1 A uracil-DNA glycosylase functions in spore development and pathogenicity of
2 *Verticillium dahliae*

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12 pathogenicity

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1 **Abstract**

2 The soil-borne fungus *Verticillium dahliae* represents notorious vascular wilt
3 pathogens and has very broad host range. Since the disease caused by *V. dahliae*
4 relies on its virulence as well as the amount of microsclerotia in the soil, large effort
5 has been taken to screen for genes functional in pathogenicity and microsclerotial
6 formation. So far DNA glycosylase has not been reported for functionality in
7 pathogenesis of any bacteria, fungi or oomycetes. Here, we described for the first time,
8 a uracil–DNA glycosylase VdUDG, which was found to be involved in not only
9 pathogenicity but also microsclerotial formation based on screening of T-DNA
10 insertion library. Transcription analysis of wild type strain and *VdUDG* gene knock-out
11 mutants suggested that VdUDG regulates transcription of a series of genes involved in
12 microsclerotial formation and pathogenesis of *V. dahliae*.

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1. Introduction

Verticillium dahliae (Kleb.) is a soil-borne fungal pathogen that is able to infect hundreds of plant species, and causes vascular wilt disease [1, 2]. In nature, the infection of *V. dahliae* initiates from the germination of resting structures called microsclerotia [3]. The germinated mycelia penetrate plant root through wounded sites or directly penetrate cortical surface by secreting cell wall degrading enzymes [4-8]. As many fungal pathogens, *V. dahliae* has to overcome plant basal defense by secreting all kinds of small proteins, also known as effectors, for successful colonization [9, 10]. Once colonized, the mycelia grow in plant xylem and produce spores, blocking water transport that results in stunting, wilting and finally dying of the whole plants [11, 12].

The melanized, multicellular microsclerotium was formed from hyphal swelling and subsequent lateral cell budding [13]. Due to the long term persisting and continuous accumulation in the soil, microsclerotia become major threat on economical crops [14-16], especially on cotton plants in China. Gene functional analysis has shown that microsclerotial formation and germination are controlled by some conserved signaling pathways such as G-protein and MAP Kinase [17, 18]. Some genes were found to be involved in not only microsclerotial development but also pathogenicity. These include genes encoding a trypsin protease, a small secreted hydrophobin, and glutamic acid rich protein [19-21]. The regulation of signaling pathways and functional genes in development and pathogenicity remain to be elucidated.

In the previous work, we constructed a T-DNA insertion library based on *V.*

1 *dahliae* strain V592, which was isolated from cotton plants in Xinjiang, China [21].
2 Phenotypic screening showed genes functionally involved in development and
3 pathogenicity [21]. Here we show one T-DNA insertion mutant reduced virulence on
4 cotton plant and showed knock-down of *VdUDG*, a gene encoding a uracil-DNA
5 glycosylase. Functional study of *VdUDG* gene suggested that it regulated spore
6 development as well as transcription of many genes encoding multiple signaling
7 pathways.

8 **2. Materials and methods**

9 *2.1. Strains and culture conditions*

10 All the *Verticillium dahliae* strains were stored as microconidial suspensions in 20%
11 glycerol at -80°C. The strains were reactivated by growing on potato dextrose agar
12 (PDA) plates at 25°C before use. Single gene knock-out mutants and gene
13 complementation transformants were grown on PDA plates containing 150 µg/ml
14 hygromycin B, and 100 µg/ml chlorimuron-ethyl, respectively.

15 *2.2. Gene cloning and constructs*

16 The T-DNA insertion site in V592 strain was verified by a thermal asymmetric
17 interlaced PCR (TAIL-PCR) following the method of Gao *et al.* [21]. For the gene
18 knockout construct, 1.5 Kb fragments from both upstream and downstream of *VdUDG*
19 gene were amplified from V592 genomic DNA with primers *VdUDG*_{up-s/a} and
20 *VdUDG*_{down-s/a}, respectively (Table 1). The amplified DNA fragments were cloned
21 into transformation vector following the strategy of Gao *et al.* [21].

2.3. Transformation, DNA isolation and southern blot

Agrobacterium tumefaciens-mediated transformation, DNA isolation and southern blot were performed following the method of Gao *et al.* [21].

2.4. RNA isolation and gene expression analysis

The conidia, mycelia, microsclerotia and a mixture of conidia and mycelia were collected for RNA isolation. Total RNAs isolation and cDNA synthesis were performed following the method of Gao *et al.* and Zhou *et al.* [21, 22]. Gene expression was analyzed by qRT-PCR with primers described in Table 1 following the method of Gao *et al.* [21]. β -tubline gene (DQ266153) was used as an endogenous gene for quantification of gene expression[23].

2.5. Phenotypic analysis

Phenotypic analysis including microsclerotial formation, conidia formation and pathogenicity analysis were performed following the method of Gao *et al.* or Zhou *et al.* [21, 22].

3. Results

3.1. A *V. dahliae* single T-DNA insertion mutant showed virulence reduction on cotton plants

In our previous study, a *V. dahliae* T-DNA insertion library was constructed for screening of genes that functional in pathogenicity [21]. According to the result of infection assay on cotton plants, one mutant strain namely d137 showed clear virulence reduction on cotton cultivar Xinluzao No.16, which is susceptible to the

1 receipt wild type strain V592. As the result shown in Fig. 1, the cotton inoculated with
2 V592 strain showed wilt disease symptom whereas the one inoculated with d137
3 strain was growing relatively healthy, similar as the mock cotton plant. Besides
4 virulence reduction the d137 strain also showed other phenotypes that related to
5 fungal development. The mutant strain formed white and very dense hyphal colony but
6 did not produce melanized microsclerotia (Fig. 2A), which is important for initiating
7 disease cycle in nature.

8 *3.2. A uracil DNA glycosylase gene was knocked out in V. dahliae d137 strain*

9 To identify the mutation site responsible for virulence reduction, we performed TAIL-P
10 CR using genome DNA of d137 as template. The PCR result indicated that T-DNA wa
11 s inserted at 155 bp downstream to stop codon of VdAG_00947 gene, which encodes
12 a uracil DNA glycosylase (Fig. 2C). This gene was therefore named *VdUDG*. A compari
13 son of sequences was performed with VdUDG and UDGs from other organisms, such
14 as *S. cerevisiae*, *B. subtilis*, *E. coli*, human and herpes viruses. The result revealed a st
15 riking similarity between VdUDG and all the other proteins, possessing 54%, 42%, 5
16 1%, 48% and 47% identical residues, respectively. Sequence alignment showed that V
17 dUDG contains a typical UDG homology domain, suggesting enzyme activity of uracil
18 DNA glycosylase (Supplementary Fig. 1). QRT-PCR analysis showed that *VdUDG* was
19 not expressed in d137 strain (Fig. 2D), suggesting that the gene was knocked down. S
20 outhern blot analysis showed that there was only one copy of T-DNA insertion in d137
21 strain (Fig. 2B), indicating that *VdUDG* gene knock-down might be due to the disruptio
22 n of 3' UTR, which was somehow necessary for gene transcription.

1 3.3. *VdUDG is differentially expressed in the development of V. dahliae*

2 According to the annotation from *V. dahliae* genome database [24], *VdUDG* encodes
3 a 386 amino acids protein. To investigate *VdUDG* expression in *V. dahliae*
4 development, RNA samples from growing mycelia, conidia and microsclerotia of V592
5 strain were isolated for qRT-PCR analysis. The result showed that *VdUDG* was highly
6 expressed in conidia whereas expressed in very low level in growing mycelia and
7 microsclerotia (Fig. 3).

8 3.4. *The biological function of VdUDG*

9 To dissect the function of *VdUDG*, the full-length gene was fully knocked out by the
10 method of homologous recombination following the method of Gao *et al.* (Fig. 4). Four
11 *V. dahliae* transformants showed reduction of microsclerotial formation (Fig. 5A), and
12 were confirmed for *VdUDG* gene knock-out by both southern blot and transcription
13 analysis (Fig. 5B and C). These mutants did not produce melanized microsclerotia in
14 consistent to d137 strain (Fig. 2A). However, the mutants produced significantly higher
15 amount of conidia in compared to the wild type strain V592 (Fig. 6B). In the induction
16 medium, mycelia of the V592 strain generated vacuolar structures, which were not
17 found in *vdudg* mutant strains (Fig. 6C).

18 3.5. *VdUDG regulates expression of a series of pathogenicity factors in V. dahliae*

19 In the infection test, *vdudg* mutant strains lost pathogenicity on cotton plants as anticip
20 ated. The *vdudg* inoculated plants grew as well as the mock control plants at four week
21 s post-inoculation. In the contrast, the plants inoculated with the wild type or gene com

1 complemented mutants showed wilt disease symptom at the same time point (Fig. 7). To elucidate the loss of pathogenicity, a series of well-known functional genes in *V. dahliae*
 2 were tested for transcription in both V592 strain and *vdudg* mutants. These genes included *VTP1*(VDAG_05650.1), *VMK1*(VDAG_09461.1), *VGB*(VDAG_00979.1), *VdNLP1*
 3 (VDAG_04701.1), *VdNLP2*(VDAG_01995.1), *VDH1*(VDAG_02273.1), *pevD1*(VDAG_02735.1), and *VdGARP1*(VDAG_08781.1) [17, 18, 20-22, 25, 26]. In compared to the
 4 wild type V592 strain, 7 of these genes, *VMK1*, *VGB*, *VdNLP1*, *VdNLP2*, *VDH1*, *pevD1* and *VdGARP1* showed significant transcriptional alteration. No significant change was
 5 as detected for *VTP1* gene (Fig. 8). This result suggested that mutation of *VdUDG* gene influenced multiple pathways that involved in development and pathogenicity of *V. dahliae*.
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12 **4. Discussion**

13 Uracil-DNA glycosylase exists in both prokaryotes and eukaryotes [27]. It functions normally in base excision repair and plays roles in DNA methylation, gene expression and cell development [28, 29]. Yet it was not reported for functionality in fungal plant
 14 pathogens. *V. dahliae* is a vascular wilt pathogen that causes annual loss on economical crops such as cotton, tomato and lettuce [2]. The disease epidemics
 15 largely relies on the amount of microsclerotia maintained in the soil [15]. To screen for virulence factors on cotton, the major economical crop in Xinjiang, China, we
 16 constructed a T-DNA insertion library of *V. dahliae* 592 (V592) strain [21]. One transformant d137 showed severe virulence reduction as well as disturbance of
 17 sporulation. Surprisingly, the T-DNA was inserted 155bp downstream of *VdUDG* gene,
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1 which was predicted to encode a uracil-DNA glycosylase. Since RT-PCR result
2 showed significant reduction of *VdUDG* gene expression in d137, T-DNA might have
3 disrupted 3'-UTR region that could be very critical for gene expression. Phenotypic
4 analysis of *VdUDG* gene knock-out mutants confirmed that this gene is indeed
5 functional in pathogenicity and spore development.

6 It is the first time that a DNA glycosylase found to be involved in virulence and spore
7 formation of pathogenic fungi. According to the property of DNA glycosylase, there
8 should be a series of genes interfered due to *VdUDG* gene disruption [30]. To date,
9 research on gene functional analysis has reported several components that regulate
10 microsclerotial formation and pathogenesis. For instance, VDB and VMK1 are two
11 components representing conserved signal transduction pathways, and involved in
12 both microsclerotial formation and pathogenicity of *V. dahliae* [17, 18], which was not
13 surprising since gene orthologs function similarly in other pathogenic fungi or
14 oomycetes [31-33]. A hydrophobin gene *VDH1* encoding a signal peptide specifically
15 participated in microsclerotial formation [20], indicating that secreted proteins also
16 contributed to *V. dahliae* development. Despite the function of these gene were well
17 elucidated, the regulation and relationship between genes remain unclear.

18 Another group of secreted proteins that determine the virulence of *V. dahliae* are
19 effectors and microbe associated molecular patterns (MAMP) that regulate plant
20 immunity [10]. So far, there is only one effector gene *Ave1* cloned in *V. dahliae*, yet it
21 does not exist in race 2 strains such as V592 and sequenced VdLs.17 [9, 24]. The
22 MAMP molecules such as necrosis and ethylene-induced proteins were functionally

1 elucidated [22, 26]. The representatives are VdNLP1 and VdNLP2 that induced plant
2 cell death and might contribute to pathogen colonization in plants. To figure out the
3 relationship between VdUDG and genes involved in microsclerotial formation as well
4 as pathogenicity, the transcription was quantified for a series of reported genes. As
5 anticipated, genes such as VMK1, VDB, VDH1 and VdGARP1 were significantly
6 downregulated in *VdUDG* gene knockout mutants. Interestingly, secreted protein
7 genes showed differential expression upon knock-down of VdUDG gene. As it shown
8 in Fig. 8, peVD1 and *VdNLP2* were down-regulated, whereas *VdNLP1* was strongly
9 up-regulated in *VdUDG* gene knockout mutants. This is also indicative for the
10 complexity of plant-*Verticillium* interactions.

11 Despite the enzyme activity, we have fully investigated the biological function of
12 VdUDG. However, more genes and signal transduction pathways influenced by uracil-
13 DNA glycosylase remain to be investigated. Our study on *VdUDG* gene would be one
14 case suggesting the importance of epigenetics on plant-pathogen interaction, and
15 hopefully a new way to search for targets of vascular wilt pathogens.

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- 3 relation to *Magnaporthe grisea* infection. Molecular plant-microbe interactions.
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5 Table 1. Primers used for gene cloning and qRT-PCR analysis

Primer	Sequences (5' 3')
<i>EGFP-s</i>	ATGGTGAGCAAGGGCGAGGAG
<i>EGFP-a</i>	TTACTTGTACAGCTCGTCCATGCCG
<i>VdUDGt-s</i>	GCGACTACCCGACCTTCA
<i>VdUDGt-a</i>	CCGTTCGTGCCATCAGC
<i>Actin-F</i>	CTGTACGGCAACATCG
<i>Actin-R</i>	GACCATATCGCCTGTG
<i>VdUDGup-s</i>	<u>GGTCTTAAU</u> TACGACTGGCTGCGCTTCAAC
<i>VdUDGup-a</i>	<u>GGCATTAAUG</u> TCATCGTGAGGAACCGAACTT
<i>VdUDGdown-s</i>	<u>GGACTTAAU</u> TAGGGCACGACGGAGTCAA
<i>VdUDGdown-a</i>	<u>GGGTTTAAU</u> AGGCAGGTACCTAGGTAGTGA
<i>VdUDG-knockout-s</i>	TCCGTTCGTGCCATCA
<i>VdUDG-knockout-a</i>	TGGGACGCAAGTGGTTT
<i>β-tubline-f</i>	TCACCAGCCGTGGCAAGGTTG
<i>β-tubline-r</i>	AGCAAAGGGCGGTCTGGACGTTG
<i>VdUDGrt-f</i>	GCGACTACCCGACCTTCA
<i>VdUDGrt-r</i>	CCGTTCGTGCCATCAGC
<i>VMK1-f</i>	CGCAGCAACGCCCTAATC
<i>VMK1-r</i>	GGCAGTGGTCATCGGAGAGGT

<i>VDH1-f</i>	CTATTGCGATTGCTCTG
<i>VDH1-r</i>	GAGCTCAAGGTTTTCGTG
<i>VdGARP1-f</i>	ATGCCGCCCAAAAAGCCCTCACCCG
<i>VdGARP1-r</i>	TTAATCACTGTCATTGCCATCCAGC
<i>VGB-s</i>	CGGTGCCTGCGATGCTTT
<i>VGB-x</i>	GGAGGTGATGCCACAGAGGA
<i>pevD1-f</i>	ATGCAGTTCACCCTCGCCG
<i>pevD1-r</i>	TTAAGCCTCGGCGGGAGC
<i>VTP1-f</i>	GCGGTGGCTGGTTCCTATCAAC
<i>VTP1-r</i>	CAACGACTTCGCCATCTGGAAG
<i>VdNLP1-f</i>	TCGGTCTTTGCCCTCGTC
<i>VdNLP1-r</i>	GCCTGGTTTGCGTTGTTC
<i>VdNLP2-f</i>	AAGCCGTACCTCAAGGTGTTCA
<i>VdNLP2-r</i>	CCGACCCAAAGTCCGTGTTCT

Mock

V592

d137

Fig. 1. Infection assay for *V. dahliae* wild type strain V592 and T-DNA insertion mutant d137 on cotton plants. The picture was taken at four weeks post-inoculation.

A

B

C

D

Fig. 2. Phenotypic and genetic analysis of *V. dahliae* d137 mutant. A. Colony morphology of d137 mutant strain in compared to wild type V592 strain. Upper figures show mycelial colonies (MY) and lower figures indicate microsclerotial formation (MS). B. Southern blot analysis for d137 genome DNA with a eGFP probe, confirming single T-DNA insertion in the mutant. C. The T-DNA insertion site in the genome of d137 strain. D. RT-PCR analysis for *VdUDG* gene expression in wild type V592 strain and d137 mutant.

Fig. 3. Quantitative RT-PCR (qRT-PCR) analysis for *VdUDG* gene expression in various tissues of *V. dahliae*. 1, conidia, 2, growing mycelia, 3, microsclerotia, 4, conidia of a *VdUDG* gene complementary transformant.

Fig. 4. Strategy to obtain *VdUDG* gene knock out mutants

Fig. 5. Colony morphology, genome and transcription analysis of *VdUDG* gene knock-out mutants. A. Colony morphology of the wild type strain V592, *vdudg* mutant and gene complement strain *vdudg/VdUDG*. B. Southern blot of DNA from wild type strain (WT) and *VdUDG* gene knock-out mutants (1 and 2). C. QRT-PCR analysis for *VdUDG* gene expression in the wild type strain and knock-out mutants. 1, negative control with water as PCR template, 2, positive control with DNA of V592 strain as template, 3 and 4, test cDNA from *vdudg* mutants.

A

B

C

Fig. 6. Characteristics of *vdudg* mutants in compared to the wild type V592. A. Microsclerotial formation on nutrient restrictive medium. B. Conidia production of mutants in compared to the wild type strain. C. Hyphal growing of the mutant and the wild type strain in different nutrient conditions.

Fig. 7. Virulence assay of *vdudg* mutant and the wild type strain V592. The picture was taken at four weeks post-inoculation.

Fig. 8. Comparison of gene expression between wild type strain V592 (white column) and *vdudg* mutant (black column).