Fungal Biology 123 (2019) 539–546

Contents lists available at ScienceDirect

Fungal Biology

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

The α-1,6-mannosyltransferase VdOCH1 plays a major role in microsclerotium formation and virulence in the soil-borne pathogen *Verticillium dahliae*

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ARTICLE INFO

Article history:
Received 4 May 2018
Received in revised form
24 April 2019
Accepted 7 May 2019
Available online 18 May 2019

Corresponding Editor: Julian Rutherford

Keywords:
Microsclerotia
Pathogenicity
Sunflower
Verticillium wilt

ABSTRACT

Sunflower yellow wilt is a widespread and destructive disease caused by the soil-borne pathogen *Verticillium dahliae* (*V. dahliae*). To better understand the pathogenesis mechanism of *V. dahliae* in sunflower, T-DNA insertion library was generated via *Agrobacterium tumefaciens* mediated transformation system (ATMT). Eight hundred positive transformants were obtained. Transformants varied in colony morphology, growth rate, conidia production and pathogenicity in sunflower compared to the wild type strain. A mutant, named VdGn3-L2, was chosen for further analysis based on its deprivation on microsclerotia formation. The flanking sequence of T-DNA insertion site of VdGn3-L2 was identified via hiTAIL-PCR, and the interrupted gene encoded an initiation-specific α-1, 6-mannosyltransferase, named as *VdOCH1*. The deletion mutant *ΔVdOCH1* was impaired in certain characteristics such as fungal growth, conidia production, and microsclerotia formation. Also, *ΔVdOCH1* mutants were more sensitive to the cell wall perturbing reagents, such as SDS and Congo red, lost their penetration ability through cellophane membrane, and exhibited dramatically decreased pathogenicity to sunflower. The impaired phenotypes could be restored to the wild type level by complementation of the deletion mutant with full-length *VdOCH1* gene. In conclusion, *VdOCH1*, encoded α-1,6-mannosyltransferase, manipulating the biological characteristics, microsclerotia formation and pathogenic ability of *V. dahliae* in sunflower.

1. Introduction

Sunflower (*Helianthus annuus* L.) is one of the main oilseed crops and the fourth largest in China, with a total cultivation area of approximately 1.2 million hectares. Inner Mongolia is the largest sunflower production region in China, accounting for around 0.7 million hectares (Lan et al., 2009). Sunflower yellow wilt, caused by the soil borne fungus *Verticillium dahliae* Kleb, has become one of the most serious threats with incidence ranging from 10 to 30 % (Ren et al., 2014). Leaf mottling and chlorosis and discoloration of vascular bundle in the infected stem are the typical symptoms (Zhang et al., 2015). *Verticillium dahliae* is, facultative parasite that can infect more than 200 dicotyledonous plant species, including potato, sunflower, eggplant, cauliflower, cotton and lettuce (Atallah et al., 2011; Davis et al., 1997; Pegg and Brady, 2002; Rowe and Powelson, 2002). It can be transmitted through conidia or microsclerotia via contaminated vegetative propagation materials, farm equipment, irrigation water, and soil (Zhang et al., 2017a). Microsclerotia, the dormant structures, can survive for at least 15 y in soil (Emilief and Bartphj, 2006; Inderbitzin and Subbarao, 2014) and it is the primary infection source in sunflower yellow wilt (He et al., 2015). Microsclerotia are composed of two layers of cells: a thin-walled hyaline layer and thick-walled melanized layer (Gordee and Porter, 1961; Griffiths, 1970). Under suitable field conditions, the microsclerotia germinate and the hyphae penetrate the roots of host plants via a narrow penetration peg and colonize the host vascular system (Ren et al., 2014; Schnathorst, 1965; Zhao et al., 2016).

A number of genes associated with microsclerotium development have been identified in *V. dahliae*. The *VDH1* gene, encoding a hydrophobic protein, was verified in its role of manipulating the development of microsclerotia. Knockout of *VDH1* gene suppressed the formation of microsclerotia but did not affect pathogenicity (Klimes et al., 2008; Klimes and Dobinson, 2006). The gene *VMK1*,

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https://doi.org/10.1016/j.funbio.2019.05.007
1878-6146/

which encodes a mitogen activated protein kinase, regulates development of microsclerotia. The *VMK1* deletion mutant led to the reduction of the amount of microsclerotia and pathogenicity (Rauyaree et al., 2005). A glutamic acid-rich protein, VdGARP1, was also confirmed to manipulate both microsclerotial development and pathogenicity (Gao et al., 2010). Other genes associated with microsclerotia formation have also been identified, including transcription factors (*VdSge1*, *Vta2*, *VdCrz1*, *VdMcm1*, *Vst1*), protein kinase genes (*VMK1*, *VdSNF*, *VdPbs2*, *VdPKAC1*) and glucose metabolism-related genes (*VdEg-1*, *VdHMGS*, *VdGPIM3*) (Maruthachalam et al., 2011; Rauyaree et al., 2005; Santhanam and Thomma, 2013; Tian et al., 2016; Tran et al., 2014; Tzima et al., 2011; Xiong et al. 2015, 2016; Sarmiento-Villamil et al., 2018). The functional study of these genes indicates that the processes associated with microsclerotia formation are rather complex, and the coordination of various genes is necessary for microsclerotia development in *V. dahliae*.

Alpha-1,6-mannosyltransferase (OCH1) plays a crucial role in the N-linked oligosaccharide glycoprotein processing. The enzyme was cloned from *Saccharomyces cerevisiae* and its homologs were then studied in detail in other fungi such as *Yarrowia lipolytica*, *Candida albicans*, *Hansenula polymorpha*, *Aspergillus fumigatus*, *Neurospora crassa* and *Kluyveromyces lactis* (Barnayverdier et al., 2004; Bates et al., 2006; Kim et al., 2006; Lambou et al., 2010; Maddi and Free, 2010; Zanni et al., 2009). The N-linked oligosaccharide is assembled at the early step of glycoprotein synthesis pathway via formation of $Man_8GlcNAc_2$ oligosaccharide in endoplasmic reticulum (ER), then, $Man_8GlcNAc_2$ is delivered to the Golgi apparatus for the addition of glycan chains (Helenius and Aebi, 2001). In yeasts, *OCH1*, which encodes alpha-1,6-mannosyltransferase, is targeted on α-1,6-mannose and transferred into the core structure of $Man_8GlcNAc_2$ in Golgi. The chain can be extended with α-1,6-linked mannoses and leads to hypermannosylated glycoproteins, thus possessing a large structure called the outer chain (Dean, 1999; Nakanishishindo et al., 1993). Such types of processing also are present in other filamentous fungi, such as *A. fumigatus* and *N. crassa* (Kotz et al., 2012; Maddi and Free, 2010).

OCH1 possess distinct functions in different fungal species. In *C. albicans*, the *OCH1* gene manipulates virulence with deletion mutants showing significant reduction in virulence, but with a normal growth rate (Bates et al., 2006). In *K. lactis*, the *OCH1* gene also affects cell wall integrity (Uccelletti et al., 2006). The same phenotypes were also observed in the *OCH1* mutants of *S. cerevisiae*, *Pichia angusta*, *Y. lipolytica*, and *Schizosaccharomyce pombe* (Barnayverdier et al., 2004; Choi et al., 2003; Kim et al., 2006; Yokoo et al., 2001). In filamentous fungi, impaired *OCH1* gene affected the cell wall integrity in *N. crassa* (Maddi and Free, 2010) and virulence in *Fusarium oxysporum f. sp. Cubense* (Li et al., 2014). However, the function of the *OCH1* gene of *V. dahliae* has not been determined.

A T-DNA insertion mutant library *V. dahliae* from sunflower was generated and mutants with phenotypes that are clearly different from the wild type strain were obtained. Analysis of the T-DNA random insertion mutant library identified *VdOCH1* encoding an alpha-1,6 -mannosyltransferase as the gene harboring a T-DNA insertion in a random mutant unable to produce microsclerotia. Targeted disruption of *VdOCH1* confirmed a major role for this mannosyltransferase in microsclerotium development and determined that it also played an important role in virulence.

2. Materials and methods

2.1. Fungal strains and growth conditions

V. dahliae strain VdGn3, isolated from diseased sunflower collected from Gannan, Heilongjian Province of China, was used as parental strain for both the production of random T-DNA insertion mutants and for targeted gene knockouts. All fungal strains were stored a −80 °C as spores suspensions in 20 % glycerol and propagated on potato dextrose agar (PDA). The same medium was used to assess the production of conidial spores and mycelium growth rates. Complete medium (CM) (Dobinson et al., 2004) and CM supplied with 0.01 % (w/v) Sodium Dodecyl Sulfate (SDS) or Congo red (CR, 40 μg/mL) were used for determining fungal cell wall integrity. Isolates were grown on wheat bran medium for 7 d at which time conidia were harvested (Zhang et al., 2017a). The strains were culutered at 25 °C.

2.2. Generation of random T-DNA insertion mutants

Agrobacterium tumefaciens (*A. tumefaciens*) strain LBA4404 containing the binary vector pCH-sGFP was kindly provided by Dr. Baolong Zhang (Jiangsu Academy of Agricultural Sciences, Nanjing, China) and was used to carry out ATMT of *V. dahliae*. The pCH-sGFP vector harbored the GFP as a report gene and hygromycin phosphotransferase (*Hph*) gene as a selection marker. The transformation of *V. dahliae* was performed as previously described with minor modifications (Dobinson et al., 2004). *A. tumefaciens* was cultured at 28 °C for 48 h in minimal medium (MM) supplemented with rifampicin (25 μg/ml) and kanamycin (50 μg/ml) and continuously shaken at 220 rpm. *A. tumefaciens* cells were collected and adjusted to $OD_{600} = 0.2$ with induction media containing 200 μM acetosyringone (AS). After incubating for an additional 6 h at 28 °C and 200 rpm, the OD_{600} reached 0.6. The bacterial culture was then mixed with an equal volume of *V. dahliae* conidia (1×10^6 conidia/mL). The mixture was sprayed onto cellophane membrane which was placed on the top of co-cultivation medium plate. After 48 h of culture in the dark, the cellophane membrane was transferred onto selection medium, which contained hygromycin B (50 μg/ml) and cefotaxime (200 μg/ml), for selection. Putative transformants were transferred onto PDA plates with hygromycin B (50 μg/ml) and incubated at 25 °C for 5 d. Those transformants that maintained stable hygromycin resistance after repeated cultivation on selective medium were stored as spore suspensions at 80 °C for further analysis.

2.3. Identification of sequences flanking T-DNA insertions

For genomic DNA isolation, a CTAB extraction method was used as previously described (Stewart and Via 1993). Mycelia (0.1–0.15 g) were scraped off 10-day-old PDA plates cultured at 25 °C and lyophilized for 24 h.

Southern blot analysis was carried out using the DIG High Prime DNA Labeling and Detection Starter Kit I according to the manufacturer's protocol (Roche, Germany). Genomic DNA was digested with *XhoI* and *EcoRV*, molecular sizes are indicated on both sides of the gene. The PCR fragment of VdOCH1F/R was used as the probe.

Total RNA was extracted from lyophilized mycelia using TRIzol reagent (Invitrogen, USA). cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). *Escherichia coli* strain Trans-T1 (TransGen Biotech, China) was used for bacterial transformation and plasmid propagation. Plasmid DNA isolation, DNA digestion with restriction enzymes, ligation reaction, and *E. coli* transformation were performed following the manufacturer's instructions. PCR amplification of DNA or cDNA fragments used for cloning was performed with PrimeSTAR® HS DNA Polymerase (Takara, Japan), while in all other PCR analysis reactions, Taq DNA polymerase (TIANGEN, China) was used.

The T-DNA mutant (VDGN3-L2) was cultured on PDA contained 50 μg/mL hygromycin B (HYG B) at 25 °C for 7 d, then harvested for genomic DNA isolation. To identify the DNA sequences flanking the

T-DNA insertion site in the mutant, high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) was performed with degenerate primers, as previously described (Table 1) (Liu and Chen, 2007). All amplicons were cloned, sequenced, and then searched against NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the genes which the T-DNA inserted into the genome.

2.4. Phylogenetic and bioinformatics analyses

The homologous OCH1 protein sequences of 16 fungi were downloaded from the GenBank database. Alignment of the protein sequences was performed with ClustalX 1.8. Phylogenetic tree was constructed using the unweighted pair group method with arithmetic average (UPGMA) in MEGA 7. Bootstrap values were expressed as a percentage of 100 replicates. The SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) was used to identify a signal peptide of *VdOCH1* and the conserved domain was searched from Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.5. Generation of VdOCH1 deletion mutant and complemented strain

The deletion mutant was generated by homologous recombination. The whole ORF was replaced by a hygromycin resistance cassette introduced into VdGn3. To generate a *VdOCH1* deletion construct, flanking sequences of *VdOCH1* gene were amplified using the genomic DNA of *V. dahliae* strain VdGn3 as template and the primers OCH1Up-F/R, which contained the *KpnI* and *XbaI* cleavage sites (Table 1). To amplify the upstream sequence, an OCH1Dn-F/R containing the *XbaI* and *HindIII* cleavage sites was used to amplify the downstream sequence of *VdOCH1*(Table 1). The upstream PCR fragment was first ligated into the pUC19 vector, to generate the pUC-OCH1Up vector. The downstream products were then ligated into pUC-OCH1Up, to obtain the vector pUC-OCH1Up-OCH1Dn. The hygromycin resistance cassette was excised from vector pUCATPH via digestion with *XbaI* and cloned into the vector pUC-OCH1Up-OCH1Dn to generate vector pUC-OCH1Up-HPH-OCH1Dn. The vector was then digested with *KpnI* and *HindIII*, and ligated into the vector pCAMBIA1300-XR by T4 DNA Ligase was used to generate the knockout vector pKOOCH1. pKOOCH1 was introduced into LBA4404 and transform *V. dahliae* strain VdGN3 to generate the knock out strain Δ*VdOCH1*.

A *VdOCH1* full length gene was introduced into the deletion mutant to generate a *VdOCH1* complementation mutant. A 3375 bp *SmaI/BamHI* fragment containing the *VdOCH1* coding sequence together with a sequence 1500 bp upstream and 700 bp downstream was amplified using the primers COCH1-F/R (Table 1). The PCR product was cloned into binary vector pCAMBIA1300-XR. The geneticin resistance cassette was generated from vector pKN-cmpacC, kindly provided by Dr. Guoqing Li (Huazhong Agricultural University, China) using *XbaI*, and cloned into vector pCAMBIA1300-OCH1, to construct complementation vector pCOMOCH1. The vector was introduced into LBA4404 and transformed the *VdOCH1* deletion strain. After selection with geneticin, the resulted positive strain was named as CΔ*VdOCH1*.

Table 1
Primers information used in this study. V = A/G/C, H = A/C/T, D = A/G/T, B = G/C/T, N = A/G/C/T.

Primer pair	Sequence (5′ to 3′)
OCH1Up-F	ggggtaccGGTCGTCAATCTCGTCATT
OCH1Up-R	gctctagaAGACGAAGAGAACAAAGGC
OCH1Dn-F	gctctagaCATTGACTGGCGCAACTTTAC
OCH1Dn-R	cccaagcttAAGATGGAAGAAGCTGGGAC
COCH1-F	TCCCCCGGGGGACACCAGATTGAGACTTTGCATGCC
COCH1R	CGCGGATCCGCGATCGTTGTTTTCGCCAATGTGACG
VdOCH1-F	CGGTCACCACCAAAAAACAAGCACC
VdOCH1-R	GGCAGATGTCGTTGAATGGACGAGC
LAD1	ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA
LAD2	ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT
LAD3	ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA
LAD4	ACGATGGACTCCAGAGCGGCCGCBDNBNNNAGGT
LAD5	ACGATGGACTCCAGAGCGGCCGCBHNDNNNGACC
AD-C	ACGATGGACTCCAGAG
RB1	CTCTCGGCATGGACGAGCTGTACA
RB2	ACGATGGACTCCAGTCGTGCCAAAGGCAATACCCTGCT
RB3	GCGGAGGAGTTCTTCGTTGCGGGTT

"ggggtacc" was the restriction site of KpnI, "gctctaga" was the restriction site of XbaI, "cccaagctt" was the restriction site of HindIII.

2.6. Fungal morphology and conidiation analysis

The colony morphology and growth rate of knockout mutants were evaluated on PDA plates. A 5 mm mycelium plug from 7-d old cultured mutant strain was placed on the center of a plate to measure the colony diameter and observe the colony morphology at intervals of 5 d. Five plates were prepared for each mutant strain and the experiment was repeated 3 times.

For conidia germination tests, spores of VdGn3, VdGn3-L2, Δ*VdOCH1* and CΔ*VdOCH1* were adjusted to 1×10^6 spores/ml and placed on sterile glass slides (five slides for each isolate). To determine germination rate, 100 conidia were examined at 6, 12, 18 and 24 h post incubation at 25 °C under 100 % humidity. The experiment was performed 3 times.

To compare conidial production, conidia were washed from 10-d old culture plates with 5 ml distilled water. A 10 μl suspension of was placed on a hemocytometer and spores were counted using a microscope. The experiment was performed 3 times.

2.7. Detection of fungal cell wall integrity

To test the fungal cell wall integrity, the strains were grown either on CM medium, CM supplied with 0.01 % (w/v) Sodium Dodecyl Sulfate (SDS) or Congo red (CR, 40 μg/mL). After inoculation for 10 d, the colony growth was observed, and the relative growth inhibition rate was calculated with the following formula: (control colony diameter - stressed colony diameter)/control colony diameter × 100 %.

2.8. Penetration assays

For penetration assays, sterilized cellophane membrane was placed on CM medium. Spore suspensions (20 μl of 1×10^6 spores/ml) were incubated on the cellophane membrane for 7 d. After the membranes were removed, hyphal growth was observed on the underlying medium to determine if the mutant strains could penetrate cellophane membrane.

2.9. Pathogenicity assays

Sunflower hybrid LD5009 (Kaifurui Seeds Company, China) was used for pathogenicity assays. The seeds were planted in six pots (12 cm diameter × 12 cm height) with five plants in each pot. During the V4 stage, the seedlings were inoculated with conidial suspension (1×10^6 spores/ml) using the root wound inoculation method (Shi et al., 1993). To obtain conidia, the strains were cultured on wheat bran medium for 7 d, then washed with distilled water and filtered through three layers of sterile cheese cloth and adjusted a final concentration of 1×10^6 conidia/ml for

inoculation. The wound root inoculation was performed by thrusting a knife into the in potting mix 5 times to wound the fibrous roots of the sunflower seedlings. Then, 100 ml of prepared conidia suspension (1×10^6 spores/ml) was poured into three soil perforations that had been made by the knife. The roots of control plants were inoculated with sterilized distilled water (SDW). All pots were kept under a 16-h photoperiod with temperatures of 20–25 °C, and 40 % relative humidity. Disease severity was rated according to a 0 to 4 scale (Flood et al., 2010): 0 indicated no evidence of disease symptoms; or 1 less than 25 %; 2 25–50 %; 3 50–75 % and 4 more than 75 % of leaves showing wilt symptoms. Disease incidence (DI) was calculated using the following formula: DI = [Σ (each rating scale × the corresponding number of seedlings in the rating)/(the highest rating scale × the total numbers of seedlings)] × 100.

The significance test of data was analyzed using ANOVA p and the Duncan's Multiple Range Test ($P < 0.05$) using SAS software (version 9.0).

3. Results

3.1. Random mutagenesis of *V. dahliae* strain VdGn3 isolated from sunflower

About 800 T-DNA insertion mutants of VdGn3 were generated by ATMT. Forty-two positive mutants were selected and screened out based on PCR identification and GFP signal under fluorescence microscopy. The comparison on the colony morphology and microsclerotia formation between positive mutants and wild type were performed on PDA medium. Mutant VDGN3-L2 (Fig. 1) which formed no microsclerotia was chosen for further analysis.

3.2. Identification of the target gene disrupted by T-DNA insertion(s) in mutant VDGN3-L2

HiTAIL-PCR was performed to identify the disrupted gene in VDGN3-L2, and the resulting 577 bp fragment was amplified. The transformed e. coli DNA sequence analysis results indicated that the T-DNA was inserted at position 705 of the nucleotide coding sequence of VDAG_02820 (Fig. 2A). The disrupted gene, *VdOCH1*, from VDAG_02820 contains an open reading frame (ORF) of 1116 bp

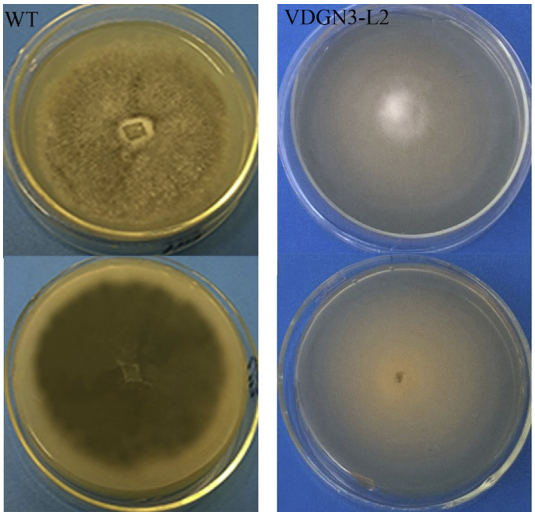

Fig. 1. Colony morphologies of *V. dahliae* wild type strain VdGn3 and T-DNA insertion mutants VDGN3-L2. The colony morphology of mutant VDGN3-L2 (Right) produces white aerial hyphae and no microsclerotia formed.

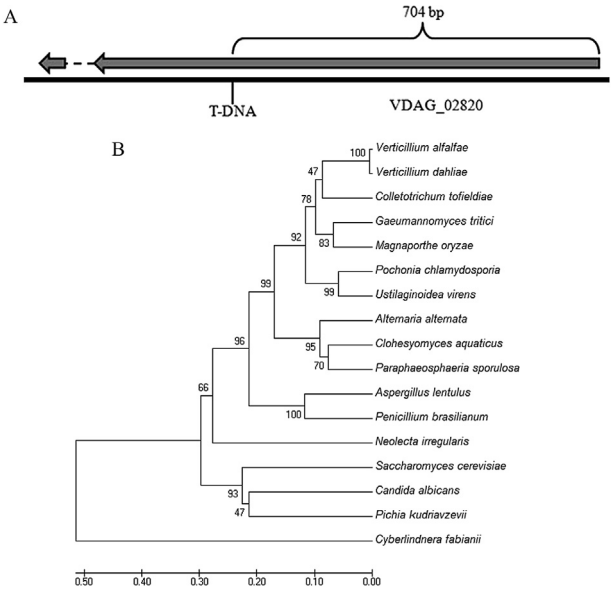

Fig. 2. Identification of the target gene disrupted by T-DNA insertion(s) in mutant VDGN3-L2. (A) A physical map of the *VdOCH1* locus and the T-DNA insertion site identified in the T-DNA insertion mutant VDGN3-L2. (B) Phylogenetic tree of VdOCH1 with homologs from other fungal species. Accession number of the homologs of VdOCH1 from other fungiis *Alternaria alternate* (OWY51525.1), *Aspergillus lentulus* (GAQ09363.1), *Candida albicans* (Q5A4E3.1), *Clohesyomyces aquaticus* (ORY17272.1), *Colletotrichum tofieldiae* (KZL78092.1), *Cyberlindnera fabianii* (ONH64818.1), *Gaeumannomyces tritici* (XP_009228105.1), *Magnaporthe oryzae* (XP_003720876.1), *Neolecta irregularis* (OLL24808.1), *Paraphaeosphaeria sporulosa* (OAG02379.1), *Penicillium brasilianum* (OOQ85703.1), *Pichia kudriavzevii* (XP_020546355.1), *Pochonia chlamydosporia* (OAQ60037.1), *Saccharomyces cerevisiae* (ONH77052.1), *Ustilaginoidea virens* (KDB14762.1), *Verticillium alfalfa* (XP_003008161.1) are included. Branch strength was tested by 100 bootstrap values.

(coding sequence of 1175 bp that is interrupted by an intron of 59 bp in length) and encodes initiation-specific alpha-1,6-mannosyltransferase with 371 amino acids. In the N-terminus of VdOCH1, a signal peptide was identified. A conserved domain of glycosyltransferase sugar-binding region containing DXD motif was also found in VdOCH1.

The blast result of VdOCH1 protein sequence showed that 75, 70 and 68 % matched the identity of the initiation-specific α-1,6-mannosyltransferase (OCH1) of *Colletotrichum tofieldiae*, *Magnaporthe oryzae* and *Ustilaginoidea virens*, respectively.

A phylogenetic tree was constructed for VdOCH1 and mapped with homologs from other fungal species; 16 fungi homologs were identified including *V. dahlia*. *V. dahlia* was classified into the same branch together with *Verticillium alfalfa*, *C. tofieldiae*, *Gaeumannomyces tritici*, *M. oryzae*, *Pochonia chlamydosporia* and *U. virens*, since their OCH1 homologs contain glycosyltransferase sugar-binding regions containing DXD motif (Fig. 2B).

3.3. Deletion and complementation of *VdOCH1*

To confirm that the phenotypic alterations displayed by the random mutant VDGN3-L2 were due to downregulation of VdOCH1 activity, targeted gene deletion mutants were generated, termed VdOCH1-1/2/3. Additionally, the WT gene was re-introduced in the mutants to generate complemented strains C*ΔVdOCH1*-1/2/3 (Fig. 3A). Additionally, these strains were fully confirmed by Southern blot analysis, where *VdOCH1* was lost in the deleted mutant and again rescued in the gene-complemented strain (Fig. 3B).

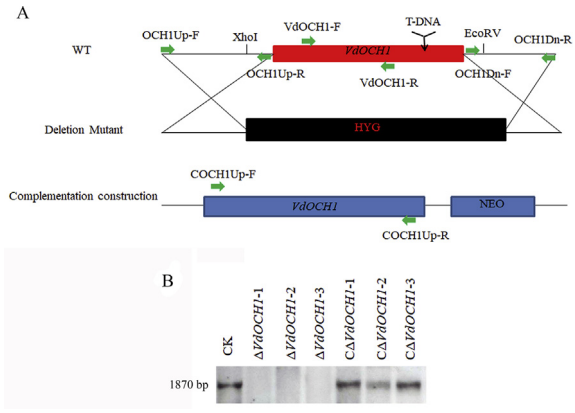

Fig. 3. Targeted gene replacement and complementation of the *VdOCH1* gene in *V. dahliae*. (A) The middle line shows the gene deletion mutant (ΔVdOCH1) with *VdOCH1* replaced by the hygromycin-resistance cassette (HygR) through homologous recombination; the bottom line indicated the map of complementation construct. The relative positions of the primers (short arrows) targeted for amplification of the linear DNA fragment employed for gene replacement and complementation. (B) Southern blot analysis of the wild-type VdGN3, deletion and complementation strains. Genomic DNA was digested with XhoI and EcoRV, molecular sizes are indicated on both sides of the gene.

3.4. Role of *VdOCH1* on the development of *V. dahlia*

To unravel the role of *VdOCH1* on the development of *V. dahlia*, growth rate and microsclerotia formation ability were tested on PDA. The deletion mutants and T-DNA insertion mutants showed a significantly reduced growth rate against the wild type, however, the mycelial growth rate was restored to the level close to wild type strain in the complemented strains (Fig. 4A). The deletion and T-DNA insertion mutants produced white aerial hyphae but no microsclerotia formation, however, the complemented strains showed a similar morphology pattern with the wild type (Fig. 4B).

Conidia production and germination rate were compared among the wild type strain and mutants. Conidia number was slightly reduced in the deletion and T-DNA insertion mutants ($3.77–4.36 \times 10^6$ spores/ml), whereas, it did not show significant differences among the complemented strains ($15.57–16.70 \times 10^6$ spores/ml) and the wild type (17.18×10^6 spores/ml) (Table 2). Almost 100 % of wild type conidia and complemented strains germinated after 24 h incubation, however, only 34 % conidia germinated in deletion mutants and 38 % in T-DNA insertion mutant within the same incubation period (Table 2). There was no obvious difference in the morphology of mycelium among all tested strains; except the conidia of the deletion and T-DNA insertion mutants were much smaller than that of the wild type and complemented strains (Table 2), suggesting that possible role of *VdOCH1* on the regulation of conidia development and germination.

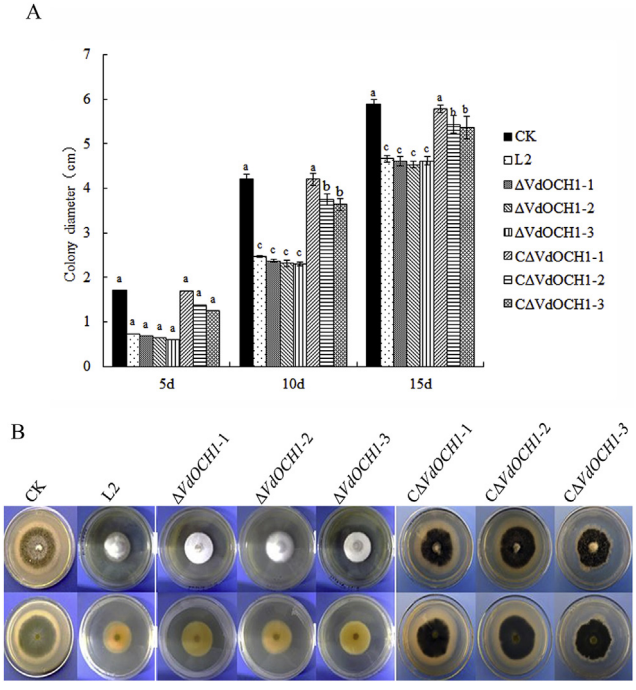

Fig. 4. The comparison of mycelia growth rate and microsclerotial development among mutants VDGN3-L2, ΔVdOCH1-1/2/3, wild type and complementation strains CΔVdOCH1-1/2/3. (A) Diagram presenting radial growth of *V. dahliae* and its mutants on minimal medium. Error bars indicate standard errors calculated for three replicates. (B) Colony morphology of *V. dahliae* wild type strain and its mutants on PDA medium (after 15 d culture).

3.5. Deletion or disruption of *VdOCH1* alters the cell wall integrity of *V. dahliae*

To analysis the possible role of *VdOCH1* on maintaining the cell wall integrity, strains were cultured on CM and CM supplied with 0.01 % (w/v) SDS or CR (40 μg/mL). Although the growth rate of all tested strains was suppressed to a certain level by SDS and CR, the deletion and T-DNA insertion mutants were much more sensitive compared with wild type and complementary strains (Fig. 5A). The relative growth inhibition rate of the mutant strains reached 81–86 % (supplied with SDS), and 76–81 % (supplied with CR) separately; whereas, for the wild type and complemented strains, it reached only 20–24 % on the medium supplied with SDS and 10–19 % with CR (Fig. 5B, C).

3.6. *VdOCH1 plays and role in penetration and colonization of the host plant*

The deletion and T-DNA insertion mutant could not grow on a CM plate, whereas, the colonies appeared on the CM plates inoculated with wild type and the complemented strains (Fig. 6). Chlorisis and etiolation were observed on the seedlings 20 d post inoculation with wild type and complemented strains. The average disease index was 43.6 and 42.3 separately; however, only a few leaves exhibited chlorisis after the sunflower seedlings were inoculated with the T-DNA insertion and deletion mutants; the average disease indices were 14.2 and 13.1 respectively (Fig. 7).

4. Discussion

ATMT was used to generate a T-DNA insertion mutagenesis library of the sunflower pathogen *V. dahliae*. Eight hundred positive mutants were obtained and 42 was checked based on their positive PCR reactions with hygromycin B primer pair and GFP signal under fluorescence microscopy. Colony morphology and microsclerotia formation of the positive mutants were variable, indicating that ATMT could be an efficient tool to create insertion mutants for genome-wide functional analysis.

Among the mutant strains, VDGN3-L2, which showed sparse white aerial hyphae and no microsclerotia formation was chosen for further analysis. The flanking sequence of T-DNA insertion site of VdGn3-L2 was identified via hiTAIL-PCR, and the interrupted gene was named as *VdOCH1*.

VdOCH1, encodes initiation-specific alpha-1,6-mannosyltransferase, contains a signal peptide and a conserved

Table 2
The comparison on conidial production, germination rate and conidial sizes among T-DNA insertion mutant VDGN3-L2, deletion mutants ΔVdOCH1-1/2/3, wild type and complementation strains CΔVdOCH1-1/2/3. Different letters in the same column indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test.

Strain	Conidial concentration (1×10^6spores/ml)	Spore germination rate (24 h) (%)	conidia size	
			Length (μm)	Width (μm)
VdGn3	17.18 ± 0.55 a	100 a	5.19 ± 0.88 a	1.88 ± 0.38 a
VdGn3-L2	4.08 ± 0.15 c	37.67 ± 2.52 b	2.16 ± 0.39 b	1.49 ± 0.17 c
ΔVdOCH1-1	3.77 ± 0.46 c	34.00 ± 2.00 bc	2.14 ± 0.34 b	1.47 ± 0.08 c
ΔVdOCH1-2	4.18 ± 0.20 c	35.67 ± 2.52 c	2.12 ± 0.40 b	1.17 ± 0.12 c
ΔVdOCH1-3	4.36 ± 0.14 c	33.00 ± 2.00 c	2.12 ± 0.43 b	1.51 ± 0.13 c
CΔVdOCH1-1	16.70 ± 0.52 a	99.67 ± 0.58 a	5.21 ± 0.77 a	1.65 ± 0.18 b
CΔVdOCH1-2	15.62 ± 0.39 b	99.00 ± 1.73 a	5.10 ± 0.77 a	1.65 ± 0.19 b
CΔVdOCH1-3	15.57 ± 0.29 b	99.33 ± 1.15 a	5.16 ± 0.73 a	1.81 ± 0.29 a

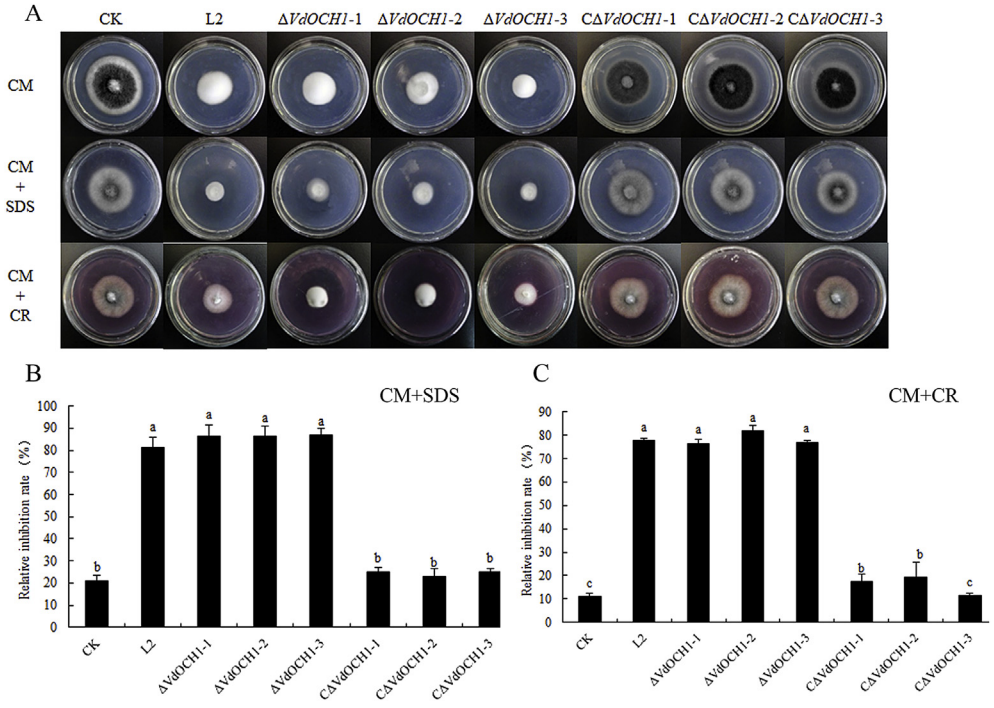

Fig. 5. Colony morphology of the indicated strain grown on complete medium (CM), CM supplemented with Sodium Dodecyl Sulfate (SDS, 0.01 % (w/v)) or Congo Red (CR, 40 lg/ml). (A) The colony morphology after incubated at 25 °C for 10 d. (B) and (C) indicated the relative inhibition rate of colonies culture on CM supplemented with SDS and CR. Error bars indicate standard errors calculated from three replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

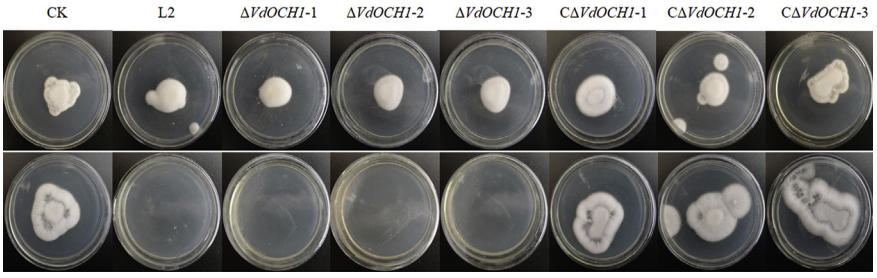

Fig. 6. Test on the penetration ability of wild type and *VdOCH1* mutants on CM medium. Sterilized cellophane sheets were placed on CM plates followed by inoculation with 20 μl spore suspension (1×10^6 spores/ml). After 7 d post incubation at 25 °C, the cellophane sheets with the fungal colony were removed carefully from plate. The presence or absence of fungal mycelia on the underlying medium was recorded after incubation for an additional 7 d at 25 °C. The colonies were observed in plate which cultured WT and complemented strain, however, this is not the case for CΔVdOCH1-1/2/3T-DNA insertion mutant VDGN3-L2 and ΔVdOCH1-1/2/3 deletion mutant.

domain (Glycosyltransferase sugar-binding region containing DXD motif). This protein showed 75, 70 and 68 % identity with the initiation-specific α-1,6-mannosyltransferase (OCH1) of *C. tofieldiae*, *M. oryzae* and *U. virens*, respectively, indicating this gene is widely distributed and may have the similar function with its homologs in different filamentous fungi. The radial growth of the deletion and T-DNA insertion mutant was reduced compared to the wild type (Fig. 4), as well as the conidia production and

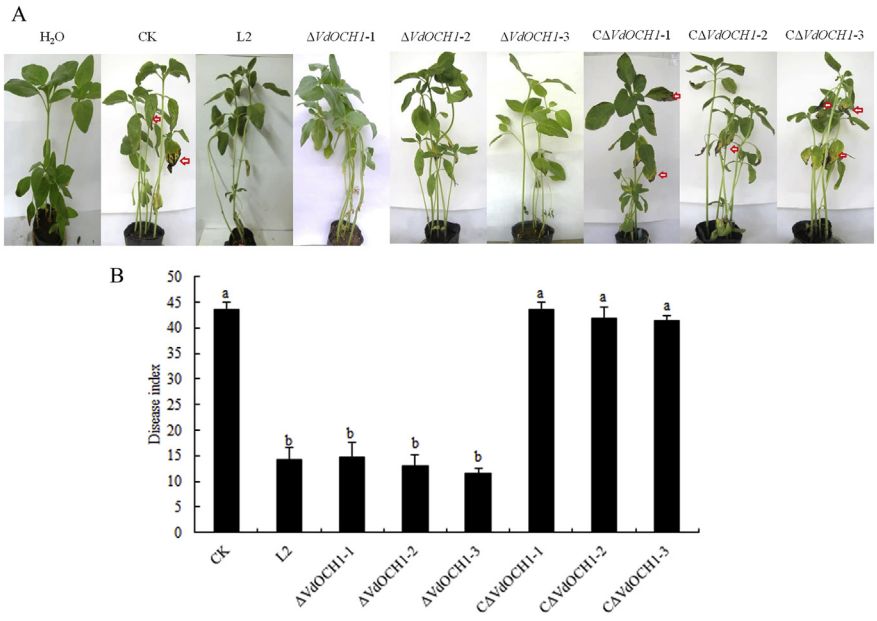

Fig. 7. Pathogenicity test of wild type strain, mutant VDGN3-L2, ΔVdOCH1-1/2/3 mutants and complemented strains CΔVdOCH1-1/2/3. (A) The symptom of sunflower seedlings after inoculation with wild type and mutants. (B) Disease index after inoculated with wild type and different kinds of mutants.

germination ability (Table 2). This is in line with the results obtained from a study on *VdMsn2* and *VdPKS1* of *V. dahliae* from smoke tree and cotton (Tian et al., 2017; Zhang et al., 2017b). The deletion mutants of both genes of *V. dahlia* exhibited reduction on the colony diameter, conidia production and germination ability (Tian et al., 2017; Zhang et al., 2017b). Conidia are an essential reinfection resource for colonization of *V. dahliae* in vascular tissues of different hosts (Emilief and Bartphj, 2006), thus, the reduction of conidia number may decrease disease severity. For instance, the deletion mutant *ΔVdsho1* not only displayed delayed spore germination ability and reduced spore production compared with the wild type and the complemented strains, but also exhibited impaired ability of attachment and invasive growth on cotton roots (Qi et al., 2016).

Microsclerotia formation defects in the deletion or insertion mutants reflect the possible regulation function of *VdOCH1* on microsclerotia development (Fig. 4). Although the microsclerotia formation is not absolutely related to the pathogenicity of fungi (Klimes and Dobinson, 2006; Tzima et al., 2010), it is still rather important for the life cycle of *V. dahliae* and disease cycle of sunflower verticillium wilt (Klosterman et al., 2009). An abundance of genes regulates the formation of microsclerotia have been cloned. These genes roughly can be classified into different groups, such as protein kinases, transcription factors and metabolism related genes (Maruthachalam et al., 2011; Rauyaree et al., 2005; Santhanam and Thomma, 2013; Tian et al., 2016; Tran et al., 2014; Tzima et al., 2011; Xiong et al. 2015, 2016; Sarmiento-Villamil et al., 2018). We determined that *VdOCH1*, a gene involved in protein glycosylation, could regulate the development of microsclerotia. Although our findings are relevant to microsclerotia development in *V. dahliae*, the precise role of *VdOCH1* in this process still needs to be determined.

Our results suggest that *ΔVdOCH1* mutants are more sensitive to the cell wall perturbing reagents, such as SDS and CR (Fig. 5), indicating that *VdOCH1* is vital for keeping the cell wall integrity similar to other fungi. In filamentous fungi, *N. crassa*, OCH1 adds an α-1,6-linked mannose residue to the N-linked oligosaccharide to provide the elements for the cross-linking of protein into the cell wall. In knockout mutants of *OCH1*, the proteins cannot be efficiently cross-linked into the cell wall of *N. crassa* (Maddi and Free, 2010), indicating the possible role of OCH1 in keeping the integrity of the cell wall. This process was also reported in *F. oxysporum f. sp. Cubense* (Li et al., 2014). Due to the lack of a-1,6-mannosyltransferase in the *FoOCH1* mutants, some cell wall proteins cannot efficiently cross-link into the glucan-chitin matrix, thus causing the low percentage of the cell wall protein in the mutant strain (Li et al., 2014). Additionally, alpha-1,6-mannosyltransferase plays a pivotal role in keeping cell wall integrity in many other fungi (Barnayverdier et al., 2004; Choi et al., 2003; Kim et al., 2006; Yokoo et al., 2001). Whether this regulation model also exists in sunflower *V. dahliae* still needs further verification.

During infection of *V. dahliae* in roots of different hosts, either appressoria or a simple penetration structure known as hyphopodia is required for the infection of *V. dahliae* (Zhao et al., 2016), suggesting penetration ability is rather important for the pathogenicity of *V. dahliae*. In our studies, *ΔVdOCH1* mutants impaired hyphal penetration ability, suggesting possible effects of *VdOCH1* on the pathogenicity. This was also verified by the pathogenicity results. The pathogenicity on sunflower of both *ΔVdOCH1* deletion and insertion mutants decreased dramatically, whereas, both the control and complementary strains showed high disease index and also clear symptoms on sunflower seedlings (Fig. 7). This indicates that knock out *VdOCH1* in *V. dahliae* could disrupt cell wall integrity, thus impeding the penetration ability of mycelium, and eventually leading to the loss of virulence of *V. dahliae* on sunflower. But how the *VdOCH1* gene manipulates such pathogenic related physiological processes, and which kind of downstream targets are involved are still to be determined. More effort is needed to unravel the regulation mechanism of *VdOCH1* in *V dahlia.*

Acknowledgements

We thank Dr. Baolong Zhang (Jiangsu Academy of Agricultural Sciences, Nanjing, China) for kindly providing the binary vector pGH-sGFP, Dr. Peibao Zhao (Liaocheng University, China) for

providing vector pUCATPH and Dr. Guoqing Li (Huazhong Agricultural University, China) for providing vector pKN-cmpacC, Prof. Mark S. Goettel (Editor of Biocontrol Science and Technology), Prof Weidong Chen (Washington State University) for kindly discussed and revising the paper. This work was funded by the China Agricultural Research System (CARS-14), Special Fund for Agro-scientific Research in the Public Interest No. 201503109, the Science and Technology Application Projects (201803048) and Inner Mongolia Science and Technology Project "Innovation of Resistance to Germplasm Resources of Sunflower Verticillium Wilt and Breeding of New Varieties".

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