Gene 626 (2017) 386–394

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Functional characterization of two bZIP transcription factors in *Verticillium dahliae*

Yulin Fang[1], Dianguang Xiong[1], Longyan Tian, Chen Tang, Yonglin Wang*, Chengming Tian

Beijing Key Laboratory for Forest Pest Control, College of Forestry, Beijing Forestry University, Beijing, China

ARTICLE INFO

Keywords:
Verticillium dahliae
bZIP transcription factor
Virulence
Oxidative response

ABSTRACT

bZIP transcription factors play various biological roles in stress responses, conidiation, and pathogenicity in pathogenic fungi. Here, we report two bZIP transcription factors (VDAG_08640 and VDAG_08676) of *Verticillium dahliae*, which were differentially expressed during microsclerotia development and induced by hydrogen peroxide as well. We find that deletion of either gene does not affect microsclerotia formation and the sensitivity to hydrogen peroxide; however, the mutants manifest decreased activity of extracellular peroxidase and laccase. Other phenotypic characterization reveals that VDAG_08676 disruption results in significant reduction of conidial production and virulence, while VDAG_08640 disruption does not lead to observable phenotypic variances compared with the wild-type strain. To elucidate whether they exhibit functional redundancy, double deletion mutants were generated. The double deletion mutants show remarkably increased sensitivity to hydrogen peroxide stress, whereas the two genes are not involved in microsclerotia formation. Taken together, our data demonstrate that a bZIP transcription factor gene VDAG_08676 is involved in the conidial production, oxidative stress response and virulence which may lay a foundation for further analysis of other bZIP transcription factors in *V. dahliae*.

1. Introduction

Verticillium dahliae is a soilborne plant pathogenic fungus. It causes Verticillium wilt on > 200 plant species, including many trees and crops. Its disease cycle starts as microsclerotia germinate to hyphae that directly penetrate into the host roots and then colonize plant xylem vessels (Klimes et al., 2015). At the end of the disease cycle, *V. dahliae* forms melanized microsclerotia which can survive in soil for a long period without any host, and microsclerotia act as new inoculum as well (Neumann and Dobinson, 2003). Due to its ability to colonize plant xylem and form microsclerotia, no curative measures are available to control the Verticillium wilt disease yet (Fradin and Thomma, 2006; Wilhelm, 1955). Recently, great progress has been made in understanding the mechanism underlying virulence, plant-pathogen interactions, and microsclerotia development, especially after the availability of the *V. dahliae* genome sequence (Klimes et al., 2015).

The basic leucine zipper (bZIP) transcription factors (TFs) represent one of the largest TF families across eukaryotes, whose domain is generally 60–80 amino acids in length. The domain consists of a basic region with relatively conserved motif sequence of N-X_7-R/K-X_9 which is responsible for DNA binding, nuclear import and a leucine zipper dimerization motif which regulates dimerization (Jakoby et al., 2002). AP1 is one of the most well-studied bZip TFs and is essential for oxidative stress response in fungi, i.e. *Saccharomyces cerevisiae* (Kuge and Jones, 1994; Schnell et al., 1992), *Magnaporthe oryzae* (Guo et al., 2011), *Ustilago maydis* (Molina and Kahmann, 2007), *Fusarium graminearum* (Montibus et al., 2013), *Cochliobolus heterostrophus* (Lev et al., 2005), *Candida albicans* (Zhang et al., 2000), and *Colletotrichum gloeosporioides* (Sun et al., 2016). Furthermore, Atf1, another bZIP TF, is possibly involved in oxidative stress as well (Balazs et al., 2010; Guo et al., 2010; Jiang et al., 2015; Lara-Rojas et al., 2011; Wilkinson et al., 1996). However, in some fungi such as *Aspergillus nidulans* and *Botrytis cinerea*, deletion of Atf1 has no effect on oxidative or osmotic stress response (Hagiwara et al., 2008; Temme et al., 2012). Remarkably, the whole bZIP TFs in *M. oryzae* have been functionally characterized (Kong et al., 2015; Tang et al., 2015), and bZIP TFs are involved in diverse phenotypes including hyphal growth, virulence, response to oxidative stress, and iron homeostasis. By contrast, single deletion of six bZIP TF genes showed no distinguishable phenotypes compared with the wild-type *M. oryzae* strain.

Abbreviations: bZIP, basic leucine zipper; TF, transcription factor; qRT-PCR, quantitative real-time PCR; PDA, potato dextrose agar; CM, complete medium; CFW, calcofluor white; BM, basal medium; ORF, open reading frame; NBT, nitrobluetetrazolium; ROS, reactive oxygen species

* Corresponding author.
E-mail address: ylwang@bjfu.edu.cn (Y. Wang).

[1] Yulin Fang and Dianguang Xiong were equal contributors.

http://dx.doi.org/10.1016/j.gene.2017.05.061
Received 7 April 2017; Received in revised form 26 May 2017; Accepted 30 May 2017
Available online 31 May 2017
0378-1119/

Twenty bZIP TFs have been identified in the *V. dahliae* genome and their expression pattern during microsclerotia formation has also been determined by RNA-Seq (Klosterman et al., 2011; Xiong et al., 2014), but none of bZIP TFs has been characterized so far. In this work, two differentially expressed bZIP TFs (VDAG_08676 homologous to Atf1 and VDAG_08640) during microsclerotia formation were selected for further study by gene knockout. Our results indicated that deletion of VDAG_08676 or VDAG_08640 showed no distinguishable differences in microsclerotia formation, sensitivity to oxidative stress, and fungal growth compared with the wild-type strain. However, the double deletion mutant of VDAG_08676 and VDAG_08640 displayed more sensitivity to oxidative stress than the wild-type strain.

2. Materials and methods

2.1. Sequence analysis

The related sequences of VDAG_08640 and VDAG_08676 were downloaded from *V. dahliae* genome database (Klosterman et al., 2011). The domain architectures were visualized using InterPro (http://www.ebi.ac.uk/interpro/). Furthermore, the putative orthologs of VDAG_08640 and VDAG_08676 in other fungi and multiple amino acid sequence alignments were performed with BLASTP program and ClustalX2.0 (Larkin et al., 2007). The phylogenetic tree was made by MEGA 6.0 (Tamura et al., 2013) based on previous work using the neighbor-joining method and the bootstrap test was replicated 1000 times.

2.2. Fungal strains and culture conditions

The *V. dahliae* strain XS11, isolated from the Fragrant Hill, Beijing, was used as a host strain and a wild-type control in this study (Wang et al., 2013). Conidia suspension in glycerol (30% v/v) stored at − 80 °C were used to start cultures. All strains were incubated on potato dextrose agar (PDA) plates at room temperature. Besides, the cultures grown on PDA plates were used to test the fungal growth rate and conidial production, and the experiment was repeated three times. Conidia were harvested from cultures grown in the fresh liquid complete medium (CM) (Dobinson et al., 1997) by filtration through two layers of Miracloth (Calbiochem, Germany). Likewise, the mycelia were prepared in liquid CM for DNA extraction. To analyze the cell wall properties, 10 μg/ml Calcofluor White (CFW) was used to stain the germinated conidia and mycelia. For stress response assays, all the strains were maintained on CM plates with $MgSO_4$, NaCl, or KCl. Microsclerotia formation was induced on a basal medium (BM) as described previously (Neumann and Dobinson, 2003; Xiong et al., 2014). To determine the sensitivity of strains to H_2O_2, 10^6 conidia of each strain were mixed with 100 ml solid CM and poured plates respectively. Subsequently, a 5 mm filter paper disk was placed on the central of plates, and then 5 μl 5% or 15% (v/v) H_2O_2 were dropped on the filter paper disk followed by another two days incubation at room temperature to measure the inhibition size. Each assay was carried out in three replicates.

2.3. Gene knockout and complementation

All the deletion mutants in this study were generated by using the split-marker method (Goswami, 2012). As for VDAG_08640 and VDAG_08676, the upstream and downstream flanking sequences were amplified with the following primer pairs: B40-5F/B40-5R,B40-3F/B40-3R, B76-5F/B76-5R and B76-3F/B76-3R (Table S1). The resulting upstream and downstream fragments were fused with a geneticin-resistant cassette by using overlap PCR with primers B40-5F/Ge-R, Ge-F/B40-3R, B76-5F/Ge-R and Ge-F/B76-3R respectively (Table S1), respectively. All the fragments were confirmed by sequencing analysis. Subsequently, the gene deletion constructs of VDAG_08640 and VDAG_08676 were directly introduced into the protoplasts of *V. dahliae* XS11, respectively. The transformants were selected on TB3 medium with 50 μg/ml geneticin. Successful gene replacement transformants of VDAG_08640 and VDAG_08676 were identified by using PCR with primers B40-O5F/B40-O3F, B40-I5F/B40-I3F, B76-O5F/B76-O3F and B76-I5F/B76-I3F, respectively.

For complementation, the fragment containing the native promoter (approx. 1.5 kb) and the entire open reading frame (ORF) of VDAG_08640 or VDAG_08676 was amplified with primers B40-5F/B40-C3F and B76-5F/B76-O3F respectively (Table S1). The resulting PCR products of VDAG_08640 or VDAG_08676 were co-transformed into the protoplasts of corresponding deletion mutants with the hygromycin-resistant cassette respectively. Successful complemented transformants were confirmed by RT-PCR with primers B40-I5F/B40-I3F for VDAG_08640 and primers B76-I5F/B76-I3F for VDAG_08676 (Table S1).

To generate the VDAG_08640 & VDAG_08676 double genes deletion mutants, the upstream and downstream flanking sequences of VDAG_08676 described above were fused with the hygromycin-resistant cassette by using overlap PCR with primers B76-5F/HY-R and YG-F/B76-3R (Table S1) respectively. The resulting constructs were transformed into the protoplasts of VDAG_08640 deletion mutant. The transformants were selected on TB3 medium with 25 μg/ml hygromycin and 50 μg/ml geneticin. Successful VDAG_08640 & VDAG_08676 double gene deletion transformants were initially screened by PCR with primers B76-I5F/B76-I3F (Table S1) and then used for Southern blot analysis. Southern blot was performed by using the DIG High Prime DNA Labeling and Detection Starter Kit I in accordance with the manufacturers' protocol (Roche, Germany). For Southern blot assays, the probe fragment was amplified with primers B76-P5F/B76-P3F and the genomic DNA was digested with *Kpn* I.

2.4. Generation of VDAG_08640-GFP and VDAG_08676-GFP fusion transformants

To identify the cellular location of VDAG_08640 and VDAG_08676, VDAG_08640-GFP fusion and VDAG_08676-GFP fusion were constructed. For VDAG_08640-GFP, a fragment containing the native promoter (approx. 1.5 kb) and coding region of VDAG_08640 without stop codon was amplified using primers B40-C5F/B40-N3F with Prime STAR HS DNA Polymerase (Takara Biotechnology (Dalian), China), and ligated into the *Sma* I-digested plasmid pKD5-GFP. The resulting plasmid was PCR amplified with primers B40-C5F/RB followed the resulting fragment was co-transformed into the protoplasts of VDAG_08640 deletion mutant with the hygromycin-resistant cassette. Successful integration transformants (VDAG_08640-GFP) were selected with GFP fluorescence and 25 μg/ml hygromycin. The same method was performed to generate the VDAG_08676-GFP fusion and VDAG_08676-GFP transformants.

2.5. Pathogenicity assays

Tobacco seedlings were inoculated with XS11 and transformants for the pathogenicity assays, using a root dip method. The inoculum was prepared by harvesting conidia from cultures grown in liquid CM for 7 days by filtration through two layers of Miracloth and suspended at 10^6 conidia/ml in sterile distilled water. The roots of the tobacco seedlings were inoculated with conidial suspensions for 10 min while control plants were mock-inoculated with sterile distilled water. Followed all inoculated tobaccos were replanted into soil. Ten tobacco seedlings were tested per strain and the experiment was repeated three times.

2.6. qRT-PCR assays

To evaluate the expression level of genes involved in oxidative responses, all strains were treated with or without 2.5 mM H_2O_2 for 1 h