

Article

# *VdPLP*, A Patatin-Like Phospholipase in *Verticillium dahliae*, Is Involved in Cell Wall Integrity and Required for Pathogenicity

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**Abstract:** The soil-borne ascomycete fungus *Verticillium dahliae* causes vascular wilt disease and can seriously diminish the yield and quality of important crops. Functional analysis of growth- and pathogenicity-related genes is essential for revealing the pathogenic molecular mechanism of *V. dahliae*. Phospholipase is an important virulence factor in fungi that hydrolyzes phospholipids into fatty acid and other lipophilic substances and is involved in hyphal development. Thus far, only a few *V. dahliae* phospholipases have been identified, and their involvement in *V. dahliae* development and pathogenicity remains unknown. In this study, the function of the patatin-like phospholipase gene in *V. dahliae* (*VdPLP*, VDAG\_00942) is characterized by generating gene knockout and complementary mutants. Vegetative growth and conidiation of *VdPLP* deletion mutants ( $\Delta VdPLP$ ) were significantly reduced compared with wild type and complementary strains, but more microsclerotia formed. The  $\Delta VdPLP$  mutants were very sensitive to the cell-wall-perturbing agents: calcofluor white (CFW) and Congo red (CR). The transcriptional level of genes related to the cell wall integrity (CWI) pathway and chitin synthesis were downregulated, suggesting that *VdPLP* has a pivotal role in the CWI pathway and chitin synthesis in *V. dahliae*.  $\Delta VdPLP$  strains were distinctly impaired in their virulence and ability to colonize *Nicotiana benthamiana* roots. Our results demonstrate that *VdPLP* regulates hyphal growth and conidial production and is involved in stabilizing the cell wall, thus mediating the pathogenicity of *V. dahliae*.

**Keywords:** *Verticillium dahliae*; patatin-like phospholipases; cell wall integrity; pathogenicity

## 1. Introduction

*Verticillium dahliae*, a soil-borne, filamentous fungal plant pathogen with wide distribution around the world, can attack many important crops such as cotton, soybean, potato, sugar beet and sunflower, causing significant crop losses annually [1,2]. It is a significant threat to cash-crop production. It also produces microsclerotia, dormant structures that can survive for many years in the soil and be induced to germinate by plant root exudates [3,4]. Hyphae colonize the plant root surface, and then form a narrow penetration peg that can penetrate the epidermal cells [5]. Hyphae grow intracellularly and intercellularly through the root cortex, enter the xylem vessels and colonize the xylem of the hypocotyl and leaves. Ultimately, water flow in the plant tissue is disrupted, causing the wilt symptoms, stunting, chlorosis and necrosis [4,6]. The molecular mechanisms underlying *V. dahliae* pathogenesis are complex and still not clear [4–8], so functional analyses of growth- and pathogenicity-related genes are crucial in developing new strategies to control *V. dahliae*.

Here, we focused on fungal phospholipases, which are involved in hyphal development, virulence and signaling. Phospholipases, key metabolic enzymes needed by all living organisms, hydrolyze phospholipids into fatty acids and lipophilic substances and are classified by the specific site of bond cleavage in the phospholipid substrates [9]. Phospholipase A enzymes hydrolyze the 1-acyl ester (PLA<sub>1</sub>) or the 2-acyl ester (PLA<sub>2</sub>) of phospholipids, and PLA enzyme actions produce free fatty acids and 2-acyl lysophospholipid (PLA<sub>1</sub>) or 1-acyl lysophospholipid (PLA<sub>2</sub>) [10,11]. Phospholipase B (PLB) enzymes possess hydrolytic activities that release sn-1 and sn-2 fatty acids from a phospholipid and PLBs catalyze the release of the remaining fatty acid linked to the lysophospholipid by lysophospholipase activity. Additionally, some PLBs can transfer a free fatty acid to a lysophospholipid and produce a phospholipid by transacylase activity [10,12,13]. Phospholipase C (PLC) catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to two intracellular messengers, inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) [10,14,15]. Phospholipase D (PLD) hydrolyzes phosphatidylcholine (PC) at second phosphodiester bond to yield phosphatidic acid (PA) and choline or ethanolamine. PLDs can also transfer the phosphatidyl moiety of the substrate of certain nucleophiles such as ethanol, which produce phosphatidylethanol (PEt) by the unique transphosphatidylation activity [9,10,16]. Lower phospholipase activities of pathogenic yeast fungi (*Candida albicans*, *Candida parapsilosis*, and *Saccharomyces cerevisiae*) reduce adherence to epithelial cells and are less lethal to mice [9,17]. During the development of *C. albicans* blastospores, phospholipase activities are concentrated at the growing tips [18]. PLC stimulates calcium release from intracellular stores and participates in hyphal extension at the growing tips in filamentous fungus *Neurospora crassa* [19]. In addition, phosphoinositide-specific PLC plays an important role in vegetative growth and cell wall regeneration in *Coprinopsis cinerea* [20].

Phospholipases are also related to signaling messengers to elicit stress tolerance and hosts immune responses in fungi [9,13,21]. The expression of phospholipases is regulated by persistent starvation [21,22]. PLA<sub>2</sub> takes part in various physiological processes, such as phospholipids hydrolysis, signal transduction, and remodeling of cell membrane. Some secreted and membrane-bound PLA<sub>2</sub> require Ca<sup>2+</sup> to perform biological activities [10]. PLC catalyzes PIP<sub>2</sub> to DAG and IP<sub>3</sub>. As second messengers, DAG and IP<sub>3</sub> play an important role in the signal transduction cascade, DAG can activate protein kinase C (PKC) and IP<sub>3</sub> can activate calcium channels [14,19]. In the symbiotic fungus *Tuber borchii*, TbSP1 is a novel Ca<sup>2+</sup>-activated phospholipase A<sub>2</sub>, which may take part in membrane remodeling and signal transduction during early stages of plant invasion [21]. PLCs cleave the glycerophosphate bond, thus are defined as phosphodiesterases [10]. PLDs hydrolyze PC to yield PA, which regulates the activation of the cAMP-specific phosphodiesterases (PDEs). PDEs and adenylyl cyclases (ACs) regulate the intracellular cAMP levels. cAMP signaling plays critical role in regulating multiple cellular responses [23,24]. In addition, high-affinity phosphodiesterase PdeH modulates intracellular cAMP levels, thereby regulates cAMP signaling, cellular development and pathogenicity of plant pathogenic fungus *Magnaporthe oryzae* [25–27]. MoMck1, as one of the components of the mitogen-activated protein kinase (MAPK), interacts with PdeH that regulates MAPK signaling pathway to regulate cell wall integrity [25,28].

The cell wall integrity (CWI) pathway can activate a compensatory salvage mechanism when the cell wall is under stress conditions [27,29]. MAPK pathways respond properly to extracellular stimuli or environmental conditions, and then regulate appropriate cellular responses [29–31]. In model yeast *S. cerevisiae*, cell wall stress is one of MAPK cascades that mediate the CWI pathway [30,31]. The MAP kinase I (Slt2/Mpk1) becomes activated under different cell wall stress conditions, such as hypoosmolality, chitin-binding agents (Calcofluor White (CFW) and Congo Red (CR)) and oxidative stress. The major components of yeast cell wall are synthesized and modified under the stress [32–34]. The response mediated by Slt2/Mpk1 is defined as CWI pathway [31,33].

The patatin-like phospholipases (PLPs) was described as lipid acyl hydrolases. PLPs catalyze the cleavage of fatty acids from membrane lipids, thereby influence membranes remodeling [35,36]. PLPs have serine-aspartate dyads and  $\alpha/\beta$  hydrolase fold, which is structurally similar to PLA<sub>2</sub>.

Meanwhile, PLPs have been shown to exhibit a PLA<sub>2</sub>-like activity [36,37]. PLPs show difference in substrate specificity. PLPs have broader substrate preference, while cytosolic PLA<sub>2</sub>s show increased substrate specificity for arachidonic acid-containing lipids [36–38]. PLPs also do not contain a lid-like structure and are limited to interfacial activation [38]. Phospholipases have been studied in some plant pathogenic fungi, such as *Fusarium graminearum* [14] and *M. oryzae* [25,26,28]. The phospholipase C (FgPLC1) is considered to be closely related to regulation of development, stress responses and pathogenicity of *F. graminearum* [14]. cAMP phosphodiesterase PdeH regulates the intracellular cAMP levels, CWI and pathogenicity of *M. oryzae* [25,26,28], while the function of the patatin-like phospholipase (encoded by *VdPLP*, VDAG\_00942) remains unclear in *V. dahliae*. The relations among *VdPLP*, cell wall integrity and fungal virulence are unknown. In this study, to investigate the function of *VdPLP*, we generated a deletion mutant ( $\Delta VdPLP$ ) of *V. dahliae* and characterized its growth and development, the stress tolerance and integrity of the cell wall, root penetration ability, and virulence in *Nicotiana benthamiana*.

## 2. Materials and Methods

### 2.1. Fungal Strains, Plant Material and Culture Conditions

*V. dahliae* strain 991 (V991, wild type (WT)) was kindly provided by Prof. Guiliang Jian from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences (IPP, CAAS). *N. benthamiana* seedlings were planted in a growth chamber with 16 h of light/8 h of dark and 60–70% relative humidity at 23–25 °C.

### 2.2. Plasmid Construction and Fungal Transformation

$\Delta VdPLP$  mutants were obtained by a homologous recombination method. The *VdPLP* knockout plasmid pGKO-*VdPLP* (pGKO2 [*EcoRI*]::*VdPLP*-5'::*neo*::*VdPLP*-3'::*pGKO2* [*HindIII*]) was constructed as in previous research [8,39]. The 2600 bp geneticin resistant cassette (*neo*) from pCAM-*neo* plasmid was amplified using primer pair neo-F/neo-R (Table 1). The 1074 bp upstream sequence of *VdPLP* were amplified from the *V. dahliae* genome with primer pairs *VdPLP*-5F/*VdPLP*-5R, and a 1092 bp downstream sequence with primer pairs *VdPLP*-3F/*VdPLP*-3R (Table 1). Vector pGKO2 [40] was linearized by double digestion with enzymes *EcoRI* and *HindIII*. Three PCR fragments were inserted into the corresponding position of linearized pGKO2 (Figure 1A) using In-Fusion enzyme (Clontech, Mountain View, CA, USA).

For generating *VdPLP* complementary plasmid pCM-*Hyg-VdPLP*, the full-length complementary DNA (cDNA) fragment of *VdPLP*, the *TrpC* promoter and *Nos* terminator were amplified from plasmid pCH-GFP and primers C-*VdPLP*-F/C-*VdPLP*-R, C-*TrpC*-F/C-*TrpC*-R and C-*Nos*-F/C-*Nos*-R (Table 1). Plasmid pCM-*Hyg* carrying the *hygromycin B* resistance gene (*hph*) was linearized with restriction enzymes *KpnI* and *XbaI*, and the fragments were inserted into plasmid pCM-*Hyg* digested with In Fusion enzyme.

Mutant strains were obtained through protoplast transformation [41]. Plasmids pGKO-*VdPLP*, pCM-*Hyg-VdPLP* and pCAM-*GFP* were used to transform protoplasts of the obtained mutants.  $\Delta VdPLP$  mutant strains were generated by inserting plasmid pGKO-*VdPLP* into the wild type strain of *V. dahliae* (Vd-wt). Plasmid pCM-*Hyg-VdPLP* was introduced into  $\Delta VdPLP$  strains to generate *VdPLP* complementary mutants ( $\Delta VdPLP$ -C). GFP-tagged strains were generated by inserting plasmid pCAM-*GFP* carrying the *eGFP* gene and *hph* gene into Vd-wt and the  $\Delta VdPLP$  strains.

### 2.3. Confirmation of *VdPLP* Disruption, Complementation of $\Delta VdPLP$ Strains and Screening for GFP-Tagged Strains

*VdPLP* knockout mutants were cultivated and selected on potato dextrose agar (PDA) plates supplied with geneticin (50 µg/mL).  $\Delta VdPLP$ -C and GFP-tagged strains were selected in the presence of hygromycin B. After single-spore isolation, all isolates were grown in complete medium (CM, 6 g/L

yeast extract, 6 g/L casein acid hydrolysate, 10 g/L sucrose) for DNA extraction. All isolates were further confirmed by genomic PCR with the specific primers. *VdPLP* was analyzed by genomic PCR with primer pair VdPLP-J-F/VdPLP-J-R (Table 1) for successful homologous recombination and with neo-J-F/neo-J-R for detecting the *neo* gene.  $\Delta VdPLP-C$  mutants were selected by genomic PCR with primer pairs VdPLP-J-F/VdPLP-J-R and hyg-F/hyg-R (Table 1) for the *hph* gene. GFP-labeled strains were examined for the *hph* gene with primer pair hyg-F/hyg-R and for fluorescence with a confocal laser scanning microscope (LSCM; LSM 700, Zeiss, Jena, Germany) using 488 nm excitation wavelength and band-pass 500 to 550 nm emission filters.

**Table 1.** List of primers used in this study.

Primer Name	Primer Sequence
neo-F	GTTTGC GGGCTGTCTTGACG
neo-R	TACCTGTGCATTCTGGGTAA
VdPLP-5F	GTACCCAATTCCGAATTCAGCGGTTCCGGGTAGTAGTAGA
VdPLP-5R	CAAGACAGCCCGCAAACGTATAACCCCGCGGACAGTAA
VdPLP-3F	CCCAGAATGCACAGGTAGACGCGCCACGACCTCAA
VdPLP-3R	ACGGTATCGATAAGCTTTGCGTGCGAACATACTCCTCAT
C-TrpC-F	TTGAAGGAGCATT TTTGGGC
C-TrpC-R	ATCGATGCTTGGGTAGAATAGGT
C-VdPLP-F	ATGCCTGTCAACGATATCCGTCT
C-VdPLP-R	CTATTCCTCGATCAGAGAGTAG
C-Nos-F	AGATGCCGACCGGGATCCACTT
C-Nos-R	TTATCTTTGCGAACCCAGGG
VdPLP-J-F	CTCGAGCGGGCCATCAAACA
VdPLP-J-R	GAGTAAGCCACCCATCTGTCCGTT
neo-J-F	GCGGTT CAGAAGCACCTCGA
neo-J-R	TATCTTTGCGAACCCAGG
VdMK1-F	CGCGCCCGAGATTATGCTGAG
VdMK1-R	CGTTGGGAGTACCGAGGATGTGAA
VdChi1-F	GCCGCGCCTGGTCATC
VdChi1-R	CGGGGTAGAGGTCGGCATCA
VdChi3-F	GGTCGGCCCTTGGAGCAGTA
VdChi3-R	CCCTTGGCAGCCTTGATGTAGC
VdChi4-F	TACGGCAAGGTTTACTCGGGTCTC
VdChi4-R	CGGTTGCCAGGCTTCGTCTTAC
VdChi7-F	CATCCTCGGCGTCACAAAGTTCTA
VdChi7-R	GCTGCCGCTGCTGGAGGTA
VdBt-F	TTCCCCGTCTCCACTTCTTCATG
VdBt-R	GACGAGATCGTTCATGTTGAACTC
Nb-actin-F	GGACCTTTATGGAAACATTGTGCTCAGT
Nb-actin-R	CCAAGATAGAACCTCCAATCCAGACAC
Vd-F	CCGCCGGTCCATCAGTCTCTCTGTTTATAC
Vd-R	CGCCTGCGGGACTCCGATGCGAGCTGTAAC

#### 2.4. Growth, Conidia Production and Microsclerotia Formation Assays

Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains were cultured in CM, conidia of each strain were harvested and filtered through a sterile 40  $\mu$ m cell strainer (Falcon, New York, NY, USA). Two microliters of  $5 \times 10^6$ /mL conidial suspension of all strains were added to the center of plates of minimal medium (MM, 1.45 g/L  $\text{KH}_2\text{PO}_4$ ; 2.05 g/L  $\text{K}_2\text{HPO}_4$ ; 0.5 g/L  $\text{NH}_4\text{NO}_3$ ; 0.01 g/L  $\text{CaCl}_2$ ; 0.6 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.3 g/L  $\text{NaCl}$ ; 0.5 mg/L  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.5 mg/L  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 0.5 mg/L  $\text{H}_3\text{BO}_3$ ; 0.25 g/L  $(\text{NH}_4)_2\text{SO}_4$ ; 0.5 mg/L  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ; 0.5 mg/L  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ; 20 g/L agar) [6] amended with different carbon sources (10 g/L xylose; galactose; pectin; or starch; or 30 g/L sucrose). Colony diameter and morphology were recorded as described previously [9]. Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains were cultured on Czapek-Dox agar (3.0 g/L  $\text{NaNO}_3$ ; 0.5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.5 g/L  $\text{KCl}$ ; 0.01 g/L  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.0 g/L  $\text{K}_2\text{HPO}_4$ ; 15 g/L agar) plates to assay conidia production as described previously [39]. For microsclerotia formation tests,  $10^6$ /mL

spores of Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains were evenly spread on basal agar modified medium (BMM, 0.2 g/L NaNO<sub>3</sub>; 0.52 g/L KCl; 0.52 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O; 1.52 g/L KH<sub>2</sub>PO<sub>4</sub>; 3 μmol/L thiamine; 0.1 μmol/L biotin; 5 g/L glucose; 15 g/L agar) plates, then incubated at 25 °C for 21 days. At seven-day intervals, microsclerotia on plates were counted [42]. Each strain was tested on 10 plates, and the assay was done three times.

### 2.5. Phenotype Assays Using Cell Wall-Disrupting Agents Calcoflour White and Congo Red

Susceptibility to cell wall-perturbing agents CFW and CR is generally tested to detect cell wall mutants of mycelial fungi [28,43]. A conidial suspension of each strain (10 μL of 5 × 10<sup>6</sup>/mL) was placed on the center of separate PDA agar plates containing 50 mg/mL CFW or 100 mg/mL CR, or without CR or CFW. Colony phenotypes were observed seven days after inoculation. The experiment was performed three times independently, using 10 plates per strain per treatment.

### 2.6. Quantitative Real-Time PCR Reaction

Cell wall reinforcement and repair in response to cell wall stress is activated by the CWI pathway, a basal cascade pathway triggered by transmembrane sensors in response to cell wall stress [44–46]. To examine the potential role of VdPLP in the CWI pathway and chitin synthesis, we analyzed the transcription levels of genes encoding mitogen-activated protein kinase kinase (*VdMKK1*, VDAG\_09823) and mitogen-activated protein kinase (*VdMK1*, VDAG\_09461) for the CWI pathway and chitin synthesis genes *VdChi1* (VDAG\_10179), *VdChi3* (VDAG\_08591), *VdChi4* (VDAG\_00419), *VdChi7* (VDAG\_01790) in the Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains, which were grown in liquid CM for 5 days. Total RNA was extracted from each strain using the RNA miniprep kit (Axygen, Union City, CA, USA) according to the user's guide. cDNAs were synthesized with the TOYOBO RT Kit (TOYOBO, Osaka, Japan). The quantitative real-time PCR (qRT-PCR) reactions were performed with the SYBR Fast qPCR kit (KAPA Biosystems, Boston, MA, USA). Specific primers for different genes were designed (Table 1). The constitutively expressed β-tubulin gene (DQ266153) was used for normalization and amplified with primers VdBt-F/VdBt-R (Table 1). The qRT-PCR reactions were performed in an ABI7500 Fast PCR thermocycler (Applied Biosystems, Foster City, CA, USA). The experiment was repeated two times.

### 2.7. Penetration Ability Assay

Penetration was assayed as described previously [47,48]. About 20 μL of 2 × 10<sup>6</sup>/mL conidial suspension of each strain were placed on cellophane laid on PDA plates, then incubated at 25 °C. After 3 days, the cellophane was removed from the plates, which were then incubated for 7 more days at 25 °C, and hyphae were observed. This assay was done three times, with five plates per strain each time.

For microscopically observing initial infection, roots of *N. benthamiana* seedlings with 6–7 true leaves were inoculated with GFP-labeled *Vd-GFP* or  $\Delta VdPLP-GFP$  strains [8]. After 3 days, the roots were vertically cut and observed on temporary slides with LSCM.

### 2.8. Pathogenicity Assays, Microscopic Observation of Initial Infection and Fungal Biomass Quantification

For pathogenicity assay experiments, roots of 10 *N. benthamiana* plants were inoculated with conidia of strain Vd-wt,  $\Delta VdPLP$  or  $\Delta VdPLP-C$  as previously described [39]. The disease symptoms were recorded at 8, 10, and 12 days post inoculation (dpi), and the mean of the disease grade was calculated as an indicator of the severity of the disease. Wilt symptoms were classified into five grades: 0, no disease symptoms; 1, wilting of fewer than two leaves; 2, wilting of three to five leaves; 3, wilting of more than five leaves; and 4, death or near death of plants [39,49].

Colonization ability of strains was assessed by quantifying the fungal biomass in *N. benthamiana* using qRT-PCR. DNA was extracted from infected plant stems using the DNAsure Plant Kit (TIANGEN, Beijing, China). The *actin* gene of *N. benthamiana* was selected as an internal standard [8]

and amplified with primer pair Nb-actin-F/Nb-actin-R (Table 1). ITS1 and ITS2 regions of the ribosomal RNA genes (Z29511) of *V. dahliae* were amplified with primer pair Vd-F/Vd-R (Table 1), which was used to quantify the fungal DNA in the mixed DNA samples. All qRT-PCR reactions were done as previously reported [39].

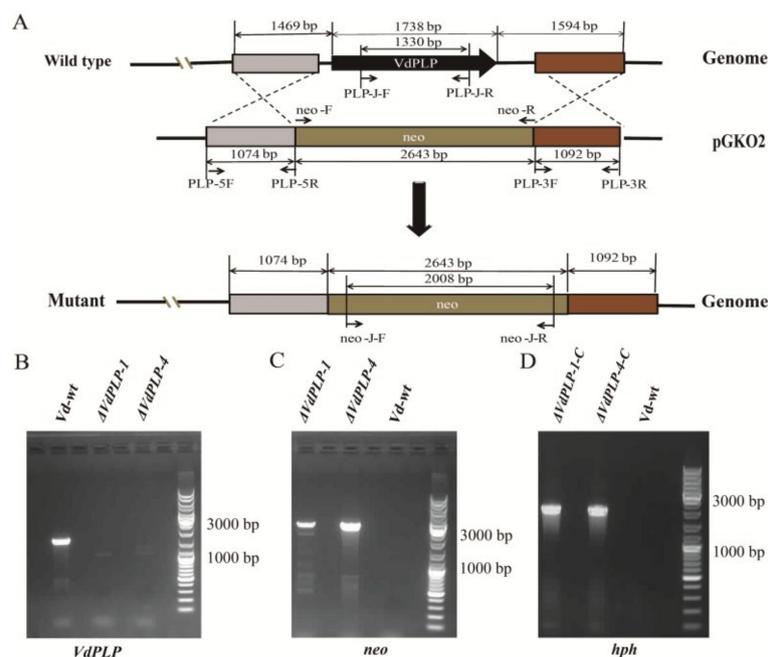
### 2.9. Statistical Analysis

Significant difference of data among the groups was analyzed using Duncan's multiple range test ( $p$ -value < 0.05) and SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA).

## 3. Results

### 3.1. Deletion and Complementation of *VdPLP* in *V. dahliae*

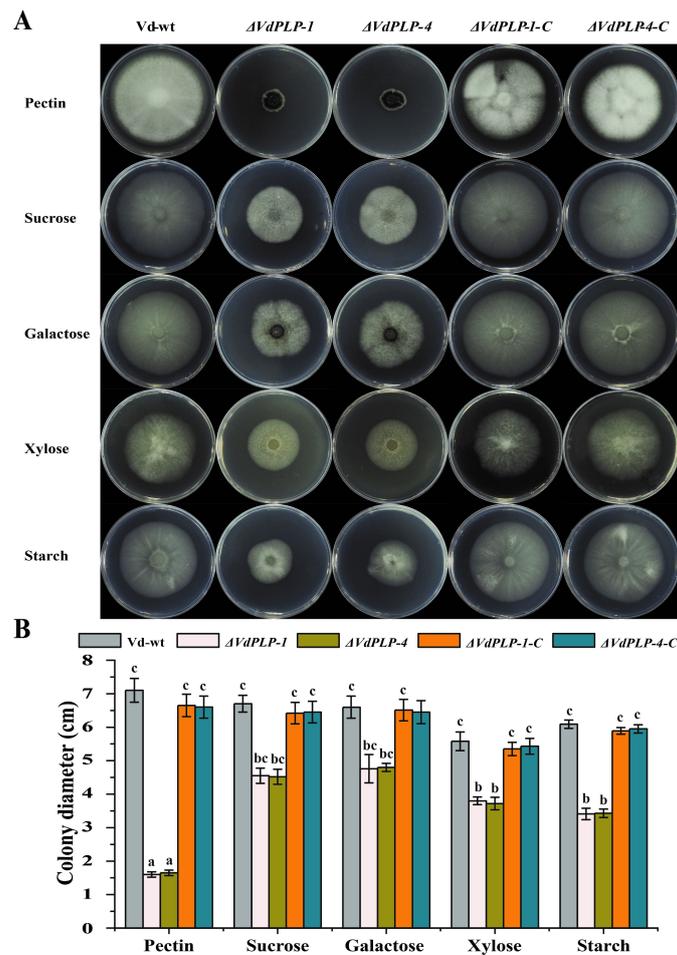
The *VdPLP* gene was replaced by the *neo* cassette in the *Vd*-wt strain. With the targeted gene replacement, geneticin resistance was generated. Transformations were confirmed by genomic PCR; homologous transformation events had occurred in 3 of 30 independent transformants of the *Vd*-wt strain. *VdPLP* deletion mutants ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ) were randomly selected for further functional analysis (Figure 1B,C). For complementation, the In-fusion-resulting plasmid was introduced into  $\Delta VdPLP-1$  and  $\Delta VdPLP-4$  strains by protoplast transformation (Figure 1D), resulting in complementary strains  $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ .



**Figure 1.** *VdPLP* gene disruption in *V. dahliae* and complementation of the  $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ . (A) Disruption scheme used for *VdPLP* in the wild type (wt) strain (*Vd*-wt); gene disruption was confirmed by PCR. Genomic DNA (gDNA) from the *Vd*-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains were used as templates for amplification with primer pairs *VdPLP*-J-F/*VdPLP*-J-R, *neo*-J-F/*neo*-J-R and *hyg*-F/*hyg*-R; (B) A 1.3-kb fragment of *VdPLP* was amplified from *Vd*-wt with primer pair *VdPLP*-J-F/*VdPLP*-J-R; (C) A 2.6 kb fragment of *neo* was amplified with primer pair *neo*-J-F/*neo*-J-R from the  $\Delta VdPLP$  mutant strains; (D) Gene complementation of the  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) was confirmed by amplification of a 1.6 kb fragment of hygromycin B resistance gene (*hph*) in PCR with primer pair *hyg*-F/*hyg*-R.

### 3.2. Radial Growth of Mycelia was Significantly Reduced in $\Delta VdPLP$ Mutants

The effects of *VdPLP* disruption on hyphal growth were defined by growing *Vd*-wt and the mutants on MM agar with different carbon source (sucrose, pectin, starch, galactose or xylose) and comparing the phenotypes of each strain. Vegetative growth of  $\Delta VdPLP$  mutants was severely retarded compared with that of *Vd*-wt,  $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$  strains, and colony diameter of the  $\Delta VdPLP$  mutants was significantly smaller than that of *Vd*-wt,  $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$  strains (Figure 2B). These results suggested that the *VdPLP* contributes to the hyphal growth of *V. dahliae* on different carbon sources.

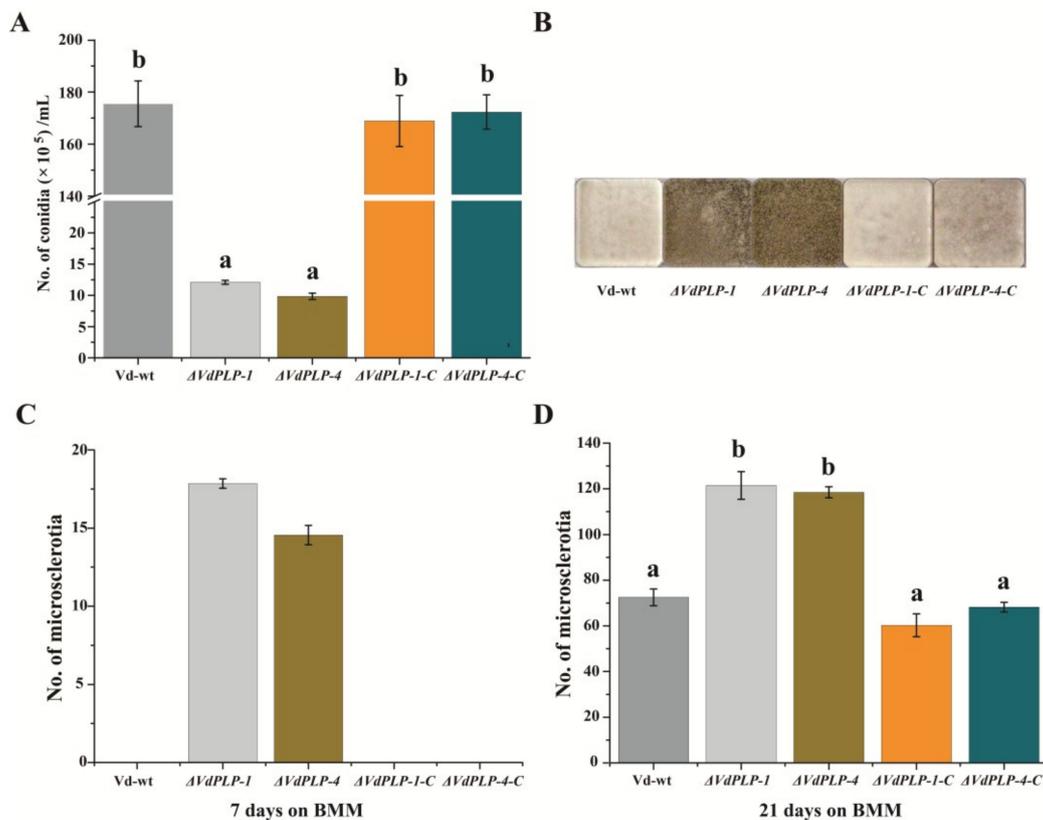


**Figure 2.** Mycelial growth of *Vd*-wt,  $\Delta VdPLP$  ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ) strains and complementary  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) strains after 15 days on minimal medium (MM) agar with different carbon sources: (A) colony morphology; and (B) colony diameters. Values represent the mean  $\pm$  standard deviation (SD) from three independent replicates. Different letters (a–c) above the bars represent the significant differences among the groups ( $p$ -value < 0.05) and the data were analyzed using Duncan's multiple range test.

### 3.3. Conidiation by $\Delta VdPLP$ Mutants Decreased Substantially and Microsclerotia Formation Increased

To check whether the deletion of *VdPLP* affects conidiation and microsclerotia formation, each strain was cultured on Czapek-Dox and BMM plates to enumerate conidia and microsclerotia after 15 days. Mutants  $\Delta VdPLP-1$  and  $\Delta VdPLP-4$  produced significantly fewer conidia on Czapek-Dox than did the *Vd*-wt and complementation strains (Figure 3A). In addition, the  $\Delta VdPLP$  mutant strains accumulated more melanin on BMM, while *Vd*-wt,  $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$  strains were light-colored and produced no melanin on BMM (Figure 3B). On BMM, the  $\Delta VdPLP$  strains

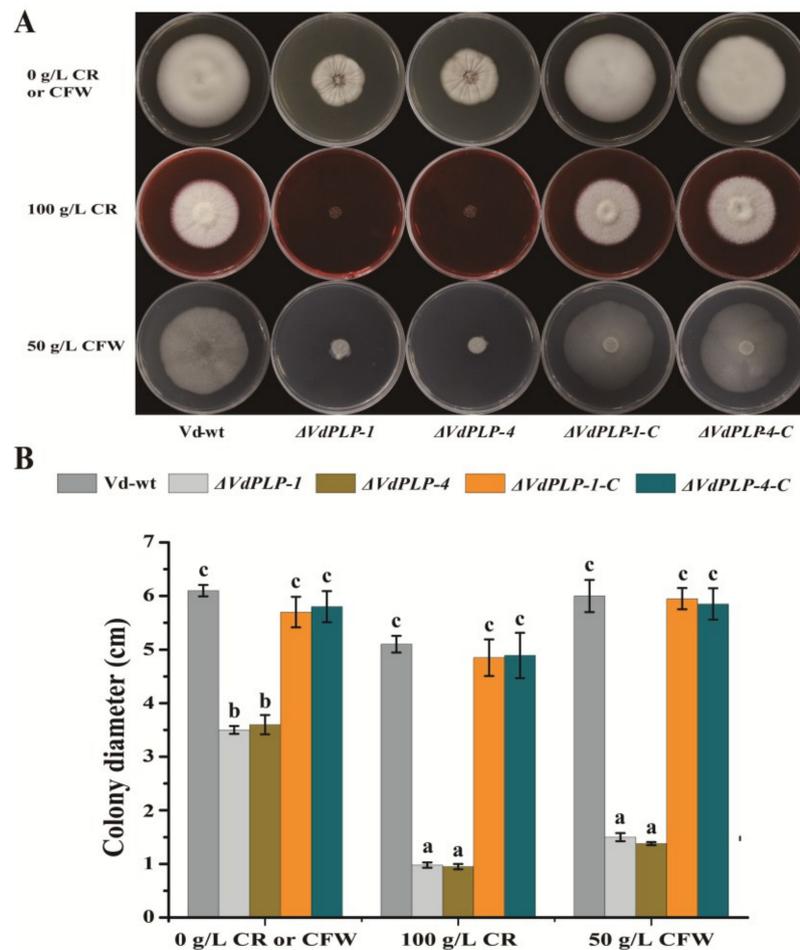
formed microsclerotia earlier and in greater numbers than did the Vd-wt and the complementation strains (Figure 3C,D). The results indicated that deletion of the *VdPLP* gene accelerated melanin and microsclerotia production in *V. dahliae*.



**Figure 3.** Deletion of *VdPLP* reduces conidial production and increases microsclerotial formation. (A) Number of conidia produced by Vd-wt,  $\Delta VdPLP$  strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), and complementary  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) after 15 days on Czapek-Dox agar. (B) Colony color of Vd-wt,  $\Delta VdPLP$  strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), and complementary  $\Delta VdPLP$  mutant ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) strains after 21 days on basal agar modified medium (BMM) agar. Mean ( $\pm$ SD) number of microsclerotia after: seven days on BMM agar (C); and 21 days on BMM agar (D). Three independent replicates were done; Different letters (a–b) above the bars represent the significant differences among the groups and were analyzed with Duncan’s multiple range test ( $p$ -value < 0.05).

### 3.4. $\Delta VdPLP$ Mutants Are Hypersensitive to Cell Wall-Perturbing Agents

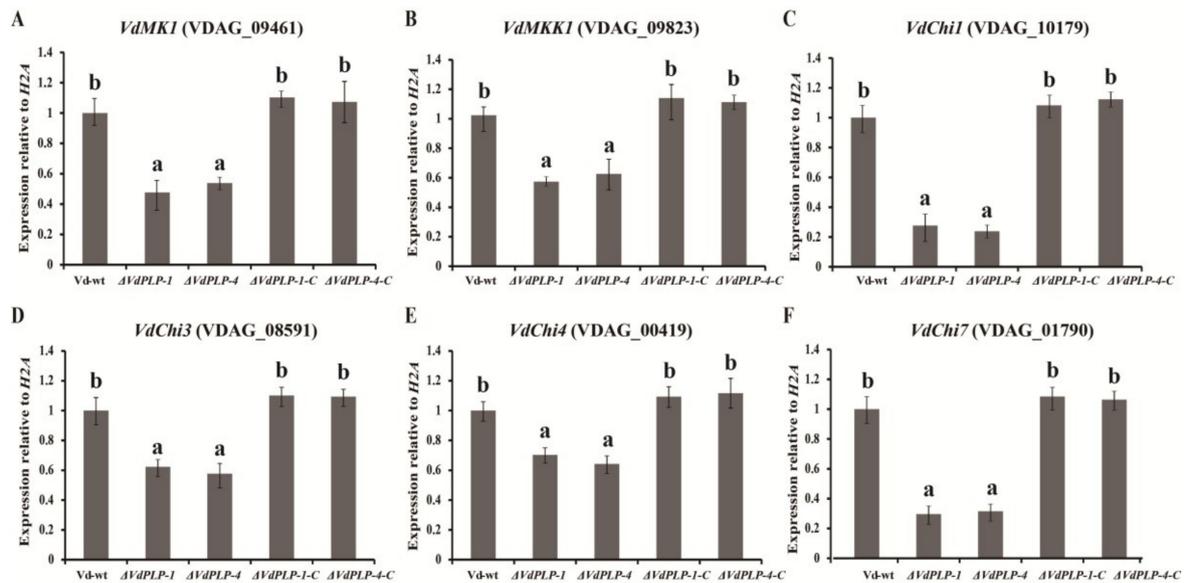
To determine whether the *VdPLP* gene was involved in maintaining cell wall integrity, we tested cell wall-perturbing agents CFW and CR for their effects on growth of the  $\Delta VdPLP$  mutant strains. On PDA plates without CR or CFW, there was no significant difference between Vd-wt and the complementary  $\Delta VdPLP$  strains in the colony diameter, but the colony diameter of  $\Delta VdPLP$  strains showed nearly 50% reduction. On PDA plates containing 100 g/L CR or 50 g/L CFW, the colony diameter of  $\Delta VdPLP$  was significantly smaller than that of Vd-wt and the complementary  $\Delta VdPLP$  strains. Vd-wt and the complementary  $\Delta VdPLP$  strains has no obvious growth defects on PDA plates with 100 g/L CR or 50 g/L CFW compared with growth on PDA without CR or CFW (Figure 4A,B). However, when 100 g/L CR or 50 g/L CFW was added to the medium, the colony diameter of  $\Delta VdPLP$  strains was significantly smaller than that of the colony on PDA without CR. These results indicated that  $\Delta VdPLP$  strains were more sensitive to CR and CFW than the wild type and complementary strains, and *VdPLP* was responsible for maintaining fungal cell wall integrity in *V. dahliae*.



**Figure 4.** Effect of cell-wall-perturbing agents calcofluor white (CFW) and Congo red (CR) on growth of Vd-wt,  $\Delta$ VdPLP ( $\Delta$ VdPLP-1 and  $\Delta$ VdPLP-4) strains, and complementary  $\Delta$ VdPLP ( $\Delta$ VdPLP-1-C and  $\Delta$ VdPLP-4-C) strains. (A) Colonies of Vd-wt,  $\Delta$ VdPLP ( $\Delta$ VdPLP-1 and  $\Delta$ VdPLP-4) mutant strains, and complementary  $\Delta$ VdPLP mutant ( $\Delta$ VdPLP-1-C and  $\Delta$ VdPLP-4-C) strains after 15 days on potato dextrose agar (PDA) plates containing 100 g/L CR or 50 g/L CFW compared with controls on PDA at 25 °C. (B) Mean ( $\pm$  SD) colony diameter after 15 days on PDA plates with 100 g/L CR or 50 g/L CFW compared with controls on PDA. Three independent replicates were done; Different letters (a–c) above the bars represent the significant differences among the groups and were analyzed with the Duncan’s multiple range test ( $p$ -value < 0.05).

### 3.5. Genes for the Calcofluor White Pathway and for Chitin Synthesis in the $\Delta$ VdPLP Mutant Were Downregulated

To further confirm that VdPLP contributes to the CWI pathway and chitin synthesis, we focused on the regulation of the CWI pathway by profiling transcript levels of the CWI pathway genes *VdMCK1* and *VdMK1* and chitin synthesis genes *VdChi1*, *VdChi3*, *VdChi4*, and *VdChi7*. In the  $\Delta$ VdPLP mutant strains, transcription of *VdMCK1*, *VdMK1*, and the chitin synthesis genes all decreased compared with the wild type strain and complementary  $\Delta$ VdPLP strains (Figure 5A–F). Downregulation of CWI and chitin synthesis genes indicated that VdPLP participates in the CWI pathway and chitin synthesis.



**Figure 5.** Expression of cell wall integrity (CWI) pathway genes (*VdMKK1* (VDAG\_09823) and *VdMK1* (VDAG\_09461)), and chitin synthesis genes (*VdChi1* (VDAG\_10179), *VdChi3* (VDAG\_08591), *VdChi4* (VDAG\_00419) and *VdChi7* (VDAG\_01790)) in the  $\Delta VdPLP$  mutant strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ) was lower than in the *V. dahliae* wild type (Vd-wt) strain and complementary  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ). Total RNA was extracted from mycelia of seven-day-old Vd-wt,  $\Delta VdPLP$  ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), and complementary  $\Delta VdPLP$  ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) strains were grown in liquid complete medium (CM). Quantitative real-time reverse-transcription PCR was used to measure expression levels: *VdMKK1* (A); *VdMK1* (B); *VdChi1* (C); *VdChi3* (D); *VdChi4* (E); and *VdChi7* (F). The  $\beta$ -tubulin gene was used as an internal standard. Values are means  $\pm$  SD of three independent experiments performed in duplicates, significant differences are indicated by letters (a,b), and data were analyzed with the Duncan's multiple range test ( $p$ -value < 0.05).

### 3.6. Penetration and Fungal Colonization of $\Delta VdPLP$ Were Impaired

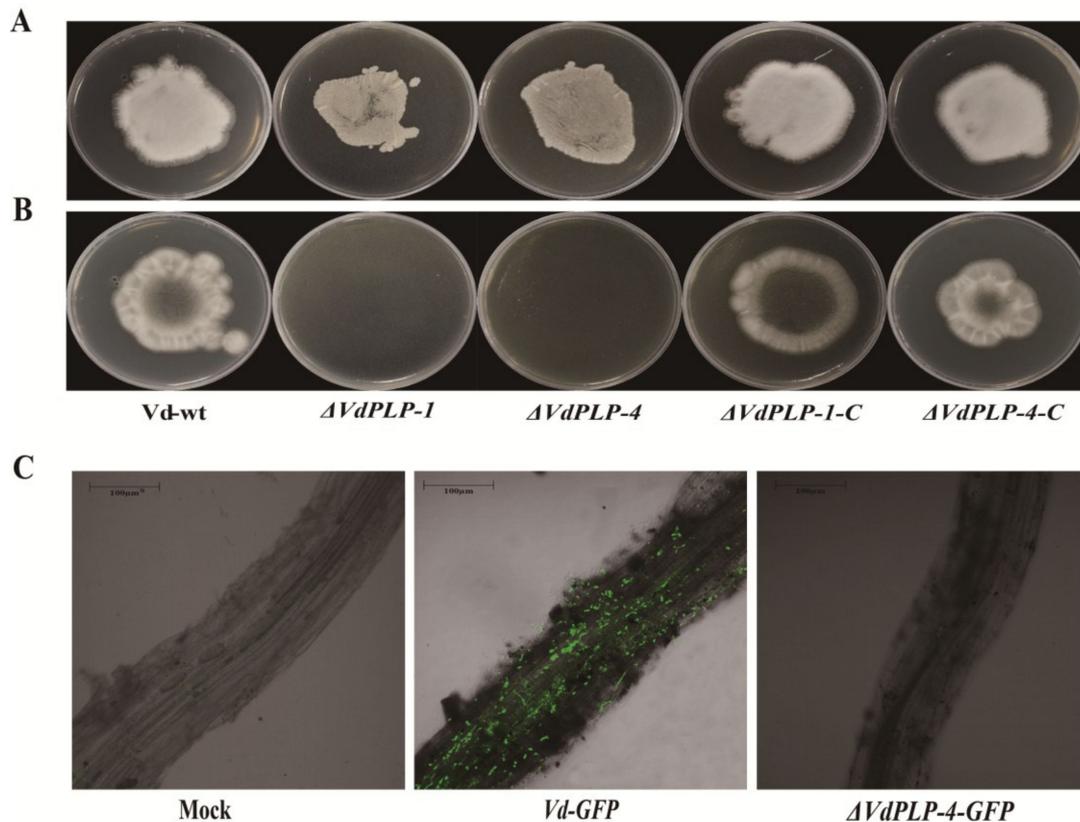
To investigate possible reasons for the significant virulence defect of  $\Delta VdPLP$  mutant strains, we analyzed penetration by the Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains. After a conidial suspension of the respective strains was placed on cellophane membranes, Vd-wt and the  $\Delta VdPLP-C$  strains penetrated and colonized the membranes, but no hyphae of the  $\Delta VdPLP$  mutant strains grew (Figure 6A,B). Thus, the deletion of *VdPLP* severely hindered the ability to penetrate the cellophane membrane.

GFP-labeled strains  $\Delta VdPLP-4-GFP$  and *Vd-GFP* constitutively express *GFP*; strong green fluorescence thus allows observation of fungal penetration and colonization of plant root tissues using LSCM. Strain *Vd-GFP* strain colonized *N. benthamiana* roots, and hyphae penetrated the epidermal and cortical cells. In contrast, few hyphae of the  $\Delta VdPLP-4-GFP$  strain grew on the root, and the strain penetrated epidermal cells (Figure 6C). These phenomena indicate that *VdPLP* contributes to penetration and colonization by *V. dahliae*.

### 3.7. *VdPLP* was Required for Fungal Pathogenicity

To verify the role of *VdPLP* in fungal pathogenicity, we inoculated roots of *N. benthamiana* seedlings with a conidial suspension of Vd-wt,  $\Delta VdPLP$ , or  $\Delta VdPLP-C$  strains. As expected, at 8, 10, and 12 dpi, plants inoculated with either Vd-wt, or the complementary strains developed significant wilting, whereas only a few chlorotic leaves appeared on plants inoculated with  $\Delta VdPLP$  strains. At 12 dpi, plants inoculated with Vd-wt or  $\Delta VdPLP-C$  strains were stunted, chlorotic, wilting or dead. In contrast, the seedlings infected with  $\Delta VdPLP$  mutants exhibited mild symptoms, with interveinal chlorosis on

the bottom 1 or 2 leaves, but no necrosis (Figure 7A,B). Furthermore, when biomass was quantified, less fungal DNA was detected from infected plants inoculated with the  $\Delta VdPLP$  strains than with Vd-wt or the  $\Delta VdPLP-C$  strains (Figure 7C). The decrease in symptoms and biomass suggest that *VdPLP* disruption reduced the virulence of *V. dahliae*.



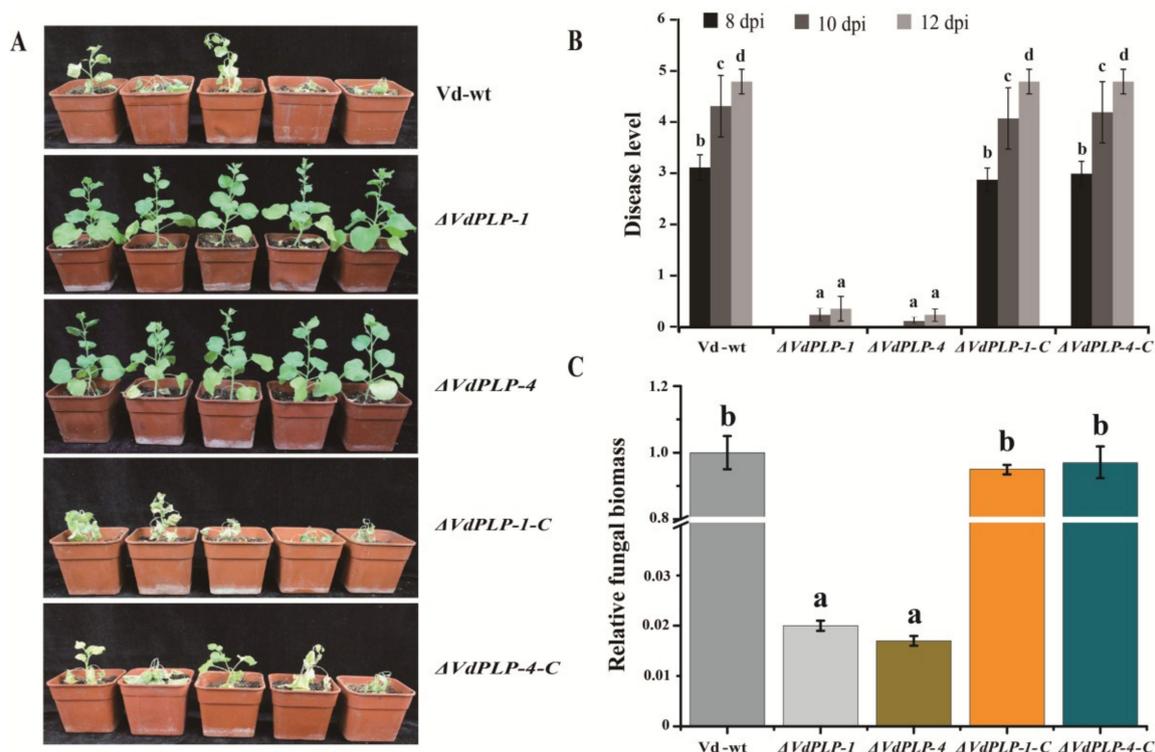
**Figure 6.** Fungal colonization and penetration assay: (A) Vd-wt,  $\Delta VdPLP$  and  $\Delta VdPLP-C$  strains grown on cellophane for three days after strains had penetrated a cellophane membrane on the agar plate and membranes were removed; (B) plates after incubation at 25 °C for seven days after cellophane were removed; and (C) fungal colonization of infected roots by the  $\Delta VdPLP-4-GFP$  mutant strains, Vd-GFP strain, and sterile water (Mock), three days after inoculation.

#### 4. Discussion

Phospholipases also regulate vegetative growth signals in filamentous fungi [9,13,21,22,50]. The cAMP-specific PDEs can be regulated by some phospholipases, which adjust intracellular cAMP level. cAMP signaling participates in numerous intracellular activities [23,24]. For example, the constitutive activation of cAMP phosphodiesterase, which can activate the cAMP pathway, influences the biosynthesis and structure of the cell wall and membrane [51,52]. Fungal phospholipases affect blastospore development [18] and participate in hyphal extension in the filamentous fungi *N. crassa* [19], *C. cinerea* [20], and *M. oryzae* [25,28]. Due to inhibition of phosphodiesterase activity in *V. dahliae*, conidial production and virulence was reduced in G protein  $\beta$  subunit gene (*VGB*) mutants [53]. Similar phenotypic changes were observed in *VdPLP* deletion mutants. Hyphal growth of  $\Delta VdPLP$  strains under different carbon sources conditions, and conidia production was drastically reduced compared with the wild type strain and complementation mutants, which suggested that impaired vegetative growth and conidial production caused by *VdPLP* deletion may be the reason for the virulence decline.

Microsclerotia, melanized survival structures that can survive in the soil for more than ten years in the absence of host plants, play a critical role in the disease cycle of *V. dahliae*, but the molecular

mechanism of their biogenesis is still not clear [54–57]. *VdPKAC1* mutants showed a decrease in fungal virulence and an increase in microsclerotial formation [58]. In contrast, microsclerotia production decreased by *V. dahliae* class II hydrophobin gene (*VDH1*) mutants [59], *Vayg1* deletion mutants [60], transmembrane mucin *Msb* (*VdMsb*) mutants [61] and MAPK mutants [62]. In the present laboratory study,  $\Delta VdPLP$  mutants produced microsclerotia earlier and in greater numbers than did *Vd*-wt and the  $\Delta VdPLP$ -C strains. Based on the above research, opposite results for mutants in different genes suggest that the molecular mechanisms of microsclerotia formation are complex. Regulatory genes such as *VdPKAC1*, *VDH1*, *Vayg1*, *VdMsb*, and dihydroxynaphthalene (DHN) melanin biosynthesis pathway [57] may regulate the microsclerotia biosynthesis. The loss of *VdPLP* likely activates microsclerotia production via participating in a signaling pathway, but the specific mechanisms of the transition from vegetative growth to resting structure development require further research.



**Figure 7.**  $\Delta VdPLP$  mutant strains were not virulent on *Nicotiana benthamiana* in pathogenicity assays. (A) Representative plants 12 days post inoculation (dpi) with *Vd*-wt,  $\Delta VdPLP$  strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), or complementary  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ); (B) Disease level on *N. benthamiana* after a 2-min root-dip in conidia of the *Vd*-wt,  $\Delta VdPLP$  strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), and complementary  $\Delta VdPLP$  mutant ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) strains at 8, 10, and 12 dpi. See methods for severity scale; (C) Relative quantification of fungal biomass in *N. benthamiana* stems inoculated with conidial suspension of *Vd*-wt,  $\Delta VdPLP$  strains ( $\Delta VdPLP-1$  and  $\Delta VdPLP-4$ ), and complementation  $\Delta VdPLP$  strains ( $\Delta VdPLP-1-C$  and  $\Delta VdPLP-4-C$ ) at 12 dpi. Values are means  $\pm$  SD from three independent replicates. Significant differences are indicated by letters (a–d), Duncan’s multiple range test ( $p$ -value < 0.05) was used to analyze significant difference between each group.

The fungal cell wall plays a critical role in maintaining cell shape, protecting against physical injury, and mediating exchanges between pathogenic fungi and their hosts [62–64]. It also provides adhesive properties and protection against host defenses, which is imperative for pathogenicity in fungi [63,65,66]. PdeH are related to cell wall integrity, stress response and virulence in *M. oryzae* and *C. albicans* [25,28,50–52]. MoPdeH targets MoMck1 of *M. oryzae*, one of the components of the MAPK

cascade, and regulates cell wall integrity and virulence [28]. Integrity of the cell wall is critical for penetration of the host and thus for fungal virulence [67]. In this study, deletion of *VdPLP* generated  $\Delta VdPLP$  mutants that were sensitive to cell wall-perturbing agents, which suggests *VdPLP* participates in stabilizing the cell wall under stress. Disruption of cell wall integrity in the *VdPLP* mutants was thus associated with the significantly reduced virulence of the  $\Delta VdPLP$  mutants.

A small G protein, Rho1, is considered to be the master regulator of CWI signaling. Rho1 integrates signals from the cell surface and regulates many outputs involved in cell wall biogenesis. GTP-bound Rho1 associates with and activate the PKC1 [47]. Polyunsaturated fatty acids like arachidonic acid (AA) released by PLPs may regulate cellular signaling and influence the activity of protein kinases [35]. MAPK pathway can be regulated by protein kinases [30,31]. Thus, PLPs could influence the MAPK pathway and CWI pathway as well. In the citrus postharvest pathogen *Penicillium digitatum*, disruption of chitin synthase (*PdChsVII*) impaired CWI integrity, causing reduced virulence [68]. Class I chitin synthases reinforce cell wall integrity under a specific cell wall stress in *C. albicans* [69]. A myosin motor domain-chitin synthases (MMD-Chs) deletion impaired fungal resistance to host defense mechanisms and colonization by invasive hyphae, possibly due to a decrease in the structural integrity and permeability of the cell wall [70]. In our research, the expression of the MAPK pathway (*VdMK1* and *VdMCK1*) was diminished in the  $\Delta VdPLP$  mutants. The CWI pathway and chitin synthesis genes were downregulated in the  $\Delta VdPLP$  strains, suggesting that *VdPLP* has a critical role in regulating the CWI pathway and chitin synthesis in *V. dahliae*. Meanwhile,  $\Delta VdPLP$  mutants cannot penetrate the cellophane membrane. GFP-labeled  $\Delta VdPLP$  mutants showed impairment in colonization. The results indicate that *VdPLP* could regulate the MAPK pathway for regulating CWI pathway and chitin synthase and affect the cell wall integrity and virulence of *V. dahliae*.

Soil-borne *V. dahliae* must penetrate and colonize the root to cause disease. The colonization of *Arabidopsis thaliana* [71], cotton [6] and tomato [72] by *V. dahliae* GFP-labeled strains has been detailed. In our research, the same strategy was used for penetration ability comparison. When the penetration ability of GFP-tagged  $\Delta VdPLP$  strains was compared with that of *Vd-GFP* strains, penetration of epidermal cells of the roots by hyphae of  $\Delta VdPLP$ -GFP strains was severely lowered as was subsequent colonization. In addition,  $\Delta VdPLP$  strains were impaired in penetration of cellophane, indicating impairment of an early phase of infection. *VdPLP* may regulate vegetative growth of *V. dahliae* in plant tissue and affect cell wall stability, and then impair pathogenicity further.

Deletion of *VdPLP* resulted in severe impairment of vegetative growth on various carbon sources and reduced conidia production. The  $\Delta VdPLP$  mutants were also severely impaired in their ability to penetrate and colonize roots of *N. benthamiana*, as shown microscopically and by a decrease in biomass compared with the wild type. The qRT-PCR results suggest that *VdPLP* participates in the CWI pathway and chitin synthesis. These results revealed the defect of  $\Delta VdPLP$  mutants to penetrate plant tissue, contributing to the impaired virulence. *VdPLP* may contribute to the full virulence of *V. dahliae* by mediating hyphal growth, the cell wall integrity and penetration process, which provides new insights into the molecular pathogenic mechanisms of *V. dahliae*.

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**Conflicts of Interest:** The authors have declared that no conflict of interest exists.

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