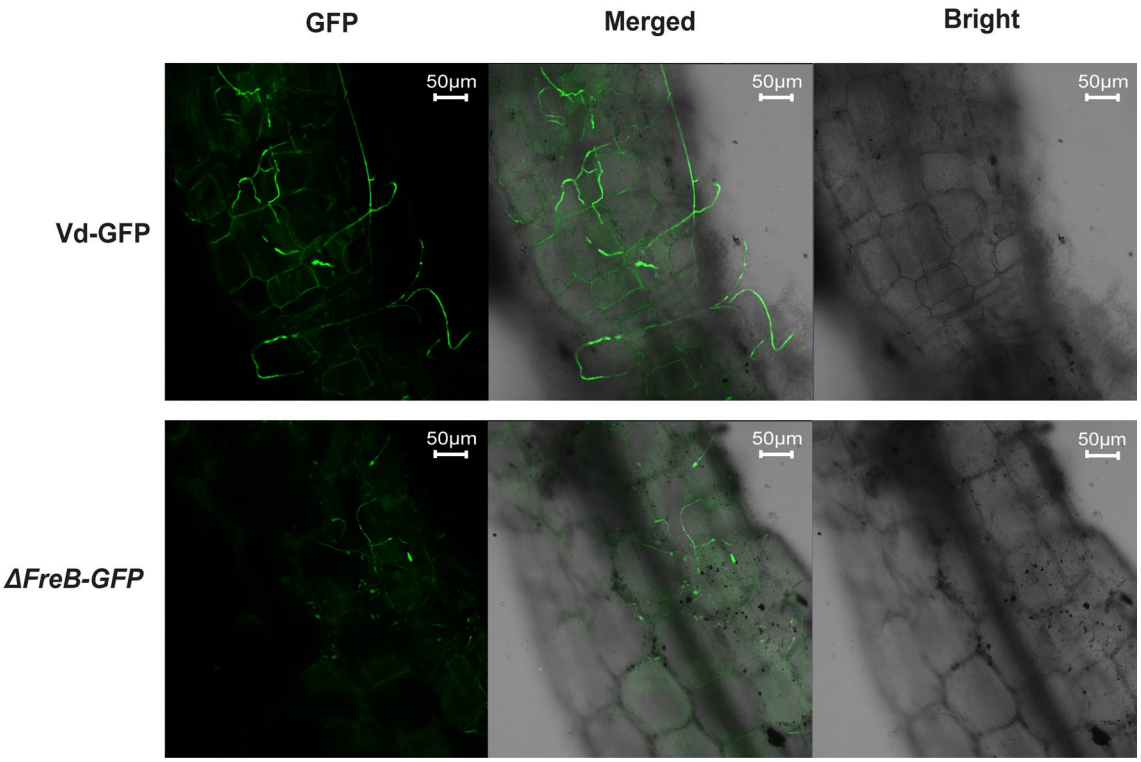


**Fig. 8** Observation of the GFP fluorescence in the roots of *N. benthamiana* seedlings inoculated with Vd-GFP and *ΔFreB-GFP. N. benthamiana* seedlings were inoculated with the spore's suspension ($10^7$/ml) of Vd-GFP and *ΔFreB-GFP* using root-dip method. The roots of the infected seedlings were collected at 7 dpi, washed with water 3–4 times and observed under confocal microscopy

of a compensatory mechanism underlying. In *C. albicans*, a compensatory mechanism was proposed when *CFL1* gene was disrupted. The deletion of *CFL1* gene resulted in elevated expression of gene responsible for alternative ferric reductases (Xu et al. 2014). In our study we also observed an increase in the expression levels of other genes related to transmembrane iron reduction and metabolism, *Frect-4, Frect-5, Frect-6* and *Met*.

Mutants with defects in iron uptake are more sensitive to oxidative stress (Achard et al. 2013). Ferric reductase *CFL1* gene in *C. albicans* was found closely associated with the oxidative stress response (Xu et al. 2014). A tight relationship was found between the iron metabolism and oxidative stress in *A. nidulans* (Eisendle et al. 2006). Strains of *Alternaria alternate*, defective in NOX, YAP1, HOG1 or NPS6 displayed increased sensitivity to oxidative stress; however, exogenous supply of iron partially rescued the fungus from oxidative stress (Chen et al. 2014). Similarly, in *A. fumigatus*, the deletion of metalloreductase *FreB* gene decreased resistance to oxidative stress (Blatzer et al. 2011). In our study, the gene deletion mutants displayed significantly increased sensitivity to oxidative stress because of the limited iron uptake impairing the iron-dependent enzymes involved in the detoxification of oxidative stress. In a previous study, *Vta2* gene has been associated with oxidative stress, decreased expression of this gene resulted in sensitive phenotypes to $H_2O_2$ stress conditions in *V. longisporum* (Tran et al. 2014). In our study the expression of this gene in gene deletion mutants, when exposed to oxidative stress, showed reduced expression level as compared to the wild type and complementary strains. Similarly decrease in the expression level of other oxidative stress related genes, *Glutathione reductase* (Han et al. 2015) and *VdNoxB* (Zhao et al. 2016), were also observed in gene deletion mutants which is a clear indication that *FreB* gene is required for the adaptation of the fungus to oxidative stress.

Availability of iron to the pathogens is considered as a prerequisite for causing pathogenicity or virulence in the host organisms (Sutak et al. 2008). Iron availability has a strong influence on the expression of major virulence factors (Saikia et al. 2014). Meanwhile, iron is pivotal for protein secretion in filamentous fungi, which are essential for fungal survival, exchanging nutrients and virulence factors (Hillmann et al. 2015; McCotter et al. 2016). Previous studies have found a close association between the iron acquisition and the virulence or pathogenicity of an organism (Cadieux et al. 2013; Moore 2013; Xu et al. 2014). The deletion of genes related to iron reduction or acquisition had a profound effect on the virulence of fungi. In our study, the deletion of *FreB* gene resulted in attenuated virulence when the virulence of gene deletion mutants was compared with the wild type and the complementary strains. The possible reason for the attenuation in the virulence can be due to the fact that iron is important for a range of key enzymes and the limited availability may diminish the activity of certain factors or enzymes that are vital for causing the virulence in *V. dahliae*.

In summary, the *FreB* function was identified in ferric metabolism and multiple pathogenicity-related traits of *V. dahliae*. In the *ΔFreB* mutants, ferric reduction and uptake were significantly inhibited. Moreover, the growth and sporulation were evidently reduced on different carbon sources and under oxidative stress. The full virulence was consequently suppressed and the inoculated plants showed less disease symptom and fungal biomass. In previous studies, the plants expressing the dsRNA against the pivotal developmental and pathogenetic genes, such as *VdH1, VdAAC*, presented effective resistance against *V. dahliae* (Su et al. 2017; Zhang et al. 2016a, b). Taken together, *FreB* mediates the carbon source utilization and adapt to oxidative stress by the regulation of ferric metabolism and is vital for the full virulence in *V. dahliae*, which would be utilized as a potential target for disease strategy.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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