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Study of the Hydrophobin Genes in Verticillium Dahliae and Characterization of the Hydrophobin Gene VDH5

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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STUDY OF THE HYDROPHOBIN GENES IN VERTICILLIUM DAHLIAE AND CHARACTERIZATION OF THE HYDROPHOBIN GENE VDH5

(Thesis format: Monograph)

by

Nadia Patricia Morales Lizcano

Graduate Program
in
Biology

A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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ABSTRACT

The broad host range, soil borne fungus *Verticillium dahliae* Kleb. is the causal agent of an economically significant vascular wilt disease. This species produces persistent resting structures, known as microsclerotia, which are the primary source of disease inoculum in the field. Five hydrophobin-like proteins (VDH1 to 5) have been identified in the genome of *V. dahliae*. The results of bioinformatics analyses suggested secretion of these proteins, and that they are all class II hydrophobins. Gene expression analyses of *VDH1* to *5* indicate that the transcript levels of the individual genes vary under different growth conditions. Additionally, the transcript levels of these genes differ from one another. These results suggest that the members of this protein family have different roles in *V. dahliae*. One of the hydrophobin genes, *VDH5*, contains an extra domain known as an extended N terminus (ENT) domain. For further functional analyses of this particular hydrophobin, *Agrobacterium tumefaciens*-mediated transformation was used to generate a targeted gene disruption (*vdh5TN*), and gene deletion mutants (*Δvdh5)*. Both *Δvdh5* and *vdh5TN* strains showed reduced spores and microsclerotial production, as well as a less aggressive pathogenic phenotype. Similarly, a strain carrying a gene lacking the ENT domain was produced (*vdh5ΔENT*). No differences in development or pathogenicity were found between the *vdh5ΔENT* and the WT strains. The response of pathogenesis related genes (*PR1*, *PR3*, and *PR5*) in tomato plants inoculated with the *Δvdh5* mutant, and with the mutant of the previously characterize *vdh1*, was also studied. The lower transcript levels of these genes in response to infection with either *vdh1* or

vdh5 mutant strain, compared to that of WT-inoculated plants, suggest that the plant is responding specifically to these two hydrophobin genes. The study and characterization of hydrophobin genes has proven their importance in developmental processes and in pathogenicity, and the knowledge produced here may lead to novel disease control methods.

Key words: *Verticillium dahliae*, hydrophobin, gene expression, conidia, microsclerotia, pathogenicity.

Cuando vayan mal las cosas,
como a veces suelen ir;
cuando ofrezca tu camino
sólo cuestas que subir;
Cuando tengas poco haber,
pero mucho que pagar,
y precises sonreír
aun teniendo que llorar;
cuando el dolor te agobie
y no puedas ya sufrir,
descansar acaso debes ...
!pero nunca desistir!
Tras las sombras de la duda,
ya plateadas, ya sombrías,
puede bien seguir el triunfo,
no el fracaso que temías;
y no es dable a tu ignorancia,
figurarte cuán cercano
puede estar el bien que anhelas
y que juzgas tan lejano.
Lucha, pues, por más que tengas
en la brega que sufrir ...
!Cuándo esté peor todo,
más debemos de insistir!

Rudyard Kipling

Para la persona que me inspiró desde pequeña a seguir el camino de la ciencia

Dr Luis Arturo Lizcano

ACKNOWLEDGEMENTS

It is a great pleasure to thank everyone who helped me during the process of my Ph.D. This thesis was possible thanks to the presence, support, and friendship of my colleagues, friends, and family.

Kathy Dobinson, more than my supervisor, you became a life example and I thank you for allowing me to do my Ph.D. in your laboratory, and for your guidance, patience, and mentorship throughout this process.

Andre Lachance, thank you for all the guidance, the advice, and the afternoons of discussion at the grad club.

Sangeeta Dhaubhadel, Jim Karagiannis, Mark Gijzen, and Cam Donly, thank you for your valuable advice and feedback throughout my research process, and for your kind words of encouragement and support during the grey hours.

Sandy Grant, thank you for your friendship, wisdom, advice, humor, and endless support.

Aiming Wang, Jamie McNeil, and to the entire Wang lab, there are not enough words to express my gratitude that I feel towards all of you. Thank you for making the transition easier and for all the help, support, advice, and overall, patience.

Dinah Qutob, thank you for your friendship, assistance, support and for pushing me to achieve my goals.

Silvie van Twest, Yinzi Li, and Aaron Simkovich, thank you for being my partners throughout this adventure.

Kuflom Kuflu, thank you for your assistance, wisdom, and support.

Behnaz Saatian, thank you for always being there for me.

To my friends in Ag Canada, thank you for your friendship, your support, the coffee times and the laughter.

Stan, thank you for always believing in me, for your support, patience, and understanding. Thank you for making my life better and for making my future shinier.

And finally, to my family:

Mamá, Papá, Tatis y Aleja, los últimos 6 años de mi vida han sido una época de aprendizaje, no solo a nivel profesional sino también a nivel personal, no habría sobrevivido sin el apoyo incondicional y el amor de todos ustedes. No me alcanzan las palabras para agradecerles por todo lo que soy hoy. Los amo.

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LIST OF ABREVIATIONS

aa	Amino acid
ATMT	*Agrobacterium tumefaciens*-mediated transformation
BM	Basal medium
cDNA	Complementary deoxyribonucleic acid
CM	Complete medium
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate
CU	Cerato-ulmin
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
ddPCR	Droplet digital polymerase chain reaction
DEPC	Diethylpyrocarbonate
DIG	Digoxygenenin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-triphosphate
dpi	Days post-inoculation
DTT	Dithiothreitol
EF1	Elongation factor 1
ENT	Extended N terminus
EST	Expressed sequence tag
EtOH	Ethanol

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gen R	Geneticin resistance
Hyg	Hygromycin B
Hyg R	Hygromycin B resistance
IM	Induction medium
KO	Knockout
LB	Lysogeny Broth
MAPK	Mitogen activated protein kinase
MCS	Microsclerotia
MM	Minimal medium
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
nr	Non redundant
O/N	Overnight
ORF	Open reading frame
OSCAR	One Step Construction of *Agrobacterium* Recombination-ready plasmids
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PI	Propidium iodide
PR	Pathogenesis related
PVPP	Polyvinyl polypyrrolidone
RH	Relative humidity

RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCPFRC	Southern Crop Protection & Food Research Centre
SDS	Sodium dodecyl sulphate
SSPE	Sodium Chloride-Sodium Phosphate-EDTA
TN	Transposon
UBI3	Ubiquitin 3
UV	Ultra-violet
VdATG8	*Verticillium dahliae* ATG 8-like protein
VdGARP1	*Verticillium dahliae* glutamic acid-rich protein
VDH	*Verticillium dahliae* hydrophobin
VGB	*Verticillium* G protein β subunit
VMK1	*Verticillium* mitogen activated protein kinase 1
Vta	*Verticillium* transcription activator of adhesion
WT	Wild-type
YFP	Yellow fluorescent protein

CHAPTER 1: Introduction and Literature Review

1.1.Verticillium spp

The genus *Verticillium* **(**order Phyllachorales, class Sordariomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi) was originally described by Nees von Essenbeck in 1816 and was named after its characteristic branched conidiophores, which form whorls of long phialides producing conidia (Pegg & Brady, 2002, Klosterman et al., 2009). The genus designation *Verticillium* was previously used in a broad sense, with approximately 190 species described. The use of molecular technology has since resulted in the transfer of several originally designated *Verticillium* species to different genera. The genus now contains ten soil-borne species that cause the devastating, vascular wilt disease known as Verticillium wilt (Inderbitzin et al., 2011).

This disease affects a wide range of plant hosts, including trees, weeds, vegetables, flowers, and major agricultural crops (Pegg & Brady, 2002). Members of the genus *Verticillium* are found worldwide, with particularly important negative consequences for crops in temperate regions, although the disease has been reported, to a lesser extent, in subtropical and tropical climates (Pegg & Brady, 2002). Verticillium wilts produce economic losses of more than 50% in crops such as cotton (Friebertshauser & DeVay, 1982), lettuce (Atallah et al., 2011), and potato (Rowe & Powelson, 2002), and up to 75% in strawberries without fumigation (Wilhelm & Paulus, 1980).

Of the ten members of the *Verticillium* genus, *V. dahliae* is the most economically important since it can affect more than 200 host plants (Pegg & Brady, 2002). Even

though they have a narrower host range, other important plant pathogens in the genus include *V. albo-atrum, V. alfalfae, V. longisporum, V. nonalfafae, V. tricorpus,* and *V. zaregamsianum* (Inderbitzin & Subbarao, 2014). Finally, *V. nubilum* causes disease in pathogenicity tests, but is not considered an important pathogen, and little is known about *V. isaacii* and *V. klebahnii* pathogenicity (Inderbitzin & Subbarao, 2014). All *Verticillium* species share the characteristic verticillate (whorled) conidiophore morphology, but differ in the types of resting structure they produce, presence of yellow-pigmented hyphae, and size of the asexual spores called conidia (Inderbitzin et al., 2011).

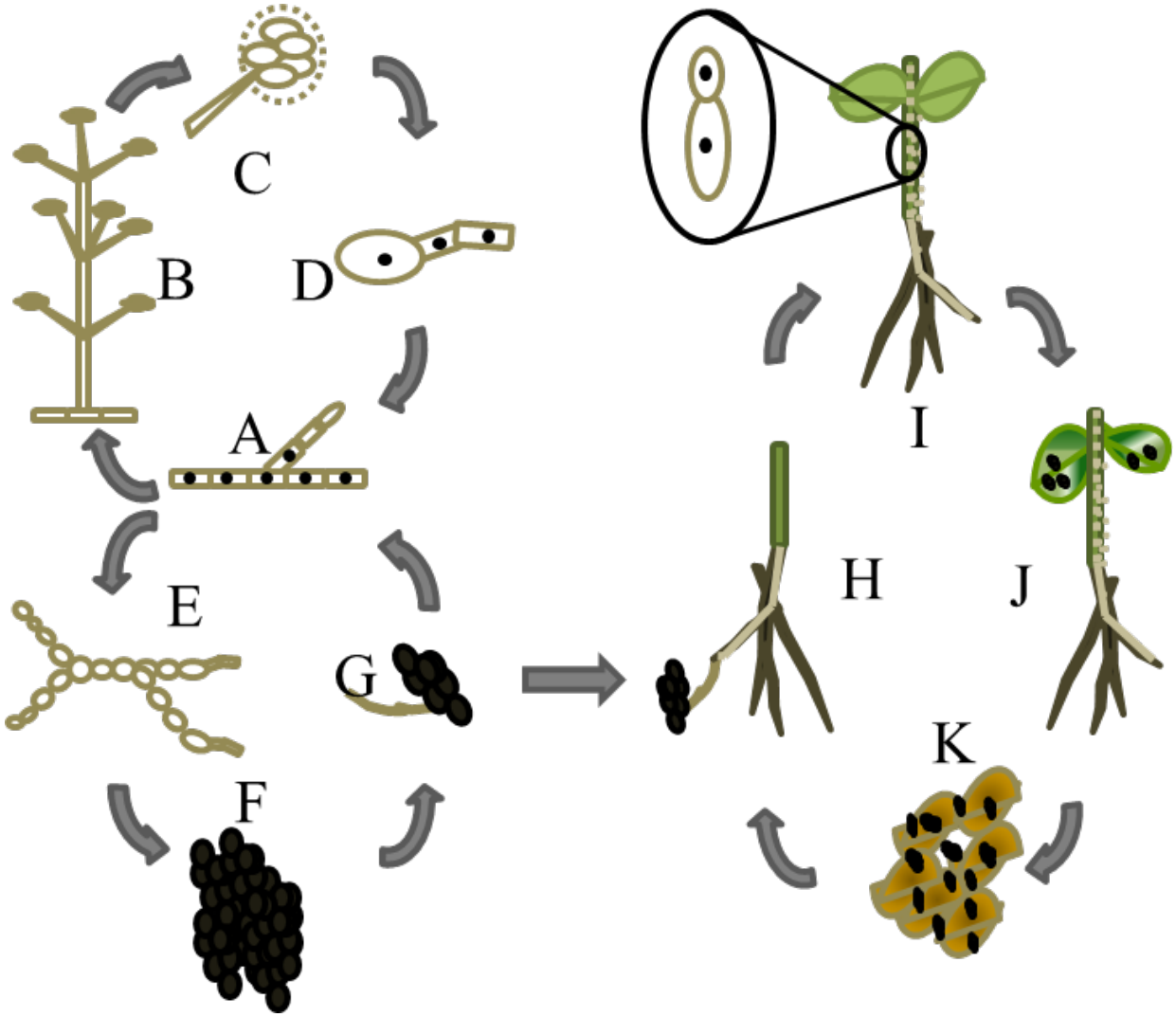
1.1.1 Life cycle of *V. dahliae*

The life cycle of *V. dahliae* (Fig.1) can be divided into three stages: the parasitic stage, the saprotrophic stage, and the dormant stage. In the parasitic stage, the plant root tip is directly penetrated by undifferentiated hyphae germinating from resting structures (i.e. microsclerotia, MCS; Dimond (1955)), and possibly also by appressoria as reported by Vallad and Subbarao (2008). Once the hyphae have grown across the endodermis and reached the xylem, asexual spores (conidia) are formed on specialized cells called conidiophores. Conidia also have the ability to produce spores by budding in a yeast-like manner. This capacity of some fungi to live as both multicellular filamentous and unicellular yeasts is known as dimorphism (Nadal et al., 2008). Fungal colonization of the vascular system occurs as spores are moved along with vascular fluid until they are trapped at vessel end walls. At this point, spores germinate and grow through the walls as mycelia, which can produce more conidia to further colonization (Fradin & Thomma, 2006). Even with the presence of vessel end walls acting as barriers that delay the

movement of conidia through the vascular system, colonization occurs rapidly, and after four days the fungus can be detected in the xylem elements (Gold & Robb, 1995, Heinz et al., 1998, Chen et al., 2004).

Figure 1: Life cycle of *V. dahliae.*

A) Polar hyphal growth that results in B) verticillate conidiophore development. C) Conidiophores produce conidia in mucilaginous heads. D) Conidia germinate and produce hyphae. E) Hyphal cells fragment further by septation, and also E) swell. Lateral budding occurs in this chain of swollen cells, and finally melanisation occurs to produce F) mature microsclerotia. G) Root exudates stimulate microsclerotial germination, and , H) the fungus penetrates the root, grows through the root cortex into the xylem elements, and colonizes the vascular system. I) In the xylem the fungus produces conidia from conidiophores and the conidia have the ability to produce spores in a yeast-like manner. J) The fungus fully colonizes the host and symptoms develop. K) Microsclerotia are produced in dead and dying plant tissue. Microsclerotia are released into the soil in plant debris.


C
B
D
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K

During the early stages of infection by *V. dahliae* some plant species will exhibit wilting when they are exposed directly to sunlight or heat, or under conditions of drought, but will recover overnight. Disease symptoms become more noticeable several weeks to months after the plant has been completely colonized (Fig. 2). Apart from wilting, these symptoms also include leaf interveinal chlorosis and necrosis, epinasty (i.e. the downward bending of leaves due to differential growth rates), premature senescence, stunting, and in highly susceptible plants complete defoliation and death (Fradin & Thomma, 2006). When the host plant begins to senesce and die, the fungus enters the saprotrophic phase, and forms microsclerotia. The dormant phase starts when microsclerotia are released into the soil when the plant tissue decomposes (Wilhelm, 1955, Schnathorst, 1981, Fradin & Thomma, 2006).

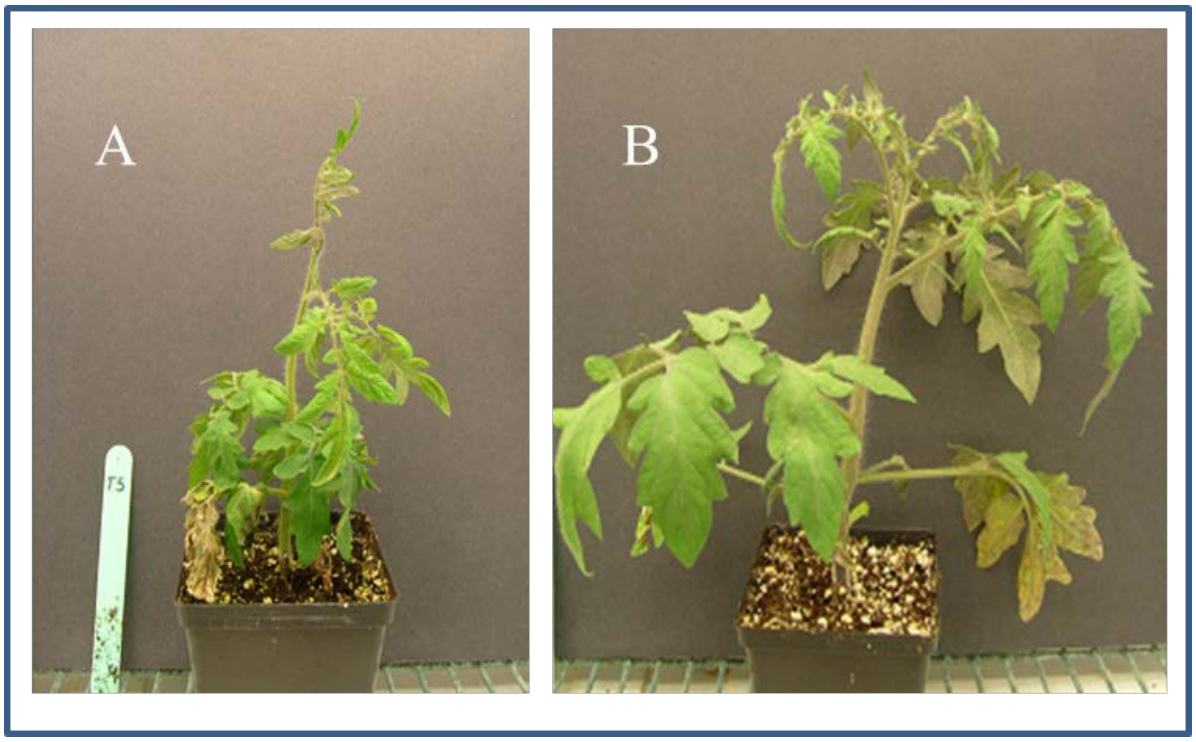


Figure 2: Verticillium wilt symptoms in Bonny Best tomato plants.

A) Bonny Best tomato plant infected with *Verticillium dahliae* wild-type strain Dvd-T5, showing typical Verticillium wilt symptoms: stunting, wilting, chlorosis, and die-back of leaves and branches. B) Mock-inoculated Bonny Best tomato plant. Both plants are approximately 6 weeks old; infection was done on two week old seedlings.

1.1.2 Microsclerotia

Microsclerotia are compact masses of spherical cells with thick, melanised walls. These structures are the main source of disease inoculum in the field, and are resistant to environmental stresses such as desiccation and UV light (Brandt, 1964, Pegg & Brady, 2002). Also, MCS can survive in the soil without a host plant for long periods of time (Wilhelm, 1955, Pegg & Brady, 2002). Microsclerotia originate from highly septate, swollen hyphae that differentiate by budding laterally to produce clusters of spherical cells. These cells originate from thin-walled, vacuolated hyaline cells that further differentiate into two different cell types: i) thick walled melanised cells with food reserves, and ii) thin walled hyaline cells ready to germinate (Gordee & Porter, 1961, Griffiths, 1970). Before microsclerotia reach full maturity, a fibrillar material and melanised particles are secreted between the cells. Fully developed microsclerotia protect a core of live cells by surrounding them with dead, heavily melanized cells (Griffiths, 1970). Melanins, pigments formed by oxidative polymerization of phenolic or indolic compounds, confer environmental protection and are essential to microsclerotial persistence (Wheeler et al., 1978, Hawke & Lazarovits, 1994, Fradin & Thomma, 2006).

Given the significance of microsclerotia in the *V. dahliae* life cycle several studies have been done to characterize the development of these structures. However, early studies, done in the 1960s and 1970s, primarily focused on the biochemistry and physiology of microsclerotial development (Wilhelm, 1955, Gordee & Porter, 1961, Brandt, 1964, Griffiths, 1970, Wheeler et al., 1978, Pegg & Brady, 2002). To date, still little is known

at the molecular level about the developmental pathways that lead to the formation of microsclerotia.

In order to study genes that may be involved in the development of microsclerotia, a cDNA library was developed from a wild-type *V. dahliae* strain isolated from a tomato plant (Dvd-T5) that was grown under conditions that induce near-synchronous development of microsclerotia (Neumann & Dobinson, 2003). From the genes identified in this study, approximately half were involved in energy, primary metabolism, and/or protein synthesis. Two genes found in the cDNA library, *VDH1* and *VDATG8*, are involved in microsclerotia development. First, the gene that encodes the class II hydrophobin VDH1 was identified in the library, and subsequently characterized. The expression of *VDH1* correlates with MCS development, and the *vdh1* mutant strain is amicrosclerotial (Klimes, 2006, Klimes & Dobinson, 2006, Klimes et al., 2008). The second gene is highly similar in sequence to the yeast macroautophagy marker gene *ATG8*. The *vdatg8* mutant is defective in microsclerotial production in *V. dahliae* Dvd-T5 (Van Twest, 2011), but no defect in microsclerotial production was detected in the cotton-infecting strain Vd8 (Zhou et al., 2013). Apart from these two genes, genes encoding a mitogen-activated protein (MAP) kinase (*VMK1*, Rauyaree et al., 2005), glutamic acid-rich protein (*VdGARP1*, Gao et al., 2010) (23) [29], G protein β subunit *(VGB*, Tzima et al., 2012), and a transcription activator of adhesion (*Vta2*, Tran et al., 2014) have also been implicated in MCS development. Mutations of either *VdGARP1* or *Vta2* result in delays in microsclerotial development (Gao et al., 2010, Tran et al., 2014). Mutations of *Vmk1* result in an amicrosclerotial phenotype, while mutations of *VGB* result in increased microsclerotial production (Rauyaree et al., 2005, Tzima et al., 2012).

1.1.3 Disease control

Verticillium wilt causes the loss of billions of dollars in crops each year, and there is limited natural disease resistance (Pegg & Brady, 2002, Fradin & Thomma, 2006). The main source of inoculum in the field is microsclerotia, which accumulate rapidly, are difficult to eradicate by fumigation, and can survive in soil without a host plant for up to 15 years. Additionally, only one MCS per gram of soil is enough to cause disease in crops like strawberry and tomato (Wilhelm, 1955, Grogan et al., 1979, Nicot & Rouse, 1987). Moreover, the fact that *Verticillium* has such a broad host range, which continuously expands as former non-hosts become susceptible (Klosterman et al., 2009), allows it to increase its inoculum reservoir not only as microsclerotia but also as hyphae and conidia inside host plants and non-host plant roots (Subbarao et al., 1995, Pegg & Brady, 2002, Atallah et al., 2011).

Usually, disease management focuses on crop rotation and pathogen avoidance, but *V. dahliae*'s wide host range (Klosterman et al., 2009) leaves few areas of agricultural land free of inoculum. The most common approaches to reduce inoculum levels are soil solarisation and chemical fumigation (Ioannou, 2000, Fradin & Thomma, 2006). However, solarisation needs special environmental conditions to work properly. In addition, while the most commonly used soil fumigant against *Verticillium spp* was methyl bromide, its use has been prohibited because of its environmental impact (Fradin & Thomma, 2006, Enebak, 2012, Korthals et al., 2014).

1.2 Hydrophobins

Hydrophobins are small, secreted proteins (approximately 100 amino acids in length) found uniquely in filamentous fungi. Hydrophobins usually exist as gene families, with an average of two to ten members (Wösten, 2001, Sunde et al., 2008, Littlejohn et al., 2012). These proteins have the ability to self-assemble spontaneously at hydrophobic-hydrophilic interfaces, and form amphipathic structures outside the fungal cell wall (Wessels, 1997, Wösten, 2001).

The amino acid sequences of different hydrophobins show very little similarity (23%) and identity (11%) (Wessels, 1994). However, the presence a characteristic conserved pattern of eight cysteine (Cys) residues that form four disulfide bridges (Cys1–Cys6, Cys2–Cys5, Cys3–Cys4, Cys7–Cys8) plus the similarity of their hydropathy patterns was used to group this proteins toghether (Wessels, 1994, Wessels, 1997, Wösten, 2001, Linder et al., 2005, Kwan et al., 2006). Based on the distance between Cys residues, hydrophobicity patterns, and solubility, these proteins have been divided in two classes (Wessels, 1997, Wösten, 2001, Linder et al., 2005). Class I hydrophobins show more variability than class II in their amino acid sequences and in the spacing between the Cys residues. Despite this variation, class I hydrophobins produce stable assemblies, insoluble monolayers in the form of fibrillar structures called rodlets. The rodlets can only be solubilized with strong acids and once the conditions are appropriate again (i.e. by removing the acids from the water), the proteins will polymerise again into rodlets (Wessels, 1997, Wösten, 2001, Bayry et al., 2012). In class II hydrophobins sequence and Cys residue spacing are more conserved than in class I. Class II hydrophobins also

have the ability to form monolayers, but these lack the rodlet morphology of class I monolayers, and can be easily solubilized with organic solvents and detergents.

Although the two-class system is generally accepted, some bioinformatics studies suggest the possibility that different and intermediate hydrophobin forms exist (Jensen et al., 2010, Seidl-Seiboth et al., 2011, Littlejohn et al., 2012). These intermediate forms, found in both *Aspergillus* and *Trichoderma*, have hydropathy patterns and a Cys spacing that is different from those of both established class I and class II hydrophobins (Jensen et al., 2010, Seidl-Seiboth et al., 2011).

1.2.1 The roles of hydrophobins in plant pathogenic fungi.

Hydrophobins have been implicated in a variety of biological functions, including development of aerial hyphae, spore production and dispersal, formation of fruiting bodies, adhesion to host surfaces, and development of penetration structures (Wessels, 1997, Wösten, 2001, Whiteford & Spanu, 2002, Linder et al., 2005, Bayry et al., 2012). The vegetative hyphae of filamentous fungi are exposed to different types of environments throughout their life cycles. When the hyphae are growing in moist environments, their surface is hydrophilic. Conversely, and in contrast to those of *Verticillium* (Klimes et al., 2008), aerial hyphae and conidia in most fungi are hydrophobic because the hydrophobic side of the amphipathic hydrophobin aggregate is exposed to the environment, while the hydrophilic side remains in contact with the cell wall polysaccharides (Wessels, 1997, Wösten, 2001, Bayry et al., 2012). This property allows hydrophobins to act as surfactants and reduce the surface tension of the substratum on which the fungus grows (Wösten, 2001). In *Magnaporthe oryzae* (causal agent of rice

blast), the hydrophobin *MPGI* is essential for attachment to the rice leaf and for the formation of appressoria, while the hydrophobin *MHP1* is involved in the latest stages of pathogenicity (Talbot et al., 1996, Kim et al., 2005). In contrast, in the corn pathogen *Fusarium verticillioides* none of the five hydrophobins studied have a role in pathogenicity in corn seedlings, and deletion of hydrophobins *HYD1* or *HYD2* causes defects in microconidial chain formation (Fuchs et al., 2004).

Hydrophobins are also thought to have important roles in the plant-pathogen interaction. Specifically, it has been hypothesized that by covering fungal structures the hydrophobins may act as a barrier to avoid recognition by the plant immune system (Wösten, 2001). On sexual and asexual spores (conidia) that are produced on aerial hyphae, the hydrophobin layer typically renders the hydrophilic surface hydrophobic and thus, moisture-resistant, resulting in dry spores that are easily dispersed through the air(Sunde et al., 2008). In plant and insect pathogens, some hydrophobins have also been reported to be pathogenicity factors, but their specific role in fungal virulence is still not fully understood (Bayry et al., 2012). For example, the mutants of the hydrophobins *MPG1* and *MHP1* of *Magnaporthe grisea* (the rice blast fungus) show reduced virulence, with loss of viability, and reduced capacity to infect and colonize susceptible rice cultivars (Talbot et al., 1996, Kim et al., 2005).

1.3 Rationale and Objectives

The characteristic host xylem colonization by *V. dahliae*, plus the resistance to environmental stresses and long term survival of MCS, make it very difficult to eradicate the *Verticillium* inoculum in soil, and within growing crops. Understanding the molecular

mechanisms involved in the *Verticillium* pathogenicity and MCS development could provide potential targets to develop new, more effective disease control strategies.

The objectives of my research centered on the study of hydrophobin genes in *V. dahliae,* and characterization of the ENT hydrophobin gene *VDH5*. The study of the hydrophobin genes involved gene expression analyses under different growth conditions, and the characterization of *VDH5* involved both targeted gene disruption and deletion, as well as deletion of the ENT domain of the gene.

Finally, in order to gain more knowledge about the interaction at a molecular level between this pathogen and its host the response of tomato plants to two of the hydrophobin genes (*VDH1* and *VDH5*) was studied by observing the expression of pathogenesis related genes (*PR1*, *PR3*, and *PR5*) in plants inoculated with the *Δvdh5* mutant, the *vdh1* strain, or the wild-type strain.

CHAPTER 2: Materials and Methods

2.1 Fungal and Bacterial Strains

The wild type strain Dvd-T5 (Dobinson et al., 1997) was the parental strain used for generation of the *VDH5* deletion (knock out (KO)) strain *Δvdh5*, *VDH5* extended N terminus (ENT) domain deletion strain, (*vdh5ΔENT*), and *VDH5*-Yellow Fluorescent Protein-tagged (*VDH5::YFP*) strains. Each strain used in this study comes from a single germinating conidium. Single-conidium cultures were generated by harvesting conidia, from aerial mycelium grown on an agar medium, with a sterile loop, and spreading them onto 4% water agar medium. After incubation for one night at 24˚C, single germinated conidia were transferred to Complete agar medium (CM (Appendix 1), van den Hondel et al., 1991, as modified by Dobinson (1995)), and incubated at 24˚C for five to seven days (Dobinson, 1995). Long-term storage of the strains was done by placing sterile 1.27 cm filter discs (Schleicher and Schuell, Keene, NH, USA) near the growing colonies. The cultures were incubated until the filters were completely covered by mycelium (Valent et al., 1991, Dobinson et al., 1996). The fungus-covered filters were then placed in sterile Petri dishes and into a desiccating chamber for approximately one month. Finally, the dried filters were stored in sterile glassine envelopes (West Waco, TX), at -20˚C over desiccant.

The fungal cultures were maintained on CM agar with the appropriate antibiotic. Hygromycin B-resistant strains were grown on CM agar with 25 µg hygromycin B mL^{-1}, while geneticin-resistant strains were grown on CM agar with 50 µg geneticin mL^{-1}. In order to achieve near-synchronous microsclerotial development, spores were spread

evenly on Basal medium (BM, appendix 1) (van den Hondel et al., 1991), as modified by Dobinson et al. (1997)) lacking antibiotics. Liquid cultures were inoculated by transferring five plugs (5 mm diameter each) of actively growing mycelium into 250 mL flasks containing 35 mL CM medium, and incubated in a Lab-Line Orbit Incubator-Shaker model G25 (Lab-Line Instruments, Inc. IL USA) at 24˚C in the dark, with shaking at 150 rpm.

Agrobacterium tumefaciens strain AGL-1 (Lazo et al., 1991) was provided by Seogchan Kang (Pennsylvania State University), and used to transform *V. dahliae* spores. All plasmids were maintained in *Escherichia coli* XL1-Blue MRF' (Stratagene). All bacterial strains were grown in Lysogeny Broth (LB, appendix 1) (Bertani, 1951) or agar medium amended with either kanamycin [50 µg mL^{-1}], ampicillin [100 µg mL^{-1}], chloramphenicol [25 µg mL^{-1}] or spectinomycin [100 µg mL^{-1}].

2.2 Bioinformatics Analyses

Each hydrophobin gene sequence was analysed using the BlastN and BlastX algorithms to search the NCBI non-redundant (nr) database. The Signal P 3.0 algorithm (Nielsen et al., 1997) was used to determine if VDH2, 3, 4 or 5 could be secreted proteins. Hydropathy plots, generated with ProtScale at the ExPASy server, and the Kyte and Doolittle scale (Kyte & Doolittle, 1982, Gasteiger et al., 2005), were used to classify *VDH2, 3, 4* and *5* as hydrophobins. Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the hydrophobin amino acid sequences, and their chromosome localization on *V. dahliae* was obtained from the

Broad Institute *Verticillium* group database (VDG, http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html).

A BlastP search with each of the hydrophobin amino acid (aa) sequences against the NCBI nr protein database was conducted and the first one hundred hits of each search were analyzed. The sequences that did not contain the features of hydrophobins (i.e. signal peptide and eight cysteine residues) were eliminated from the analysis. At the end 80 protein sequences were used to generate a phylogenetic tree, with the MEGA 5 program (Tamura et al., 2011), using the Neighbor-Joining algorithm, and a bootstrap analysis of 500 replicates.

2.3 Nucleic Acid Isolation

2.3.1 Bacterial plasmid and fungal DNA extraction

Bacterial cultures were grown overnight (O/N) at 37ºC, with shaking at 200 rpm, in LB amended with the appropriate antibiotic. Plasmids were extracted with the QIAprep Miniprep Kit (Qiagen Sciences, Maryland 20874, USA) following the manufacturer's instructions.

To extract genomic DNA from *V. dahliae* a glass bead breakage method (Dobinson, 1995) was used. Small plugs of cultures grown on CM agar were transferred to liquid CM, and grown for 4 to 6 days at 24ºC, with shaking at 150 rpm. Spores were separated from mycelia by filtering through sterile Miracloth (Calbiochem, La Jolla, CA, USA). The spores were then pelleted by centrifugation at 11872 g for 10 min at 4ºC. The medium was completely removed, and the spores were resuspended in 200 µL spore-

breaking buffer (SBB, appendix 1). Glass beads of approximately 0.5 mm diameter were added, and the suspension was vortexed for a total of 3 min, alternating between 30 sec vortexing and 30 sec standing on ice. Spore DNA was extracted by adding an additional 200 µL SBB and 300 µL phenol-chloroform (1:1) followed by an additional 30 sec vortex and a 5-minute centrifugation at 13500 g. The aqueous phase was transferred to a new microfuge tube and the procedure was repeated with 200 µL of phenol-chloroform (1:1), and chloroform. DNA was precipitated from the aqueous phase by the addition of 0.5 volume 7.5 M ammonium acetate, and two volumes 100% ethanol (EtOH), followed by overnight incubation at -20ºC. Finally, the precipitated DNA was centrifuged for 30 min at 13500g, 4˚C, and the pellet was washed with EtOH 70% and dissolved in TE buffer containing RNaseA (1 µg mL^{-1}).

2.3.2 Fungal RNA extraction

For fungal RNA isolation, Trizol® Reagent (Invitrogen Canada Inc., Burlington, ON, Canada)was used following the manufacturer's instructions, as modified by Neumann and Dobinson (2003)). In brief, CM agar plates covered with cellulose membranes (Research Products International Corp., Mount Prospect, IL, USA), or flasks with 35 mL of CM were inoculated with 10^5 spores and incubated at 24ºC for 4 days (flasks shaking at 150 rpm). To harvest the cultures from the agar plates, the cellophane was lifted from the plates and flash-frozen in liquid nitrogen, and the mycelium was subsequently ground using a pre-chilled mortar and pestle. Liquid cultures were filtered through sterile Miracloth (Calbiochem, La Jolla, CA, USA) in order to separate the mycelia from conidia. The filtrate was centrifuged (11872 g for 10 min) at 4ºC to pellet conidia. After

removing the supernatant, the conidia were ground in liquid nitrogen. Ground fungal material was resuspended in 5 mL Trizol® Reagent. After five min incubation at room temperature, one mL chloroform was added and the suspension was vortexed, incubated for three min at room temperature, and centrifuged for 15 min at 12000 g at 4ºC. RNA was precipitated from the aqueous phase by adding 2.5 mL of isopropanol and incubating at room temperature for 10 min. The samples were then centrifuged at 4˚C/1200 g for 10 min. The RNA pellet was washed with 75% ethanol and air dried. RNA was finally dissolved in 50 µL water treated with 0.1% diethylpyrocarbonate **(**DEPC).

2.3.3 Plant RNA extraction

For plant RNA extraction, 14 to 19 day-old tomato plants were harvested, and immediately flash frozen in liquid nitrogen. Tissue that was not immediately processed was stored at -80˚C. Frozen plant tissue was ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. RNA extraction was done with Trizol® Reagent following the manufacturer's instructions, adding to the Trizol® Reagent 1% polyvinyl polypyrrolidone (PVPP) and 2% dithiothreitol (DTT) in order to reduce and absorb polyphenols, abundant in plant tissues, that interfere with the quality of nucleic acids (Xiong et al., 2011).

2.4 Polymerase Chain Reaction Amplifications (PCR)

Two DNA polymerases were used regularly for PCR amplification from genomic DNA, cDNA and plasmid templates: Platinum Taq polymerase (Invitrogen, Canada Inc.,

Burlington, ON, Canada), and Phusion Hot Start II DNA Polymerase (Thermo Scientific, Ottawa, ON, Canada).

Unless otherwise specified, reactions (25 µL) with Platinum Taq polymerase contained 10 ng genomic DNA or 1 ng plasmid DNA, 1 X buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 5.12 pmoles of each primer, and 0.5 U Platinum Taq polymerase. Amplification was done with an initial denaturation of the template at 94ºC for 2 min, followed by 30 cycles: 94ºC for 45 sec, 60 to 65ºC annealing for 45 sec, 72ºC extension for 1 min/kilobase (kb), and a final 5 min extension at 72ºC.

Unless otherwise specified, reactions (25 µL) with Phusion Hot Start II DNA Polymerase contained 50 ng genomic or 1 ng plasmid DNA, 1 X buffer HF, 250 µM each dNTP 12.5 pmoles of each primer, 3% DMSO, and 0.5 U Phusion Hot Start II DNA Polymerase. Amplification was done with an initial denaturation of the template at 98ºC, for 30 sec, followed by 35 cycles: 98ºC for 10 sec, 60 to 65ºC annealing for 30 sec, 72ºC for 30 sec/kb, and a final 10 min extension at 72ºC.

All amplification reactions were done using a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems®). One tube in which the template DNA was replaced with sterile distilled water was used as a negative control for all PCR experiments.

2.5 Gene Expression Assays

Expression of the *V. dahliae* hydrophobin genes was assessed by droplet digital PCR (ddPCR) using FAM-BHQ dual-labelled probes. RNA was extracted from WT strain

Dvd-T5 cultures grown for four days under various conditions, including in liquid or on agar versions of a nutrient-rich medium (CM), or a nutrient-limited medium (BM), as described above. Expression of the hydrophobin genes in tomato plants inoculated with *V. dahliae* Dvd-T5 (WT) at zero to five days post inoculation (dpi) was also assessed.

Expression of tomato pathogenesis related (PR) 1, 3, and 5 genes, in plants inoculated with *V. dahliae* Dvd-T5 (WT), VDAT85-3 (*Δvdh5*), or VDAT2-17 (*vdh1*), and grown for an additional one to five dpi, was evaluated by reverse transcription quantitative PCR (RT-qPCR). The primers and probes used in each assay are listed in Table 1.

Table 1: List of primers and probes used in gene expression assays

Primers and probes names*	Type of assay	Remarks
pBeta tub Fw/pBeta tub Rv Beta tub Probe	ddPCR	*V. dahliae* Tubulin beta chain gene.Used as reference gene for hydrophobin gene expression during growth on agar and in liquid media.
pActin Fw1/pActin Rv1 Actin Probe	ddPCR	*V. dahliae* Actin gene. Tested as reference gene for ddPCR hydrophobin gene expression during growth on agar and in liquid media.
VDH1 Fw2/VDH1 Rv2 Vdh1 Probe	ddPCR	*V. dahliae* hydrophobin 1 gene.
VDH2 Fw3/VDH2 Rv1 Vdh2 Probe	ddPCR	*V. dahliae* hydrophobin 2 gene.
pVdh3 Fw2/ VDH3 RV1 Vdh3 Probe	ddPCR	*V. dahliae* hydrophobin 3 gene.
pVdh4 Fw/pVdh4 Rv Vdh4 Probe	ddPCR	*V. dahliae* hydrophobin 4 gene.
pVdh5 Fw/pVdh5 Rv Vdh5 Probe	ddPCR	*V. dahliae* hydrophobin 5 gene.
LepUBI3 Fw1/LepUBI3 Rv1 Le UBI3 Probe 1	ddPCR	Tomato glyceraldehyde 3- ubiquitin 3. Used as reference gene for *in planta* assays.
LepGAPDH Fw2/LepGAPDH Rv2 Le GAPDH probe	ddPCR	Tomato glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Used as reference gene for *in planta* assays.

Table 1 (continued). List of primers and probes used in gene expression assays

TomEF1FW/TomEF1RV	RT q PCR	Tomato elongation factor 1 gene. Used as reference gene for Pathogenesis Related gene expression in inoculated plants assay.
Sl PR1 FW2/ Sl PR1 RV1	RT q PCR	Tomato pathogenesis-related protein 1
Sl PR3 FW1/ Sl PR3 RV1	RT q PCR	Tomato pathogenesis-related protein 3
Sl PR5 FW1/ Sl PR5 RV2	RT q PCR	Tomato pathogenesis-related protein 5

* The sequences of all primers and probes are provided in Appendix 2

2.5.1 Reverse transcription quantitative PCR (RT-qPCR)

One µg of RNA, pre-treated with amplification grade DNAse I, was reverse transcribed with SuperScript II reverse transcriptase and oligo (dT) 12-18 primer (Invitrogen Canada Inc., Burlington, ON, Canada) according to supplier's protocols.

Expression of tomato plant PR genes was assessed by RT-qPCR. Standard curves were generated with serial 5-fold dilutions of template cDNA. Each 10 µL amplification reaction contained 1X SsoFast™ EvaGreen® super mix (BioRad, Hercules, CA, USA), 6 pmoles of each primer, and cDNA template (various dilutions). PCRs were done in 96 well WHT-CLR hard shell microtitre plates sealed with Microseal® 'B' Film (BioRad, Hercules, CA, USA). Amplification was done using a CFX96 Real Time System (C1000 Thermal Cycler, BioRad, Hercules, CA, USA), with an initial 2 min denaturation at 95 ˚C followed by 40 cycles of 5 sec denaturation at 95˚C and 15 sec annealing at 62˚C. The melting curve started at 75˚C, and increased by 0.2˚C increments for 10 sec to 95˚C. Relative transcript abundance was calculated using the ΔΔCT method (BioRad CFX manager software version 3.0), relative to Tomato Elongation factor 1 gene (*EF1*), and normalized against the plant inoculated with the WT strain.

The efficiency of the primers and probes for ddPCR was assessed by RT-qPCR. Standard curves were generated with 5-fold dilutions of template cDNA. Each 10 µL amplification reaction contained 1 X SsoAdvanced™ Universal Probes Supermix (BioRad, Hercules, CA, USA), 9 pmoles of each primer, 2.5 pmoles fluorogenic probe, and cDNA template (various dilutions). PCRs were done in 96 well WHT-CLR hard shell microtitre plates

sealed with Microseal® 'B' Film (BioRad, Hercules, CA, USA). Amplification was done using a CFX96 Real Time System (C1000 Thermal Cycler, (BioRad, Hercules, CA, USA), with an initial 3-minute denaturation at 95˚C followed by 40 cycles of 15 sec at 95˚C and 15 sec annealing/extension at 60˚C. The efficiency was calculated with BioRad CFX manager software version 3.0, and an efficiency between 90 and 110 was considered satisfactory for the designed primers/probe set to be used in the droplet digital PCR procedure.

2.5.2 Droplet digital PCR (ddPCR)

For the ddPCR, a 20 µL reaction mixture containing 1X ddPCR™ Supermix for Probes (Bio-Rad), 18 pmoles each primer, and 5 pmoles probe and cDNA template was mixed with 70 µL of ddPCR droplet generation oil in an eight-channel disposable droplet generator. The resulting 40 µL emulsion (containing the initial 20 µL reaction mix partitioned into approximately 20 000 monodispersed droplets) was transferred to a 96-well polypropylene plate (twin.tec PCR plates Eppendorf, Mississauga, ON, Canada), and the plate was sealed with foil. Amplifications were done in a T100 Thermal Cycler (BioRad, Hercules, CA, USA), with an initial 10 min template denaturation step at 95ºC, followed by 45 cycles: 94ºC for 30 sec and 60ºC annealing for one min, ending at 98˚C for 10 min. After completion of the PCR amplification the fluorescence of each individual droplet was read in a QX100 droplet reader (BioRad, Hercules, CA, USA), with a fluorescence positive or negative signal indicating whether or not the DNA target was present in that particular droplet after partitioning (Hindson et al., 2011).

2.6 Sequence Analysis

DNA sequencing was done by Eurofins (Louisville, KY, USA), or at the sequencing facility of Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre (SCPFRC) in London, ON, the latter using an ABI prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), with fluorescence-dye-labeled terminators, and the Sanger dideoxy-chain terminating method (Sanger et al., 1977) coupled with capillary electrophoresis.

2.7 Production of Gene Deletion, Complement, and Fluorescent-Protein Tagged Gene Transformation Vectors

2.7.1 Construction of *VDH5* gene deletion plasmid

The OSCAR (One Step Construction of *Agrobacterium* Recombination-ready plasmids) methodology (Paz et al., 2011), based on the Invitrogen Gateway ® system, was used to generate the *VDH5*gene deletion vector. Briefly, the 5' flanking region of the *VDH5* ORF was amplified with the primers OscarVDH5 1 and OscarVDH5 2 (Appendix 2), and the 3' flanking region of the *VDH5* ORF with the primers OscarVDH5 3 and OscarVDH5 4 (Appendix 2). The amplicons were cloned, using BP clonase ® (Invitrogen Canada Inc., Burlington, ON, Canada), into the *Agrobacterium tumefaciens*-mediated transformation (ATMT) binary vector. The resulting gene deletion vector contains the *hph* gene, which encodes hygromycin B phosphotransferase (conferring resistance to hygromycin B), surrounded by the flanking regions of *VDH5*.

2.7.2 Construction of *vdh5TN* disruption and *VDH5* complement vectors

To construct the *vdh5TN* and *VDH5* complementation vectors a PCR was done with the primers Vdh5 Fw I (Appendix 2), annealing 1839 bases upstream, and Vdh5 Rv II (Appendix 2), annealing 977 bases downstream of the Vdh5 WT ORF. The amplicon generated by PCR was purified using either the QIAquick PCR Purification for PCR mix or the QIAquick Gel Extraction Kit for gel bands (Qiagen Sciences, Maryland 20874, USA). Once the desired size amplicon was obtained it was cloned into a pGEM®-T Easy Vector (Promega Corporation, Madison, WI, USA), and electroporated into *E. coli* XL1-Blue MRF' using a MicroPulser Electroporation Apparatus (Bio-Rad, Hercules, CA, USA). The presence of the correct insert was verified by sequencing. The plasmid was then cut with the restriction enzyme EcoRI and the resulting insert fragment was sub-cloned into EcoRI-digested binary vector pDHt (Mullins et al., 2001). The cloned gene was then subjected to *in vitro* transposon mutagenesis as described by Dobinson et al. (2004), using the EZ::TN system (Epicentre Technologies, Madison, WI, USA). Briefly, a PCR using the pMOD PCR Fw and Rv primers (Appendix 2, Epicentre Technologies, Madison WI) was done to amplify the transposon sequence from the ATMT vector pSK848, which contains a geneticin-resistance gene. The amplified transposon was inserted into the *VDH5*-carrying binary vector by the EZ-Tn5 Transposase (Epicentre Technologies, Madison WI), according to the manufacturer's directions. Mutagenized plasmids were propagated in *E. coli* strain XL1-Blue MRF'. For the *vdh5TN* disruption vector, clones containing the transposon in the middle of the VDH5 ORF were identified by PCR amplification using the primers VDH5 FW2 and VDH5 RV2 (Appendix 2), and sequencing with primers pMOD sequencing Fw and Rv (Appendix 2, Epicentre

Technologies, Madison WI) . Similarly, for the *VDH5* complement vector, clones containing the transposon inserted downstream of the *VDH5* open reading frame (ORF) were identified by PCR amplification using the primers Vdh5 TN screen Fw 2 and Vdh5 TN Screen Rv1 (Appendix 2), and confirmed by sequencing with primers pMOD sequencing Fw and Rv (Appendix 2, Epicentre Technologies, Madison WI) .

2.7.3 Construction of *VDH5-ΔENT* domain vector

To construct the *VDH5-ΔENT* vector, PCR amplifications were first done of i) the sequence from 1839 bp upstream of the *VDH5* ORF to the beginning of the ENT domain (ENT 1 fragment), using primers Vdh5 Fw I and VDH5 ENT Rv (Appendix 2), and ii) the sequence from the end of the ENT domain to 977 bp downstream of the *VDH5* ORF, with primers VDH5 ENT Fw and Vdh5 Rv II (Appendix 2, ENT 2 fragment). Both VDH5 ENT Fw and Rv primers contain a BamHI restriction sequence in their 5' termini. The purified amplicons generated by the PCR were then cloned into a pGEM®-T Easy Vector and the resulting plasmids electroporated into *E. coli* XL1-Blue MRF'. In order to verify the amplification accuracy the cloned amplicons were sequenced. The plasmid containing the ENT1 fragment was cut with PstI and BamHI to release the ENT1 fragment, and the plasmid containing the ENT 2 fragment was likewise cut with EcoRI and BamHI. The two ENT fragments were then ligated into pDHt (Mullins et al., 2001) that had been digested with both EcoRI and PstI. Clones containing the correct plasmid were identified by digestion with EcoRI and BamHI, and by sequencing. The selectable marker (geneticin resistance gene) was inserted into the plasmid by *in vitro* transposon mutagenesis (Dobinson et al., 2004) as described above.

2.7.4 Construction of VDH5::Yellow Fluorescent Protein (YFP) fusion vector

To construct the *VDH5::YFP* fluorescent fusion gene-containing strain the sequence from 1720 bp upstream of the *VDH5* ORF to the codon immediately before the *VDH5* stop codon was amplified using primers FP Vdh5 prom Fw2 and FL-VDH5 Rv (Appendix 2). The purified PCR amplicon was cloned into pGEM®-T Easy Vector and electroporated into *E. coli* XL1-Blue MRF'. Correct sequence of the amplicon was verified by sequencing as described above. Once the sequence was verified, a PCR with the primers attB_F1 and attB_R1 (Appendix 2) was done to incorporate the full length attB adaptor sequences at the ends of the original product. The sequence was cloned into the pFPL-Yh binary vector (Gong et al., 2014) by doing a BP recombination reaction using BP clonase ® (Invitrogen Canada Inc., Burlington, ON, Canada), followed by an LR recombination reaction using LR clonase ® (Invitrogen Canada Inc., Burlington, ON, Canada). The resulting vector pVdh5-YFP was used for *Agrobacterium tumefaciens*-mediated transformation (ATMT).

2.7.5 *Verticillium dahliae* transformation: *Agrobacterium tumefaciens*-mediated transformation (ATMT)

Vectors p*Δvdh5*, p*vdh5TN*, p*vdh5ΔENT*, p*VDH5::YFP*, and p*VDH5*-complement were transferred into *A. tumefaciens* for transformation of the appropriate *V. dahliae* strains (i.e. p*ΔVDH5*, p*vdh5TN* p*VDH5ΔENT*, p*VDH5::YFP* were used to transform Dvd-T5 (WT), and p*VDH5*-complement was used to transform VDAT85-3 (*ΔVDH5*)) as described by Mullins et al.,(2001), following modifications by Dobinson et al. (2004)). Briefly, *A. tumefaciens* strain AGL-1, containing one of the vectors described above, was

grown at 28°C for 2 days in minimal medium (MM; Hooykas et al., 1979) supplemented with kanamycin (50 µg/mL). The cells were then diluted in induction medium (IM, Bundock et al., 1995) to an OD_{600} of 0.15, and further incubated for an additional 6 h at 28°C. The culture was then mixed with an equal volume of a Dvd-T5 conidial suspension (10^6 conidia per mL) generated from a 4 day-old liquid culture. Two hundred µL of the mixed suspension was plated onto 0.45 µm pore, 45-mm diameter nitrocellulose filters (Whatman, Hillsboro, OR, USA) that were overlaid onto co-cultivation medium amended with 200 µM aceto-syringone. The plates were incubated at 24°C for 2 days, and then the filters were transferred to selective medium (CM containing 25 µg hygromycin B/mL or 50µg geneticin/mL as the selection agent, as well as 100 µg moxalactam/mL and 200 µM cefotaxime to kill the *A. tumefaciens* cells). PCR and Southern blot hybridization analyses were used to identify transformant strains.

Southern blots were done as previously described by Dobinson et al. (1998)). Five hundred ng genomic DNA was digested with specific restriction enzymes. The digested fragments, plus 1 ng of plasmid DNA as a positive control, were separated by size via electrophoresis through a 0.8% agarose gel. The gel was then soaked in 0.25 N HCl for 10 min, in Southern denaturing buffer for 30 min, and finally soaked in neutralizing buffer for 30 min. The fragmented genome was then transferred, by capillary blotting with 20 X sodium chloride-sodium phosphate-EDTA (SSPE) pH 7.4, to a Hybond N+ membrane (Amersham Biosciences, Baie d'Urfé, QC), where the DNA was finally fixed by UV cross-linking (CL-1000 UV Crosslinker, UVP INC, CA, USA). For prehybridization, the membrane was submerged in prehybridization buffer (Appendix 1) for two hours at 65˚C (VWR® Hybridization Oven, Model 5420, VWR International).

Hybridization was done by transferring the membrane into hybridization buffer (Appendix 1) and incubating at 65˚C overnight. The next day the membrane was washed three times in low stringency buffer (Appendix 1) and then three times in high stringency buffer (Appendix 1) at 65˚C for 20 min each time. The membrane was incubated for 10 min at 37˚C with the chemiluminescent substrate CSPD® (4-methoxyspiro{1,2-dioxetane-3,2'-(5'- chloro) tricyclo[3.3.1.13,7]decan}-4-yl)phenylphosphate) (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's directions, with the following modifications: i) digoxigenin (DIG) antibody was diluted 1:20000, and ii) CSPD was diluted 1:4000 (Klimes, 2006). The membrane was then exposed overnight to X-ray film (Curix Ultra UV-G-Plus Medical X-Ray film, Belgium) to visualize the probe hybridized with the target.

For the construction of DIG-labelled DNA hybridization probes, DIG labelled dUTPs were incorporated into the probes by PCR amplification of *V. dahliae* genomic DNA using the DIG Labelling Mix (Roche Molecular Diagnostics). Primers OSCAR VDH5 3 and OSCAR VDH5 4 (Appendix 2) were used to amplify and DIG label an 882 bp probe of the *VDH5* ORF 3'flanking region. This 3'flank region probe was used on the Southern blot screening of the *vdh5 V. dahliae* transformants. Primers Vdh5 TN screen Fw 2 and OSCAR VDH5 2 (Appendix 2) were used to amplify and DIG label a 1089 bp probe of the *VDH5* ORF 5'flanking region. The 5'flank region probe was used for Southern blot screening of the *VDH5ΔENT*, and *VDH5*-complement *V. dahliae* transformants.

2.8 Strain Characterization

2.8.1 Fungal staining and confocal microscopy

Localization of VDH5::YFP on *V. dahliae* structures was done with a Leica TCS SP2 confocal laser scanning inverted microscope (Leica Microsystems, Wetzlar, Germany) fitted with a 63X water immersion objective. DAPI (4′,6-diamidino-2-phenylindole , Sigma-Aldrich, Inc, Oakville, ON, Canada) nuclear staining was done by placing mycelia, spores, and conidia in a 50 µg mL^{-1} DAPI/0.1 M phosphate buffer pH 7 solution for 1 min at room temperature and washing twice in 0.1 M phosphate buffer pH 7. Propidium Iodide (PI, Life Technologies Inc. Burlington, ON, Canada) staining was done by placing the fungal tissue in a 300 µg/mL solution of PI for 30 sec, followed by washing two times with sterile water. YFP and PI fluorescence were visualized with an argon laser at excitation and emission wavelengths of approximately 514 /560 nm and 570 /660 nm respectively. DAPI fluorescence was detected with a 405 nm laser diode with approximate 359 nm excitation and 461 nm emission wavelengths. Images were captured using the integrated Leica software (Leica Microsystems, Wetzler, Germany), and were analysed using the Leica Application Suite for Advanced Fluorescence (LAS AF) (Leica Microsystem, V2.3.5 build 5379).

2.8.2 Morphological studies

Radial growth of *V. dahliae* was measured after 7, 14 and 21 days growth on CM or BM agar. Each experiment was done four times, with 3 technical replicates for each time point.

For spore production assays the fungus was grown on CM agar, and five plugs (5 mm diameter) of actively growing mycelium (i.e. at the edge of a growing colony) were used to inoculate 35 mL of liquid CM. Liquid cultures were grown for 4-5 days at 24˚C, with shaking at 150 rpm. Spores were separated from the liquid/mycelium by filtering the culture as described previously. Spores were counted with a haemocytometer (Double Neubauer Counting Chamber, Hausser Scientific, VWR International). The experiment was done 3 times, with 3 technical replicates each time.

Production of MCS was assessed visually by culturing the strains on BM agar for ten weeks.

Colony wettability was assessed as described by Talbot et al. (1993), by placing 200 μL drops of sterile, distilled water for 12 hours at room temperature onto the surface of colonies grown on CM agar.

To study conidial germination, cellophane squares (one cm^2) were overlaid onto CM and BM agar plates. Each square was inoculated with 10 uL of a suspension containing 10^7 conidia mL^{-1}, and incubated at 24˚C. Three cellophane squares were transferred onto a haemocytometer and approximately 100 spores were counted on each square. The percentage of germinating spores was assessed at 2, 4, 6, 8 and 10 hours post inoculation. The experiment was done three times.

2.8.3 Pathogenicity tests

Bonny Best tomato plants were used to assess pathogenicity of *V. dahliae* strains. Tomato seeds were germinated and grown in sand under the following environmental

conditions: 15:9 hours light:dark photoperiod, 25˚C:24˚C temperature, and 70% relative humidity (RH). Fourteen-day-old seedlings were gently extracted from the sand, and their roots rinsed in water. The roots were then immersed in 10 mL inoculum of 5×10^7 spores mL^{-1} in 0.5% gelatin for two min; control (mock-inoculated) plant roots were immersed in 0.5% gelatin (Dobinson et al., 1996). Inoculated seedlings were then transplanted into 10 cm^2 pots with Pro-Mix BX Mycorrhizae (Premier Horticulture, Rivière-du-Loup, QC, Canada), and were grown under the conditions described above. Disease severity was scored at 28 days-post-inoculation (dpi) using a previously defined numerical rating system (see Table 2) in which a score of 0 is given to a healthy plant, and a dying or dead plant is given a score of five (Klimes & Dobinson, 2006). Plant fresh weight was also assessed as measurement of overall growth.

2.9 Statistical Analyses

Statistical analyses were done in order to determine the significance of gene expression or strain differences observed in gene expression experiments, colony growth, spore production, and pathogenicity.

One-way ANOVA was performed using the SigmaPlot software (SigmaPlot; Systat Software, San Jose, CA). A significance value of 0.05 was used for α, and all data were tested for normality prior to the ANOVA. The Holm-Sidak method, an adjunct to the one-way ANOVA, was used for multiple (within-experiment) comparison of the expression of the five hydrophobin genes.

Table 2: Rating system of disease progression.

Score	Disease symptoms
0	Negligible chlorosis or wilting
1	Chlorosis and wilting and/or curling in individual leaves
2	Necrosis in leaves
3	At least one branch dead
4	Wilt and/or chlorosis in upper leaves, and/or two or more branches dead
5	Plant dead, or all leaves and most of stem necrotic

Adapted from Klimes & Dobinson, 2006

CHAPTER 3: Results

3.1 Classification of *V. dahliae* Hydrophobins.

Hydrophobins are proteins unique to filamentous fungi, which have been classified into two classes according to sequence characteristics and hydrophobicity profiles (Wösten, 2001). Class I hydrophobins have been found in both Ascomycota and Basidiomycota while class II proteins have been found only in the Ascomycota (Kubicek et al., 2008).

Five hydrophobin-like genes have been identified in the genome of *V. dahliae* VdLs.17 and are herein designated as *VDH1, 2, 3, 4*, and *5*, respectively (Appendix 3). Of these, *VDH1* was previously characterized, and classified as a class II hydrophobin (Klimes, 2006, Klimes & Dobinson, 2006). In order to classify the other four hydrophobin-like sequences the amino acid (aa) structure of the proteins was analyzed. All four sequences have a putative signal peptide at their N terminus and eight Cys residues in the typical hydrophobin arrangement (i.e. CX-CCX-CX-CX-CCX-CX; Fig. 3). It was also evident that VDH5 has an extra domain composed primarily of glycine and asparagine repeats (Fig. 3). This type of domain (named the Extended N-terminus; ENT) is not common in hydrophobins but is not unique to VDH5 (Kubicek et al., 2008).

In order to assign the proteins to either class I or class II hydrophobins, the hydrophobicity pattern of each sequence was assessed using the Kyte and Doolitle hydropathy scale (Kyte & Doolittle, 1982), which assigns a positive value to each hydrophobic aa and a negative value if the aa is hydrophilic. In general, the profiles of class I hydrophobins have more hydrophobic regions when compared with the profiles of

class II hydrophobins. Also, the Cys doublets in class I hydrophobins are usually followed by hydrophilic residues, while in class II hydrophobins, these Cys doublets are followed generally by hydrophobic residues (Mgbeahuruike et al., 2013).

The patterns obtained for VDH2, 3, 4 and 5 were compared with the profiles of previously characterized class I (MPG1) and class II (VDH1) hydrophobin sequences. The resulting data are presented in the hydropathy plots shown in Figure 4, and suggested that VDH2, 3, 4 and 5 are class II hydrophobins (Fig. 4). Additionally, it was noted that the VDH5 ENT domain is highly hydrophilic (Fig.4). Finally, the distance between the Cys residues in the hydrophobin protein sequences was assessed. The spacing pattern according to Kershaw and Talbot (1998, Table 3) was compared with that observed in the *V. dahliae* sequences (Table 4). Based on both the hydrophobicity patterns and the cysteine spacing patterns it was possible to classify all four of these proteins as class II hydrophobins.

Table 3: Consensus of cysteine spacing in class I and class II hydrophobins according to Kershaw and Talbot 1998

Class	Consensus cysteine distance
Class I	CN5-7 CCN19-39 CN8-23 CN5 CCN6-18 CN2-13
Class II	CN9-10 CCN11 CN16 CN8-9 CCN10 CN6-7

Table 4: Cysteine spacing and classification of *V. dahliae* hydrophobins

Hydrophobin	Spacing between cysteine residues	Class
VDH1	CN9 CCN11 CN15 CN6 CCN10 CN6	Class II
VDH2	CN10 CCN11 CN16 CN8 CCN10 CN5	Class II
VDH3	CN8 CCN11 CN16 CN8 CCN10 CN8	Class II
VDH4	CN8 CCN11 CN15 CN8 CCN10 CN6	Class II
VDH5	CN9 CCN11 CN16 CN8 CCN10 CN8	Class II

Figure 3: Alignment of the five hydrophobins of *V. dahliae*.

Sequences were aligned with Clustal W and visualized in BioEdit. The signal peptide is highlighted in blue and the characteristic eight Cysteine residues are highlighted in red. The boxed amino acids are identical among the hydrophobin sequences. *= fully conserved residue, : = conservative change, . = semiconservative change

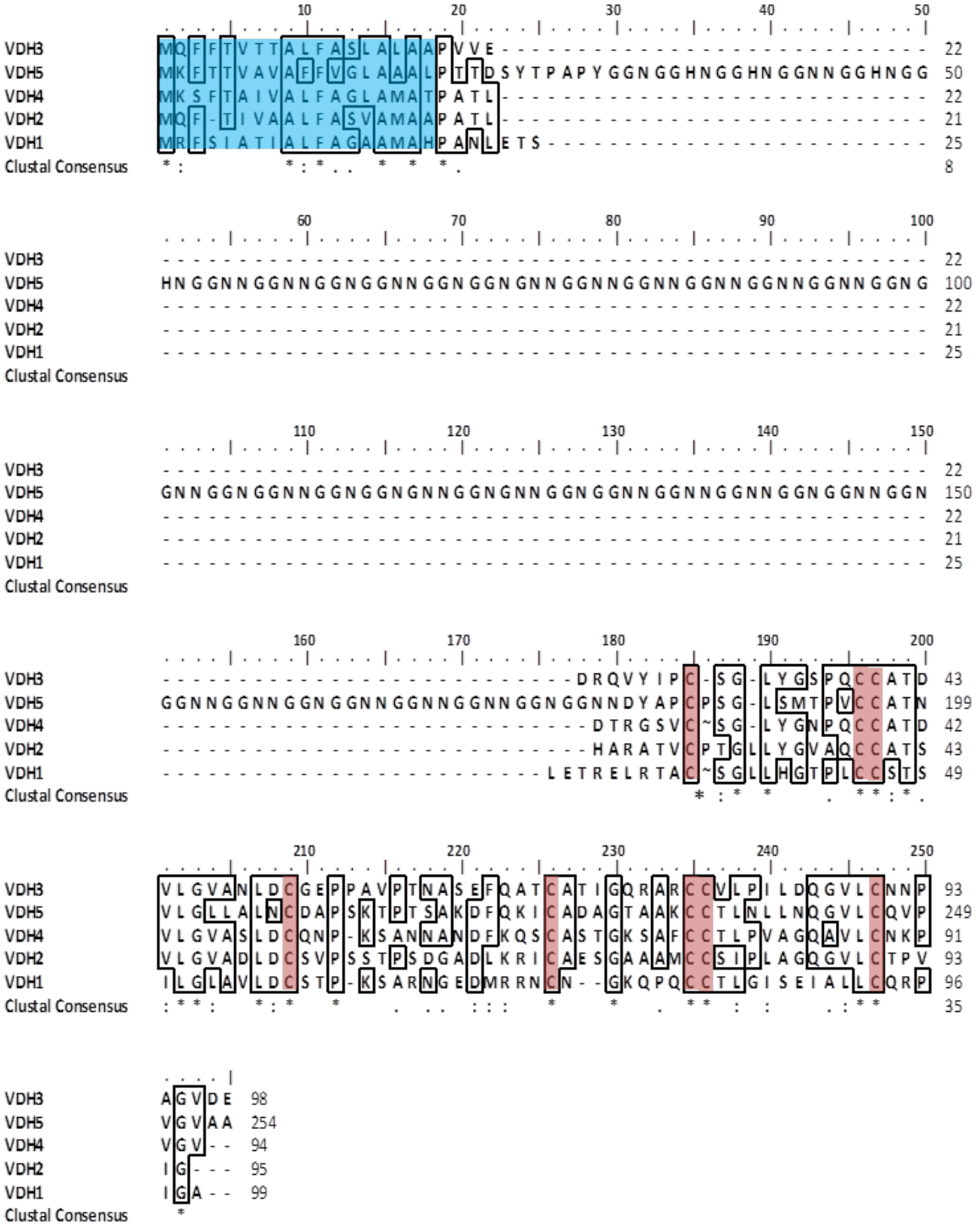
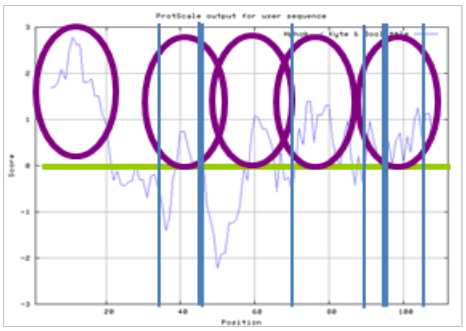

VDH3
VDH5
VDH4
VDH2
VDH1
Clustal Consensus

Figure 4: Kyte and Doolittle hydropathy plots for the *V. dahliae* hydrophobins.

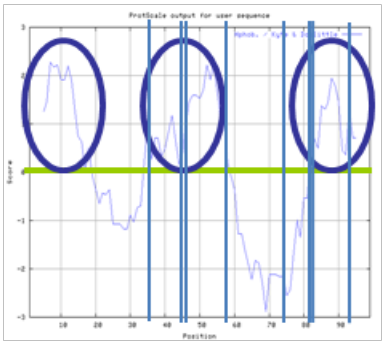
A) Examples of hydrophobin class I and class II hydropathy plots. B) Hydropathy plots for the four unclassified hydrophobins of *V. dahliae*. C) Hydropathy plot of *V. dahliae* chitin synthase is shown for comparison. The light blue diagonal bars represent the position of Cys residues. Window size = 9.

A

Class I Hydrophobin: MPG1 (*M. grisea*)

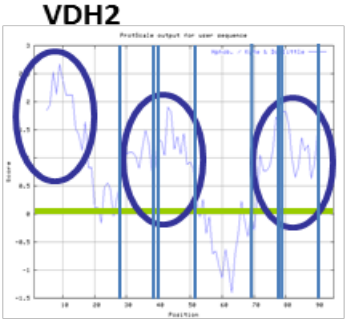


Class II Hydrophobin: VDH1 (*V. dahliae*)

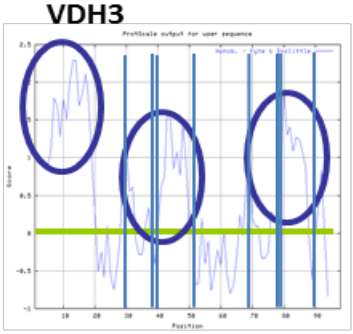


B

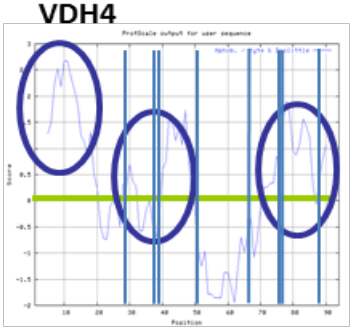
VDH2



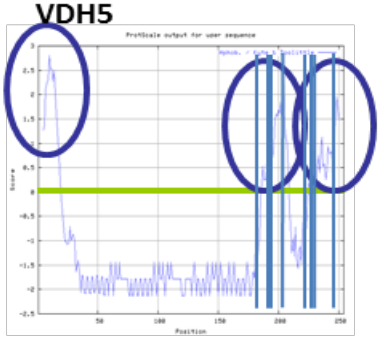
VDH3



VDH4

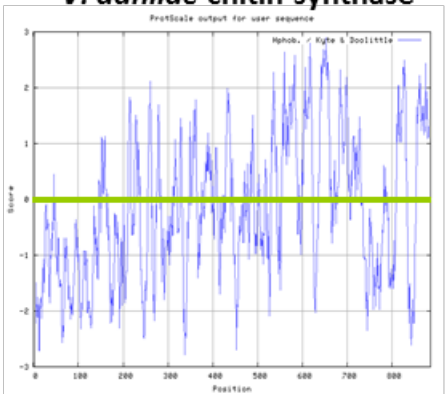


VDH5



C

V. dahliae chitin synthase



3.2 Blast P Analyses of *V. dahliae* Hydrophobins.

A BlastP analysis was performed using each of the *V. dahliae* hydrophobin amino acid sequences as query against the NCBI nr protein database. The 80 sequences obtained in the BlastP analyses contained 44 sequences from plant pathogenic fungi, 29 from plant symbionts, and seven from entomopathogenic fungi (Appendix 4). These were used to construct a neighbor-joining tree. The results of this analysis suggest that both *V. dahliae* and *V. albo-atrum* have orthologs of VDH1, 2, and 3, whereas VDH4 and 5 are unique to *V. dahliae* (Fig. 5). Finally, it was interesting to notice that VDH1, 2, 3, 4, and 5 are in different clades and do not cluster together (Fig. 5).

3.3 Features of the *V. dahliae* Hydrophobin Upstream DNA Sequences.

In order to characterize the upstream regulatory regions of the *V. dahliae* hydrophobin genes, a region 1000 bp upstream of the start codon of the genes was analysed (Fig. 6). The analysis showed the location of typical TATA boxes (TATAAA) at -170 bp region as well as several heat shock factor binding elements (NGAAN) for all the genes (Åkerfelt et al., 2010), and GATA (WGATAR, Ko and Engel, 1990) and NIT (TATCTA, Fu and Marzluf, 1990)elements for *VDH1* and *VDH3* (Fig.6).

Figure 5: Neighbor-joining tree of class II hydrophobin sequences.

Similarity of eighty class II hydrophobin amino acid sequences that were found by BlastP analysis to be similar to *V. dahliae* hydrophobins was analysed by the Neighbor-Joining method (bootstrap = 500). *V. dahliae* hydrophobins are marked by red arrows. *V. albo-atrum* hydrophobins are marked with blue circles. Ants mark hydrophobin sequences of entomopathogenic fungi. Branch lengths scale = 0.05 amino acid substitutions per site. Full species names are given in Appendix 4.

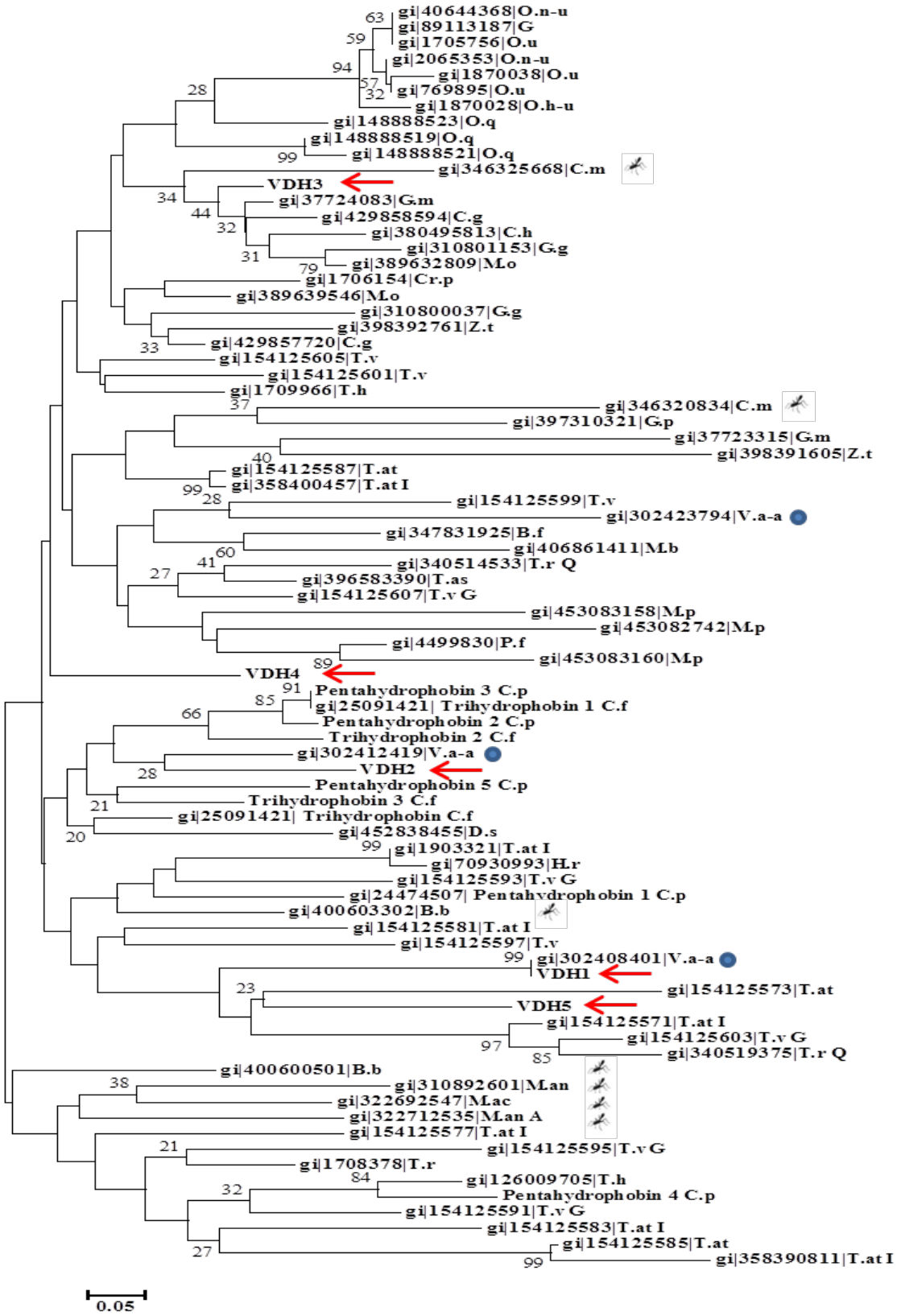
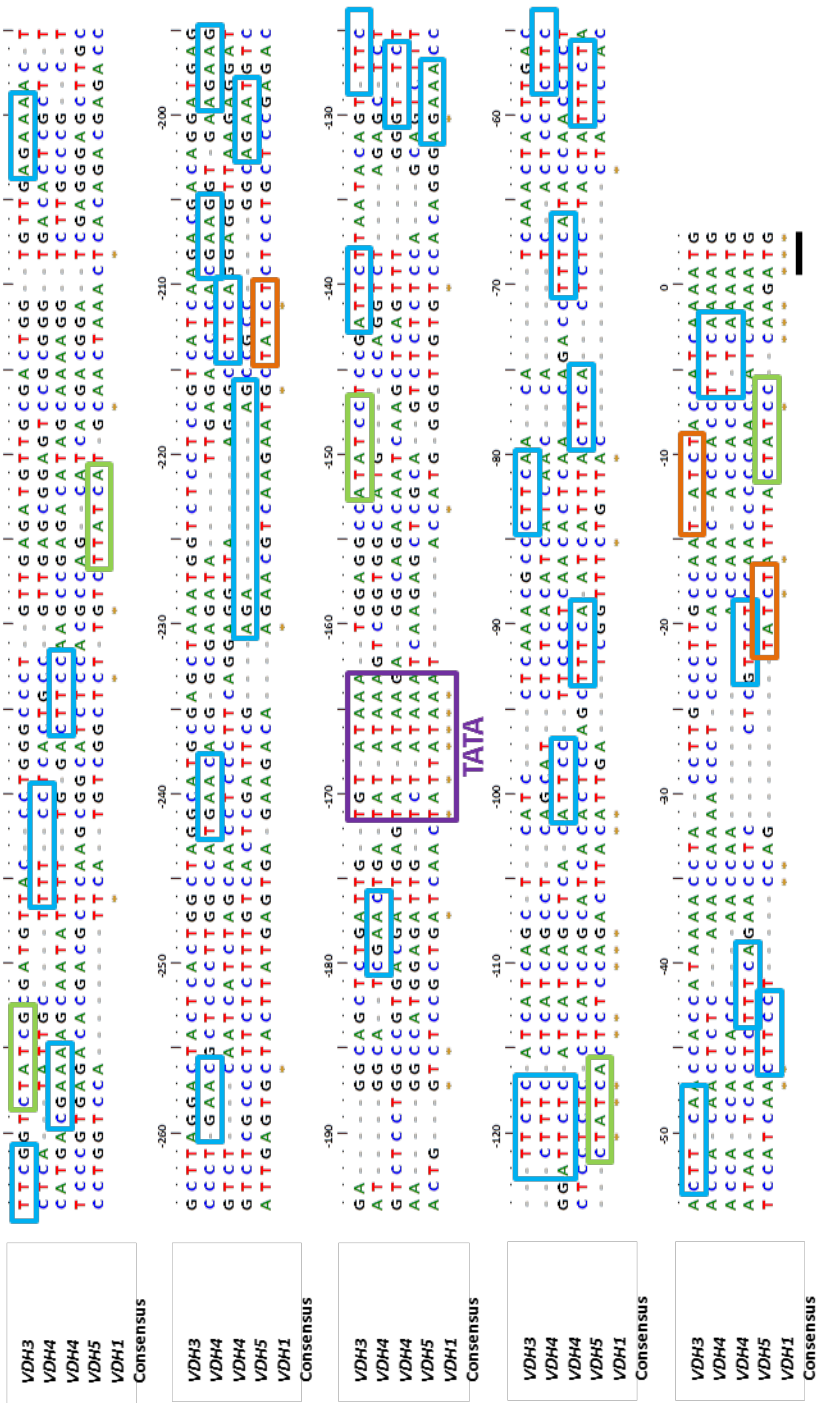

gi|40644368|O.n-u
gi|89113187|G
gi|1705756|O.u
gi|2065353|O.n-u
gi|1870038|O.u
gi|769895|O.u
gi|1870028|O.h-u
gi|148888523|O.q
gi|148888519|O.q
gi|148888521|O.q
gi|346325668|C.m
VDH3
gi|37724083|G.m
gi|429858594|C.g
gi|380495813|C.h
gi|310801153|G.g
gi|389632809|M.o
gi|1706154|Cr.p
gi|389639546|M.o
gi|310800037|G.g
gi|398392761|Z.t
gi|429857720|C.g
gi|154125605|T.v
gi|154125601|T.v
gi|1709966|T.h
gi|346320834|C.m
gi|397310321|G.p
gi|37723315|G.m
gi|398391605|Z.t
gi|154125587|T.at
gi|358400457|T.at I
gi|154125599|T.v
gi|302423794|V.a-a
gi|347831925|B.f
gi|406861411|M.b
gi|340514533|T.r Q
gi|396583390|T.as
gi|154125607|T.v G
gi|453083158|M.p
gi|453082742|M.p
gi|4499830|P.f
gi|453083160|M.p
VDH4
Pentahydrophobin 3 C.p
gi|25091421| Trihydrophobin 1 C.f
Pentahydrophobin 2 C.p
Trihydrophobin 2 C.f
gi|302412419|V.a-a
VDH2
Pentahydrophobin 5 C.p
Trihydrophobin 3 C.f
gi|25091421| Trihydrophobin C.f
gi|452838455|D.s
gi|1903321|T.at I
gi|70930993|H.r
gi|154125593|T.v G
gi|24474507| Pentahydrophobin 1 C.p
gi|400603302|B.b
gi|154125581|T.at I
gi|154125597|T.v
gi|302408401|V.a-a
VDH1
gi|154125573|T.at
VDH5
gi|154125571|T.at I
gi|154125603|T.v G
gi|340519375|T.r Q
gi|400600501|B.b
gi|310892601|M.an
gi|322692547|M.ac
gi|322712535|M.an A
gi|154125577|T.at I
gi|154125595|T.v G
gi|1708378|T.r
gi|126009705|T.h
Pentahydrophobin 4 C.p
gi|154125591|T.v G
gi|154125583|T.at I
gi|154125585|T.at
gi|358390811|T.at I
0.05

Figure 6: Alignment of sequences upstream of *V. dahliae* hydrophobin genes. **S**equences were aligned with Clustal W and visualized in BioEdit. *= fully conserved residue. The putative transcription start site (ATG) is underlined, and putative TATA, heat shock factor, GATA, and NIT elements are boxed.


VDH3
VDH4
VDH4
VDH5
VDH1
Consensus
TATA
Heat shock factor
GATA
NIT

3.4 Expression Analyses of *V.dahliae* Hydrophobins.

Droplet digital PCR (ddPCR) was used to analyse the expression pattern of *V. dahliae* hydrophobin genes. First, hydrophobin gene expression in relation to overall nutrient availability was studied. In order to do this, RNA was extracted from four-day axenic cultures of the WT *V. dahliae* Dvd-T5 strain grown on nutrient-rich (CM) agar or nitrogen-limited (BM) agar (Fig. 7). The *V. dahliae β-tubulin* and *Actin* genes were intended to be used as reference genes for the comparison of gene expression between different experiments. However, because the expression of these genes was not consistent between the different environments, they were not used in further analyses. Since ddPCR has the ability to quantify the absolute number of molecules present within a sample, the use of a reference gene is optional, and therefore, the comparison of the hydrophobin gene expression was only done within each experimental condition.

The transcripts for all five hydrophobin genes were detected when the fungus was grown on either CM or BM agar. In both growth conditions, the transcript levels for *VDH1*, *VDH2* and *VDH3* were significantly lower than the transcript levels of *VDH4* and *VDH5* (Fig. 7, Tables 5 and 6). Hydrophobin gene expression was also measured using RNA extracted from spores or mycelia of axenic cultures of *V. dahliae* Dvd-T5 grown in liquid CM for four days (Fig. 8). The expression of *β-tubulin* and *Actin* genes was measured for reference. Surprisingly, the expression of actin in mycelia was approximately half the expression found in spores ($P<0.001$), while β-tubulin expression was not significantly different in the two cell types ($P = 0.164$**;** Fig. 8).

The expression of *VDH1, 2, 4* and *5* genes was significantly higher in mycelium when compared to that in spores (Fig. 8, Tables 7 and 8). Also, contrary to the other four hydrophobin genes, and even though the expression of *VDH3* was very low in both cell types, its expression was approximately 10 times higher in spores than in mycelia, (Fig. 8).

To assess the expression of the hydrophobin genes during interaction of *V. dahliae* with tomato plants, RNA was extracted from Dvd-T5-inoculated tomato plants as described in the Materials and Methods. Tomato *GAPDH* and *UBI3* genes were intended to be used as reference genes for this experiment. However, the levels of transcripts for both genes were unexpectedly very low after one day following inoculation with the fungus and remained so until day 5 post-inoculation (Fig. 9A). Immediately after inoculation (0 dpi), only the transcripts of *VDH2, 3,* and *5* were detected (Fig. 9B). *VDH1* transcripts were detected at 1dpi but *VDH2* transcripts were not, and neither of these genes were detected at 2dpi (Fig. 9B). Expression of *VDH3* was not detected at 3, 4 or 5 dpi (Fig. 9B). The expression of *VDH4* was not detected immediately after inoculation (0 dpi), but its transcripts were detected from day 1 until day 5 post inoculation. Finally, at 3 and 4 dpi, the expression levels of *VDH5* were significantly higher than those of the other four hydrophobins (Fig. 9B, Tables 9 to 14).

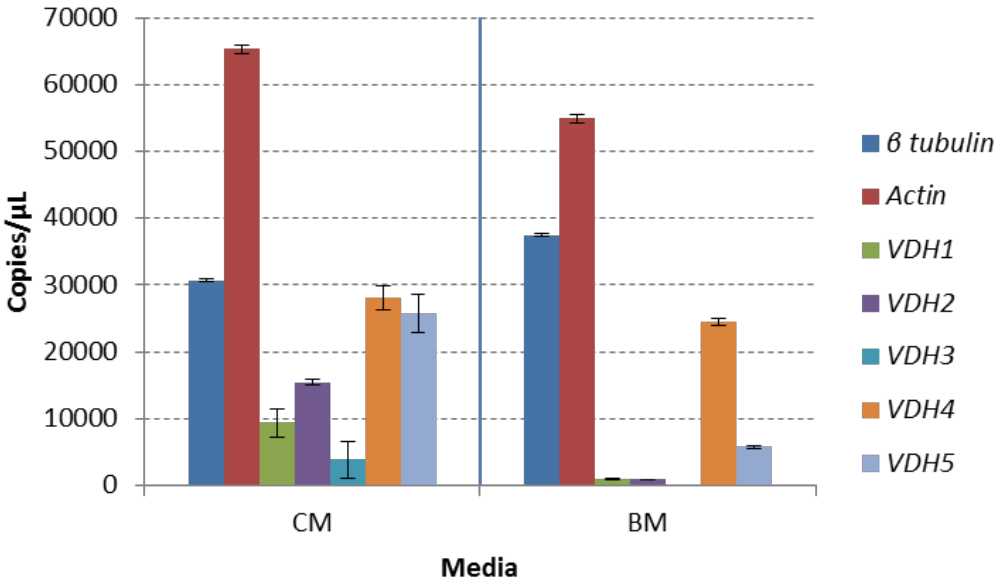


Figure 7: Expression of *V. dahliae* hydrophobin genes during axenic growth.

Droplet digital PCR analysis of *V. dahliae β-tubulin*, *Actin*, and hydrophobin gene transcript levels in Dvd-T5 cultures grown at 24˚C for four days on Complete medium (CM) agar or Basal medium (BM) agar. Error bars represent standard error; n=6.

Table 5: One-way ANOVA for ddPCR of hydrophobin gene expression in axenic cultures of WT *V. dahliae* grown on solid Complete medium.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.124	No
VDH1 vs. VDH3	0.161	No
VDH1 vs. VDH4	<0.001	Yes
VDH1 vs. VDH5	<0.001	Yes
VDH2 vs. VDH3	<0.001	Yes
VDH2 vs. VDH4	<0.001	Yes
VDH2 vs. VDH5	0.002	Yes
VDH3 vs. VDH4	<0.001	Yes
VDH3 vs. VDH5	<0.001	Yes
VDH4 vs. VDH5	0.369	No

* A gene expression P value <0.05 is considered to be significantly different

Table 6: One-way ANOVA for ddPCR of hydrophobin gene expression in WT *V. dahliae* cultures grown on solid Basal medium.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.932	No
VDH1 vs. VDH3	0.243	No
VDH1 vs. VDH4	<0.001	Yes
VDH1 vs. VDH5	<0.001	Yes
VDH2 vs. VDH3	0.198	No
VDH2 vs. VDH4	<0.001	Yes
VDH2 vs. VDH5	<0.001	Yes
VDH3 vs. VDH4	<0.001	Yes
VDH3 vs. VDH5	<0.001	Yes
VDH4 vs. VDH5	<0.001	Yes

* A gene expression P value <0.05 is considered to be significantly different

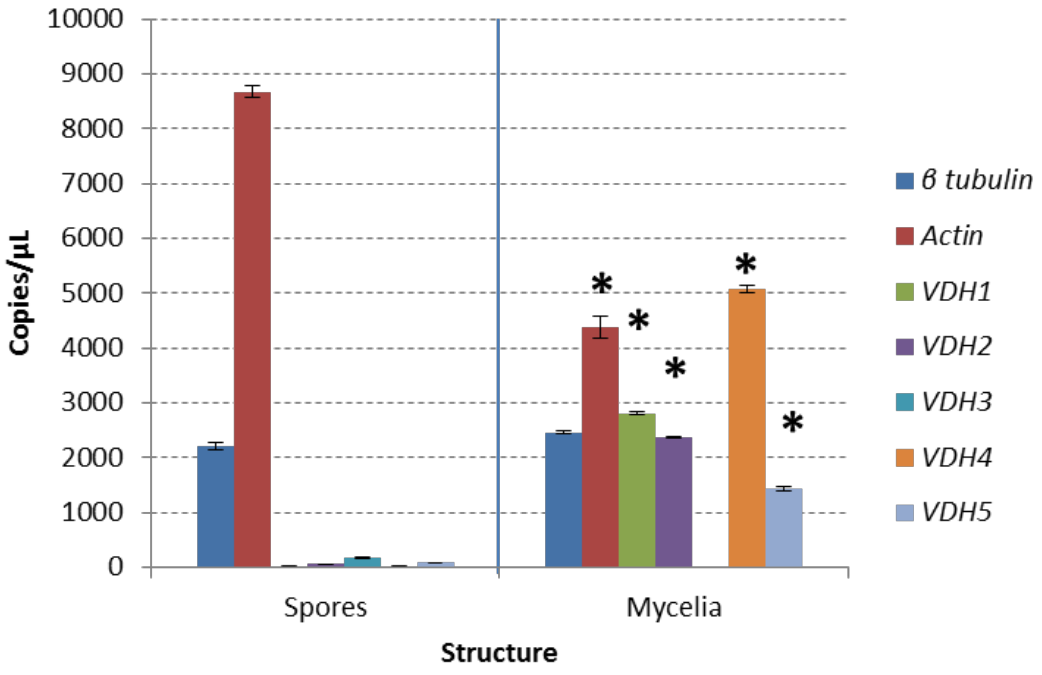


Figure 8: Expression of *V. dahliae* hydrophobin genes in spores and mycelia.

Droplet digital PCR of *V. dahliae β-tubulin*, *Actin*, and hydrophobin gene transcript levels in Dvd-T5 spores and mycelia of cultures grown, with shaking at 24˚C, for four days in liquid Complete medium. Asterisks mark the statistically significant differences between expression of each gene in mycelia compared to its expression in spores. ($P<0.05$, one-way ANOVA; Error bars are standard error; n=6).

Table 7: One-way ANOVA for ddPCR of hydrophobin gene expression in spores of WT *V. dahliae* cultures grown in liquid Complete medium.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.946	No
VDH1 vs. VDH3	0.326	No
VDH1 vs. VDH4	0.972	No
VDH1 vs. VDH5	0.905	No
VDH2 vs. VDH3	0.632	No
VDH2 vs. VDH4	0.974	No
VDH2 vs. VDH5	0.904	No
VDH3 vs. VDH4	0.333	No
VDH3 vs. VDH5	0.849	No
VDH4 vs. VDH5	0.929	No

* A gene expression P value <0.05 is considered to be significantly different

Table 8: One-way ANOVA for ddPCR of hydrophobin gene expression in mycelia of WT *V. dahliae* cultures grown in liquid Complete medium.

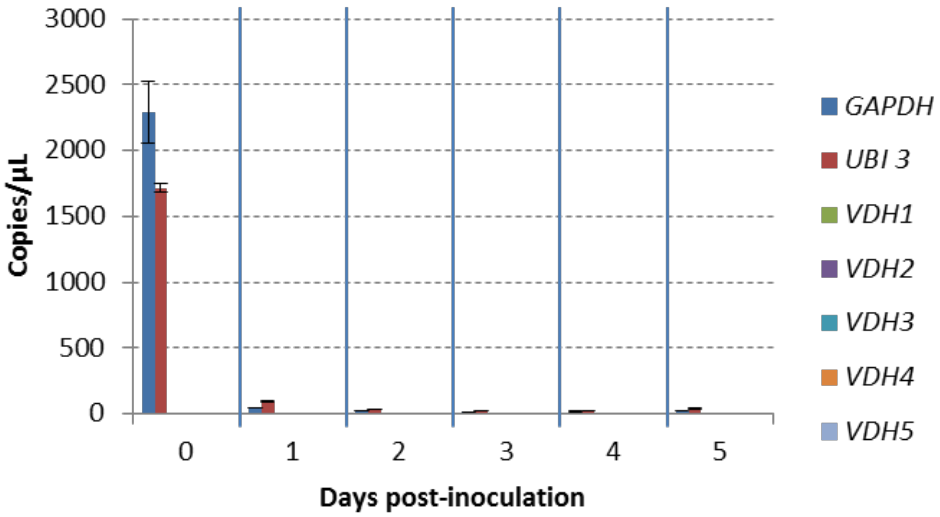
Comparison	**P value**	**P<0.05***
VDH1 vs. VDH2	0.002	Yes
VDH1 vs. VDH3	<0.001	Yes
VDH1 vs. VDH4	<0.001	Yes
VDH1 vs. VDH5	<0.001	Yes
VDH2 vs. VDH3	<0.001	Yes
VDH2 vs. VDH4	<0.001	Yes
VDH2 vs. VDH5	<0.001	Yes
VDH3 vs. VDH4	<0.001	Yes
VDH3 vs. VDH5	<0.001	Yes
VDH4 vs. VDH5	<0.001	Yes

* A gene expression P value <0.05 is considered to be significantly different

Figure 9: Expression of V. *dahliae* hydrophobin genes *in planta*.

Droplet digital PCR analysis of transcript levels of tomato *GAPDH* and *UBI3*, and *V. dahliae* hydrophobin genes in Bonny Best tomato plants inoculated with Dvd-T5. The gene expression levels were assessed immediately after inoculation (0 dpi), and one to five days following inoculation. A) Scale set to emphasize the expression of tomato *GAPDH* and *UBI3*. B) *V. dahliae* hydrophobin expression. (Bars represent standard error; n=6)

A


3000
2500
2000
1500
1000
500
0
Copies/µL
0
1
2
3
4
5
Days post-inoculation
GAPDH
UBI 3
VDH1
VDH2
VDH3
VDH4
VDH5

B

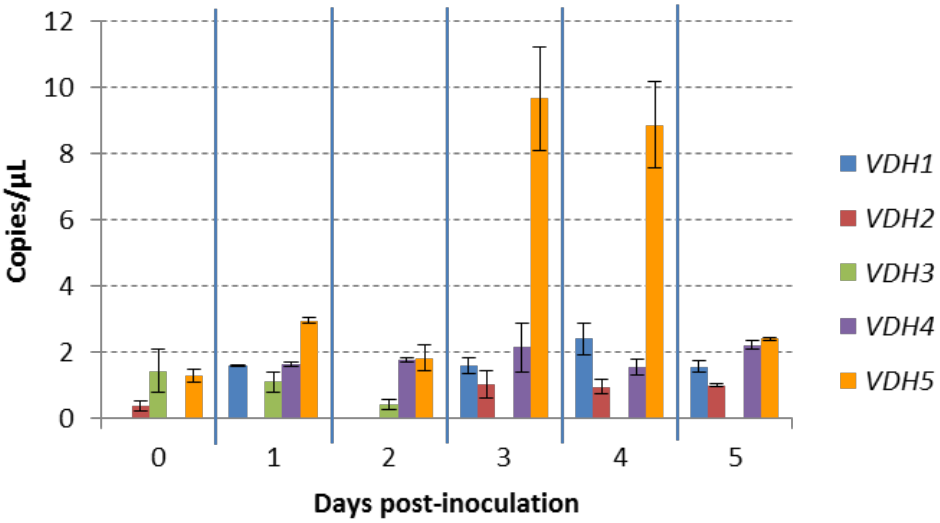

12
10
8
6
4
2
0
Copies/µL
0
1
2
3
4
5
Days post-inoculation
VDH1
VDH2
VDH3
VDH4
VDH5

Table 9: One-way ANOVA for ddPCR of hydrophobin gene expression in WT *V. dahliae*-infected plants at 0 days post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	1	No
VDH1 vs. VDH3	1	No
VDH1 vs. VDH4	1	No
VDH1 vs. VDH5	1	No
VDH2 vs. VDH3	1	No
VDH2 vs. VDH4	1	No
VDH2 vs. VDH5	1	No
VDH3 vs. VDH4	1	No
VDH3 vs. VDH5	1	No
VDH4 vs. VDH5	1	No

* A gene expression P value <0.05 is considered to be significantly different

Table 10: One-way ANOVA for ddPCR of hydrophobin gene expression in WT *V. dahliae*-infected plants at 1 day post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.998	No
VDH1 vs. VDH3	0.997	No
VDH1 vs. VDH4	0.988	No
VDH1 vs. VDH5	0.989	No
VDH2 vs. VDH3	0.994	No
VDH2 vs. VDH4	0.999	No
VDH2 vs. VDH5	0.944	No
VDH3 vs. VDH4	1	No
VDH3 vs. VDH5	0.996	No
VDH4 vs. VDH5	0.976	No

* A gene expression P value <0.05 is considered to be significantly different

Table 11: One-way ANOVA for ddPCR of hydrophobin gene expression in WT *V. dahliae*-infected planta at 2 days post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	1	No
VDH1 vs. VDH3	0.98	No
VDH1 vs. VDH4	0.679	No
VDH1 vs. VDH5	0.708	No
VDH2 vs. VDH3	0.994	No
VDH2 vs. VDH4	0.63	No
VDH2 vs. VDH5	0.746	No
VDH3 vs. VDH4	0.75	No
VDH3 vs. VDH5	0.8	No
VDH4 vs. VDH5	1	No

* A gene expression P value <0.05 is considered to be significantly different

Table 12: One-way ANOVA for ddPCR of hydrophobin gene expression in WT *V. dahliae*-infected planta at 3 days post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.767	No
VDH1 vs. VDH3	0.954	No
VDH1 vs. VDH4	0.88	No
VDH1 vs. VDH5	0.001	Yes
VDH2 vs. VDH3	0.929	No
VDH2 vs. VDH4	0.953	No
VDH2 vs. VDH5	<0.001	Yes
VDH3 vs. VDH4	0.793	No
VDH3 vs. VDH5	<0.001	Yes
VDH4 vs. VDH5	0.003	Yes

* A gene expression P value <0.05 is considered to be significantly different

Table 13: One-way ANOVA for ddPCR of hydrophobin genes expression in WT *V. dahliae*-infected plants at 4 days post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.896	No
VDH1 vs. VDH3	0.715	No
VDH1 vs. VDH4	0.861	No
VDH1 vs. VDH5	0.003	Yes
VDH2 vs. VDH3	0.944	No
VDH2 vs. VDH4	0.886	No
VDH2 vs. VDH5	<0.001	Yes
VDH3 vs. VDH4	0.93	No
VDH3 vs. VDH5	<0.001	Yes
VDH4 vs. VDH5	<0.001	Yes

* A gene expression P value <0.05 is considered to be significantly different

Table 14: One-way ANOVA for ddPCR of hydrophobin genes expression in WT *V. dahliae*-infected plants at 5 days post inoculation.

Comparison	P value	P<0.05*
VDH1 vs. VDH2	0.952	No
VDH1 vs. VDH3	0.982	No
VDH1 vs. VDH4	0.989	No
VDH1 vs. VDH4	0.987	No
VDH1 vs. VDH5	0.953	No
VDH2 vs. VDH3	0.977	No
VDH2 vs. VDH5	0.974	No
VDH3 vs. VDH4	0.881	No
VDH3 vs. VDH5	0.887	No
VDH4 vs. VDH5	0.946	No

* A gene expression P value <0.05 is considered to be significantly different

3.5 *VDH5* Roles in *V. dahliae* Dvd-T5.

3.5.1 Differences of *VDH5* between *V.dahliae* Dvd-T5 and VdLs.17 strains.

In spite of a sexual stage not having been described for *Verticillium* spp., there is much genetic diversity among and within these species. For this reason, the differences between the *VDH5* genes of *V. dahliae* strains Dvd-T5 (WT strain used throughout this study) and VdLs.17 (the reference genome strain) were assessed. At the nucleotide level, only one difference, in the signal peptide at position 22, "A" in the VdLs.17 sequence, and "G" in Dvd-T5, was identified (Fig. 10). More differences were evident throughout the ENT domain region, including not only nucleotide changes, but also insertions and deletions (Fig. 10). Finally, no variation was observed at the 3' end of the sequence (Fig. 10). On the amino acid level, a conservative change was observed in the signal peptide (Ile8Val) but no changes were observed in the hydrophobin domain (Fig 11). The ENT domain displayed the most variation, and was 14 amino acids longer in the Dvd-T5 sequence than in the VdLs.17 sequence (Fig 11).

Figure 10: Differences between the VDH5 sequences in VdLs.17 (V. dahliae reference genome strain) and Dvd-T5 (wild type strain used in this study).

Sequences were aligned with Clustal W and visualized in BioEdit. The ENT domain is highlighted in purple, and the introns are highlighted in green. The boxed nucleotides are identical in the two sequences.

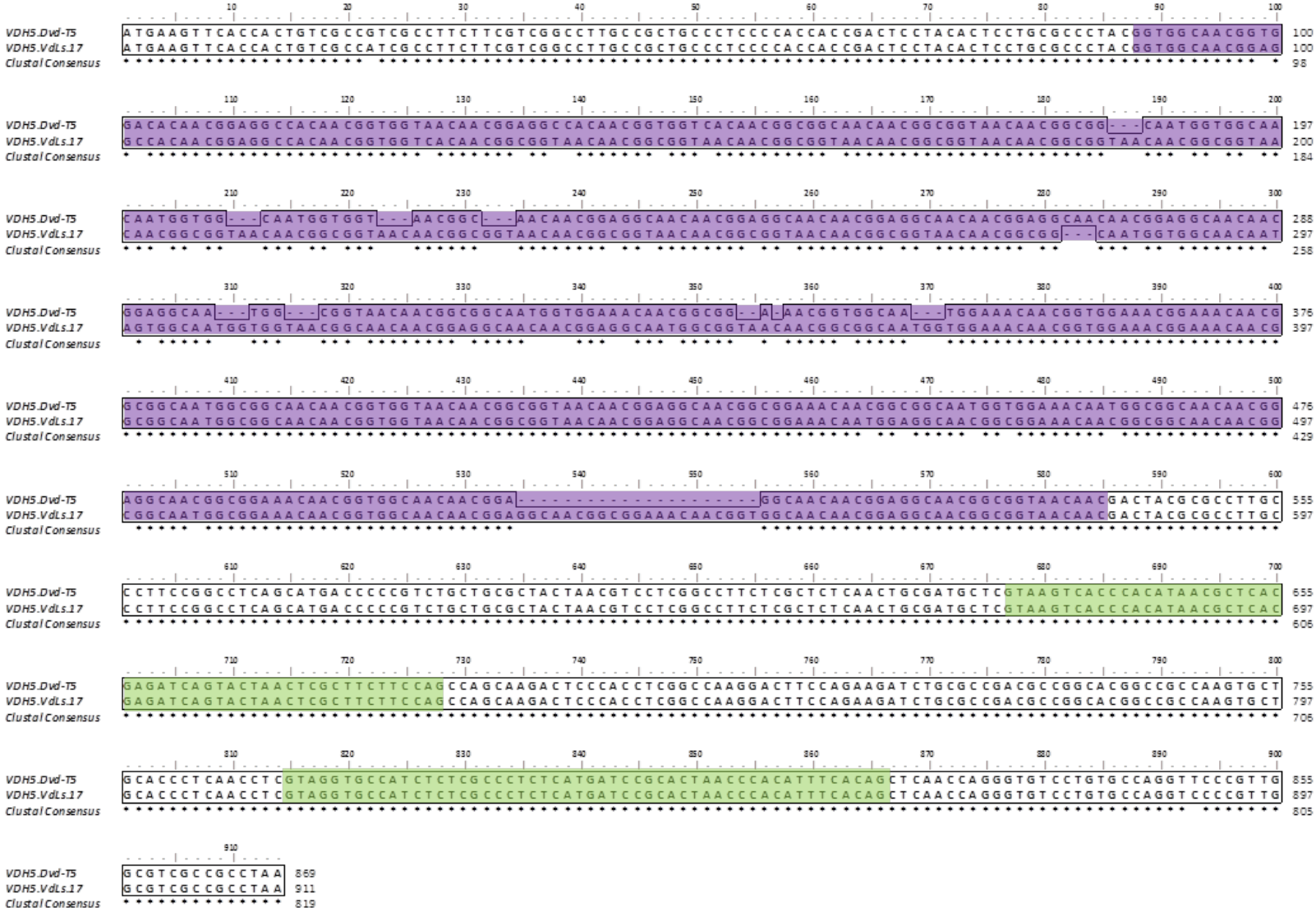

VDH5.Dvd-T5
VDH5.Vdls.17
Clustal Consensus

Figure 11: Comparison of VDH5 protein sequences inVdLs.17 (*V. dahliae* reference genome strain) and Dvd-T5 (wild type strain used in this study).

Sequences were aligned with Clustal W and visualized in BioEdit. The signal peptide is highlighted in blue, the ENT domain is highlighted in purple, and the characteristic eight cysteine residues are marked in red. The boxed amino acids are identical among the hydrophobin sequences. *= fully conserved residue, : = conservative change, . = semiconservative change.

3.5.2 Localization of Vdh5 *V.dahliae* Dvd-T5

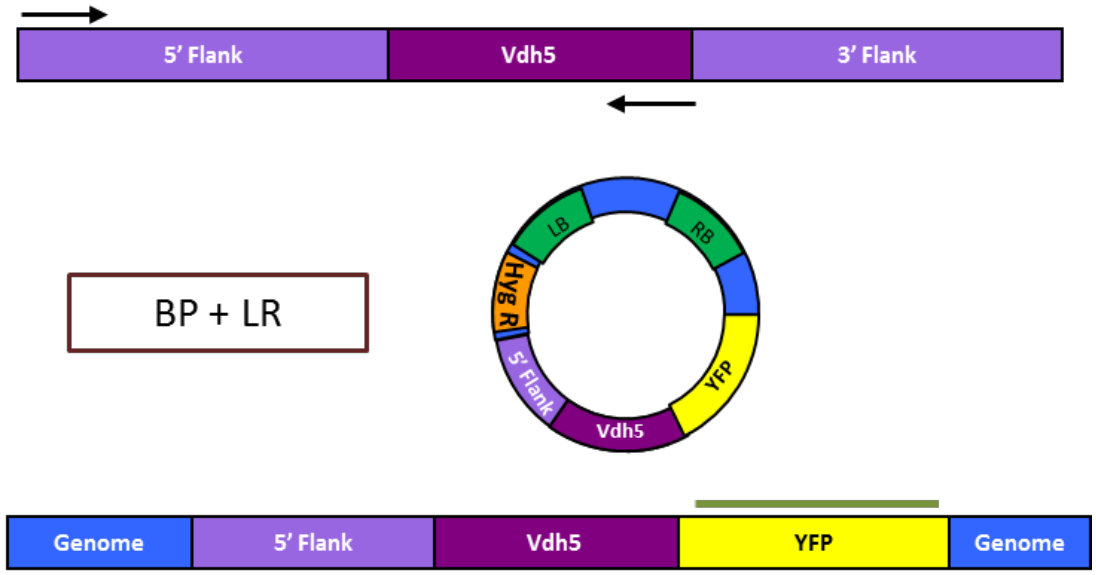
In order to study the localization of Vdh5 in *V. dahliae*, a vector was constructed that carried a gene encoding a VDH5::YFP fusion protein vector, and used to transform the WT strain Dvd-T5 (Fig. 12A). Hyg-resistant transformants were screened for single insertions by Southern blot analysis, and two of these, VDAT 98-8 and VDAT98-13, were used for subsequent analyses (fig 12B). The results obtained by these two independent transformant strains were consistent with each other, and for clarity figures 13, 14, and 15 show only the results for VDAT 98-8.

Confocal laser scanning microscopy was used to assess the localization of the fusion protein in the two independent transformants. Yellow fluorescence was detected in the cytoplasm of hyphae, spores, and in chains of swollen cells, the first precursors to the MCS (Fig. 13). In order to confirm the cytoplasmic localization of the fluorescent fused protein, the *VDH5::YFP* strain was stained with either a nuclear stain (4′,6-diamidino-2-phenylindole, DAPI) or a cell membrane stain (Propidium Iodide, PI). Figure 14 shows that VDH5::YFP did not co-localise with the nucleus. The staining with PI further revealed that the VDH5::YFP protein was detected in the pores of the hyphal septa, confirming its localization in the cytoplasm (Fig 15).

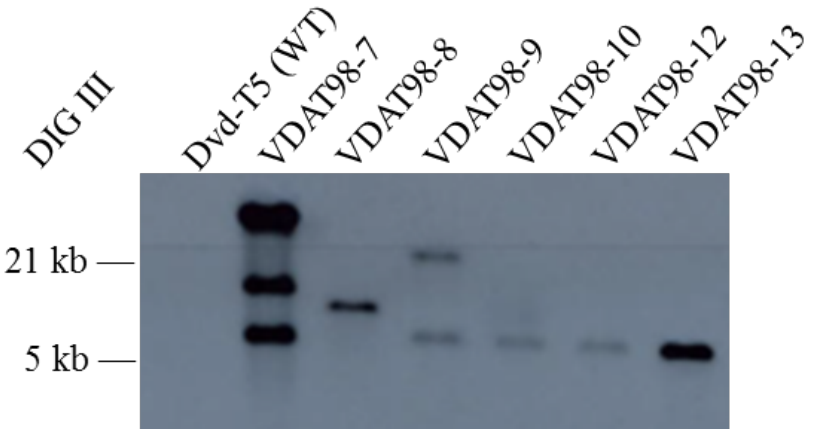
Figure 12: Generation of VDH5::YFP gene fusion vector.

A) For creation of the fusion vector a sequence from approximately two kb upstream of the ORF of *VDH5* to the codon immediately before the stop codon of the *VDH5* ORF was amplified. The black arrows in the first diagram represent the primers used in the PCR amplification. Using the gateway system the amplicon was incorporated into the final binary vector used for *Agrobacteriuim* mediated transformation of *V. dahliae* Dvd-T5 spores. B) Southern blot hybridization of the *VDH5::YFP* transformants, using a probe complementary to YFP (green bar in bottom figure of panel A).

A


5' Flank
Vdh5
3' Flank
BP + LR
LB
RB
Hyg R
5' Flank
YFP
Vdh5
Genome
5' Flank
Vdh5
YFP
Genome

B


DIG III
Dvd-T5 (WT)
VDAT98-7
VDAT98-8
VDAT98-9
VDAT98-10
VDAT98-12
VDAT98-13
21 kb
5 kb

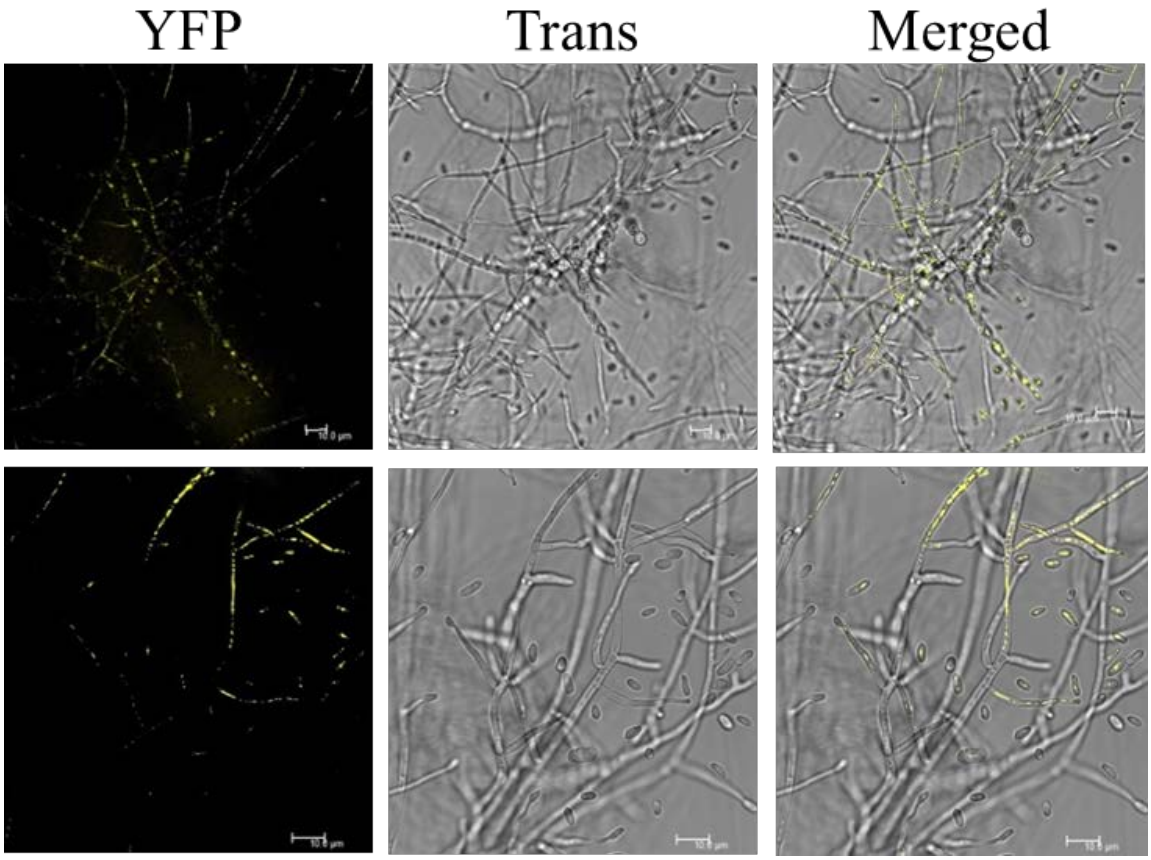


Figure 13: Confocal scanning laser micrographs of VDH5::YFP.

VDH5::YFP is localized to hyphae, spores and chains of swollen cells. YFP = YFP channel, Trans = transmitted light image. Scale bar = 10µm. YFP was visualized with an argon laser with an excitation and emission wavelengths of approximately 514 /560 nm.

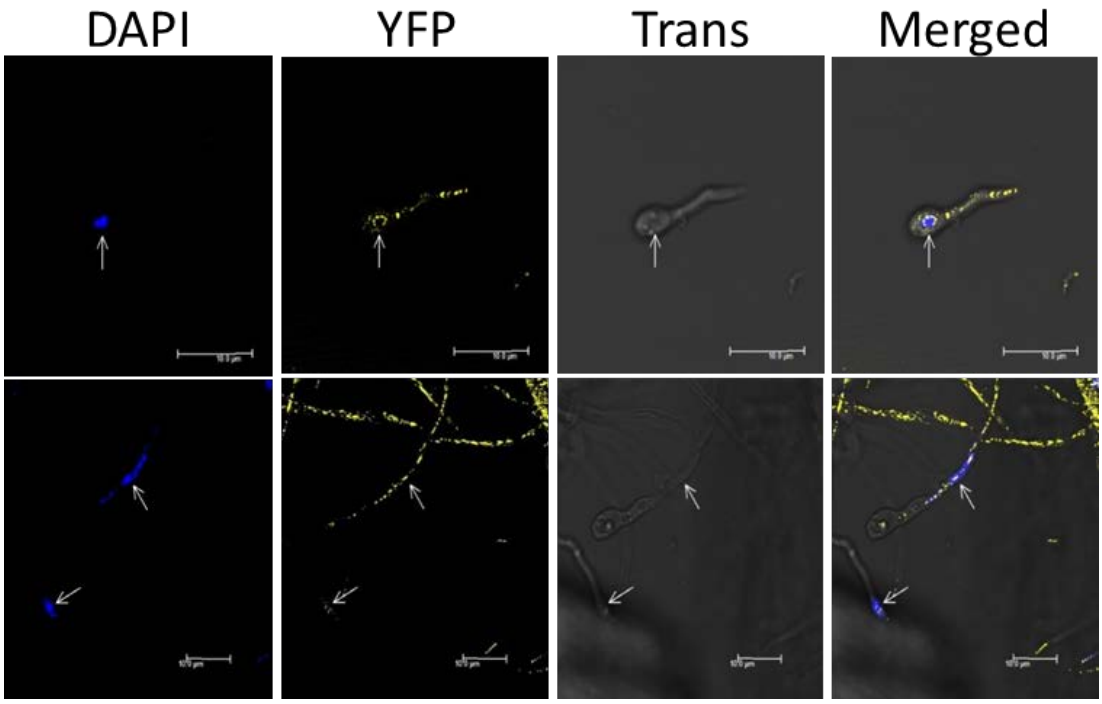


Figure 14: Confocal scanning laser microscopy of VDH5::YFP strain stained with DAPI.

VDH5::YFP does not show nuclear localization. DAPI = DAPI channel, YFP = YFP channel, Trans = transmitted light image. Scale bar = 10μm. Arrows point to nuclei. YFP was visualized using an argon laser, with excitation and emission wavelengths of approximately 514 and 560 nm, respectively. DAPI fluorescence was measured using a 405 nm laser diode, with approximately 359 nm excitation and 461 nm emission wavelengths, respectively.

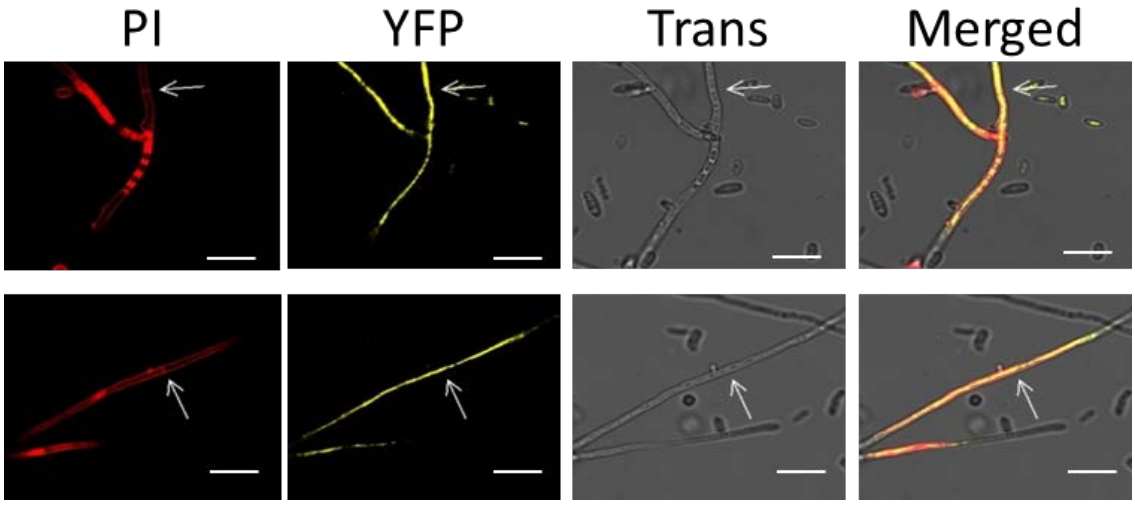


Figure 15: Confocal scanning laser microscopy of VDH5::YFP strain stained with propidium iodide.

PI = propidium iodide channel, YFP = YFP channel, Trans = transmitted light image. Scale bar = 10µm. Arrows point to septa. YFP and PI fluorescence were visualized with an argon laser, with excitation and emission wavelengths of approximately 514 /560 nm and 570 /660 nm respectively.

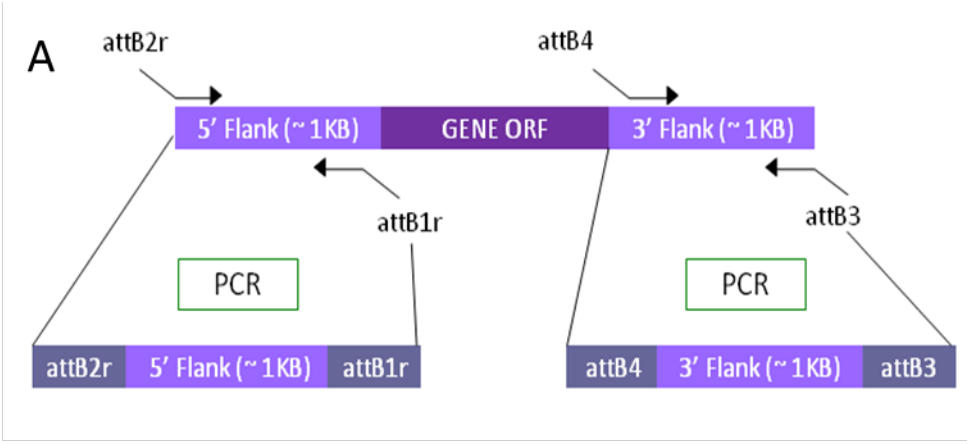
3.5.3 Generation of the Δ*vdh5*, *vdh5TN*, *vdh5ΔENT*, and complementation strains.

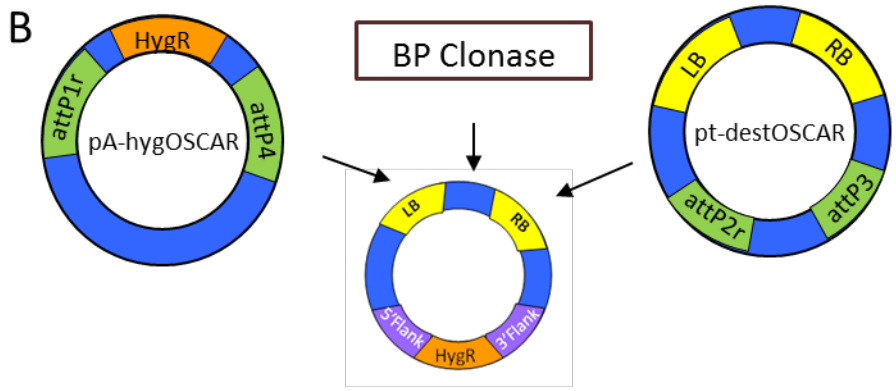
In order to study the effect of *VDH5* disruption in fungal development and pathogenicity a *Δvdh5* plasmid was generated to replace the *VDH5* ORF with the hygromycin B-resistance (HygR) gene, as described in the Materials and Methods (Fig. 16A and B). The construct was introduced into *V. dahliae* Dvd-T5 by *Agrobacterium tumefaciens*-mediated transformation as described in Mullins et al. (2001)), and integrated into the genome of *V. dahliae* in the *VDH5* locus either by homologous recombination (Fig. 16C) or ectopically at random locations. Forty independent HygR transformants were produced, and screened for true *VDH5* deletion events by PCR and Southern Blot hybridization. Figure 17A and B shows an example of ten typical transformant screening results obtained. Only one true homologous recombination event was detected (VDAT85-3), whereas transformants with two or more ectopic insertions were common (Fig. 17B).

Because I was only able to identify one deletion mutant strain for *VDH5*, a *vdh5TN* disruption vector was constructed, as described in the Materials and Methods section, by transposing a geneticin-resistant gene into the middle of the *VDH5* ORF (Fig 18). Eight independent GenR transformants were generated by *Agrobacteriuim tumefaciens*-mediated transformation (ATMT), and screened by Southern hybridization. Five of these transformants were shown to carry the GenR-tagged gene homologously recombined into the *VDH5* locus (Fig 19), and two of these, VDAT 97-2.2 and VDAT97-9.1, were used for subsequent analyses.

Figure 16: Generation of *VDH5* gene deletion vector.

A) Flanking regions of the *VDH5* ORF were amplified with primers containing specific attB recombination regions. B) The amplified fragments were incorporated by the BP Clonase into the final binary vector used for *Agrobacterium* mediated transformation of *V. dahliae* Dvd-T5 spores. C) Integration of the deletion cassette into *Verticillium* genome by homologous recombination. Blue arrows represent the primers used for PCR screening of transformants, and the green line represents the probe used in the southern hybridization analysis.


A
attB2r
attB4
5' Flank (~1KB)
GENE ORF
3' Flank (~1KB)
attB1r
attB3
PCR
PCR
attB2r
5' Flank (~1KB)
attB1r
attB4
3' Flank (~1KB)
attB3


B
HygR
attP1r
attP4
pA-hygOSCAR
BP Clonase
LB
RB
pt-destOSCAR
attP2r
attP3
LB
RB
5'Flank
HygR
3'Flank

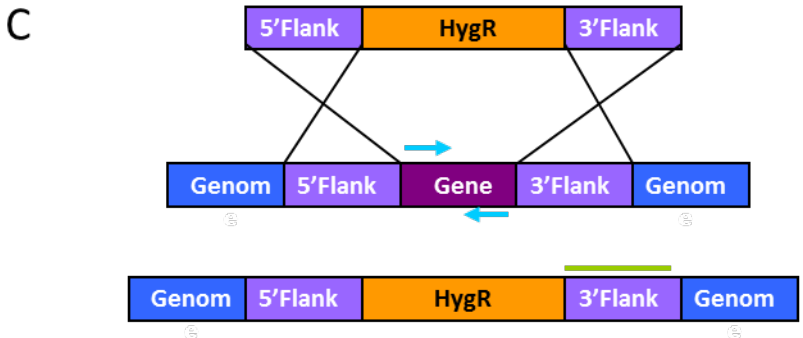
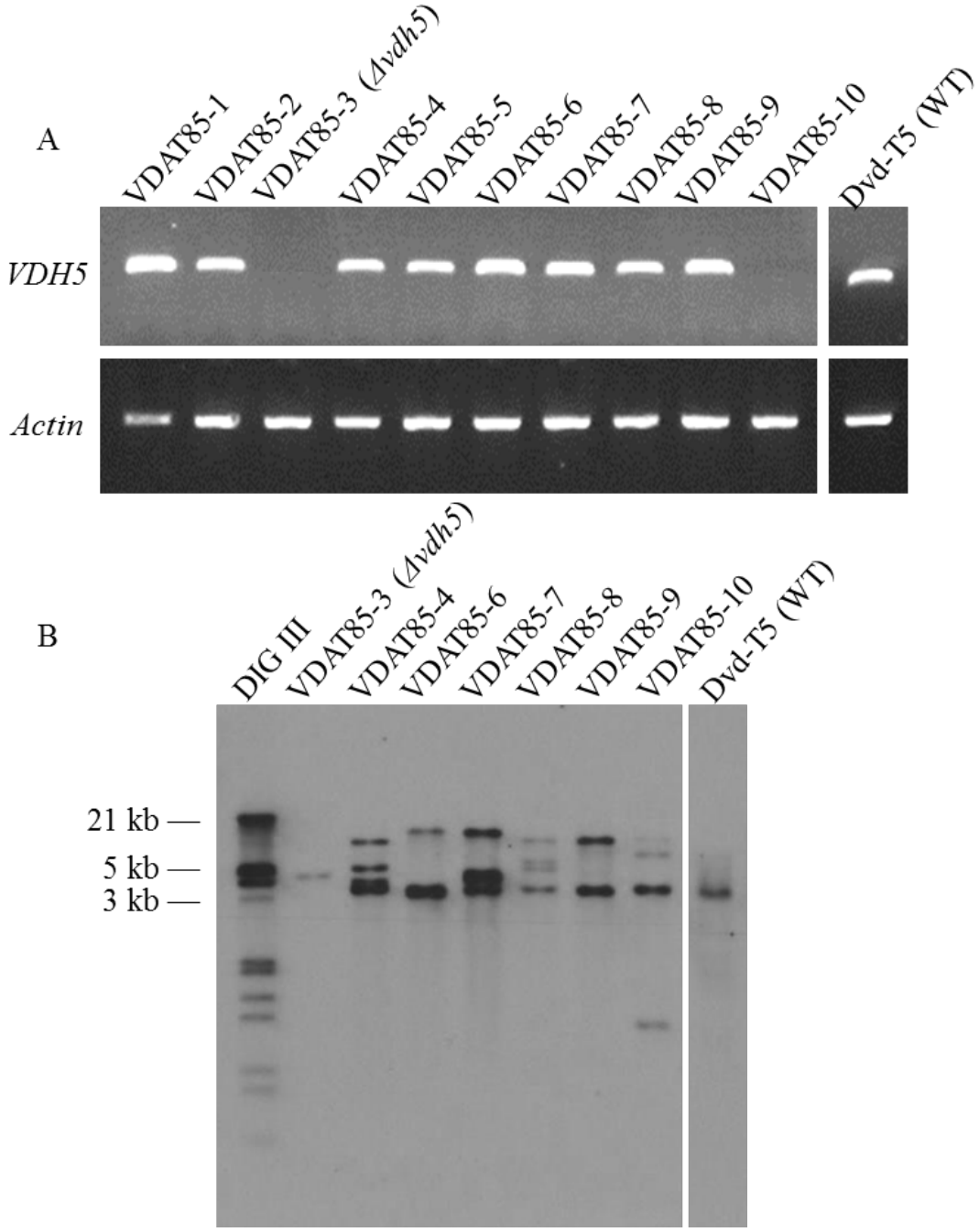

C
5'Flank
HygR
3'Flank
Genom
5'Flank
Gene
3'Flank
Genom
Genom
5'Flank
HygR
3'Flank
Genom

Figure 17: PCR and Southern hybridization data for the transformants with the *VDH5* deletion construct.

A. PCR analysis of the different *VDH5* deletion construct transformants with primers annealing inside the ORF of the *VDH5* gene. *Actin* was used as an amplification control. B. Southern blot hybridization of the transformants with a probe complementary to the 3' flank of the *VDH5* ORF.


A
VDAT85-1
VDAT85-2
VDAT85-3 (Δvdh5)
VDAT85-4
VDAT85-5
VDAT85-6
VDAT85-7
VDAT85-8
VDAT85-9
VDAT85-10
Dvd-T5 (WT)
VDH5
Actin
B
DIG III
VDAT85-3 (Δvdh5)
VDAT85-4
VDAT85-6
VDAT85-7
VDAT85-8
VDAT85-9
VDAT85-10
Dvd-T5 (WT)
21 kb
5 kb
3 kb

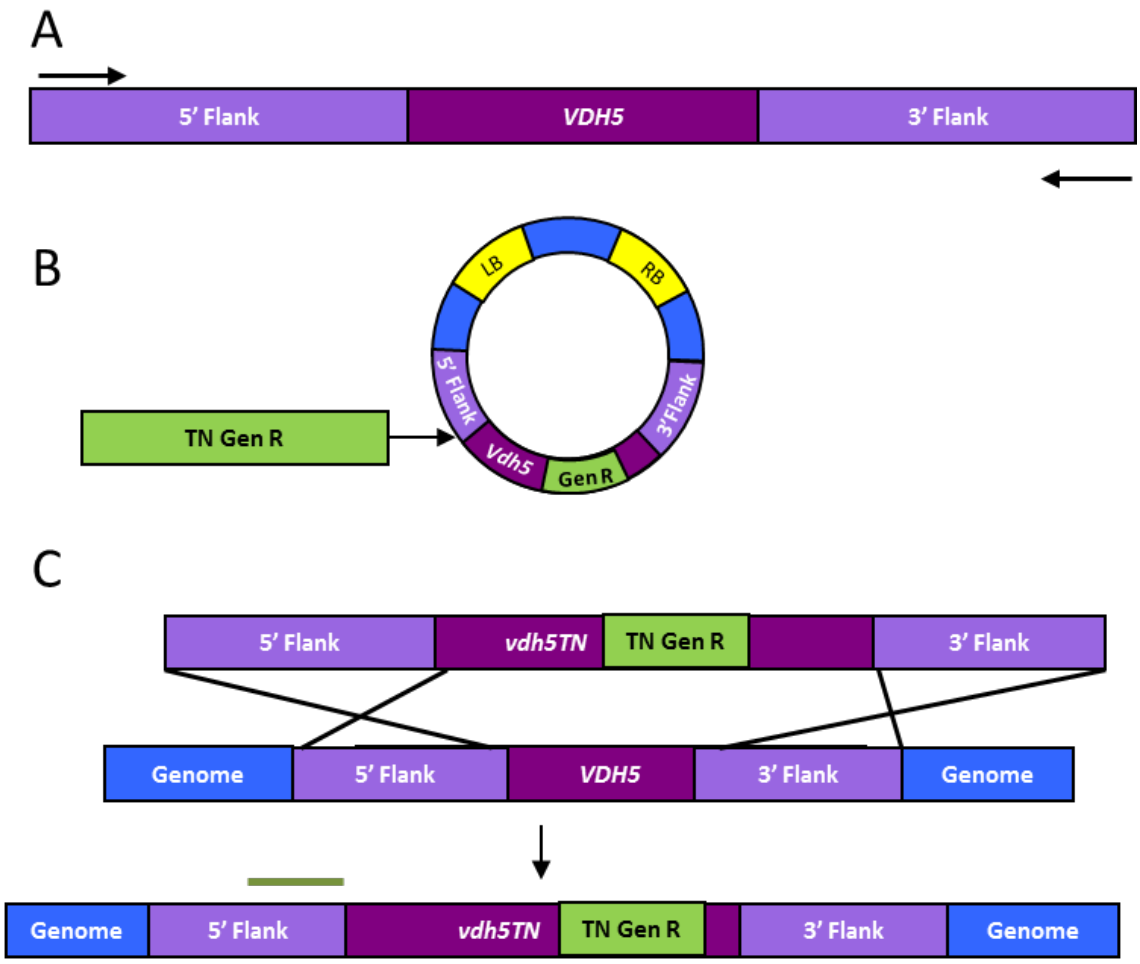


Figure 18: Strategy for production of the *vdh5TN* null strain.

A) A sequence from approximately two kb upstream of the ORF of *VDH5* to approximately one Kb downstream the*VDH5* ORF, was amplified. B) The amplicon was ligated into a binary plasmid vector. The plasmid was subjected to *in vitro* TN mutagenesis, and a construct with the TN insertion inside the *VDH5* ORF was used for *Agrobacteriuim tumefaciens* mediated transformation into *V. dahliae* Dvd-T5 spores. C) Integration of the *vdh5TN* cassette into the *Verticillium* genome by homologous recombination. The green line represents the 5' flanking sequence probe used in the Southern hybridization analysis.

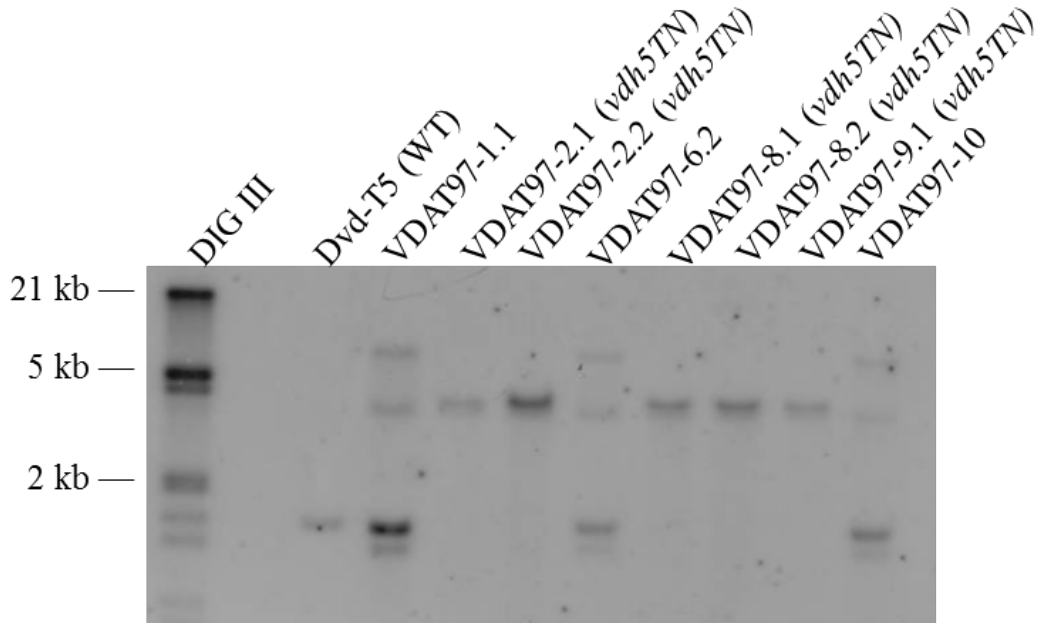


Figure 19: Southern hybridization data for the transformants with the *vdh5TN* cassette.

Southern blot hybridization of the *vdh5TN* transformants with a probe complementary to the 5' flanking sequence of the *VDH5* ORF.

The *vdh5ΔENT* vector was constructed as described in the Materials and Methods. First, sequences were amplified from 1) approximately two Kb upstream of the *VDH5* ORF to the beginning of the ENT domain, and 2) immediately after the ENT domain to approximately one Kb downstream of the*VDH5* ORF. The two amplicons were then ligated together, resulting in the production of a gene construct lacking the ENT domain (Fig. 20A). The *vdh5ΔENT* construct was ligated into the binary vector pDHt (Mullins et al., 2001) and subjected to *in vitro* transposon (TN) mutagenesis (Fig 20B). The mutagenized vector, having the TN (containing the geneticin-resistant gene, GenR) insertion approximately one Kb upstream of the *vdh5ΔENT* sequence, was used for ATMT into *V. dahliae* Dvd-T5 as described above. Homologous recombination events were screened for by PCR and Southern blot analysis of the transformants, and the results for 14 independent GenR transformants are shown in Figure 21A and C. Ten putative true transformants were identified by PCR screening (Fig. 21A), and two, VDAT95-4.2 and VDAT95-9.1, were confirmed by Southern blot (Fig. 21C) and used for subsequent analyses.

To generate a complemented strain the *VDH5* deletion mutant was transformed by ATMT with the WT *VDH5* gene containing a Gen R gene approximately one kb upstream the WT ORF. Fourteen independent GenR transformants were generated, and screened by PCR. All transformants appeared to carry the GenR-tagged gene homologously recombined into the *VDH5* locus (Fig. 21B), and two of these, VDAT 94-2.1 and VDAT94-10.1, were further confirmed by Southern Blot analysis (Fig. 21C) and used for subsequent analyses.

Figure 20: Generation of VDH5-ENT domain deletion vector.

A) Sequences from approximately two kb upstream of the ORF of *VDH5* to the beginning of the ENT domain, and from immediately after the ENT domain until approximately one Kb downstream the*VDH5* ORF, were amplified. The two amplicons were then digested with BamHI, which cutting sites were incorporated in the primers, and the ligation of the digested amplicons resulted in the removal of the ENT region. B) The *vdh5ΔENT* fragment was ligated into a binary vector. The plasmid was subjected to in vitro TN mutagenesis, and a construct with the TN insertion approximately one Kb upstream of the *vdh5ΔENT* sequence was used for *Agrobacteriuim tumefaciens* mediated transformation into *V. dahliae* Dvd-T5 spores. C) Integration of the ENT deletion cassette into the *Verticillium* genome by homologous recombination. Blue arrows represent the primers used for PCR screening of transformants, and the green line represents the probe used in the Southern hybridization analysis.

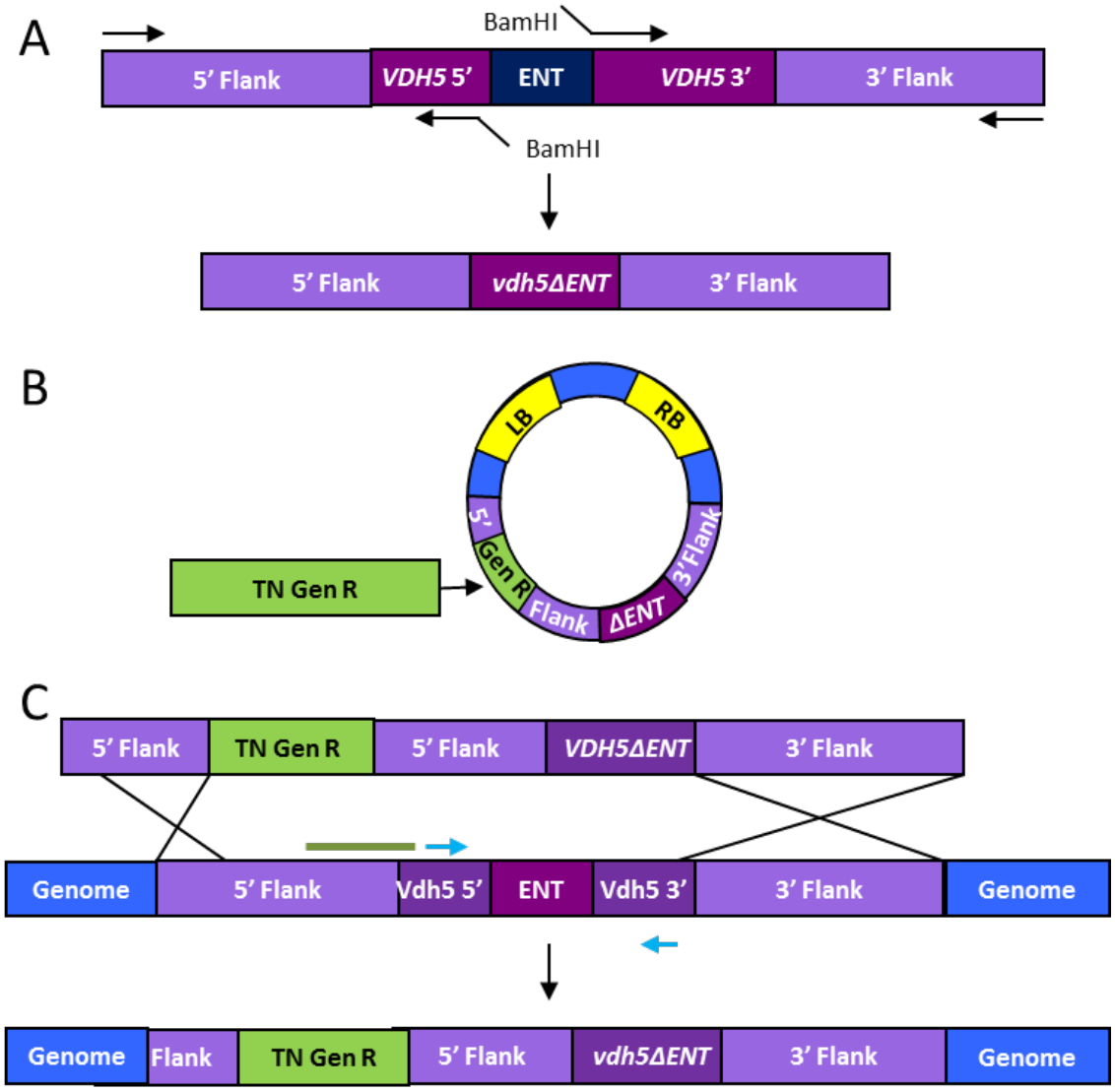
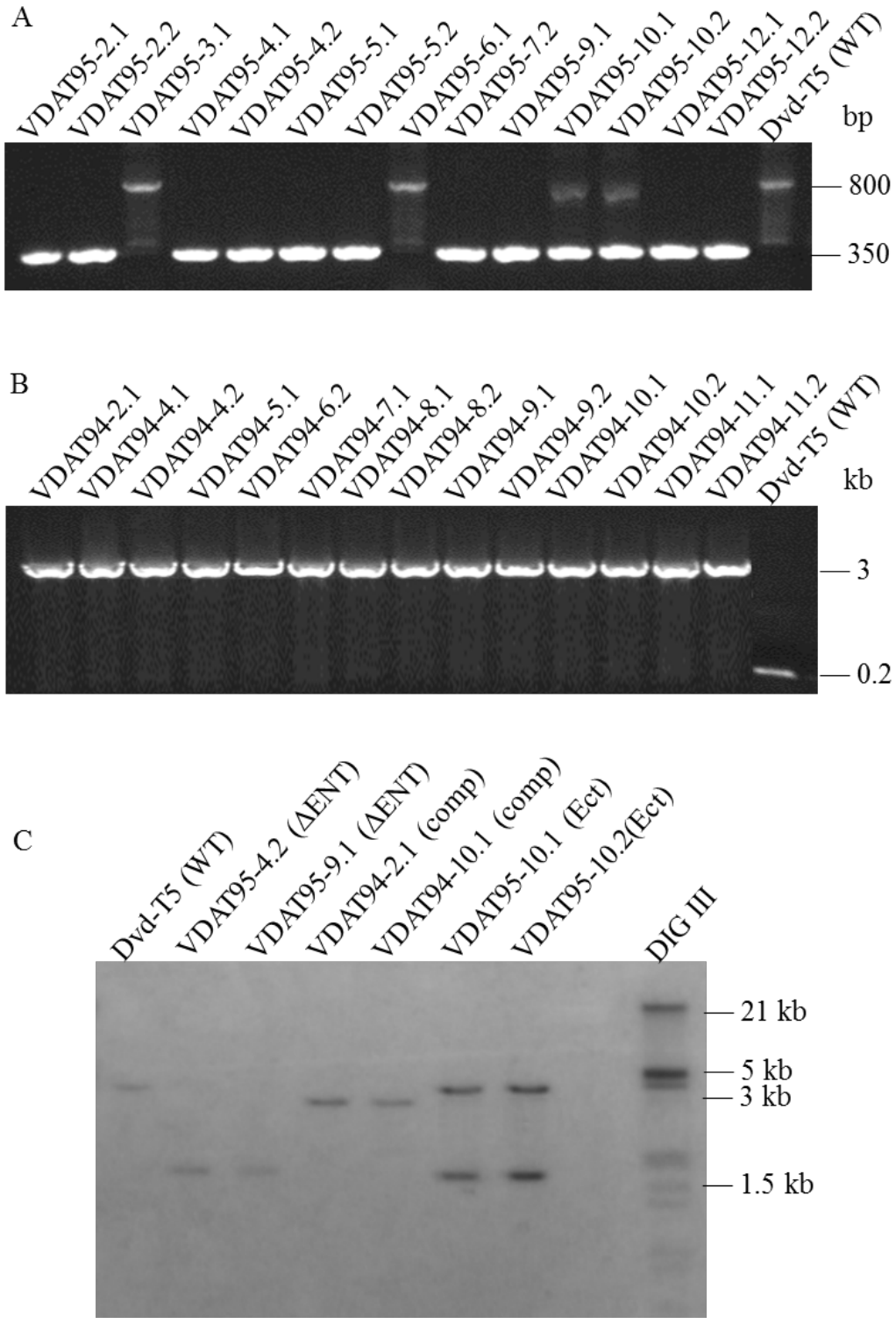

A
BamHI
5' Flank
VDH5 5'
ENT
VDH5 3'
3' Flank
BamHI
5' Flank
vdh5ΔENT
3' Flank
B
LB
RB
5'
Gen R
Flank
ΔENT
3' Flank
TN Gen R
C
5' Flank
TN Gen R
5' Flank
VDH5ΔENT
3' Flank
Genome
5' Flank
Vdh5 5'
ENT
Vdh5 3'
3' Flank
Genome
Genome
Flank
TN Gen R
5' Flank
vdh5ΔENT
3' Flank
Genome

Figure 21: PCR and Southern hybridization data for the transformants with the *VDH5ΔENT* and complement transformants.

A) PCR analysis of the different ENT deletion transformants with primers annealing inside the ORF of the *VDH5* gene. B) PCR analysis of the different complement transformants with primers annealing around the TN insertion site. C) Southern blot hybridization of the *VDH5ΔENT* and the complement transformants with a probe complementary to the 5' flanking sequence of the *VDH5* ORF.


A
VDAT95-2.1
VDAT95-2.2
VDAT95-3.1
VDAT95-4.1
VDAT95-4.2
VDAT95-5.1
VDAT95-5.2
VDAT95-6.1
VDAT95-7.2
VDAT95-9.1
VDAT95-10.1
VDAT95-10.2
VDAT95-12.1
VDAT95-12.2
Dvd-T5 (WT)
bp
800
350
B
VDAT94-2.1
VDAT94-4.1
VDAT94-4.2
VDAT94-5.1
VDAT94-6.2
VDAT94-7.1
VDAT94-8.1
VDAT94-8.2
VDAT94-9.1
VDAT94-9.2
VDAT94-10.1
VDAT94-10.2
VDAT94-11.1
VDAT94-11.2
Dvd-T5 (WT)
kb
3
0.2
C
Dvd-T5 (WT)
VDAT95-4.2 (ΔENT)
VDAT95-9.1 (ΔENT)
VDAT94-2.1 (comp)
VDAT94-10.1 (comp)
VDAT95-10.1 (Ect)
VDAT95-10.2(Ect)
DIG III
21 kb
5 kb
3 kb
1.5 kb

3.5.4 Effects of the deletion of the *VDH5*-ENT domain or deletion of the full-length *VDH5* on fungal development and pathogenicity.

In order to study the effect of the partial (*vdh5ΔENT*) and complete *VDH5* deletions in development and pathogenicity, the one *Δvdh5* mutant, two *vdh5ΔENT* mutants, two ectopic insertion transformants (one from the deletion construct and one from the insertion construct), and two complementation construct-carrying strains were used in further analyses. The results obtained by the independent transformant strains were consistent with each other, and for clarity the figures show the results for only one of each of these different types of recombinant strains.

The *VDH5* deletion mutant (*Δvdh5*) and the targeted gene disruption mutant (*vdh5TN*) showed no evidence of microsclerotia at 21 dpi when grown axenically on CM or BM agar, and introducing the WT gene into the *Δvdh5* strain recovered the WT microsclerotia phenotype (Fig. 22, Comp). No difference in microsclerotial development was evident between the WT strain (Dvd-T5), the *vdh5ΔENT* strain, or the *Δvdh5* ectopic insertion transformants (Fig. 22, Ect). Also, light microscopy suggested that the *VDH5* deletion mutant has delayed microsclerotial development, since fully melanised microsclerotia were not evident until approximately 60 dpi (Fig. 23).

Another change that was evident with the *VDH5* deletion mutant was that the colony radius appeared to be smaller when compared to the WT strain; however, this reduction was not statistically significant (Fig. 24).

Figure 22: Colony morphologies of Dvd-T5 (WT), *Δvdh5, vdh5TN, vdh5ΔENT,* Complement (Comp) of *Δvdh5* with WT *VDH5,* and ectopic *Δvdh5* insertion (Ect) strains from the *Δvdh5* transformation.

Cultures were grown for 14 days on solid CM at 24˚C. Photos were taken of the upper (Top) or underside (Bottom) of the plates.

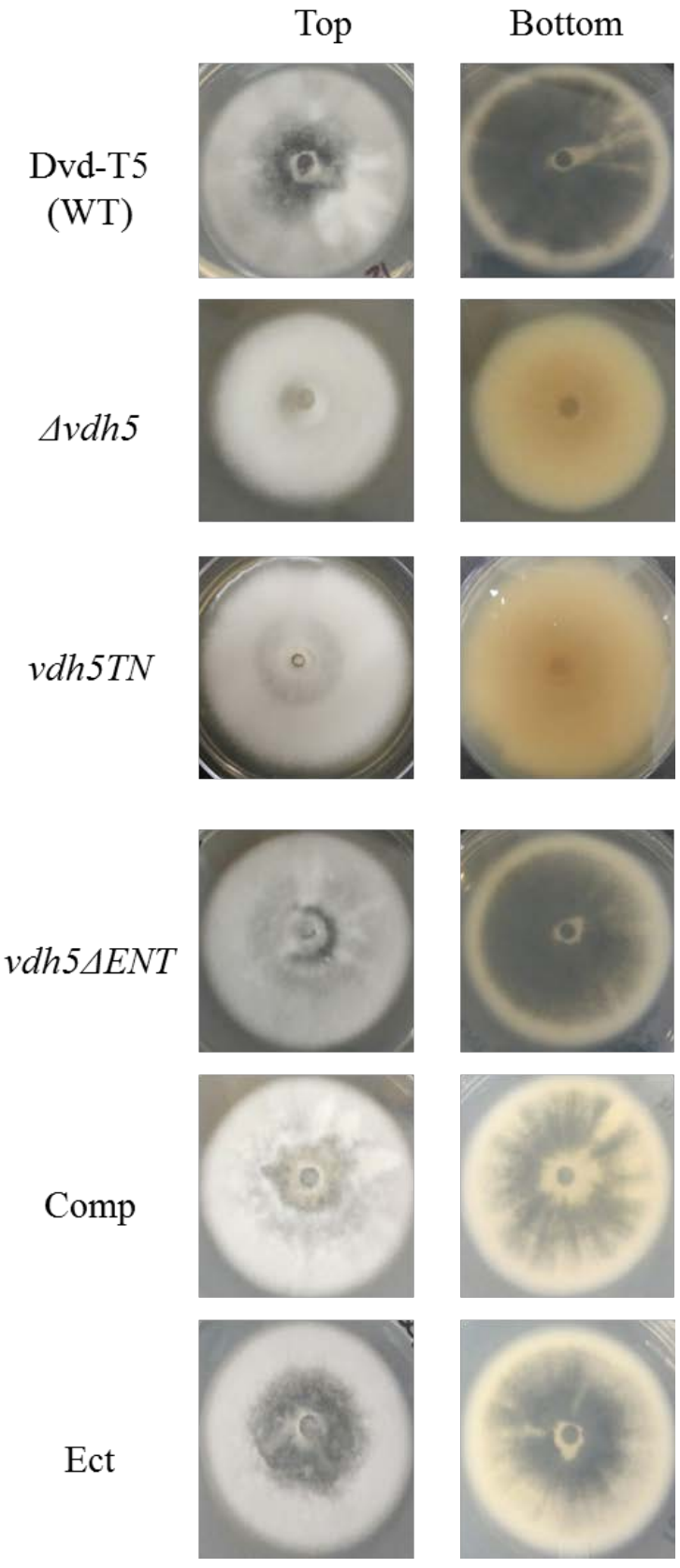
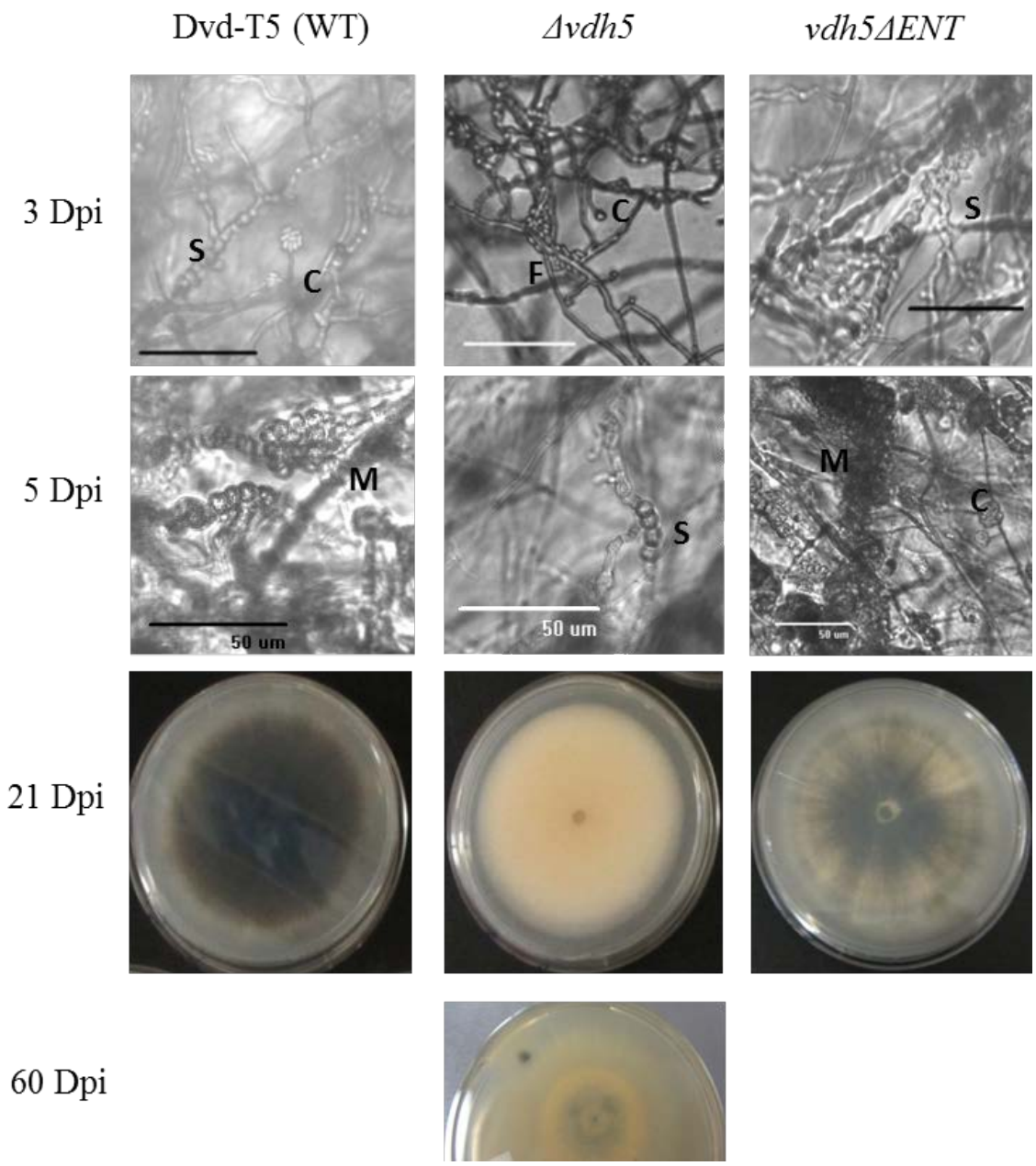

Top
Bottom
Dvd-T5 (WT)
Δvdh5
vdh5TN
vdh5ΔENT
Comp
Ect

Figure 23: Microsclerotial development in *V. dahliae* Dvd-T5 (WT), *Δvdh5*, and *vdh5ΔENT* strains.

Deletion of *VDH5* causes delayed microsclerotial development. Micrographs of the development of MCS at three and five days post inoculation (dpi, scale bars: 50 µm), showing conidiophores (C), fusing hyphae/conidia (F), swollen, septate hyphae (S) and mature microsclerotia (M). Lower panels show colony morphologies at 21 dpi and, for *Δvdh5*, at 60 dpi. Cultures were grown on CM agar at 24 ˚C.


Dvd-T5 (WT)
Δvdh5
vdh5ΔENT
3 Dpi
5 Dpi
21 Dpi
60 Dpi
S
C
F
M
50 um

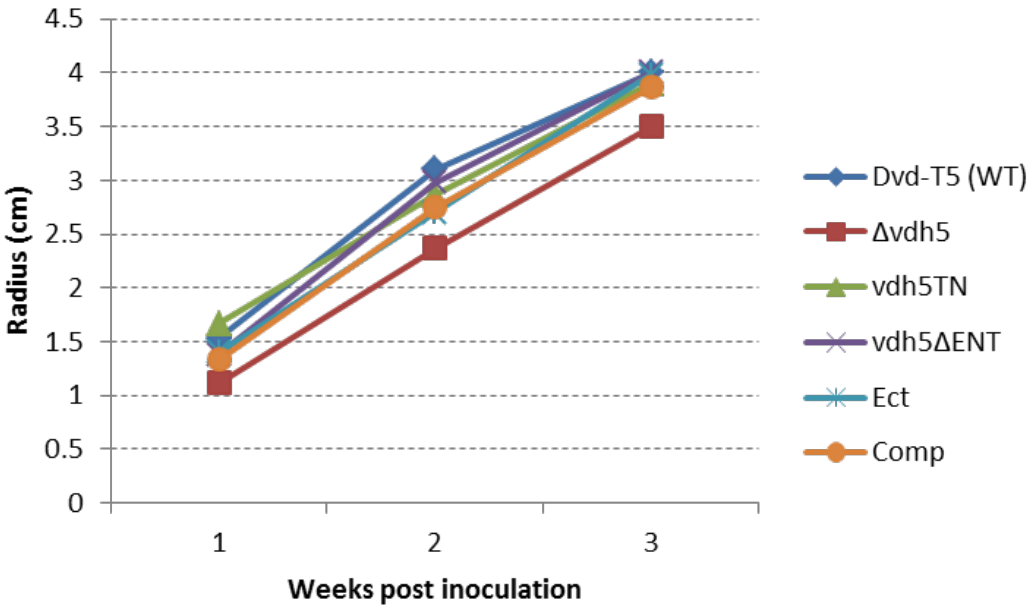


Figure 24: Radial growth of Dvd-T5 (WT), *Δvdh5*, *vdh5TN*, *vdh5ΔENT*, complement (Comp) and ectopic *Δvdh5* insertion (Ect) *V. dahliae* strains.

Cultures were grown at 24˚C on CM. One-way ANOVA was performed, and no significant difference in radial growth between *Δvdh5* and the other strains was detected. Bars represent standard error (n=9)

Aerial hyphae and conidia of filamentous fungi are generally hydrophobic, and it is believed that this feature is due to the presence of hydrophobins (Bayry et al., 2012). For this reason, the hydrophobicity of the mycelium of WT and *VDH5* mutant strains was tested by placing a 200 µL droplet of water onto the surface of the growing margins of two-week old axenic cultures grown on CM agar. Twelve hours later, the drops remained on the surface of all strains, and no visible difference in droplet shape was detected between the WT, and the Δ*vdh5, vdh5TN, and vdh5ΔENT* strains (Fig. 25).

The number of conidia from mycelium produced by the WT or the *VDH5* mutant strains was also assessed as described in the Material and Methods. The *Δvdh5* and the *vdh5TN* strains showed a significant reduction in the number of conidia produced, when compared to that of the WT, and the addition of the complementation construct to the *ΔVDH5* mutant failed to rescue this phenotype (Fig. 26). No difference in the number of spores produced by the WT and the *vdh5ΔENT* or the ectopic insertion strains was observed (Fig. 26). No difference in conidial germination was observed between the WT and any of the mutant strains (data not shown).

Finally, I assessed the role of *VDH5* in pathogenicity on tomato. The *Δvdh5* strain has a defect in pathogenicity on tomato root dip-inoculated plants. The plants inoculated with that strain showed lower symptom severity, and were less stunted in comparison to the WT-inoculated plants. Nonetheless, disease symptoms were clearly evident when compared to the lack of disease observed for the mock inoculated tomato plants (Fig. 27A and B). The addition of the complementation construct to the *Δvdh5* strain restored WT pathogenicity (Fig. 27A and B). No difference was observed in the severity of disease

caused by the *vdh5ΔENT* and ectopic strains in comparison to that caused by the WT (Fig. 27A and B).

As a quantitative way of measuring stunting, the fresh weight of the plants was determined. The results correlate with the disease scoring assessment (i.e. higher disease score was associated with more negative effects on plant growth), with the *Δvdh5*-inoculated plants having an average weight between that of the WT inoculated plants, and the mock inoculated plants (Fig. 27C).

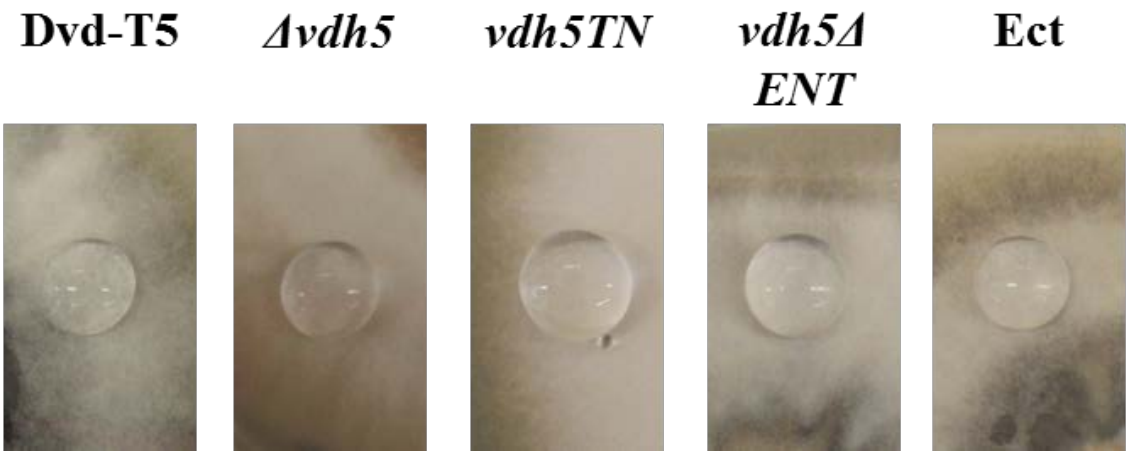


Figure 25: Images of colony wettability assay for Dvd-T5 (WT), *Δvdh5*, *vdh5TN*, *vdh5ΔENT*, and *Δvdh5* ectopic insertion *V. dahliae* strains.

Colony wettability was assessed by placing onto the surface of the growing margins of 14 day old cultures a 200 µL drop of water. Photographs were taken 12 hours after droplet placement. Cultures were grown on solid Complete medium at 24 ˚C.

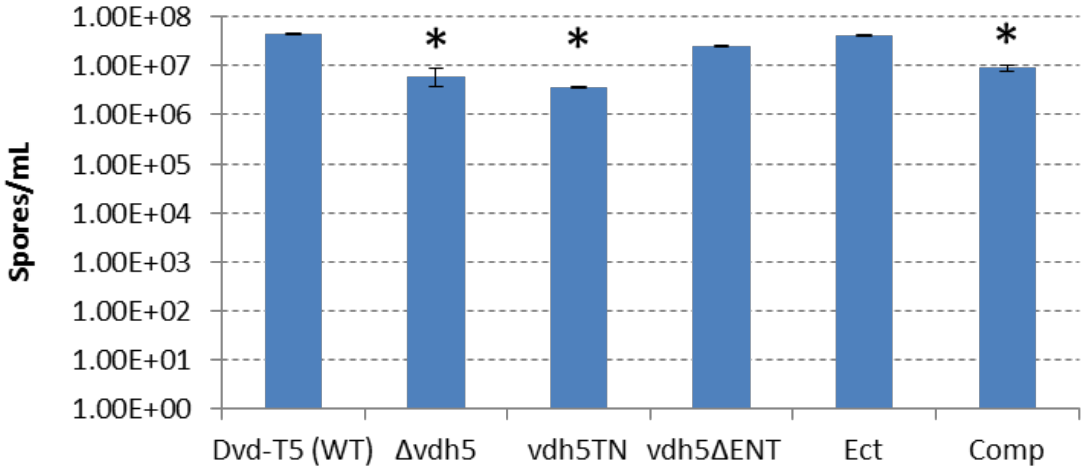
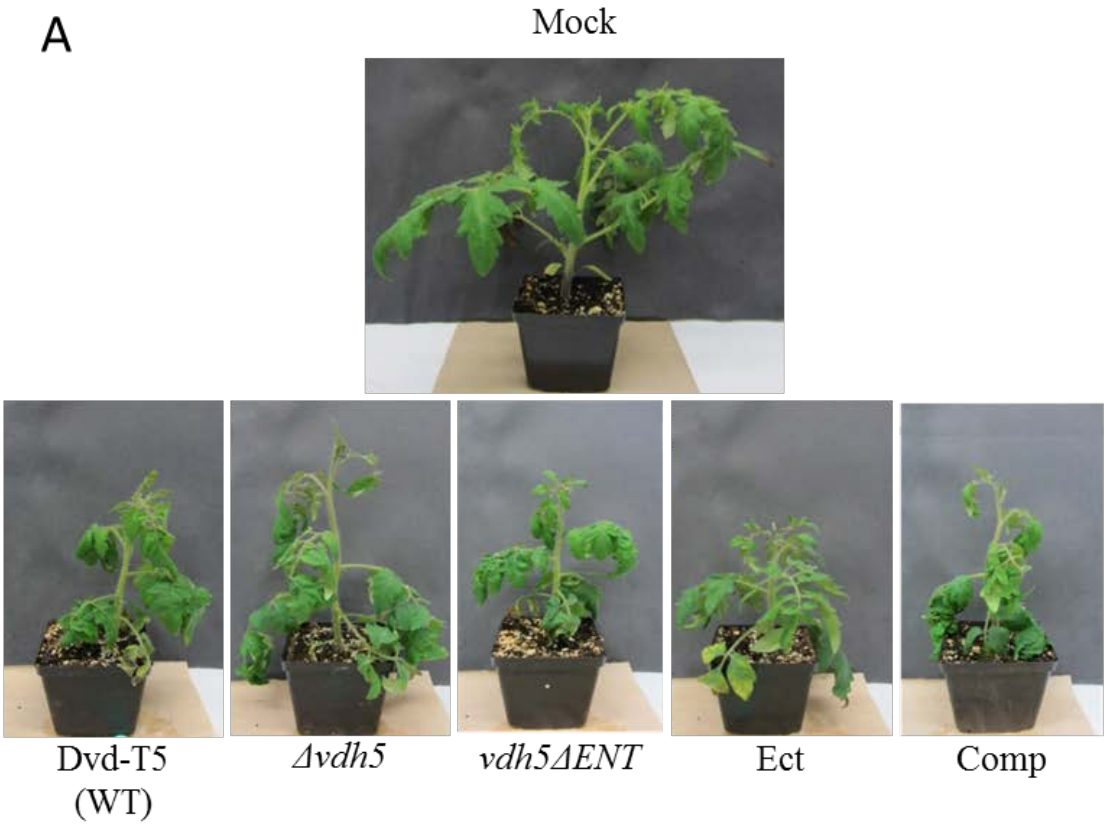


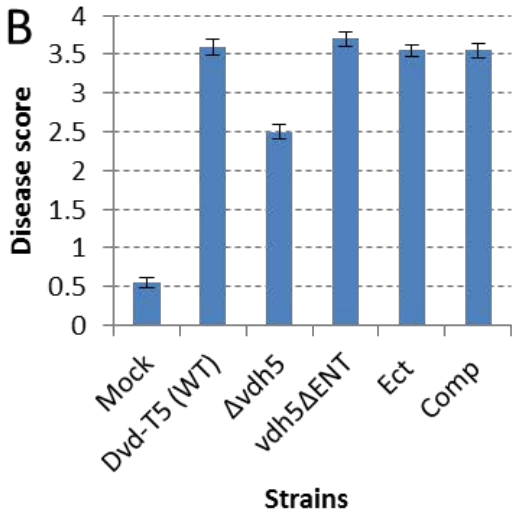
Figure 26: Spore production from actively growing mycelia of *V. dahliae*.

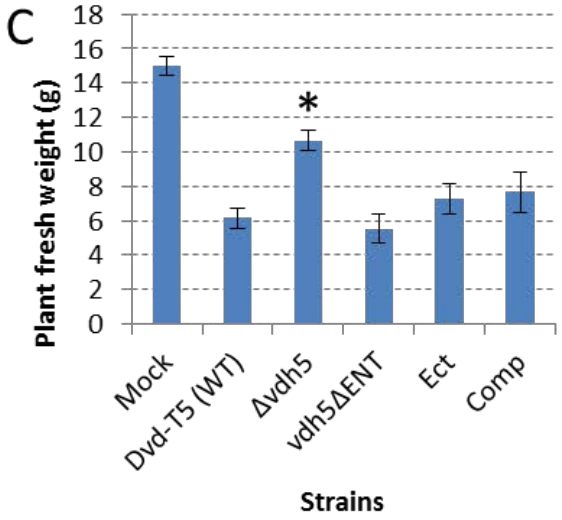
Spore production during growth in liquid CM was assessed for WT, *Δvdh5*, *vdh5TN*, vdh5ΔENT, complement (Comp), and *Δvdh5* ectopic insertion (Ect) strains. Data are the means of three biological replicates, each with three technical replicates. Asterisks mark statistically significant differences of spore production compared to that of the WT strain ($P<0.05$, One-way ANOVA; Standard error bars are shown (n=9)

Figure 27: Pathogenicity assay

Plants inoculated with *Δvdh5* show a slower disease progression when compared to those inoculated with the WT strain. A) Bonny Best tomato plants inoculated with Dvd-T5 (WT), *Δvdh5*, *vdh5ΔENT*, *Δvdh5* ectopic insertion (Ect), complement (Comp) strains, or mock-inoculated (Mock) at 28 dpi. B) Average disease severity, scored visually at four weeks after inoculation. C) Average weight of plants at four weeks post inoculation. The experiment was done three times with five plants each time. The weights of all the fungal-strain inoculated plants were significantly different from the weights of the mock-inoculated plants, and an asterisk marks the statistically significant difference of fresh weight of *Δvdh5*-inoculated plants compared to those inoculated with the WT strain ($P<0.05$ One-way ANOVA; Bars represent standard error; n=30).


A
Mock
Dvd-T5 (WT)
Δvdh5
vdh5ΔENT
Ect
Comp


B
Disease score
4
3.5
3
2.5
2
1.5
1
0.5
0
Mock
Dvd-T5 (WT)
Δvdh5
vdh5ΔENT
Ect
Comp
Strains


C
Plant fresh weight (g)
18
16
14
12
10
8
6
4
2
0
*
Mock
Dvd-T5 (WT)
Δvdh5
vdh5ΔENT
Ect
Comp
Strains

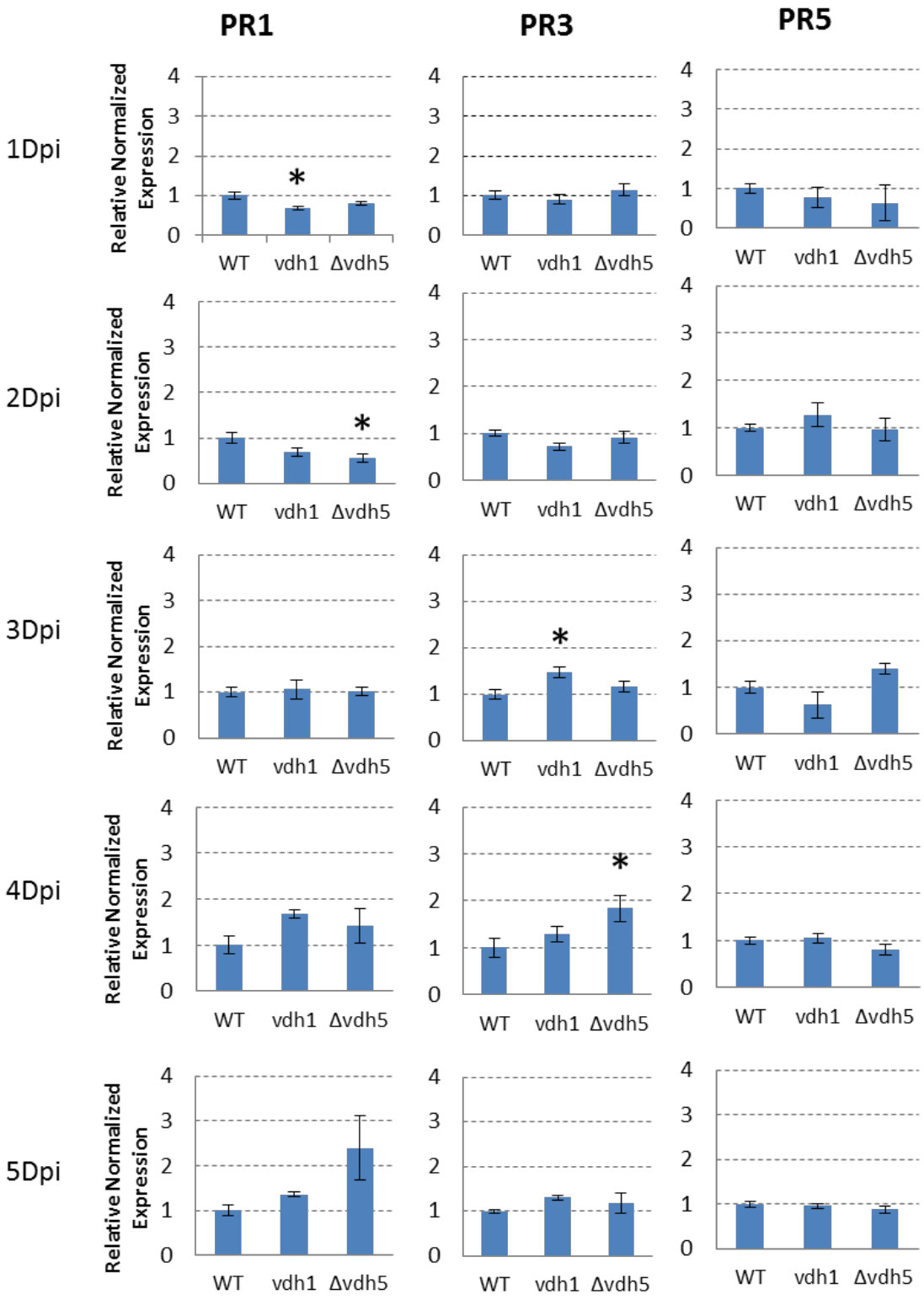
3.6 Expression of Tomato Pathogenesis Related Genes in Response to Infection With the *Δvdh5* and *VDH1* Disruption Strains.

Three pathogenesis related genes (*PR1, 3* and *5)* were previously shown to be highly expressed upon infection of lettuce with *V. dahliae* (Klosterman et al., 2011a). I have therefore assessed the expression of these genes in Bonny Best tomatoes inoculated with the *Δvdh5* strain, which produces disease more slowly than does WT Dvd-T5, and the *VDH1* disruption mutant (*vdh1*), which produces disease more rapidly (Klimes, 2006, Klimes & Dobinson, 2006). Initially this experiment was designed so that gene expression would be normalized against the gene expression in mock-inoculated plants, and in relation to expression of the tomato elongation factor 1 gene (*EF1*). However, it became evident that the expression of *EF1* was 1000 times lower when the plants were inoculated with any of the *V. dahliae* strains, than in the mock-inoculated plants. Given this, the data analysis was performed without the mock inoculation data, but relative to expression of the tomato *EF1*, and gene expression was normalized against the expression of each PR gene in plants infected by the WT *V. dahliae* strain.

The resulting data showed that at 1 and 2 dpi expression levels for *PR1* were lower in plants inoculated with either of the hydrophobin mutants than in plants inoculated with the WT, but were higher at 4 and 5 dpi than in WT-infected plants. The *PR3* expression levels were higher in plants inoculated with the *VDH1* disruption mutant when compared with the WT inoculated plants at 3 dpi. Plants inoculated with *Δvdh5* strain had higher levels of PR3 at 4 dpi compared to that of WT or *vdh1* inoculated plants. No significant changes were observed for the expression level of *PR5.* (Fig 28).

Figure 28: Expression of tomato Pathogenesis Related (PR) genes in response to inoculation with different strains of *V. dahliae*.

Quantitative reverse transcriptase (qRT)-PCR analysis was done of *PR1, PR3,* and *PR5* transcript levels in Bonny Best tomato plants inoculated with *V. dahliae* Dvd-T5 (WT), VDAT85-3 (*Δvdh5*), or VDAT2-17(*vdh1*). Transcript levels were measured at one to five days post inoculation (dpi), relative to those of the tomato *Elongation Factor 1* gene (*EF1*), and were normalized against the expression in plants inoculated with the WT strain. Asterisks mark the statistically significant different gene expression in plants inoculated with either *Δvdh5* or *vdh1*, as compared to those inoculated with the WT strain. ($P<0.05$ One-way ANOVA; Standard error bars are shown; n=9).


PR1
PR3
PR5
1Dpi
2Dpi
3Dpi
4Dpi
5Dpi
Relative Normalized Expression
0
1
2
3
4
WT
vdh1
Δvdh5
*

CHAPTER 4: Discussion

4.1*Verticillium dahliae* has Five Class II Hydrophobins

Hydrophobins are proteins that play diverse roles in the fungal life cycle (Kubicek et al., 2008, Linder, 2009). Analyses of different fungal genomes show that hydrophobin genes usually exist as gene families containing two to ten members (Sunde et al., 2008, Bayry et al., 2012). However, up to 33 hydrophobins have been reported in the mushroom *Coprinus cinereus* (Bayry et al., 2012), and 40 in *Trametes versicolor* (Mgbeahuruike et al., 2013).

Five hydrophobin-like genes were found in the genome of *V. dahliae* strain VdLs.17 (Klosterman et al., 2011c). Of these, *VDH1* was previously characterized as a true class II hydrophobin with a role in the development of microsclerotia (Klimes, 2006, Klimes & Dobinson, 2006). My analysis of the amino acid sequences of the uncharacterized four hydrophobins confirms their classification as hydrophobins. Each one of them has a putative signal peptide at their N terminus, and eight cysteine (Cys) residues in the C terminus, in a pattern that is characteristic of hydrophobins (i.e. CX-CCX-CX-CX-CCX-CX).

Although both class I and class II hydrophobins are found in Ascomycota (Wösten, 2001, Seidl-Seiboth et al., 2011), I have found only class II hydrophobins in *V. dahliae*. Class I hydrophobins have been found as rodlet layers in fungal dry spores (Sunde et al., 2008) but the conidia in *V. dahliae* is hydrophilic. It has also been proposed that only the class I hydrophobins may have a role in the formation of fruiting bodies (Mgbeahuruike et al.,

2013). If this is the case, since *V. dahliae* does not produce fruiting bodies, it is not surprising that it may only have class II hydrophobins in its repertoire.

4.2 The Hydrophobins From *V. dahliae* Are Similar to Hydrophobins from Plant-Interacting Fungi and Entomopathogenic Fungi.

Most of the 80 hydrophobin sequences obtained from the BlastP analysis performed against the *V. dahliae* hydrophobin amino acid sequences belong to other ascomycetous plant-associated fungi. Surprisingly, the blast search also retrieved as well a group of hydrophobin sequences from entomopathogenic fungi. What all these fungi have in common is that they interact with other living organisms, by penetrating them, growing inside them, and producing spores or resting structures to start the colonization cycle again. It could be inferred then that these proteins might have important roles during the above processes.

One of the most accepted theories to explain the diversity of hydrophobins is the birth and death model, which states that new gene copies evolve through gene duplication and diversification through spontaneous mutation (Kubicek et al., 2008). New genes with vital functions are retained while the ones that do not provide any added benefit or, on the contrary have deleterious effects, are eliminated (Kubicek et al., 2008). The phylogenetic results for the genera *Claviceps, Ophiostoma,* and *Trichoderma,* in which hydrophobins from each of these distantly related organisms cluster in a single clade, can be explained by such purifying selection. In contrast, the hydrophobins from *Verticillium* did not group together in one clade, suggesting that these proteins are the result of rapid

diversification or duplication events that happened before speciation, and have had a long time to accumulate changes in their sequences.

Only three of the five *V. dahliae* hydrophobins, VDH1, 2, and 3, appear to have orthologs in *V. albo-atrum* (recently reclassified as *V. alfalfae*, Inderbitzin et al., 2011). Some of the differences between these two species are the type of resting structure produced by each, the host ranges of the two species, and their genome sizes. While *V. dahliae* produces microsclerotia, affects more than 200 hosts in at least 14 families, and has a genome size of 33.83 Mb, *V. alfalfae* produces dark resting mycelium, has so far only been isolated from *Medicago sativa,* and its genome size is 32.83 Mb (Pegg & Brady, 2002, Inderbitzin et al., 2011, Klosterman et al., 2011c, Inderbitzin & Subbarao, 2014). No significant correlation between genome size and number of hydrophobin-encoding genes in a fungus has been found (Mgbeahuruike et al., 2013), suggesting that the discrepancy found in the number of hydrophobins between *V. dahliae* and *V. alfalfae* may be due to their differences in lifestyle, and not merely their genome size difference. Some hydrophobins needed for microsclerotial development in *V. dahliae* may be absent from *V. alfalfae*, which does not produce microsclerotia. Also, it could be speculated that a larger number of hydrophobins helps *V. dahliae* to colonize a wider, more variable range of hosts, by producing a barrier to hide the pathogen surface from the plant immune system, as has been reported by Aimanianda et al. (2009) who suggested that the hydrophobin layer was essential for preventing the recognition of airborne fungal spores by immune cell lines.

4.3 Analyses of the Promoter Regions of *V. dahliae* Hydrophobin Genes Suggest Expression in Response to Stress and Nitrogen Deficiency.

Sequence analyses of the regions upstream of the *V. dahliae* hydrophobin ORFs revealed the presence of several regulatory elements. All five genes have several putative binding sites for heat shock factors. Heat shock elements are sequences that mediate the transcription of heat shock protein genes, and are typically present in multiple copies upstream of these genes. The expression of these proteins is induced in response to stresses such as heat, oxidative stress, heavy metals, and toxins (Åkerfelt et al., 2010). The presence of these heat shock elements in the hydrophobin genes in *V. dahliae* suggests that the hydrophobin genes may respond also to different environmental stresses. In addition to protection against different stresses, heat shock factors are involved in different physiological functions (Åkerfelt et al., 2010). The presence of heat shock factor elements in the promoter region of hydrophobins has been reported previously for the hydrophobin *MPG1* gene in *Magnaporthe grisea.* However, the expression of the *MPG1* gene was not altered in response to pH, osmotic, or oxidative stress (Soanes et al., 2002), suggesting that these regulatory elements are not functional in *M. grisea*.

GATA transcription factors regulate the expression of genes necessary for nitrogen uptake from different nitrogen sources (Wong et al., 2008). In the ascomycetes *Aspergillus nidulans* and *Neurospora crassa,* for example, the GATA factors AreA and Nit2, respectively, regulate the ability to utilize nitrogen from different sources (Arst Jr & Cove, 1973, Marzluf, 1997). Five putative GATA binding sites, specifically NIT sites, were found in the promoter region of *VDH1*, and three for *VDH3*. The presence of

GATA/NIT elements in the promoter region of hydrophobins has been described previously for the hydrophobin genes *MPG1* (Soanes et al., 2002), and *VDH1* (Klimes, 2006). The expression of *MPG1* was induced upon nitrogen starvation, (Soanes et al., 2002), while no changes in *VDH1* expression were detected with nitrogen limitation (Klimes et al., 2008). Given these contradictory results, the importance, if any, of these putative GATA binding sites in the promoter region of *V. dahliae* hydrophobin genes will need to be addressed in future experiments.

4.4 The Hydrophobin Genes in *V. dahliae* are Differentially Expressed.

The expression of the *V. dahliae* hydrophobin genes was assessed under different growth conditions. Firstly, WT *V. dahliae* was grown for four days on CM agar or BM agar. After four days post inoculation on CM agar, the fungus was growing actively, conidiophores were developed, spores were being produced, and MCS were developing. Under these growth conditions, transcription of all five hydrophobin genes was detected. *VDH3* displayed the lowest levels of transcripts, and *VDH4* and *VDH5* (absent *in V. alfalfae*) the highest. It could be speculated then, that *VDH4* and *5* might have roles in mycelial growth, spore production, and/or microsclerotial development.

It has been reported that production of microsclerotia in *Verticillium* increases at higher C:N ratios (Wyllie & DeVay, 1970). Having less than 3% the amount of nitrogen found in CM, BM has a higher C:N ratio than does CM, and consistent with the abovementioned report, the development of microsclerotia is accelerated when cultures are grown on BM (Neumann & Dobinson, 2003). After four days growth under these conditions, the transcript levels of all hydrophobin genes except *VDH4* were considerably

lower when compared to the levels found when the fungus was growing on CM agar. These results suggest therefore that *VDH4* may play a role during the latter stages of microsclerotial development, when the other four hydrophobins are no longer needed.

The expression of the hydrophobin genes in liquid CM cultures was also measured after four days growth. Liquid medium supports both mycelial growth and spore production, and allows separation of the two structures, making possible the study of the hydrophobin gene expression profiles in each structure. Surprisingly, the expression of these genes in spores was very low, ranging from 20 to 400 copies/µL, in contrast to 2000 to 8000 for the reference genes. The hydrophobin genes with highest expression in spores were *VDH3* followed by *VDH5*, suggesting a role for these genes in spore viability and/or germination. These results are consistent with a study on hydrophobins in *Botrytis cinerea* in which three hydrophobin genes showed low expression in conidia when detectable (Mosbach et al., 2011).

When the hydrophobin genes expression was studied in *V. dahliae* mycelium, the gene transcript levels, for all genes but *VDH3*, were higher than those found in spores, in a pattern similar to the one observed in solid CM, with *VDH4* having the highest expression. This result suggests a role for *VDH3* in spores survival and/or germination, and for *VDH1, 2, 4,* and *5* in hyphal growth and production of conidia by conidiophores.

The last condition in which expression of the hydrophobins genes was assessed was during *in planta* growth. For such analysis, two-week old tomato seedlings were inoculated with WT *V. dahliae,* and RNA from the infected plants was extracted from 0 to 5 dpi. As expected, given the differences in biomass between the plant and the fungal

inoculum, the detected levels of fungal gene expression were extremely low when compared to those of the reference genes (i.e. plant genes). Nonetheless, I was able to detect expression of the hydrophobin genes. Immediately after inoculation (0 dpi) the expression profile of the hydrophobin genes resembled that found in conidia harvested from cultures grown in liquid. This result was expected, since a suspension of liquid-grown conidia was used as inoculum to infect the plants. In general, the *in planta* data revealed a dynamic expression profile for the hydrophobin genes, suggesting that the different genes are important in different stages of infection and colonization of the host. The more or less consistent expression levels of *VDH1*, *2* and *4* throughout the course of the experiment suggest a role of these genes throughout the pathogenicity process. The drop in expression of *VDH3* at day 2 post inoculation suggests once more that this hydrophobin's role may be limited to conidial survival and/or germination. The significantly higher expression at the end of the studied time period of *VDH5* compared to the levels seen for the other hydrophobins suggests an important role of this protein in plant xylem colonization and pathogenicity. These results are consistent with the results obtained in the pathogenicity analyses in which plants inoculated with the *Δvdh5* strain displayed a slower disease progression when compared to plants inoculated with the WT strain.

4.5 VDH5 Localizes to the Cytoplasm in Spores, Mycelia, and Microsclerotial Initials.

In order to study the localization of VDH5 in *V. dahliae*, a fusion protein construct containing VDH5 with YFP linked to the C-terminus was constructed and transformed

into Dvd-T5. Confocal microscopy analyses showed what appears to be YFP expression in the cytoplasm of hyphae, spores, and swollen cells (MCS developmental precursors). The distribution of YFP did not appear constant throughout the structures and so nuclear staining with DAPI was also done. The results confirmed that VDH5::YFP does not have a nuclear localization. Finally, in order to see if the YFP-tagged hydrophobin might be localized to the cell wall, the fungus was stained with propidium iodide . There was no co-localization of the YFP with cell wall-specific dye. From the abovementioned results I concluded that VDH5 is localized to spores, mycelia, and microsclerotial initials. The apparent cytoplasmic (i.e. not secreted) localization of such a class II hydrophobin is uncommon. One possibility to explain the observed intracellular pattern is that I was only observing hydrophobins that have not yet been secreted, or secreted and diffused throughout the medium. It is also possible that the fusion of the fluorescent tag to this particular hydrophobin has altered its ability to be secreted, and therefore it remains in the cytoplasm. Whether this fusion does indeed result in an inability to be secreted, and could render the hydrophobin non-functional, has yet to be studied. Even though a functional characterization of the ENT domain (present in VDH5) has not been done, it has been hypothesised that the ENT domain might mediate cell wall binding, given its resemblance to a module of plant bimolecular proteins (Lora et al., 1995). If in fact the ENT domain mediates cell wall binding, this would be consistent with the results obtained with the *VDH5::YFP* strain and the cytoplasmic localization of the fusion protein.

Interestingly, the intensity of fluorescence displayed by the *VDH5::YFP* strain was not as strong as might have been expected given the results obtained previously with a

fluorescence-tagged *VDH1* gene construct (Klimes et al., 2008). However, in contrast to the VDH5-YFP construct, the fluorescent tag in the *VDH1* construct was fused to the promoter region of *VDH1* and not directly to the protein. Given that the transcript levels of *VDH5* were higher than those of *VDH1* under all the conditions studied here, the small amount of YFP-tagged VDH5 I observed may be explained by post-transcriptional and/or post-translational regulation. A post-translational type of regulation has been suggested in a study in a class I hydrophobins from *Trichoderma atroviride* by differential splicing in response to changes in nutritional and environmental conditions (Seidl-Seiboth et al., 2011).

4.6 Deletion of *VDH5* Affects spore Production and Pathogenicity in *V. dahliae*.

Hydrophobins are involved in numerous developmental processes (Bayry et al., 2012). Studies of hydrophobin mutants have shown that even though sometimes the function of the different genes is redundant, in general the different proteins have different roles. Hydrophobins play a role in the development of resting structures. For example, in *V. dahliae*, a mutation of the class II hydrophobin gene *VDH1* leads to the inability to produce MCS (Klimes & Dobinson, 2006). Additionally, a recent study showed that double and triple hydrophobin mutations in *Botrytis cinerea* lead to the production of aberrant sclerotia and defective apothecia (Terhem & van Kan, 2014). These findings are consistent with the results observed here in which deletion of *VDH5* resulted in a delayed production of MCS.

Hydrophobins have also been shown to have a role in sporulation. The deletion of the hydrophobin genes *HFB2* from *Trichoderma reesei* (Askolin et al., 2005), and *MPG1*

and *MHP1* from *Magnaporthe oryzae* (Talbot et al., 1996, Kim et al., 2005) result in reduced sporulation. Such was also the case observed in this study with the *Δvdh5* strain.

Finally, several hydrophobins have been implicated in pathogenicity. The class II hydrophobin Cerato-ulmi from *Ophiostoma ulmi*, for example, favors the adhesion of the fungus to its insect vector, and therefore helps the spread of Dutch Elm Disease (Whiteford & Spanu, 2002), while both *MPG1* and *MHP1* mutants show defects in appresorium development and pathogenicity (Talbot et al., 1993, Kim et al., 2005). Consistent with this information, the results in my study suggest that *VDH5* has a role in pathogenicity, since the *VDH5* deletion strain showed a slower disease progression when compared to that of the WT strain. This defect may be linked to the defect in spore production observed in the *Δvdh5* strain.

4.7 The ENT Domain of *VDH5* does not Have a Function in Development or Pathogenicity.

Comparing the primary structure of the five hydrophobins in *V. dahliae*, it became evident that one of the hydrophobins, VDH5, had an extra domain that was absent from the other hydrophobins. This Extended N-Terminus (ENT) domain has been reported in a few other hydrophobin genes (Lora et al., 1995, Kubicek et al., 2008). An ENT domain is also found in *QID3*, a cell wall protein gene from *Trichoderma harzianum* (Lora et al., 1995), the hydrophobin gene *HV_21a* from *Hypocrea virens*, *HA_2c* from *Hypocrea atroviridis* (Kubicek et al., 2008), and *HCF6* from *Cladosporium fulvum* (Nielsen et al., 2001). A similar domain is also present between the hydrophobin domains in the multi-hydrophobin gene *CFTH1* of *Claviceps fusiformis* (De Vries et al., 1999).

No differences between the *vdh5ΔENT* and the WT strains were observed in any of the development or pathogenicity studies conducted, suggesting that the ENT domain is not important for development or pathogenicity. These results suggest, rather, that the domain is either a remnant of what once was a multi-hydrophobin gene, or that it may have arisen from duplications of the GGNN tandem repeats.

There can be two reasons why fungi have multiple hydrophobins in their genomes. The function of hydrophobins could be so important that redundancy will ensure the reproductive success of the organism. Alternatively, it could be that each hydrophobin has a specific, unique role. In the case of *V. dahliae,* the expression profiles of the hydrophobin genes, the fact that the genes have different promoter elements, and the results found in development and pathogenicity assays with the mutants of *VDH1* and *VDH5* point toward the second possibility.

4.8 *Verticillium dahliae* Manipulates Host Gene Expression

It has been previously shown that the pathogenesis related genes *PR1, PR3* and *PR5* are highly expressed in tomato plants infected with WT *V. dahliae* (Tjamos et al., 2005, Klosterman et al., 2011a). Since I observed a marked difference in the rate of disease progress in plants inoculated with the *Δvdh5*, *vdh1* or WT strains, I decided to study the response of the three PR genes in tomato in response to infection with the different *V. dahliae* strains.

It was evident that expression of *PR1* was significantly lower at 1 and 2 dpi in plants inoculated with either hydrophobin mutant than in WT-infected plants. Given that PR1

has antifungal activity (Stintzi et al., 1993), the lower expression of *PR1* observed in plants inoculated with the *vdh1* null mutant compared to those inoculated with the WT may explain the faster disease development observed in the pathogenicity tests with this mutant (Klimes, 2006, Klimes & Dobinson, 2006). One would, however, expect opposite results in response to inoculations with *Δvdh5* given that it produces a slower disease progression in plants compared to those inoculated with the WT. It might be suggested then, that the pathogenicity phenotype observed when *Δvdh5* was inoculated onto tomato plants is due to its defect in spore production rather than to differences in the host defence responses.

Contrary to what was observed with *PR1* the expression levels of *PR3* were significantly higher in plants inoculated with the *vdh1* and the *Δvdh5*strains at 3 and 4 dpi respectively. *PR3* genes encode for chitinases-lysozymes and have antifungal activity (Stintzi et al., 1993). The higher expression of *PR3* observed in plants inoculated with both the *vdh1* or the *Δvdh5* mutant strains compared to those inoculated with the WT suggest that the presence of VDH1 and VDH5 might suppress the expression of this gene by the host plant.

From the analysis of the expression of the hydrophobin gene *in planta* it became evident that the levels of tomato *GAPDH* and *UBI3* genes dropped dramatically one day after inoculation when compared to the levels observed immediately after inoculation. These results are similar to those obtained by Xie et al. (2013) in which expression levels of cotton *GAPDH* and *Ubiquitin* genes decreased after 72 hours post inoculation with *V. dahliae*. A similar pattern was observed with the levels of *EF1* in tomato plants

inoculated with the WT, *Δvdh5*, or *vdh1* strains. It appears that the levels of tomato *EF1* is down-regulated after infection with any of the *V. dahliae* strains. These results support the view that *V. dahliae* not only causes wilting symptoms by preventing water and nutrient movement through the xylem, but may be also regulating growth at a molecular level, by altering the expression of genes involved in plant growth and metabolism.

4.9 Conclusions and Future Studies

The study presented here has added to our understanding about the molecular mechanisms involved in development and pathogenicity of *V. dahliae*. The gene expression profiles generated in this study are useful in suggesting potential roles for the hydrophobin genes that merit further characterization. The characterization of *VDH5* achieved by the generation of mutants by two different techniques (target disruption and deletion) allowed me to compare the benefits and difficulties of each of the methodologies, creating more options for future gene studies. Also, the use of newly developed PCR techniques (ddPCR) enabled the comparison of expression of multiple genes during individual conditions, in a way that was limited before. It was because of the use of this new PCR system that the down regulation of plant genes was visible, suggesting the possibility that wilt pathogens have a stronger role in host gene manipulation than previously known.

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APPENDICES

APPENDIX 1: Composition of growth media, buffers and solutions.

Media

Lysogeny Broth (LB) medium (Bertani, 1951) contains peptone 10g L^{-1}, yeast extract 5g L^{-1} and NaCl 5g L^{-1}.

Complete medium (CM, van den Hondel et al., 1991) (84) [52] modified by Dobinson (1995)) contains 1X nitrate salts ($NaNO_3$ (6 g L^{-1}), KCl (0.52 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.52 g L^{-1}), KH_2PO_4 (1.52 g L^{-1})), 1X trace elements ($ZnSO_4 \cdot 7H_2O$ (0.022 g L^{-1}), H_3BO_3 (.011 g L^{-1}), $MnCl_2 \cdot 4H_2O$ (0.005 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.005 g L^{-1}), $CoCl_2 \cdot 6H_2O$ (.0017 g L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.0016 g L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (.0015 g L^{-1}), Na_4EDTA (0.05 g L^{-1}), glucose (10 g L^{-1}), peptone (2 g L^{-1}), yeast extract (1 g L^{-1}), casamino acids (1 g L^{-1}) and 1X vitamin solution (biotin, pyridoxine, thiamine, riboflavin, *p*-aminobenzoic acid, nicotinic acid, all at 0.01% (w/v)).

Basal medium (BM, van den Hondel et al., 1991) (84) [52] modified by Dobinson et al. (1997)) contains glucose (10 g L^{-1}), sodium nitrate (0.2 g L^{-1}), 1x potassium salts (KCl 0.52 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.52 g L^{-1}, and KH_2PO_4 1.52 g L^{-1}), 3 μM thiamine HCl, and 0.1 μM biotin.

Minimal medium (MM, Hooykaas et al., 1979) (33)[69] used for *Agrobacterium tumefaciens*-mediated transformation (ATMT) contains K_2HPO_4 (2 g L^{-1}), KH_2PO_4 (1.45 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.6 g L^{-1}), NaCl (0.3 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.001% weight/volume

(w/v)), glucose (0.02% (w/v)), $FeSO_4$ (0.0001% (w/v)), $ZnSO_4 \cdot 7H_2O$ (100 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.5 mg L^{-1}), H_3BO_3 (0.5 mg L^{-1}), $MnSO_4 \cdot H_2O$ (0.5 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.5 mg L^{-1}), NH_4NO_3 (0.05% (w/v)).

Induction medium (IM, Bundock et al., 1995) (9)[70] used for ATMT contains K_2HPO_4 (2g L^{-1}), KH_2PO_4 (1.45 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.6g L^{-1}), NaCl (0.3 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.001% weight/volume (w/v)), $FeSO_4$ (0.0001% (w/v)), $ZnSO_4 \cdot 7H_2O$ (100 mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.5 mg L^{-1}), H_3BO_3 (0.5 mg L^{-1}), $MnSO_4 \cdot H_2O$ (0.5 mg L^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.5 mg L^{-1}), 10 mM glucose, 200 uM acetosyringone.

Co-cultivation medium used for ATMT contains IM, with 5 mM instead of 10 mM glucose

Buffers and solutions

Spore breaking buffer (SBB) contains 0.5M NaCl, 0.2M Tris pH 7.5, 10 mM EDTA and 1% SDS.

Southern blot denaturing buffer contains 0.4N NaOH, 0.8N NaCl.

Southern blot neutralizing buffer contains 1.5M NaCl, 0.5M Tris-HCl pH 7.6.

20X sodium chloride-sodium phosphate-EDTA (SSPE) pH7.4 contains 3.6M NaCl, 0.2M $NaH_2PO_4 \cdot H_2O$, 0.02M EDTA.

Southern Blot prehybridization buffer contains 6X SSPE, 1% skim milk, 0.5% sodium dodecyl sulfate (SDS) and 50 µg salmon sperm DNA mL^{-1}.

Southern blot hybridization buffer contains 6X SSPE, 1% blocking reagent, 0,5% SDS and 10 ng DIG-labeled DNA hybridization specific probe.

Southern blot low stringency washing buffer contains 2X SSPE, 0.1%SDS and 0.1% sodium pyrophosphate.

Southern blot high stringency washing buffer contains 0.2X SSPE, 0.1% SDS and 0.1% sodium pyrophosphate

APPENDIX 2: Primers and probes used in this study

Primer name	Primer sequence 5'→ 3'
pBeta tub Fw	CGCATGATGGCTACCTT
pBeta tub Rv	TCGTCGGAGTTCTCAAC
Beta tub Probe	FAM-CCTCGCCCAAGGTTTCCGACAC-BHQ1
pActin Fw1	CAAGCGTGGTATCCTTAC
pActin Rv1	GTGGTGCCAAATCTTCTC
Actin Probe	FAM-CCATCGAGCACGGTGTCGTCAC-BHQ1
VDH1 Fw2	ACCCAGCCAACCTCGAAACATCC
VDH1 Rv2	GCCTCATGTCCTCGCCGTTCC
Vdh1 Probe	FAM-TCTGGCCGTTCTAGACTGCTCTACC-BHQ1
VDH2 Fw3	GCGTTGCCGATCTGGACTGC
VDH2 Rv1	CTGGCCGGCGAGAGGGATG
Vdh2 Probe	FAM-CCGACCTCAAGAGGATCTGTGCCG-BHQ1
pVdh3 Fw2	AGGACCGCCAAGTCTAC
VDH3 RV1	GCGGGGTTGTTGCAGAGGACAC
Vdh3 Probe	FAM-CTCAGTGCTGCGCGACCGAT-BHQ1
pVdh4 Fw	CCAACGACTTCAAGCAGAG
pVdh4 Rv	GGCTTGTTGCAGAGGAC
Vdh4 Probe	FAM-CACCGGCAAGTCGGCCTTCT-BHQ1
pVdh5 Fw	CTGCGCTACTAACGTCCT
pVdh5 Rv	CCGAGGTGGGAGTCTTG

Vdh5 Probe	FAM-TCGCTCTCAACTGCGATGCTCC-BHQ1
LepUBI3 Fw1	GTGTTGCAGTTCTATAAGGT
LepUBI3 Rv1	TCCAGCCTTGTTGTAAAC
Le UBI3 Probe 1	FAM-CAGAGGCTTCGTAAGGAGTGCCC-BHQ1
LepGAPDH Fw2	GTGGTGCCAAGAAGGTT
LepGAPDH Rv2	CGTTGTGCAACTAGCATT
Le GAPDH probe	FAM-TCCCATGTTTGTTGTGGGTGTCA-BHQ1
TomEF1FW	GGAACTTGAGAAGGAGCCTAAG
TomEF1RV	CAACACCAACAGCAACAGTCT
Sl PR1 FW2	CACAAGCTCAAAACTCCCCTCAAG
Sl PR1 RV1	CACCCGTTGTTGCACCTGACC
Sl PR3 FW1	TGGTTGTTCATCGGATTTGTGTTG
Sl PR3 RV1	TGTGACATGAGCGAAGAAAGCAGC
Sl PR5 FW1	GCCACTTTCGAGGTACGCAACAAC
Sl PR5 RV2	GTCGGGGCGAAAGTCATTGG
OscarVDH5 1	GGGGACAGCTTTCTTGTACAAAGTGGAA
	GTTACCCTCGACAGCCGCCTACTC
OscarVDH5 2	GGGGACTGCTTTTTTGTACAAACTTGT
	TTTGATGGTTGGGGGTTGGAGAA
OscarVDH5 3	GGGGACAACTTTGTATAGAAAAGTTGTT
	CCCATCTCGGACTGGCAACATAC
OscarVDH5 4	GGGGACAACTTTGTATAATAAAGTTGT

	CAGCCTCATTTGGTGTCTTTGCC
Vdh5 Fw I	TGATGCTGTCTTGTGTCGCTACGC
Vdh5 Rv II	ACGGCAGCCTCATTTGGTGTCT
*pMOD PCR Fw	ATTCAGGCTGCGCAACTGT
*pMOD PCR Rv	GTCAGTGAGCGAGGAAGCGGAAG
VDH5 FW2	CCCACCACCGACTCCTACACTCC
VDH5 RV2	GGGAACCTGGCACAGGACACC
Vdh5 TN screen Fw 2	CGGCGGAGGACGTTGAGATG
Vdh5 TN Screen Rv1	AGTAGGCGGCTGTCGAGGGTAA
*pMOD sequencing Fw	GCCAACGACTACGCACTAGCCAAC
*pMOD sequencing Rv	GAGCCAATATGCGAGAACACCCGAGAA
VDH5 ENT Rv	CGC**GGATCC**GTAGGGCGCAGGAGTGTAGGAGTC
VDH5 ENT Fw	CGC**GGATCC**GACTACGCGCCTTGCCCTTCC
FP Vdh5 prom Fw2	AAAAAGCAGGCTTAGCGGCGAGGATAGACAGAAGAAG
FL-VDH5 Rv	AGAAAGCTGGGTAGGCGGCGACGCCAACG
attB_F1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB_R1	GGGGACCACTTTGTACAAGAAAGCTGGGT

Underlined sequences in the four OSCAR, FP Vdh5 prom Fw2, and FL-VDH5 Rv primers are gateway recombination sequences. **Bold** sequences in VDH5 ENT Rv, and VDH5 ENT Rv are BamHI restriction recognition sequences.

*primers obtained from EZ::TN system (Epicentre Technologies, Madison, WI, USA)

All other primers where designed for this study.

APPENDIX 3: Genome locations of the *Verticillium dahliae* hydrophobin genes

Gene	Locus
VDH1	VDAG_02273.1
VDH2	VDAG_08956.1
VDH3	VDAG_07838.1
VDH4	VDAG_01586.1
VDH5	VDAG_07851.1

Verticillium group Database - Broad Institute :

http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html

APPENDIX 4: Amino acid sequences found with the BlastP analyses

Gene Bank ID	Organism (Abbreviation used on the Neighbor-joining tree)
	Plant Pathogen
gi|347831925|	*Botryotinia fuckeliana* (B.f)
gi|25091421	*Claviceps fusiformis** (C.f)
gi|24474507|	*Claviceps purpurea*** (C.p)
gi|429858594|	*Colletotrichum gloeosporioides* Nara gc5 (C.g)
gi|429857720|	*Colletotrichum gloeosporioides* Nara gc5 (C.g)
gi|380495813|	*Colletotrichum higginsianum* (C.h)
gi|1706154|	*Cryphonectria parasitica* (Cr.p)
gi|452838455|	*Dothistroma septosporum* NZE10 (D.s)
gi|397310321|	*Geosmithia pallida* (G.p)
gi|89113187|	*Geosmithia sp*. IVV7 (G)
gi|37724083|	*Gibberella moniliformis* (G.m)
gi|37723315|	*Gibberella moniliformis* (G.m)
gi|310801153|	*Glomerella graminicola* M1.001 (G.g)
gi|310800037|	*Glomerella graminicola* M1.001 (G.g)
gi|389639546|	*Magnaporthe oryzae* 70-15 (M.o)
gi|389632809|	*Magnaporthe oryzae* 70-15 gb1 (M.o)
gi|406861411|	*Marssonina brunnea* f. sp. multigermtubi (M.b)
gi|453083160|	*Mycosphaerella populorum* SO2202 (M.p)
gi|453083158|	*Mycosphaerella populorum* SO2202 (M.p)
gi|453082742|	*Mycosphaerella populorum* SO2202 (M.p)
gi|1870028|	*Ophiostoma himal-ulmi* (O.h-u)
gi|40644368|	*Ophiostoma novo-ulmi* (O.n-u)
gi|2065353|	*Ophiostoma novo-ulmi* (O.n-u)
gi|148888523|	*Ophiostoma quercus* (O.q)

gi|148888521|	*Ophiostoma quercus* (O.q)
gi|148888519|	*Ophiostoma quercus* (O.q)
gi|769895|	*Ophiostoma ulmi* (O.u)
gi|1870038|	*Ophiostoma ulmi* (O.u)
gi|4499830|	*Passalora fulva* (P.f)
gi|302423794|	*Verticillium albo-atrum* VaMs.102 (V.a-a)
gi|302412419|	*Verticillium albo-atrum* VaMs.102 (V.a-a)
gi|302408401|	*Verticillium albo-atrum* VaMs.102 (V.a-a)
gi|398392761|	*Zymoseptoria tritici* IPO323 (Z.t)
gi|398391605|	*Zymoseptoria tritici* IPO323 (Z.t)

Plant Symbiont	
gi|70930993|	*Hypocrea rufa* (H.r)
gi|396583390|	*Trichoderma asperellum* (T.as)
gi|154125587|	*Trichoderma atroviride* (T.at)
gi|154125585|	*Trichoderma atroviride* (T.at)
gi|154125573|	*Trichoderma atroviride* (T.at)
gi|358400457|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|358390811|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|1903321|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|154125583|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|154125581|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|154125577|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|154125571|	*Trichoderma atroviride* IMI 206040 (T.at I)
gi|1709966|	*Trichoderma harzianum* (T.h)
gi|126009705|	*Trichoderma harzianum* (T.h)
gi|6647555|	*Trichoderma reesei* (T.r)
gi|1708378|	*Trichoderma reesei* (T.r)
gi|340519375|	*Trichoderma reesei* QM6a (T.r Q)
gi|340514533|	*Trichoderma reesei* QM6a (T.r Q)

gi|154125605|	*Trichoderma virens* (T.v)
gi|154125599|	*Trichoderma virens* (T.v)
gi|154125597|	*Trichoderma virens* (T.v)
gi|154125595|	*Trichoderma virens* Gv29-8 (T.v G)
gi|154125593|	*Trichoderma virens* Gv29-8 (T.v G)
gi|154125591|	*Trichoderma virens* Gv29-8 (T.v G)
gi|154125607|	*Trichoderma virens* Gv29-8 (T.v G)
gi|154125603|	*Trichoderma virens* Gv29-8 (T.v G)
gi|154125601|	*Trichoderma virens* Gv29-8 (T.v G)

	Insect Pathogen
gi|400603302|	*Beauveria bassiana* ARSEF 2860 (B.b)
gi|400600501|	*Beauveria bassiana* ARSEF 2860 (B.b)
gi|346325668|	*Cordyceps militaris* CM01 (C.m)
gi|346320834|	*Cordyceps militaris* CM01 (C.m)
gi|322692547|	*Metarhizium acridum* CQMa 102 (M.ac)
gi|310892601|	*Metarhizium anisopliae* (M.an)
gi|322712535|	*Metarhizium anisopliae* ARSEF 23 (M.an A)

* Trihydrophobin

** Pentahydrophobin

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