Insertional mutagenesis in the vascular wilt pathogen *Verticillium dahliae*

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This research was conducted under the auspices of
the Graduate School of Experimental Plant Sciences

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday, January 20 2014
at 4 p.m. in the Aula.

Parthasarathy Santhanam
Insertional mutagenesis in the vascular wilt pathogen *Verticillium dahliae*,
124 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in English and Dutch

ISBN: 978-94-6173-825-7

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CHAPTER 1

General Introduction

Introduction

Depending on the type of parasitism and the infection strategy, plant pathogens are generally categorized into two groups; biotrophs and necrotrophs. Biotrophic pathogens, such as those that cause rust and mildew diseases (Mendgen et al., 2002; Perfect et al., 2001), feed on nutrients provided by living host cells to complete their life cycle and generally only cause disease on one, or a few related, plant species. In contrast, necrotrophic pathogens first kill their host cells and then metabolize their contents. Many necrotrophic species, such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (van Kan, 2006; Bolton et al., 2007), have a broad host range and cell death is induced by (an array of) toxins that target common substrates that are shared among various plant species. Other necrotrophs, such as *Stagonospora nodorum* and *Alternaria* spp. (Solomon et al., 2006; Thomma et al., 2003), have narrow host ranges and produce host-selective toxins. Since biotrophs and necrotrophs are not strictly separated classes but rather form a continuum, in addition to biotrophs and necrotrops also hemibiotrophs are recognized such as *Mycosphaerella graminicola* and *Moniliophthora perniciosa* (Orton et al., 2011; Meinhardt et al., 2008). During host colonization, hemibiotrophs initially display a biotrophic life style which is followed by transition to a necrotrophic stage when they produce toxins to kill host cells (Agrios 2005).

Vascular wilt diseases caused by soil-borne pathogens are among the most devastating plant diseases worldwide, affecting annual crops and woody perennials. Symptom development proceeds acropetally (from bottom to top). Stunting, wilting, chlorosis, vascular browning and necrosis are the primary disease symptoms. A combination of the environment, virulence of the pathogen and nutritional status of the host plant determines the severity of symptom development (Nino-Liu et al., 2006; Chatterjee et al., 2008). Vascular wilt diseases are caused by fungal (4 main genera), bacterial (7 main genera) and oomycete (one main genus) pathogens. The four main fungal genera containing vascular wilt pathogens are *Ceratocystis* (vascular wilts of oak, cacao and eucalyptus), *Fusarium* (broad host range), *Ophiostoma* (wilting of elm tree) and *Verticillium* (broad host range). Vascular wilt pathogens overwinter in the soil, on plant debris, in watercourses or in insect vectors (Fradin and Thomma, 2006; Nino-Liu et al., 2006; Michielse and Rep, 2009; Klosterman et al., 2011). Most of the vascular wilt pathogens enter host plants through natural openings, or via wounds or cracks, except for a few bacterial (*Xylellea fastidiosa, Pantoea stewartii* and *Erwinia tracheiphila*) and fungal (*Ceratocystis* and *Ophiostoma*)

species, which are transmitted through beetles (Nadarasah and Stavrinides, 2011; Roper, 2011).

Verticillium dahliae

The *Verticillium* genus contains hemibiotrophic vascular wilt pathogens with a wide host range. Although *V. longisporum* infects various hosts belonging only to the Cruciferaceae, *V. dahliae* and *V. albo-atrum* cause vascular wilt diseases on over 200 dicotyledonous species in temperate and subtropical regions, including herbs, annuals and woody plants. In addition, the *Verticillium* genus contains 7 species that have been characterized as saprophytic or narrow host range pathogens that are only weakly pathogenic on plant hosts (Inderbitzin et al., 2011). *Verticillium* wilt diseases are difficult to control due to the longevity of resting structures, the broad host ranges of the pathogen, and inability of fungicides to eliminate the fungi that have colonised xylem tissue. Disease symptoms vary among plant species, and there is no unique symptom common to all infected plants (Fradin and Thomma, 2006). Dark coloured, thick walled resting structures, called microsclerotia, of *Verticillium* are induced to germinate by root exudates. Upon germination, the germination tube penetrates the root through the cortex or a wound site. The hyphae then grow into the xylem vessels where they produce large numbers of conidia which are transported acropetally with the water flow, eventually colonizing the whole plant (Agrios, 2005). Either extensive fungal growth in the xylem vessels, or vessel blocking by gums and tyloses as result of plant defence, blocks water and mineral transportation in the xylem which results in wilting of leaves, yellowing of the stem and finally the whole plant may wilt and die (Yadeta and Thomma, 2013). As the plant starts to dry, microsclerotia are produced. The morphological events during microsclerotia formation have been studied extensively by using both light and electron microscopy. During initial stages of microclerotia development, the hyphae swell and form numerous septa. Later, each septate cell enlarges and becomes spherical. In the final stage, melanin granules are deposited that give a black appearance to the microsclerotia which can stay viable in the soil for more than 20 years (Agrios, 2005). Because of the persistence of the resting structures, the broad host range of the pathogen, and lack of natural resistance in many plant species, control of *Verticillium* wilt is difficult (Pegg and Brady 2002).

Pathogenicity genes of *Verticillium dahliae*

Pathogenicity is the capability of the pathogen to cause disease (qualitative), while virulence refers to the degree of aggressiveness of the pathogen (quantitative).

Relatively few pathogenicity and virulence genes of *V. dahliae* are known. During colonization, the pathogen assimilates nutrients by degradation of plant cell wall components (Pegg and Brady 2002). *V. dahliae* produces a variety of plant cell wall-degrading enzymes (CWDEs), including pectinases, polysaccharidases, and proteinases, which likely serve this purpose (Bidochka et al., 1999; Dobinson et al.,

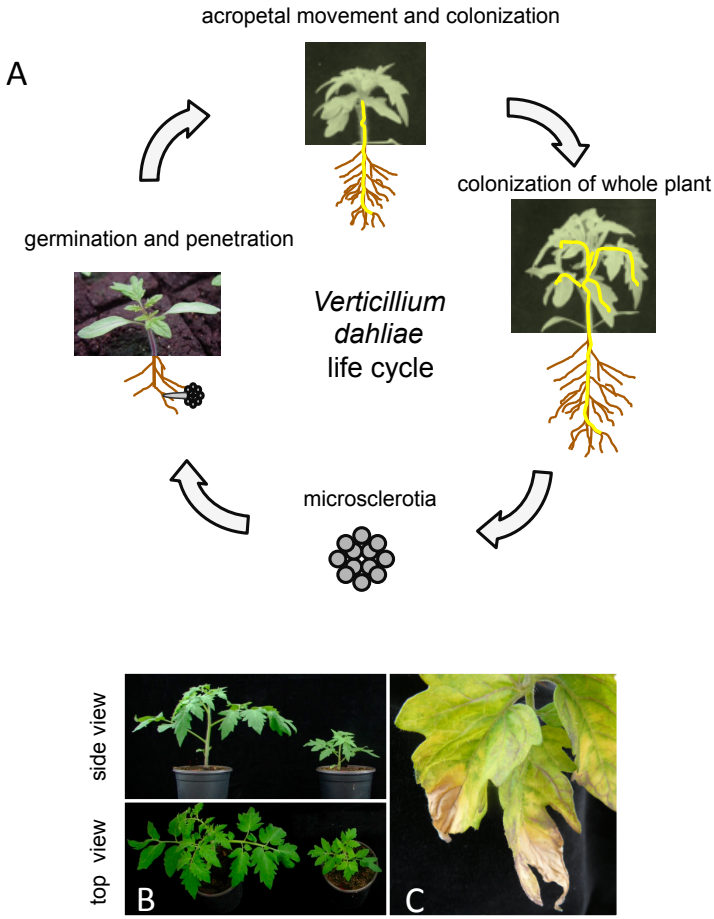

Figure 1. Life cycle of the fungal vascular wilt pathogen *Verticillium dahliae.* **A.** Resting structures, microsclerotia, germinate and penetrate plant roots via wounds or cracks. Once inside the plant, the fungus reaches the vascular tissue and colonizes the xylem. Conidiospores are transported acropetally and colonize the whole plant. Death and decay of the host plant release microsclerotia back in the soil. **B.** 10-day-old tomato seedlings that are mock-inoculated (left) or inoculated with conidiospores of wild type *V. dahliae* (right) are shown in the top panel (side view) and bottom panel (top view). **C.** Leaf of a tomato plant infected with *V. dahliae* showing typical symptoms of infection, such as chlorosis and necrosis.

1997). It was shown that the *V. dahliae* sucrose non-fermenting 1 protein (SNF1) regulates CWDE expression as well as virulence on tomato and eggplant (Tzima et al., 2011). Targeted disruption of the trypsin protease gene *VTP1* using *Agrobacterium tumefaciens*-mediated transformation (ATMT) did not affect *in vitro*

Table 1. Pathogenicity and virulence factors of *V. dahliae*

Pathogenicity factor	Function	Reference
CWDE	Degradation of plant cell wall components	Dobinson et al., 1997 and Bidochka et al., 1999
VTP1	No effect on pathogenicity	Dobinson et al., 2004
VMK1	Microsclerotia formation, required for virulence	Rauyaree et al., 2005
VDH1	Microsclerotia formation, required for virulence	Klimes and Dobinson 2006
VdGLO1	No effect on pathogenicity	Klimes et al., 2006
VdGARP	Microsclerotia formation, required for virulence	Gao et al., 2010
VdPKAC1	Required for conidia production, ethylene biosynthesis and virulence on eggplant and tomato plants	Tzima et al., 2010
VdSNF1	Regulates CWDE expression as well as virulence on tomato and eggplant	Tzima et al., 2011
VdGT	Required for virulence in *N. benthamiana* but not in lettuce	Klosterman et al., 2011
VGB	Required for virulence in tomato and radial growth	Tzima et al., 2012
Ave1	Genuine effector of *V. dahliae* recoganized by tomato Ve1	de Jonge et al., 2012
VdNLP1	Cytotoxic, required for vegetative growth and virulence (Arabidopsis, tomato and *N. benthamiana*)	Wang et al., 2004, Zhou et al., 2012 and this thesis
VdNLP2	Cytotoxic, required for virulence on Arabidopsis, and tomato	this thesis
VdSge1	Radial growth, conidiation and affects candidate effector gene expression	this thesis
VdNSR/ER	Required for the biosynthesis of UDP-rhamanose, nucleotide rhamanose contaning exoploysaccaride are required for adhesion on tomato roots and required for virulence on tomato and *N. benthamiana.*	this thesis

growth or pathogenicity in tomato plants (Dobinson et al., 2004). Deletion of a homolog of a hydrophobin gene (*VDH1*) in *V. dahliae* resulted in impaired pathogenicity on tomato and reduced microsclerotia formation (Klimes and Dobinson, 2006). Moreover, Klimes et al. (2006) showed that the *V. dahliae* glyoxalase I gene (*VdGLO1*) is not required for pathogenicity or *in vitro* growth. Disruption of a mitogen-activated protein kinase gene (*VMK1*) and a glutamic acid-rich protein (*VdGARP*) resulted in reduced virulence and the mutants also displayed reduced microsclerotia formation (Rauyaree et al., 2005; Gao et al., 2010). *VdPKAC1* encodes cyclic AMP-dependent protein kinase A which was shown to be required for conidia production, ethylene biosynthesis and virulence on eggplant and tomato plants (Tzima et al., 2010). Disruption of the G protein β subunit gene (*VGB*) resulted in reduced virulence, decreased ethylene production, increased production of microsclerotia and conidia and elongated growth *in vitro* when compared to the wild type strain (Tzima et al., 2012). *V. dahliae* secretes a necrosis and ethylene -inducing protein (VdNEP). The protein toxin induces cell death in *N. benthamina* upon infiltration and triggers reactive oxygen production and *PR* gene expression in *A. thaliana.* Moreover, VdNEP induces wilting in detached cotton leaves (Wang et al., 2004). The *V. dahliae* effector Ave1, which is recognized by the Ve1 immune receptor of tomato, was identified through comparative genomics using a collection of *V. dahliae* strains. Ave1 was shown to be required for full virulence on tomato plants lacking the *Ve1* resistance gene, although its mode of action remains unclear thus far (de Jonge et al., 2012).

Verticillium genome analysis

The *V. dahliae* genome contains 10,535 predicted protein-encoding genes on 8 chromosomes with a total genome size of 33.8 Mb (Klosterman et al., 2011). On the one hand, orthologs of well-characterized effectors like the SIX proteins, proteins with RXLR domains and Avrs, reported to be produced by *Fusarium oxysporum, Phytophthora infestans and Cladosporium fulvum*, are absent in the genome of *V. dahliae.* On the other hand, particular gene families which are known to play roles in pathogenesis in other species, like LysM effectors, NLPs and polysaccharide lyases, have under gone expansion in *V. dahliae*. The *V. dahliae* genome contains 8 NLP genes, while most of the fungal genomes sequenced till now carry two to three NLP genes. Polysaccharide lyases digest pectin and the fungus utilizes the breakdown products as carbon source. All fungal vascular wilt pathogens belong to different subclasses of sordariomycetes, and among the sequenced sordariomycetes (*Fusarium graminearum, Fusarium oxysporum, Fusarium verticillioides, Hypocera jecorina, Magnaporthe grisea, Neurospora crassa, Podospora anserina, Verticillium albo-*

atrum and *Verticillium dahliae*) *V. dahliae* secretes the highest number, and most diverse types, of polysaccharide lyases, including pectate lyases and rhamnogalactoranan lyases. Klosterman et al. (2011) identified 14 wilt pathogen-specific genes that are present in *V. dahliae, V. albo-atrum* and *F. oxysporum*, and that are absent in the related non-vascular plant pathogenic species *F. solani*, *F. graminearum*, and *F. verticilliodes*. One of the candidates showed homology to a bacterial glucan glucosyltransferase that is involved in the production of osmoregulated glucans to maintain osmolarity of periplasmic space. Interestingly, there are no homologues in other sequenced eukaryotes except for *Metarhizium anisopliae*, a fungal pathogen of insects. Knock-out of the glucosyltransferase homolog in *V. dahliae* resulted in reduced virulence on *N. benthamiana*, but not on lettuce (Klosterman et al., 2011).

Functional genomics to identify pathogenicity and virulence factors

The study of gene function in phytopathogenic fungi has advanced over recent years due to the increased availability of whole genome sequences (http://www.genomesonline.org/cgi-bin/GOLD/index.cgi). In addition, tools for gene functional analysis like random mutagenesis, transcriptomics, RNA interference (RNAi), proteomics, metabolomics, comparative genomics and targeted mutagenesis are used to unravel mechanisms involved in pathogenicity. Random T-DNA insertional mutagenesis is a powerful forward genetic tool to identify genes involved in plant-pathogen interactions. The main advantage is that it does not require prior knowledge of a fungal genome, although the availability of a whole genome sequence is advantageous for the identification of the T-DNA insertion site. Random mutagenesis through *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been widely used for transforming plant pathogenic fungi, such as *Botrytis cinerea, Colletotrichum lagenarium, Fusarium oxysporum, Magnaporthe oryzae, Mycosphaerella graminicola, Venturia inaequalis* and *Verticillium dahliae* (Rolland et al., 2003; Tsuji et al., 2003; Khang et al., 2005; Jeon et al., 2007; Zwiers et al., 2001; Fitzgerald et al., 2003; Gao et al., 2010). The advantage of ATMT over other transformation techniques restriction enzyme-mediated integration (REMI) and polyethylene-glycol (PEG) mediated transformation, are the flexibility with respect to starting material (conidia or mycelium) for transformation, higher transformation efficiencies and higher percentages of transformants with a single T-DNA insertions (Mullins et al., 2001). RNA interference (RNAi) and targeted gene deletions are the most widely used reverse genetics techniques to identify the function of candidate

genes. RNAi is a technique in which double-stranded RNA (dsRNA) triggers the degradation of a homologous mRNA, resulting in silencing of target gene expression. It was accidently discovered in petunia while trying to overexpress a chalcone synthase gene, which resulted in gene silencing (Napoli et al., 1990). RNAi has been shown to be an efficient system for high-throughput functional genomics in many organisms, including nematodes and drosophila (Kamath et al., 2003; Boutros et al., 2004). Successful RNA-mediated gene silencing has also been shown in filamentous plant pathogens such as *C. fulvum, M. oryzae* and *P. infestans* (Bolton et al., 2008; Kadotani et al., 2003; van West et al., 1999).

RNAi has a number of advantages when compared with targeted gene deletion, such as: 1) RNAi works at the mRNA level and hence the efficiency is not affected by the presence of non-transformed nuclei (Weld et al., 2006). 2) If there is sequence conservation among genes of a gene family, one RNAi construct can be used to knockdown an entire gene family. 3) RNAi can be used to down-regulate the expression of target genes especially when knockout is lethal (Goldoni et al., 2004). Limitations of RNAi technique are that RNAi down-regulates the expression of the target gene, and thus that the extent of down-regulation is variable. Consequently, when the down-regulation efficiency is low it is necessary to screen large numbers transformants (Mouyna et al., 2004 and Spiering et al., 2005). Another limitation of RNAi is the occurrence of off-target gene silencing.

Another way to explore gene function is targeted deletion of the gene of interest by homologous recombination. Homologous recombination involves a reciprocal exchange of DNA sequences between a vector and a target locus in a genome. This approach was first used in *Saccharomyces cerevisiae* to decipher gene function (Bundock et al., 1995). Since then, this method has been applied in several phytopathogens. In *S. cerevisiae* deletion of all known open reading frames has been carried out to generate a functional genomic resource (Shoemaker et al., 1996). In *S. cerevisiae*, 50 bp of sequence that is homologous to the flanking regions of the target sequence is enough for efficient homologous recombination, but in most filamentous fungi a longer stretch of homology is required for efficient homologous recombination (Bahler et al., 1998; Hynes 1996). Efficiency of homologous recombination is determined by the length of the flanking region, the GC content of the target sequence and the location of the target gene on the chromosome (Nelson et al., 2003; Michielse et al., 2005).

Genome sequencing projects are generating sequence data at an exponential rate. One of the best ways to quickly identify pathogenicity and virulence factors is to compare

the genomic sequences of multiple strains of pathogenic and non-pathogenic relatives (de Jonge et al., 2012; Gan et al., 2013). Using comparative population genomics and transcriptomics de Jonge et al. (2012) identified the *V. dahliae* avirulence factor Ave1 which is recognized by the tomato immune receptor Ve1. Comparative genomics and transcriptomics analysis of *Colletotrichum higginsianum* (pathogen of Arabidopsis) and *Colletotrichum graminicola* (pathogen of maize) revealed the expansion of genes encoding secreted effectors, pectin degrading enzymes and secondary metabolism enzymes in *C. higginsianum* (O'Connell et al., 2012). Moreover, genome-wide expression profiling resulted in the identification of genes that are linked to pathogenic transitions (O'Connell et al., 2012). Comparative genomics in combination with transcriptomics is a powerful tool to quickly identify potential pathogenicity and virulence factors (de Jonge et al., 2012; O'Connell et al., 2012; Gan et al., 2013).

Objective and scope of this thesis

The molecular mechanism of pathogenicity of *V. dahliae* and other vascular wilt pathogens is not well understood. The primary objective of the research described in this thesis is to identify genes that are of importance for the virulence of *V. dahliae*, using tomato as a model plant. Fungal genes that are required to initiate disease on susceptible host plant species remain largely unknown. In this thesis, ATMT was used to generate 900 insertional mutants in *V. dahliae*. Transformants resulting from ATMT were screened for altered pathogenicity on tomato. From 80 selected transformants that consistently displayed reduced virulence, the sequence flanking the T-DNA was amplified using inverse PCR, and one of the identified genes was further characterized. In this thesis, we utilized both forward and reverse genetic approaches to identify pathogenicity and virulence factors of *V. dahliae.*

In **chapter 2**, we have used ATMT to generate random T-DNA insertional transformants and screened the transformants for altered pathogenicity on susceptible tomato. Furthermore, we characterized one of the candidate genes, *VdNRS/ER*, which has homology to bacterial rhamnose synthases. In **chapter 3**, we investigated the role of the *V. dahliae* homolog of *F. oxysporum* Sge1 and assessed its role in vegetative growth, virulence and regulation of effector gene expression. *VdSge1* was deleted in wild type *V. dahliae* using ATMT and the resulting transformants were assessed for vegetative growth, conidiation and altered pathogenicity on tomato plants. In addition, the transformants were cultured under conditions that mimic *in planta* growth and the regulation of potential effector gene expression was assessed. **Chapter 4** describes the expanded *V. dahliae NLP* family and the study of potential functional diversification.

Out of eight NLPs, only two displayed cytolytic activity (NLP1 and NLP2). *NLP1* and *NLP2* deletion strains were assessed for *in vitro* growth and conidiospore production. Furthermore, effects of deletion of *NLP1* and *NLP2* on pathogenicity was assessed by inoculation on tomato, Arabidopsis and *N. benthamiana.* Finally, in **Chapter 5** the results obtained in this thesis are discussed in a broader context.

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CHAPTER 2

Random Mutagenesis Identifies a Rhamnose Synthase Required for Pathogenicity of *Verticillium dahliae*

ABSTRACT

Verticillium dahliae causes vascular wilt diseases in over 200 dicotyledonous plant species, including economically important crops. To identify novel molecular mechanisms underlying the pathogenesis of *Verticillium dahliae*, we used *Agrobacterium tumefaciens*-mediated transformation (ATMT) to generate 900 random T-DNA insertion mutants. All 900 transformants were screened for reduced virulence by root dip inoculation and we identified 80 transformants that consistently displayed reduced virulence. Inverse PCR resulted in the identification of 55 potential pathogenicity or virulence genes. Of the 55 pathogenicity or virulence genes, 17 were homologous to previously identified pathogenicity and virulence factors of plant and animal pathogens and the remaining were previously unknown pathogenicity or virulence factors based on PHI database blasts. In one of the random mutants, the T-DNA was inserted 56 bp upstream of the coding region of a nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER). NRS/ER shows homology to a bacterial rmlD substrate binding domain which is involved in biosysnthesis of the nucleotide sugar dTDP-rhamnose. In bacteria, dTDP-rhamnose is a precursor of L-rhamnose, which is required for the virulence of pathogenic bacteria such as *Salmonella enterica*, *Vibrio cholerae* and *Streptococcus mutans*. Targeted deletion of *VdNRS/ER* resulted in loss of pathogenicity on tomato and *N. benthamiana*, while the vegetative growth and sporulation were unaffected *in vitro*. Moreover, the *VdNRS/ER* deletion mutants showed impaired attachment to tomato roots. These results demonstrate that VdNRS/ER function is essential for pathogenesis of *V. dahliae* on tomato, and suggest that rhamnose plays a critical role in attachment to plant tissues.

INTRODUCTION

Vascular wilt diseases caused by soil-borne pathogens are among the most devastating plant diseases worldwide. The fungus *Verticillium dahliae* causes vascular wilt diseases in over 200 dicotyledonous plant species, including important crops such as eggplant, lettuce, olive, spinach and tomato (Fradin and Thomma , 2006; Klosterman et al., 2009). Triggered by root exudates the melanised resting structures, microsclerotia, germinate and penetrate roots through the root tips, wounds, or sites of lateral root formation (Fradin and Thomma, 2006). After crossing the cortex, hyphae of the fungus grow into the xylem vessels. The mycelium remains exclusively in these vessels and produces conidia which are transported acropetally with the water flow in the xylem throughout the plant. Once senescing, microsclerotia are produced that are released into the soil upon tissue decomposition (Wilhelm, 1955). Typical symptoms of plants infected with *V. dahliae* comprise stunting, wilting, chlorosis and vascular browning (Pegg *et al*., 2002).

Verticillium wilt diseases are difficult to control due to the longevity of the microsclerotia and inability of fungicides to eliminate the fungus once it has entered the xylem tissues of the host plant (Wilhelm, 1955; Fradin and Thomma, 2006). Moreover, crop rotation is ineffective due to the broad host range of *V. dahliae*. The only effective control measure, soil fumigation, is expensive and has harmful environmental effects (Rowe *et al*., 1987). As a consequence, genetic resistance is presently preferred to control *Verticillium* wilt diseases. Importantly, *Verticillium* resistance has been described in several plant species, including crops (Schaible *et al*., 1951; Bolek *et al*., 2005; Simko *et al*., 2004; Hayes *et al*., 2011). From tomato (*Solanum lycopersicum*) a *Verticillium* resistance gene has been cloned, *Ve1*, that mediates resistance to strains of *V. dahliae* that have been assigned to race 1 (Fradin *et al*., 2009). Interestingly, putative *Ve1* orthologs have been identified within (Chai *et al*., 2003; Fei *et al*., 2004; Simko *et al*., 2004; Zhang et al., 2012) and outside the Solanaceae family (Vining and Davis, 2009; Hayes *et al*., 2011), suggesting that Ve1-mediated *Verticillium* resistance may be widespread in plants (Thomma et al., 2011). Moreover, it was recently demonstrated that transfer of tomato *Ve1* mediates resistance against race 1 *Verticillium* strains in Arabidopsis (Fradin *et al*., 2011). Recently, through comparative population genomic sequencing, the Ave1 elicitor that activates Ve1-mediated resistance was identified (de Jonge *et al*., 2012). Interestingly, Ave1 contributes to virulence on susceptible tomato plants, and homologous proteins were found in a handful of plant pathogenic fungi (de Jonge *et al*., 2012).

So far, relatively few *V. dahliae* pathogenicity and virulence genes are known (Fradin and Thomma, 2006; Klosterman *et al*., 2011). These comprise genes encoding plant cell wall-degrading enzymes (CWDEs), although functional redundancy typically complicates investigations into the contribution of individual CWDEs to virulence (Fradin and Thomma, 2006; Klosterman *et al*., 2011). However, it was demonstrated that the *V. dahliae* sucrose nonfermenting 1 protein (SNF1) regulates CWDE expression as well as virulence on tomato and eggplant (Tzima *et al*., 2010). Other characterized virulence genes include *VMK1* that encodes a mitogen-activated protein kinase, and *VdGARP* that encodes a glutamic acid-rich protein, both of which are required for microsclerotia formation as well as for virulence, although the manner through which these genes contribute to virulence has not been characterized (Rauyaree *et al*., 2005; Gao *et al*., 2010). Furthermore, the cyclic AMP-dependent protein kinase A (VdPKAC1) acts in conidia production, ethylene biosynthesis and virulence on eggplant and tomato plants (Tzima *et al*., 2010). In addition, analysis of the *V. dahliae* genome sequence for wilt pathogen-specific genes resulted in the identification of a glucosyltransferase gene that is closely related to bacterial enzymes that are involved in production of osmoregulated periplasmic glucans in response to osmotic stress (Klosterman *et al.*, 2011). Analyses of glucosyltransferase gene deletion mutants in *V. dahliae* revealed decreased virulence on *Nicotiana benthamiana*, but not on lettuce (Klosterman *et al*., 2011). Furthermore, necrosis and ethylene-inducing protein (NEP1)-like proteins (NLPs) are produced by many pathogenic bacteria, fungi, and oomycetes, and generally induce cell death in dicotyledonous plants through plasma membrane permeabilization (Ottmann *et al*., 2009). Whereas the NLP family of *V. dahliae* is expanded when compared to other filamentous fungi, only two family members display cytotoxic activity and were recently shown to be required for pathogenicity on tomato, Arabidopsis and *Nicotiana benthamiana*, but not on cotton (Wang *et al*., 2004; Zhou et al., 2012; Santhanam et al., 2012). Finally, the *V. dahliae* transcriptional regulator Sge1 was recently shown to be required for radial growth, production of asexual conidiospores, and pathogenicity on tomato. In contrast to its homolog in *Fusarium oxysporum*, which is required for effector gene expression *in planta*, VdSge1 differentially regulates *V. dahliae* effector gene expression, as it is not required for induction of Ave1, nor for induction of the cytotoxic NLPs that are required for pathogenicity (Santhanam and Thomma, 2012).

Understanding the molecular mechanisms behind *Verticillium*-host interactions is of fundamental importance to design novel control strategies. An effective way to identify novel pathogenicity or virulence genes is random mutagenesis followed by screening of the mutants for reduced virulence on a particular host plant (Jeon *et al*., 2007). *Agrobacterium tumefaciens*-mediated transformation (ATMT) is commonly used to transform filamentous fungi (de Groot

et al., 1998). The advantage of ATMT over more conventional techniques such as polyethylene glycol (PEG)-mediated transformation and restriction enzyme-mediated integration (REMI) are the generally higher transformation efficiency, the higher percentage of single copy insertions, and an increased chance to identify the mutated genes (Meyer *et al*., 2003; Michielse *et al*., 2005). Genes associated with reduced virulence have successfully been identified by screening random T-DNA mutants generated through ATMT of several plant pathogenic fungi (Giesbert *et al*., 2011; Hüser *et al*., 2009; Michielse *et al*., 2009; Münch *et al*., 2011; Mullins *et al*., 2001; Jeon *et al*., 2007). In this study, we report on the identification of pathogenicity and virulence genes in *V. dahliae* through ATMT. A collection of 900 transformants of *V. dahliae* was generated and tested for reduced virulence on susceptible tomato plants. Candidates with reduced virulence were selected and T-DNA insertion sites were identified.

RESULTS

Identification of random *V. dahliae* transformants with reduced virulence

In this study, we generated 900 random T-DNA insertion mutants of *V. dahliae* by transforming conidiospores through ATMT. All transformants were assessed for reduced virulence on tomato seedlings by root-dip inoculation and scored for typical disease symptoms, including stunting, wilting, chlorosis and necorosis, for up to 14 days post inoculation (dpi). Seedlings that exhibited reduced *Verticillium* wilt symptoms when compared with plants inoculated with wild type *V. dahliae* were selected, and the corresponding *V. dahliae* mutants were retained for rescreening. Out of 900 transformants, 200 were selected for rescreening. Subsequently, after calibration of the conidial concentration in the inoculum to 10^6 conidia/mL, all 200 transformants were assessed twice for reduced virulence on tomato for up to 21 dpi. Eventually, 80 transformants consistently exhibited reduced virulence and were selected for further analysis (Figure 1).

Isolation of T-DNA flanking regions in selected *V. dahliae* transformants

Inverse PCR (iPCR) was carried out on the 80 selected transformants that consistently displayed reduced virulence to determine the T-DNA insertion sites. The genomic borders flanking the T-DNA were amplified, sequenced, and the resulting sequences were queried against the *V. dahliae* genome (Klosterman *et al*., 2011). In this manner,

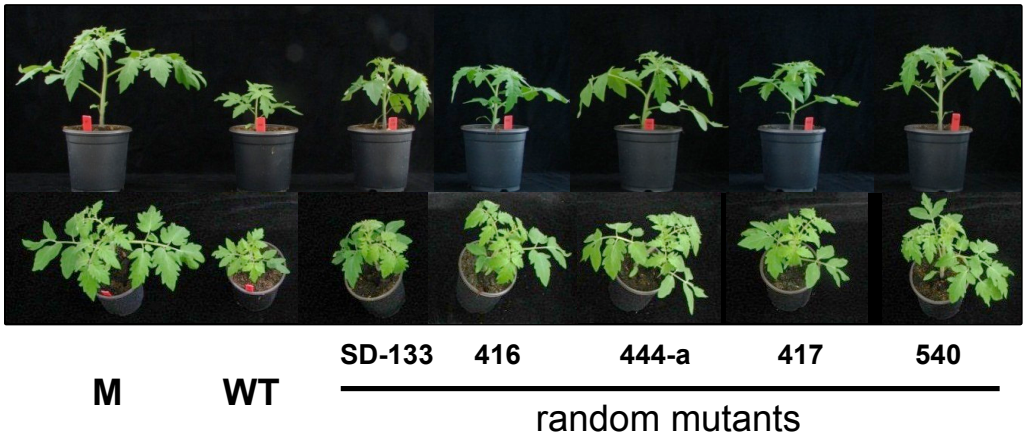


Figure 1. Typical assay to identify transformants with reduced virulence. Ten-day-old tomato seedlings were mock-inoculated (M) or inoculated with conidiospores of wild-type *V. dahliae* (WT) or random T-DNA insertion mutants. At 21 days post inoculation the plants were scored for disease development by comparing plants inoculated wild-type *V. dahliae* with those inoculated with the T-DNA insertion mutants. Side (upper panel) and top (bottom panel) of plants inoculated with five T-DNA insertion mutants that are impaired in aggressiveness (upper panel) and the top (bottom panel).

sequences flanking the T-DNA were determined for 65 mutants, while T-DNA flanking regions repeatedly could not be amplified from any of the borders of the remaining 15 mutants. Of the 65 flanking sequences, 12 sequences corresponded to the backbone of the vector that was used for ATMT while 10 sequences could not unambiguously be assigned to a single genomic location. The remaining 43 sequences resulted in single genomic hits, allowing to determine the T-DNA integration sites. In total, 12 insertions were found within predicted open reading frames (ORFs; Table 1), while the remaining 31 insertions were found in intergenic regions. For these 31 insertions the predicted genes flanking the T-DNA were identified. In two cases we found that in two transformants the T-DNA was integrated in the same intergenic region, albeit at a slightly different location, resulting in the identification of the same flanking genes. Furthermore, in one case there was no gene predicted downstream of the insertion site on the sequence contig. In total, this resulted in the identification of 56 candidate genes from the 31 mutants with insertions in the intergenic regions. In order to reduce this number, and try to determine which of the two genes flanking a particular insertion site in the intergenic region is likely causal to the reduced virulence phenotype, *in planta* expression of the flanking genes was assessed making use of previously generated RNAseq data of *V. dahliae* infected *Nicotiana benthamiana* plants (de Jonge et al., 2012; Faino et al., 2012).

Table 1. List of candidate genes identified based on the T-DNA insertion sites in the random mutants that showed compromised virulence on tomato.

T-DNA insertion within the CDS											
Mutant	**Chr[a]**		**Locus**	**Annotation**	**Expression *in planta***	**CDS[d]**					
SD-133	1		VDAG_04418	Ran GTPase activating protein	+	-					
407	2		VDAG_00500	carboxypeptidase S1	+	-					
657-2	3		VDAG_04769	phospho-2-dehydro-3-deoxyheptonate aldolase	+	-					
174	4		VDAG_05684	hypothetical protein	+	-					
375	5		VDAG_07314	copper amine oxidase	+	-					
211	5		VDAG_06985	leucine rich-repeat protein	+	-					
48	6		VDAG_08150	caib baif family enzyme	-	-					
618	6		VDAG_08151	hypothetical protein	+	-					
659	7		VDAG_02200	prolyl aminopeptidase	+	-					
444-B	8		VDAG_08830	acetate kinase	+	-					
367	8		VDAG_06253	cyclic nucleotide-binding protein	+	-					
416	Us		VDAG_10292	hypothetical protein	+	-					
T-DNA insertion in intergenic region											
		Upstream direction					**Downstream direction**				
Mutant	**Chr[a]**	**Dis.**	**Locus**	**Annotation**	**Expression *in planta***	**CDS[d]**	**Dis.**	**Locus**	**Annotation**	**Expression *in planta***	**CDS[d]**
59	Us	47	VDAG_10192	benzoate monooxygenase cytochrome P450	+	-	1565	VDAG_10193	cellulose-binding family II	+	-
584	5	51	VDAG_09007	hypothetical protein	+	-	1221	VDAG_09008	alcohol dehydrogenase	+	-
389	8	56	VDAG_06010	epimerase/ hydratase	+	-	2363	VDAG_06011	squalene synthetase	+	-
199	4	64	VDAG_05141	potassium transporter	-	-	703	VDAG_05142	Sodium ion /H ion antiporter	+	-
550	3	78	VDAG_04591	Calmodulin	+	-	667	VDAG_04592	AP-1 complex subunit gamma	+	-
417	7	88	VDAG_01651	F-box domain-protein	+	-	731	VDAG_01652	hypothetical protein	-	-
SD-107	2	165	VDAG_07796	Hydrolase	+	-	1586	VDAG_07797	sterol desaturase family	+	-
SD-7	7	215	VDAG_02014	Myb DNA binding protein	+	-	1004	VDAG_02015	hypothetical protein	-	-
646	Us	246	VDAG_10191	methyltransferase domain-	+	-	422	VDAG_10192	benzoate 4-monooxygenase	-	-

646	Us	246	VDAG_10191	methyltransferase domain-containing protein	+	-	422	VDAG_10192	benzoate 4-monooxygenase cytochrome P450	-	-
SD-102	2	270	VDAG_00599	histidinol-phosphate aminotransferase	+	-	746	VDAG_00600	acetyl esterase	+	-
SD-89	5	335	VDAG_07703	transcription factor Cys6	+	-	423	VDAG_07704	pathway-specific nitrogen regulator	+	-
143	1	374	VDAG_00843	hypothetical protein	-	-	100	VDAG_00844	exosome complex exonuclease	+	-
575	6	466	VDAG_03431	hypothetical protein	-	-	1040	VDAG_03432	DUF 221	+	
182	7	470	VDAG_09439	DNA repair protein	-	-	257	VDAG_09440	SGT1-like protein	+	-
44	8	545	VDAG_08030	deuterolysin metalloprotease	-	-	669	VDAG_08031	FES CIP4 domain- protein	+	-
540	7	622	VDAG_01960	KH domain- protein	+	-	336	VDAG_01961	duf974 domain-containing protein	-	-
SD-94	3	716	VDAG_04810	FMN binding protein	+	-	1014	VDAG_04811	hypothetical protein	+	100
534	5	737	VDAG_09007	hypothetical protein	+	-	535	VDAG_09008	alcohol dehydrogenase	+	-
525	7	895	VDAG_02343	cell wall glucanase	+	100	71	VDAG_02344	phosphoribosylglycinamide formyltransferase	+	-
438	Us	977	VDAG_10534	CTP synthase	+	-		no further predicted ORF in this scaffold			
374	8	1023	VDAG_06275	hypothetical protein	+		279	VDAG_06276	NAD-dependent malic enzyme	+	-
75	4	1060	VDAG_09054	ubiquitin fusion degradation protein	+	-	196	VDAG_09055	hypothetical protein	+	-
SD-78	5	1214	VDAG_07011	mannosyltransferase complex	+	-	1914	VDAG_07012	fungal specific transcription factor	+	-
412	5	1647	VDAG_09301	calcium-transporting ATPase	+	-	1459	VDAG_09302	glycoside hydrolase	+	100
402	7	1788	VDAG_01891	hypothetical protein	+	400	1318	VDAG_01892	hypothetical protein	-	100
11	2	1960	VDAG_08003	hypothetical protein	-	100	4132	VDAG_08004	transcriptional activator XlnR	+	100
414	5	2048	VDAG_08987	phosphatidylinositol 4-kinase LSB6	+	200	3330	VDAG_08988	methyltransferase	-	-
459-1	1	2485	VDAG_00838	hypothetical protein	+	100	1784	VDAG_00839	transcription initiation factor	+	-
78	1	2691	VDAG_01460	transcription factor VIB	+	-	8182	VDAG_01461	ring finger protein	+	100
73	1	2721	VDAG_01460	transcription factor VIB	+	-	8152	VDAG_01461	ring finger protein	+	100
622	1	5167	VDAG_04426	hypothetical protein	-	3000	101	VDAG_04427	serine threonine kinase	+	-

[a] Chr = chromosome; Us = unpositioned scaffold;

[b] Distance from the T-DNA to the coding sequence (CDS). CDS indicates that the T-DNA is in the CDS.

[c] Evidence for *in planta* expression.

[d] Evidence for expression in the region between T-DNA and the closest ORF. Distances from T-DNA are indicated in base pairs (bp).

For 13 mutants, this revealed that only one of the two flanking genes is expressed during infection, disqualifying the 13 candidate genes that are not expressed. Thus, 55 candidate genes in total qualified as potential pathogenicity or virulence genes (Table 1). Subsequently, the 55 candidate genes were queried against the pathogen-host interaction (PHI) database that contains experimentally verified pathogenicity and virulence factors of plant and animal pathogens (Winnenburg *et al*., 2008). This resulted in the identification of homologues ($E<10^{-6}$) for 17 candidate genes (Table 2).

Functional analysis of VdNRS/ER identifies UDP-rhamnose as virulence factor

In random mutant 389, the T-DNA was integrated 56 bp upstream of the coding region of a nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER) homolog. NRS/ERs show similarity with the bacterial rmlD substrate binding domain, which is involved in biosynthesis of the nucleotide sugar dTDP-rhamnose. This dTDP-rhamnose is the precursor of L-rhamnose, which has been implicated in virulence of pathogenic bacteria such as *Salmonella enterica*, *Vibrio cholerae* and *Streptococcus mutans* (Giraud et al. 2000). In contrast to bacteria that produce dTDP-rhamnose in a three-step pathway, plants follow a two-step pathway to synthesize UDP-rhamnose (Oka et al. 2007). And it was recently shown that UDP-rhamnose is also present in glycans isolated from the rice pathogen *Magnaporthe oryzae* and from the broad host range pathogen *Botrytis cinerea*, although a role for UDP-rhamnose in fungal virulence has not yet been demonstrated (Martinez et al. 2012).

The first CDS downstream of the T-DNA insertion site is found only at 2.4 kb, encoding a squalene synthetase (VDAG_06011). Squalene synthetases have been implicated in sterol and triterpene biosynthesis, but considering the distance between the T-DNA insertion and the CDS of the squalene synthetase it is unlikely that this gene is affected and causal to the impared pathogenicity phenotype of the RM-389 mutant. To assess whether any genes have been missed by the automated gene prediction in the region between the T-DNA insertion and the squalene synthetase CDS, RNAseq reads from samples of *V. dahliae*-infected *N. benthamiana* (de Jonge et al., 2012; Faino et al., 2012) were queried for this region, but no reads were found to map, suggesting that no *in planta* transcribed genes that have been missed by the gene prediction are present in this region. Finally, with real-time PCR on genomic DNA, using the single copy *Ave1* gene as a reference (de Jonge et al., 2012) it was

Table 2. Homologues identified in the PHI database among the 55 potential *V. dahliae* pathogenicity and virulence genes.

Locus	Annotation	Species	Host	Pathogenicity	E-value
VDAG_00500	carboxypeptidase S1	*Ustilago maydis*	Plant	Unaffected	1.E-55
VDAG_00839	transcription initiation factor	*Candida albicans*	Animal	Loss	2.E-14
VDAG_00844	exosome complex exonuclease	*Candida albicans*	Animal	Reduced	5.E-06
VDAG_01960	KH domain- protein	*Calviceps purpurea*	Plant	Unaffected	2.E-10
VDAG_02014	Myb DNA binding protein	*Magnaporthe grisea*	Plant	Loss	6.E-14
VDAG_02343	cell wall glucanase	*Magnaporthe grisea*	Plant	Reduced	3.E-30
VDAG_04427	serine threonine kinase	*Cryptococcus neoformans*	Animal	Reduced	1.E-151
VDAG_04591	calmodulin	*Candida albicans*	Animal	Reduced	4.E-20
VDAG_06253	cyclic nucleotide-binding protein	*Cryptococcus neoformans*	Animal	Increased	5.E-11
VDAG_07012	fungal specific transcription factor	*Fusarium oxysporum*	Plant	Loss	2.E-14
VDAG_07704	pathway-specific nitrogen regulator	*Fusarium oxysporum*	Plant	Unaffected	1.E-08
VDAG_08004	transcriptional activator XlnR	*Fusarium oxysporum*	Plant	Unaffected	0.E+00
VDAG_08031	FES CIP4 domain- protein	*Candida albicans*	Animal	Loss	8.E-07
VDAG_09008	alcohol dehydrogenase	*Cryptococcus neoformans*	Animal	Reduced	1.E-09
VDAG_09301	calcium-transporting ATPase	*Candida albicans*	Animal	Reduced	3.E-95
VDAG_10191	methyltransferase domain-containing protein	*Aspergillus fumigatus*	Animal	Reduced	2.E-34
VDAG_10193	cellulose-binding family II	*Fusarium oxysporum*	Plant	Unaffected	2.E-07

determined that only a single T-DNA insertion was present in the genome of the RM-389 mutant. Thus, considering all of the above, functional analysis of the *NRS/ER* gene was pursued.

VdNRS/ER is required for *V. dahliae* pathogenicity

Targeted replacement of *VdNRS/ER* by a hygromycin resistance cassette through homologous recombination was pursued in wild-type *V. dahliae*, and gene deletion was verified by PCR. Several independent *VdNRS/ER* deletion mutants were obtained, of which two (Δ*6010-1* and Δ*6010-2*) were used for further analysis in this study (Supplemental Figure 1). To assess the role of *VdNRS/ER* in *V. dahliae* vegetative growth and conidiospore production, radial growth and sporulation of the *VdNRS/ER* deletion mutants on potato dextrose agar was assessed together with the random mutant (RM-389) and an ectopic transformant. This analysis showed that growth and conidiation was not markedly affected upon *VdNRS/ER* deletion (Figure 2).

Subsequently, the role of *VdNRS/ER* in *V. dahliae* pathogenicity was addressed. To this end, the expression of *VdNRS/ER* was assessed in wild-type *V. dahliae* during infection of tomato plants in a time course harvested at regular

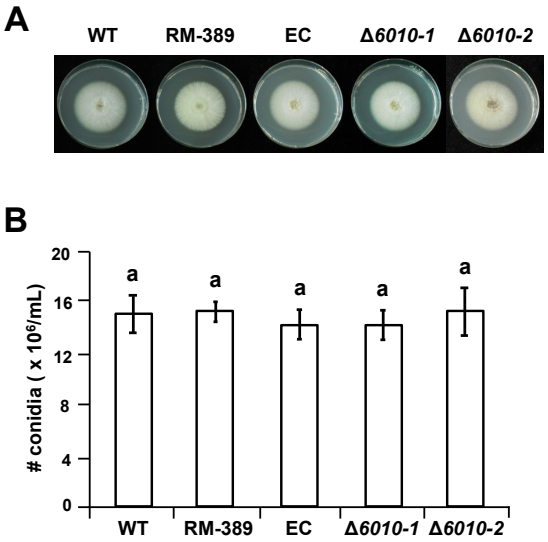


Figure 2. Targeted deletion of *VdNRS/ER* does not impair growth and conidiogenesis. A) Radial growth and colony morphology of wild type *Verticillium dahliae* (WT), random transformant RM-389, an ectopic transformant (EC) and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) after 7 days of incubation on PDA medium at 22°C. B) Average number of conidia produced after 7 days of growth on PDA medium based on two independent experiments. Letters indicate significant differences ($P<0.05$) calculated with the Student's t test.

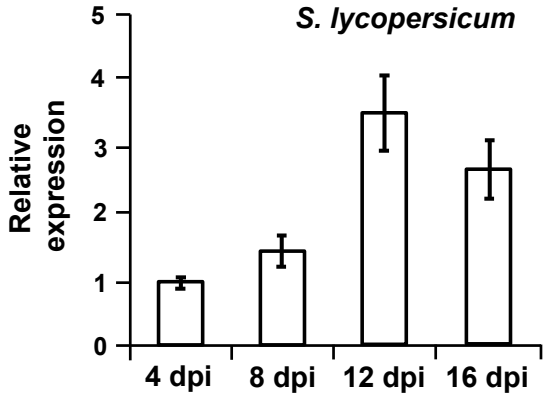


Figure 3. Expression of *VdNRS/ER* during infection of *Verticillium dahliae* on tomato. Ten-day-old tomato (*S. lycopersicum*) cultivar MoneyMaker plants were root-inoculated with *V. dahliae* and plants were harvested at regular intervals from 4 to 16 days post inoculation (dpi). After RNA isolation and cDNA synthesis, real-time PCR was performed to determine the relative expression levels of *VdNRS/ER* using the *V. dahliae* elongation factor 1-alpha gene as a reference. Expression at 4 dpi is set to one for all panels.

Intervals after inoculation, showing that *VdNRS/ER* is induced during *in planta* growth with a peak in expression around 12 days post inoculation (Figure 3). Subsequently, the *VdNRS/ER* deletion mutants were inoculated on tomato plants alongside the random mutant (RM-389) and an ectopic transformant to investigate the role of *VdNRS/ER* in fungal virulence. While inoculation of the plants with the wild-type *V. dahliae* strain and the ectopic transformant resulted in the development of symptoms of Verticillium wilt disease that include stunting of the plants and wilting, inoculation with RM-398 as well as with the *VdNRS/ER* deletion mutants did not result in symptom development on any of the two species throughout the assay up to 21 days post inoculation (Figure 4A). This was confirmed upon measuring of the surface area of the foliage of the plants inoculated with the various fungal genotypes, as plants that were inoculated with *VdNRS/ER* deletion strains developed similar foliage areas as mock-inoculated plants and plants inoculated with RM-389, while the amount of foliage developed by plants that were inoculated with the wild-type *V. dahliae* strain and the ectopic transformant was significantly reduced (Figure 5A). Fungal outgrowth assays upon plating of stem sections harvested from the hypocotyls of the inoculated plants demonstrated that, while the ectopic transformants and the wild-type strain had extensively colonized the stems of the tomato plants, the *VdNRS/ER* deletion strains and RM-389 were not able to colonize the xylem vessels of the inoculated tomato plants (Figure 4A). This finding was confirmed by real-time PCR quantification of fungal biomass for the various genotypes (Figure 5B). Importantly, pathogenicity was restored upon complementation of the *VdNRS/ER* deletion strains with a genomic construct containing the wild-type *VdNRS/ER* locus

(Supplemental Figure 2). Thus, it can be concluded that *VdNRS/ER* is required for pathogenicity on tomato.

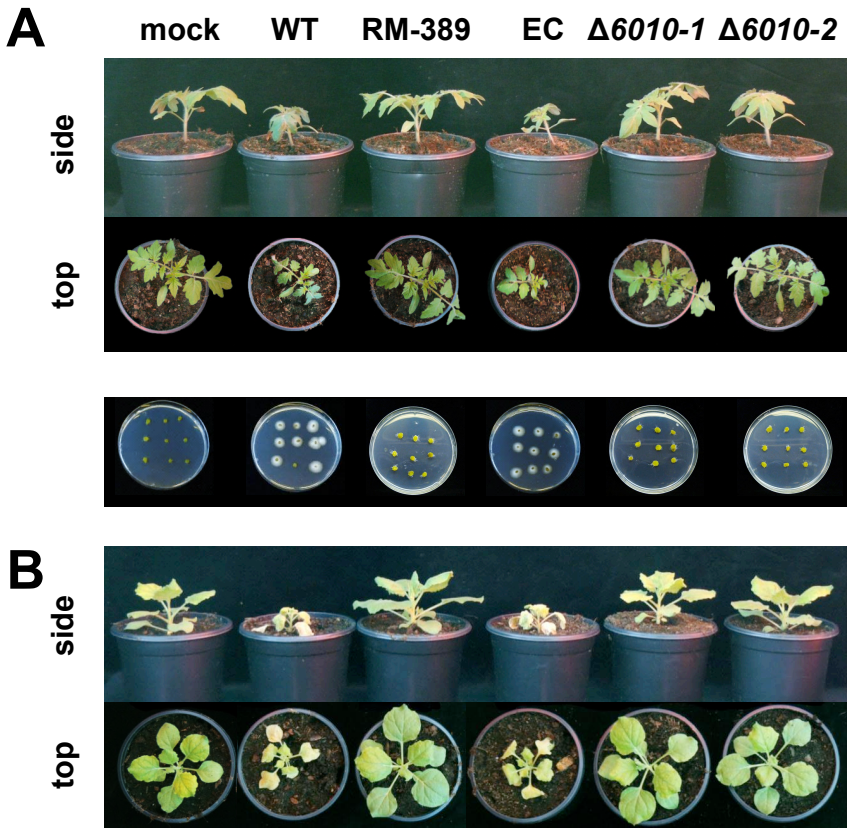


Figure 4. *VdNRS/ER* is required for pathogenicity of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. A) Top and side view of tomato cultivar MoneyMaker plants that were mock-inoculated (mock), or inoculated with wild type *V. dahliae* (WT), random transformant RM-389, an ectopic transformant (EC), and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) at 14 dpi. Fungal outgrowth at 7 days after plating of stem sections harvested at 14 days post inoculation is shown at the bottom of the panel. B) Top and side view of *N. benthamiana* plants inoculated as specified for panel A.

To investigate whether the observed role of *VdNRS/ER* in pathogenicity is confined only to tomato, or also extends to other host species, we tested the pathogenicity of the *VdNRS/ER* deletion strains on the Solanaceous model plant *Nicotiana benthamiana*. Similar to tomato, targeted deletion of *VdNRS/ER* resulted in compromised pathogenicity, as also these plants remained devoid of *Verticillium* wilt symptoms (Figure 4B). Again, loss of pathogenicity was confirmed by measurement

of the surface area of the foliage of the plants inoculated with the various fungal genotypes (Figure 5C). And also in this case, loss of pathogenicity was confirmed by real-time PCR quantification of fungal biomass for the various genotypes (Figure 5D). Collectively, these data not only confirm that the impaired pathogenicity of RM-389 on tomato can be attributed to the *VdNRS/ER* gene, but also that *VdNRS/ER* is required for pathogenicity of *V. dahliae* on multiple host plants.

VdNRS/ER is not required for cell wall integrity and osmotic stress resistance

To examine the role of *VdNRS/ER* in cell wall integrity and osmotic stress resistance, the random mutant (RM-389), the *VdNRS/ER* deletion mutants and the ectopic transformant were grown on minimal medium supplemented with a concentration range of congo red, sodium chloride, mannitol and sorbitol, and after seven days the colony diameter was measured (Figure 6). Our analyses showed that the mutant and wild type strains were equally sensitive to all the tested components, suggesting that *VdNRS/ER* is not involved in cell wall integrity and osmotic stress resistance.

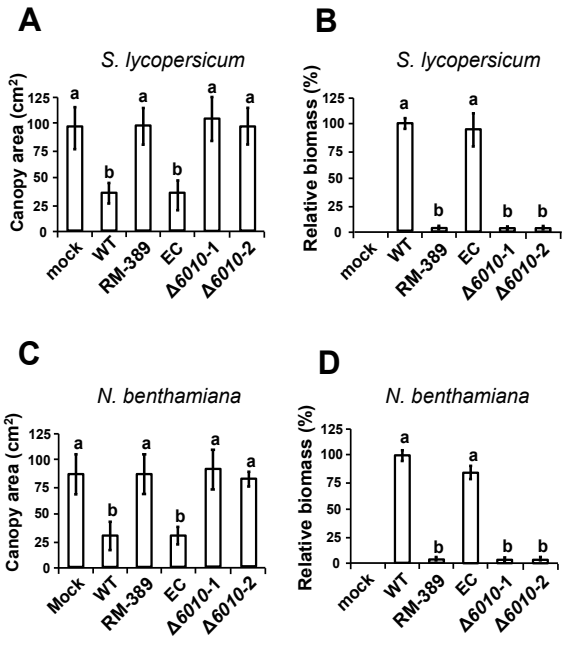


Figure 5. *VdNRS/ER* is required for pathogenicity of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. A) Average canopy area of 6 tomato plants, and (B) real-time PCR quantification of fungal biomass at 14 days after mock-inoculation (mock), or inoculation with wild type *Verticillium dahliae* (WT), random transformant (RM-389), an ectopic transformant (EC), and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*). Different letter labels indicate significant differences (P<0.05). C) Average canopy area of 6 *N. benthamiana* plants, and (D) real-time PCR quantification of fungal biomass upon inoculation as specified for panels A and B.

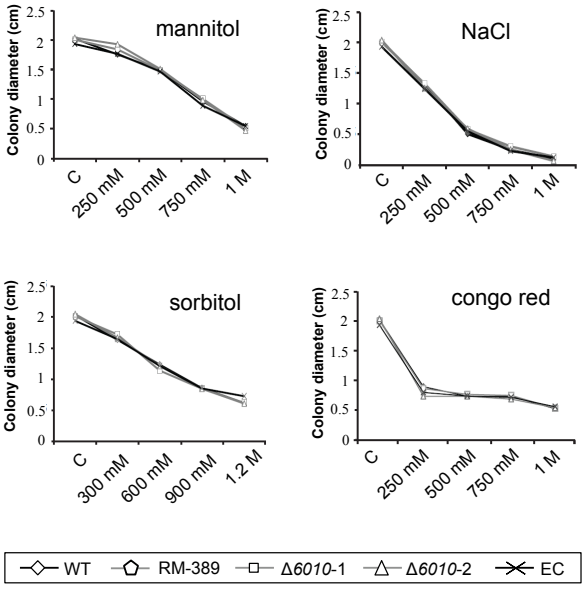


Figure 6. Targeted deletion of *VdNRS/ER* does not affect cell wall integrity and osmotic stress resistance**.** Stress sensitivity assays were performed by placing a xx µL droplet (10^6 conidia/mL) of wild type *V. dahliae* (WT), an ectopic transformant (EC) and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) on Czapek-dox medium (C), or Czapek-dox supplemented with congo red (250 mM, 500 mM, 750 mM and 1 M), NaCl (250 mM, 500 mM, 750 mM and 1 M), mannitol (250 mM, 500 mM, 750 mM and 1 M), or sorbitol (300 mM, 600 mM, 900 mM, 1.2 M). The colony diameter was measured after 7 days of incubation at 22°C.

VdNRS/ER is required for conidiospore attachment to tomato roots

To examine the role of *VdNRS/ER* in the initial stages of infection, roots of ten-day-old tomato plants were inoculated in a hydroponics solution with conidiospores of the random mutant (RM-389), the *VdNRS/ER* deletion mutants and the ectopic transformant, and subsequently incubated for five days. After incubation, the tomato roots were inspected using a binocular microscope, showing that the spores of the wild type and ectopic strains had efficiently attached to the root surface resulting in extensive fungal growth on the tomato roots (Figure 7A). In contrast, little fungal growth was observed on roots inoculated with the random mutant (RM-389) and the *VdNRS/ER* deletion mutants (Figure 7A). The reduced fungal biomass of the random mutant (RM-389) and the *VdNRS/ER* deletion mutants on the tomato roots was confirmed by quantitative PCR (Figure 7B). Collectively, these data suggest that *VdNRS/ER* affects attachment of fungal conidiospores to the root surface.

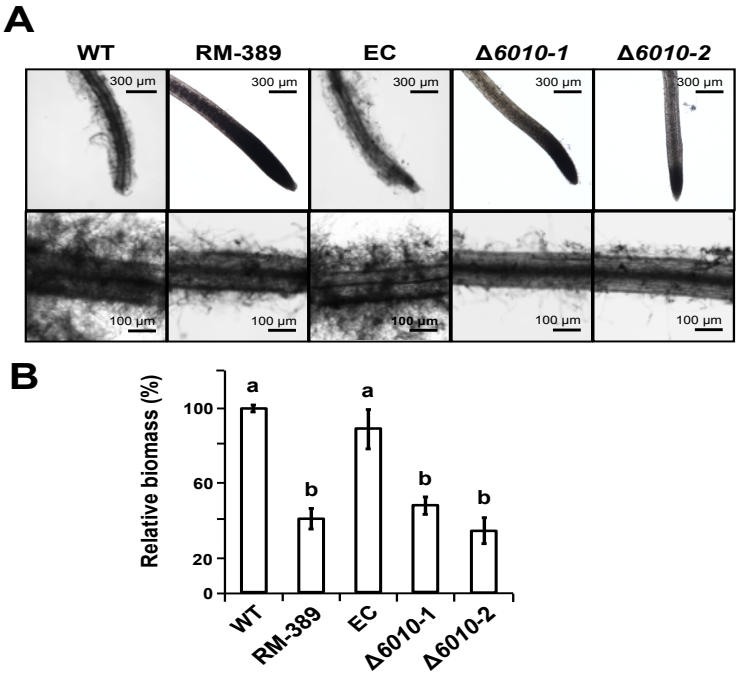


Figure 7. Fungal attachment to tomato roots. Roots of ten-day-old tomato cultivar MoneyMaker seedlings were immersed in one-fifth PDB containing 10^6 conidia/mL of wild type *V. dahliae* (WT), random transformant (RM-389), an ectopic transformant (EC) and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) for 72-96 h. A) Roots were rinsed with water and photographed under a microscope. B) Real-time PCR quantification of fungal biomass on the roots. Different letter labels indicate significant differences ($P<0.05$).

DISCUSSION

Random mutagenesis through ATMT followed by inoculation of the generated mutants on host plants has been widely used to identify pathogenicity or virulence genes in several plant pathogens (Giesbert *et al*., 2011; Hüser *et al*., 2009; Michielse *et al*., 2009; Münch *et al*., 2011; Mullins *et al*., 2001; Jeon *et al*., 2007). In this manuscript, we report on the generation of a library of random T-DNA insertion mutants in *V. dahliae* that were constructed through ATMT in order to identify pathogenicity and virulence genes. Out of 900 random T-DNA transformants, 80 were selected based on reproducible defects in virulence using root dip inoculation on tomato, and T-DNA insertion sites were identified using iPCR. In total, 12 insertions were found within predicted open reading frames, while 31 insertions were found in

intergenic regions. Based on *in planta* expression of potential candidate genes, 55 genes were qualified as potential pathogenicity or virulence genes.

In random mutant 389, the T-DNA was integrated 56 bp upstream of the coding region of a nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER). NRS/ERs show similarity to the bacterial rmlD substrate binding domain, which is involved in biosynthesis of dTDP-rhamnose. In Gram negative bacteria such as *S. enterica*, *V. cholerae* or *Escherichia coli*, L-rhamnose is an important residue in the O-antigen of lipopolysaccharides, which are essential for resistance to serum killing and for colonization (Chiang and Mekalanos 1999). In mycobacteria such as *Mycobacterium tuberculosis*, L-rhamnose maintains the structural integrity of the cell wall through connecting the inner peptidoglycan layer to the arabinogalactan polysaccharides (Giraud et al. 2000). Moreover, recent studies identified two genes encoding UG4,6-Dh and U4k6dG-ER enzymes from *Magnaporthe oryzae* and *Botrytis cinerea* involved in UDP-rhamnose formation (Martinez et al. 2012).

The VdNRS/ER identified in *V. dahliae* also showed high homology to the *M. oryzae* and *B. cinerea* U4k6dG-ER enzyme. We have shown that targeted deletion of *Vd NRS/ER* contributes to loss of *V. dahliae* pathogenicity on various host plants. The role of rhamnose-containing macromolecules in cell walls on oxidative stress tolerance in fungi is largely unknown. *VdNRS/ER* deletion strains were able to grow and sporulate without any visible defects. This suggests that UDP-rhamnose containing glycoproteins or exopolysacharides are not essential for vegetative growth and sporulation. In addition, the growth rate of *VdNRS/ER* knock-out mutants was not affected in the presence of inducers of cell wall stress or osmotic stress, suggesting that the UDP-rhamnose containing glycoproteins or exopolysacharides are not required for cell wall and osmotic stress tolerance. To determine whether *VdNRS/ER* participates during surface colonization, we compared the ability of wild-type and knock-out mutants to successfully surface colonize tomato roots. Tomato roots immersed in wild-type and ectopic conidial suspension became completely covered with fungal propagules. In contrast, knock-out conidial suspensions failed to attach efficiently on tomato roots. For the first time we show that UDP-rhamnose containing macromolecules are required for the attachment of fungal spores on to tomato roots and required for pathogenicity.

Based on the findings from this study, we propose a model to explain the contribution of UDP-rhamnose to the virulence of *V. dahliae* during infection on host plants. Glucose is converted into UDP-rhamnose in a two step pathway by VdUG4,6-Dh and VdNRS/ER in the cytoplasm and then attached to glycoproteins or exopolysaccharides that are then secreted. The rhamnose containing glycoproteins or exopolysaccharides play a role in the attachment of spores on the root surface, resulting in successful colonization of host plants. VdNRS/ER deletion strains are

unable to synthesize UDP-rhamnose and hence, glycoproteins or exopolysaccharides are not fully functional and the fungal spores are unable to attach efficiently, which results in impaired pathogenicity.

MATERIALS AND METHODS

Agrobacterium tumefaciens mediated transformation (ATMT)

The binary vector (pBHt2) harboring the hygromycin B resistance gene (*hph*) under control of the *Aspergillus nidulans trpC* promoter was used for ATMT (Mullins *et al*., 2001). This vector was introduced to *A. tumefaciens* strain SK1044 to transform conidia of race 1 *V. dahliae* strain JR2 (Fradin *et al*., 2009). To this end, *A. tumefaciens* was grown at 28°C for 2 days in minimal medium supplemented with kanamycin (25 µg/mL). The *A. tumefaciens* cells were diluted to an OD_{600} of 0.15 in induction medium (IM), supplemented with 200 µM acetosyringone (AS). The cells were grown for an additional six hours before mixing them with an equal volume of *V. dahliae* conidiospore suspension (10^6 conidia/mL). 200 µl of this mixture was plated onto a Hybond-N^+ filter placed on induction medium supplemented with 200 µM acetosyringone. The plates were incubated in the dark at room temperature for 48 h after which the filter was transferred onto a selection plate (PDA supplemented with 50 µg/mL of hygromycin B and 200 µM of cefotaxime). After 10 to 14 days, individual transformants were transferred to 24-well culture plates containing one mL of selection medium and incubated for 7 to 10 days. Spores from these cultures were stored in 30% glycerol at -80°C until further analysis.

Plant inoculations

Pathogenicity assays were performed as described previously (Santhanam, 2012). Briefly, individual transformants were sub-cultured in six-welled culture plates for 7 to 10 days. Subsequently, 10 glass beads (~3 mm) and 3 mL of tap water were added to each of the wells and the plates were sealed. The conidiospores were released by shaking the plates for 15 min at 200 rpm on a reciprocal shaker after which the sealing was removed. The roots of 10-day-old tomato (*Solanum lycopersicum* cv. Moneymaker) seedlings were rinsed in water and dipped into the conidiospore suspension for 5 minutes. Seedlings were replanted in soil and scored for symptom development (wilting and stunting) up to 14 days. Seedlings that exhibited reduced *Verticillium* wilt symptoms when compared to inoculation with wild type *V. dahliae*

inoculated plants were identified and the corresponding mutants were retained for rescreening.

During rescreening, the infection assay was carried out essentially as described above, with the modification that the conidiospore concentration of the transformants was calibrated to 10^6 spores/mL. The rescreening was repeated at least two times for each of the mutants that was retained in the initial screen.

Identification of T-DNA insertion sites

Genomic sequences flanking the T-DNA were isolated from the selected transformants with inverse PCR (iPCR) as described previously (Meng *et al*., 2007, Santhanam, 2012). Essentially, 500 ng of genomic DNA was digested overnight with *Msp*I or *Nco*I and heat-inactivated at 65°C for 20 min. Subsequently, 50 µL of ligation mix (10 µL of 10X T4 DNA ligase buffer, 5 units of T4 DNA ligase and 38 µL of H_2O) was added and incubated overnight at 15°C. Next, the DNA was precipitated and dissolved in 50 µL of demineralized water. The genomic region flanking the T-DNA was amplified in 50 µL reaction mix using 2 µL of ligation product, 1 µL of each primer (Table 1), 1X PCR buffer, 0.6 µL dNTP mix (10 mM), 0.8 units GO Taq DNA polymerase and 35.25 µL water. Cycling conditions consisted of 1 cycle for 2 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 3 min at 72°C, and a final extension step for 10 min at 72°C. PCR products were purified and sequenced. The obtained sequences were used as query to blast against the *V. dahliae* genome (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/Blast.html).

Targeted mutagenesis and complementation

To generate a *VdNRS/ER* deletion construct, sequences flanking the *VdNRS/ER* coding sequence were amplified from genomic DNA of *V. dahliae* strain JR2 using the primers KO-6010-LF with KO-6010-LR to amplify the left border, and KO-6010-RF with KO-6010-RR to amplify the right border, respectively (Supplemental Table 1). The resulting amplicons were cloned into pRF-HU2 as described previously (Frandsen et al 2008). *A. tumefaciens*-mediated transformation of *V. dahliae* was performed as described previously (Santhanam, 2012), and transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime (Duchefa, Haarlem, The Netherlands), 50 µg/mL of hygromycin (Duchefa, Haarlem, The Netherlands). Homologous recombination was verified by PCR.

To generate a *VdNRS/ER* complementation construct, a 2404 bp *Eco*RI/*Pac*I fragment containing the VDAG_06010 coding sequence with 1000 bp upstream and 450 bp downstream sequence was amplified from *V. dahliae* strain JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al 2008). Complementation transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime and 100 µg/mL phleomycin (InvivoGen, San Diego, USA).

Growth, conidiogenesis and stress assays

Radial growth was monitored by placing a 2 µL droplet of a conidial suspension of 10^6 conidiospores/mL in the centre of PDA or Czapek-dox medium, incubated at 22°C and measurement of the colony diameter after 10 days. For quantification of conidiospore production, 5 mL of water was added to the culture and a conidial suspension was prepared by gently rubbing the mycelium. A ten-fold dilution of the conidial suspension was counted using a haemocytometer. Stress sensitivity assays were performed by placing a 2 µL droplet with 10^6 conidiospores/mL of wild type *V. dahliae,* two *VdNRS/ER* deletion strains and an ectopic transformant in the centre of a Czapek-dox plate supplemented with congo red (250 mM, 500 mM, 750 mM and 1 M), NaCl (250 mM, 500 mM, 750 mM and 1 M), mannitol (250 mM, 500 mM, 750 mM and 1 M), or sorbitol (300 mM, 600 mM, 900 mM, 1.2 M), and incubated at 22ºC. Plates were photographed at 7 dpi and the colony diameter was measured using ImageJ software.

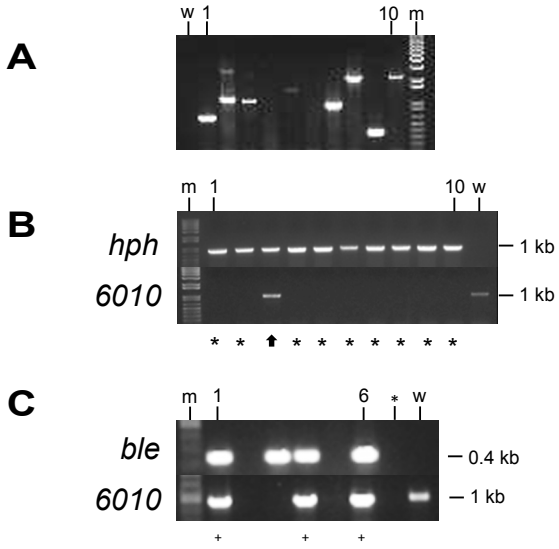
Adhesion assay

The root adhesion assay was performed as described by Di Pietro et al. (2001). Briefly, the roots of 10-day-old tomato seedlings were rinsed in water and placed in Erlenmeyer flasks containing a suspension of 10^6 conidia/mL in one-fifth PDB and incubated at 22°C and 100 r.p.m. Fungal adhesion to the root surface was observed macroscopically from 3 up to 5 days post inoculation. The experiments were performed three times with similar results.

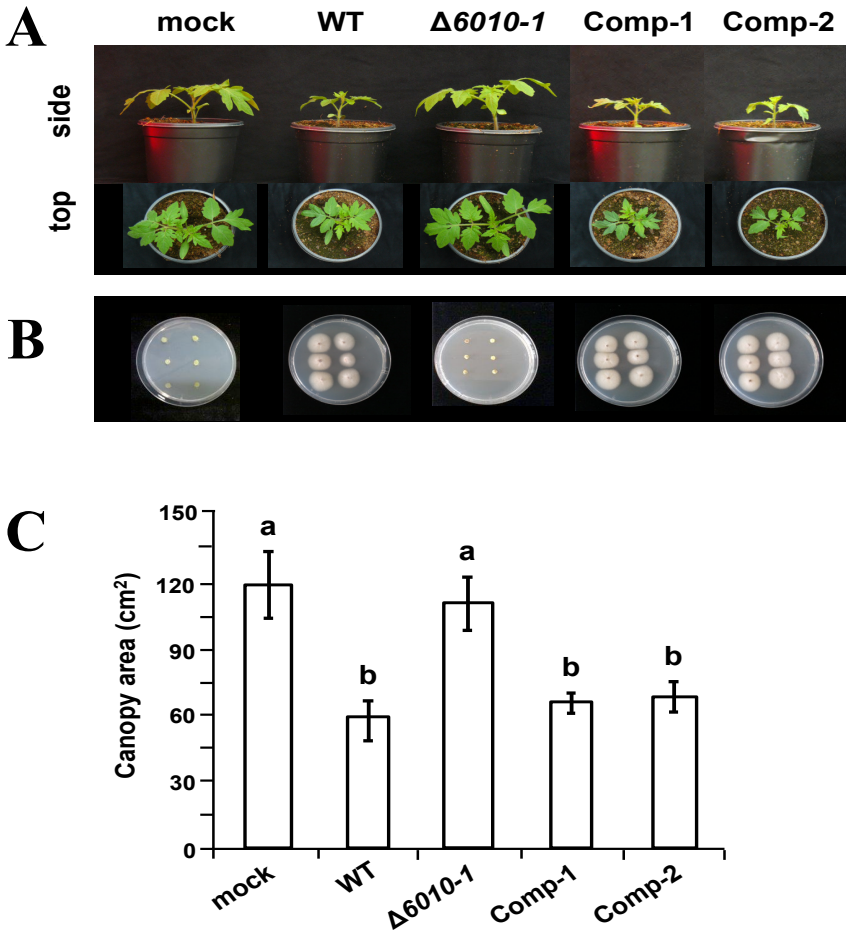
ACKNOWLEDGEMENTS

The authors thank Md. Tohidul Islam, Mojtaba Keykha Saber, Grardy van den Berg, Hugo de Vries and Emilie Fradin for technical assistance, and Bert Essenstam and Henk Smid for excellent plant care.

SUPPLEMENTAL FIGURES AND TABLES



Supplemental Figure 1. Rescue of flanking sequences, verification of deletion and complementation strains by PCR. A) Amplification of the T-DNA flanking sequences from wild type *V. dahliae* (w), 10 independent random transformants (lanes 1 to 10), and the marker (m) is indicated. B) Amplification of the hygromycin resistance gene (*hph*) and *VdNRS/ER* from 10 independent transformants (lanes 1 to 10) and wild type *V. dahliae* (w). The marker (m) is indicated. True deletion strains are marked with asterisks, while the ectopic transformant is marked with an arrow. C) Amplification of the zeocin resistance gene (*ble*) and *VdNRS/ER* from 5 independent complementation strains (lanes 1 to 5), a *VdNRS/ER* deletion strain (*) and wild type *V. dahliae* (w). The marker (m), and true complementation strains (+) are indicated.



Supplemental Figure 2. Complementation of *VdNRS/ER* deletion strain restores pathogenicity on tomato**.** A) Side and top view of tomato cultivar MoneyMaker plants inoculated with wild type *Verticillium dahliae* (WT), a *VdNRS/ER* deletion strain (Δ*6010-1*) and two complementation strains (comp-1 and comp-2), or mock-inoculated at 14 days post inoculation. B) Fungal outgrowth at 7 days after plating of stem sections harvested at 14 days post inoculation. C) Average canopy area of 6 plants at 14 days after inoculation as described for panel A. Different letter labels indicate significant differences ($P<0.05$).

Supplemental Table 1. Primers used in this study

Primer	Sequence (5'- 3')	Remarks
MLBF	GGATTTTGGTTTTAGGAATTAGA	*Msp*I left border, forward
MLBR	AATTCGGCGTTAATTCAGTACA	*Msp*I left border, reverse
MLSeq	TCAGTACATTAAAAACGTCCGCAA	*Msp*I left border, sequencing
MRBF	CAACTGTTGGGAAGGGCGATC	*Msp*I right border, forward
MRBR	CAGCCTGAATGGCGAATGCTA	*Msp*I right border, reverse
MRSeq	GAATGCTAGAGCAGCTTGAGCT	*Msp*I right border, sequencing
NLBF	AGTGTATTGACCGATTCCTTGC	*Nco*I left border, forward
NLBR	AGGGTTCCTATAGGGTTTCGCTCATG	*Nco*I left border, reverse
NLSeq	GAATTAATTCGGCGTTAATTCAGT	*Nco*I left border, sequencing
NRBF	CGTTATGTTTATCGGCACTTTG	*Nco*I right border, forward
NRBR	GGCACTGGCCGTCGTTTTACAAC	*Nco*I right border, reverse
NRSeq	CCCTTCCCAACAGTTGCGCA	*Nco*I right border, sequencing
KO-6010-LF	GGTCTTAAUACTGCGCTGATGACCTCAC	Left border, forward
KO-6010-LR	GGCATTAAUCAGTGGATGCTGTTGTCGAT	Left border, reverse
KO-6010-RF	GGACTTAAUAATGCGAAATGCGTAAGGAG	Right border, forward
KO-6010-RR	GGGTTTAAUGCGGTAGGCCTTCTTGATCT	Right border, reverse
6010-F	GCGGCCGCATGTCAGTTACGAACGGTGC	Full length, forward
6010-R	CCATGGTCAATTAACACCAGCAGCCTT	Full length, reverse
6010 -comp_F	GAATTCTGCGCTGATGACCTCACC	Complementation, forward
6010 -comp_R	TTAATTAACCGCCTTGGATCACCAC	Complementation, reverse
qPCR-6010-F	ACACGATCCTCCACG ATCTC	Real-time PCR, forward
qPCR-6010-R	GCGAGAAGTTCTTCCAGGTG	Real-time PCR, reverse
VdELF1-a-F	CCATTGATATCGCACTGTGG	Real-time PCR, forward
VdELF1-a-F	TGGAGATACCAGCCTCGAAC	Real-time PCR, reverse
qPCR-Hyg-F	ATAGGTCAGGCTCTCGCTGA	Real-time PCR, forward
qPCR-Hyg-R	GATGTAGGAGGGCGTGGATA	Real-time PCR, reverse

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CHAPTER 3

Verticillium dahliae Sge1 Differentially Regulates Expression of Candidate Effector Genes

Published as:
Santhanam, P., and Thomma, B. P. H. J. 2012. *Verticillium dahliae* Sge1 differentially regulates expression of candidate effector genes. Mol. Plant-Microbe Interact. 26:249-256.

ABSTRACT

The ascomycete fungus *Verticillium dahliae* causes vascular wilt diseases in hundreds of dicotyledonous plant species. However, thus far only few *V. dahliae* effectors have been identified, and regulators of pathogenicity remain unknown. In this study, we investigated the role of the *V. dahliae* homolog of Sge1, a transcriptional regulator that was previously implicated in pathogenicity and effector gene expression in *Fusarium oxysporum*. We show that *V. dahliae Sge1* (*VdSge1*) is required for radial growth and production of asexual conidiospores, as *VdSge1* deletion strains display reduced radial growth and reduced conidia production. We furthermore show that *VdSge1* deletion strains have lost pathogenicity on tomato. Remarkably, VdSge1 is not required for induction of *Ave1*, the gene encoding the recently identified *V. dahliae* effector that activates resistance mediated by the Ve1 immune receptor in tomato. Further assessment of the role of *VdSge1* in the induction of the nine most highly *in planta* induced genes that encode putative effectors revealed differential activity. Whereas the expression of one putative effector gene in addition to *Ave1* was not affected by *VdSge1* deletion, *VdSge1* appeared to be required for the expression of six putative effector genes, whereas two of the putative effectors genes were found to be negatively regulated by *VdSge1*. In conclusion, our data suggest that *VdSge1* differentially regulates *V. dahliae* effector gene expression.

INTRODUCTION

As all multicellular organisms have evolved immune systems to withstand microbial attack, immunity to infection by microbes that generally occur in their habitats is common. Nevertheless, particular microbes still developed into pathogens, which can be attributed to their deployment of effectors; secreted molecules that target the host's physiology such that it becomes susceptible to infection, often through direct suppression of immune responses (de Jonge et al. 2011). It can be anticipated that the expression of effector genes and other genes that are involved in pathogenicity or virulence are under tight regulation. Typically, fungal effectors are not, or lowly, expressed outside the host and are induced upon host penetration. Since it was found that some effectors are induced by nitrogen, it was proposed that nitrogen acts as *in planta* trigger of effector gene induction (van den Ackerveken et al. 1993). However, we now know that many effector genes do not respond to nitrogen starvation (Bolton and Thomma 2008). Thus, the *in planta* signals that trigger effector gene induction remain largely unknown. Unfortunately, also regulators of effector gene expression are generally unknown (de Jonge et al. 2011).

Recently, a transcriptional regulator that is important for early infection was identified in the root invading tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* as Sge1. This transcriptional regulator was found to be localized in the nucleus, required for pathogenicity, and required for the *in planta* expression of various effector genes during conditions that mimic growth *in planta* (Michielse et al. 2009). Furthermore, *Sge1* mutants showed significantly reduced conidia production and an altered metabolic profile (Michielse et al. 2009). Interestingly, Sge1 orthologs occur widely in fungi and family members are involved in morphological switching in dimorphic fungi. In the human pathogens *Candida albicans* and *Histoplasma capsulatum*, the transcriptional regulators Wor1 and Ryp1, respectively, were identified as major regulators of a morphological switch that is associated with disease causing ability. Whereas *C. albicans* Wor1 regulates the switch from white to opaque cell type, *H. capsulatum* Ryp1 governs the transition from yeast to filamentous growth. Similar to Sge1, Wor1 and Ryp1 are localized in the nucleus and control expression of phase-specific genes (Huang et al. 2006; Nguyen and Sil 2008; Srikantha et al. 2006; Zordan et al. 2006).

After the functional characterization of *Fusarium oxysporum* Sge1 as a regulator of parasitic growth, homologs in other plant pathogens have been studied, revealing that these homologs also act as regulators of pathogenicity. In the necrotrophic plant pathogen *Botrytis cinerea*, the FoSge1 homolog BcReg1 is required for conidiation and pathogenicity on bean leaves. Although *BcReg1* mutants

were able to penetrate host plant tissue, they were unable to establish necrotic lesions, possibly due to their inability to produce sesquiterpene and polyketide toxins. Similarly, the homolog Fgp1 of the wheat and barley pathogen *F. graminearum* regulates asexual and sexual spore formation as well as pathogenicity, which correlated with the lack of trichothecene mycotoxin production (Jonkers et al. 2012).

The ascomycete fungus *Verticillium dahliae* causes vascular wilt diseases in hundreds of dicotyledonous plant species (Fradin *et al*. 2006; Klosterman *et al*. 2009). The host range of *V. dahliae* also includes tomato, which is colonized in a similar fashion as by *F. oxysporum* f. sp. *lycopersici*. Triggered by root exudates, the melanised resting structures that reside in the soil germinate and penetrate roots of susceptible host plants. After crossing the cortex, hyphae of the fungus grow into the xylem vessels. The mycelium remains exclusively in these vessels and produces conidia which are transported acropetally with the water flow in the xylem throughout the plant. Once senescing, microsclerotia are produced that are released into the soil upon tissue decomposition. Compared to *F. oxysporum* f. sp. *lycopersici*, for which several effectors that are secreted in the xylem during host colonization have been identified as the so-called SIX proteins, only few *V. dahliae* effectors have been identified. Among these are plant cell wall-degrading enzymes (CWDEs) of which expression is regulated through the sucrose nonfermenting 1 protein (SNF1) (Tzima et al. 2010). Based on a query of the *V. dahliae* genome sequence, 127 (conserved) hypothetical proteins were designated as small (<400 amino acids), cysteine-rich (>4 cysteine residues) effector candidates, but no homologs of any of the *F. oxysporum* SIX effectors were identified (Klosterman et al. 2011). The only bona fide effector that has recently been characterized is the Ave1 effector that activates Ve1-mediated immunity in race 1-resistant tomato plants (de Jonge et al. 2012). This Ave1 effector is a secreted, small (134 aa), protein with 4 cysteines that is required for full virulence on tomato plants lacking *Ve1*, and the corresponding gene is strongly induced during host colonization, thus conforming to a typical effector protein (de Jonge et al. 2012). In this study, we investigated the role of the *V. dahliae* homolog of *F. oxysporum* Sge1 and assessed its role in pathogen virulence and regulation of effector gene expression.

RESULTS

Identification of VdSge1

To identify the *V. dahliae* ortholog of the *F. oxysporum* transcriptional regulator Sge1, the genome of *V. dahliae* strain VdLs17 was queried with BlastP. In this

manner, a single homolog, VDAG_ 06298.1 (E-value: 0), was identified which was tentatively called *VdSge1*. The *VdSge1* gene has an open reading frame (ORF) of 1533 nucleotides that is not interrupted by introns, as was verified by reverse-transcription polymerase chain reaction, and encodes a protein of 510 amino acids (aa). The length of the various homologs that have been functionally analyzed varies considerably, as *F. oxysporum* Sge1 is 330 aa, *F. graminearum* Fgp1 is 342 aa, *H. capsulatum* Ryp1 is 487 aa, *B. cinerea* Reg1 is 506 aa, *S. pombe* Gti1 is 720 aa and *C. albicans* Wor1 is 785 aa (Michielse et al. 2009; Jonkers et al. 2012; Nguyen et al. 2008; Michielse et al. 2011; Caspari 1997; Huang et al. 2006).

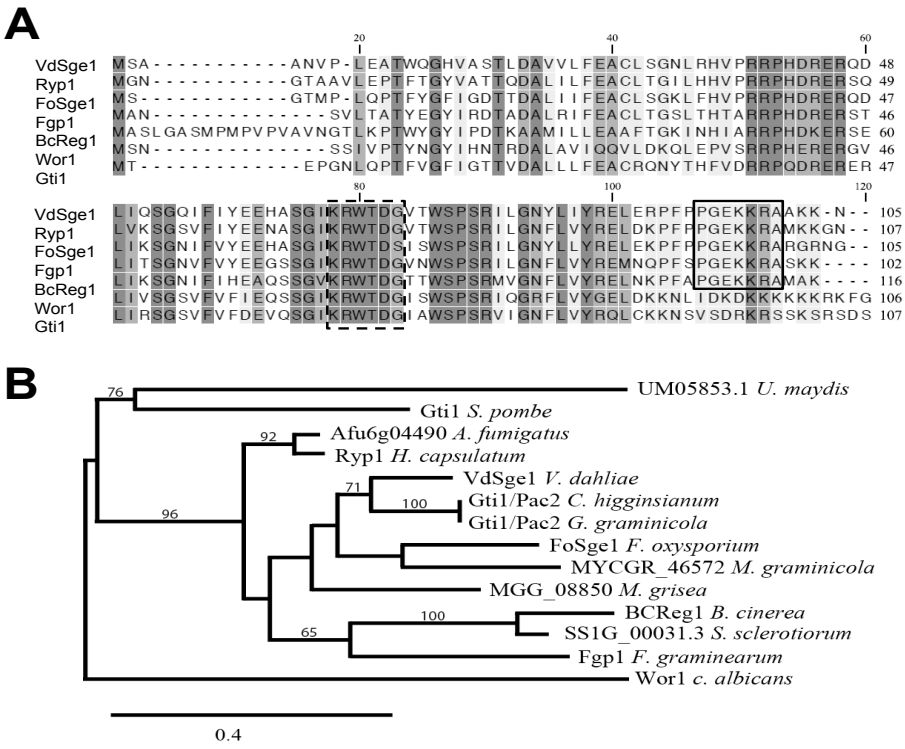


Figure 1. Alignment of VdSge1 with homologs from other fungal species. **A)** Amino acid sequence alignment of the N-terminal region of VdSge1 and of the homologs from *Histoplasma capsulatum* (Ryp1), *Fusarium oxysporum* (FoSge1), *F. graminearum* (Fgp1), *Botrytis cinerea* (BcReg1), *Candida albicans* (Wor1) and *Schizosaccharomyces pombe* (Gti1). Conserved residues are shaded. The box with the dashed line indicates the conserved phosphorylation site, while the box with the solid line indicates the predicted nuclear localization signals. **B)** Phylogenetic tree of VdSge1 with homologs from other fungal species. In addition to the homologs described above, also homologs from *Aspergillus fumigatus, Colletotrichum higginsianum, Glomerella graminicola, Magnaporthe grisea, Mycosphaerella graminicola, Sclerotinia sclerotiorum* and *Ustilago maydis* are included. Bootstrap percentages over 60% are indicated at the nodes.

Alignment of the N-terminal part, which is the most conserved part of the protein (Michielse et al. 2009; 2011), demonstrated that the TOS9 (COG5037) and the Gti1_Pac2 family domain (Pfam09729) motifs which are found in all orthologs analyzed thus far, are also present in VdSge1 (Figure 1). In addition, the VdSge1 protein shares a putative protein kinase A phosphorylation site (+66 to +71; KRWTDG) and a nuclear localization signal (+94 to +100; PPGEKKR) with several other homologs (Figure 1), suggesting that VdSge1 is nuclear localized.

VdSge1 is required for radial growth and conidia production

Targeted replacement of *VdSge1* by a hygromycin resistance cassette through homologous recombination was pursued, and gene deletion was verified by PCR. Several independent *VdSge1* deletion mutants were obtained, of which two were used for further analysis in this study (Supplemental Figure 1). It has previously been shown that the Sge1 orthologs from the plant pathogens *F. oxysporum*, *F. graminearum* and *B. cinerea* were not required for vegetative growth *in vitro* (Michielse et al. 2009; 2011; Jonkers et al. 2012). However, both orthologs were found to play a role in conidiation as deletion mutants in *F. oxysporum* and *F. graminearum* produced significantly reduced numbers of conidia, whereas deletion strains in *B. cinerea* did not produce conidia at all. Remarkably, the *VdSge1* deletion mutants showed clearly reduced radial growth on potato dextrose agar medium when compared with the wild-type strain and ectopic transformants (Figure 2A).

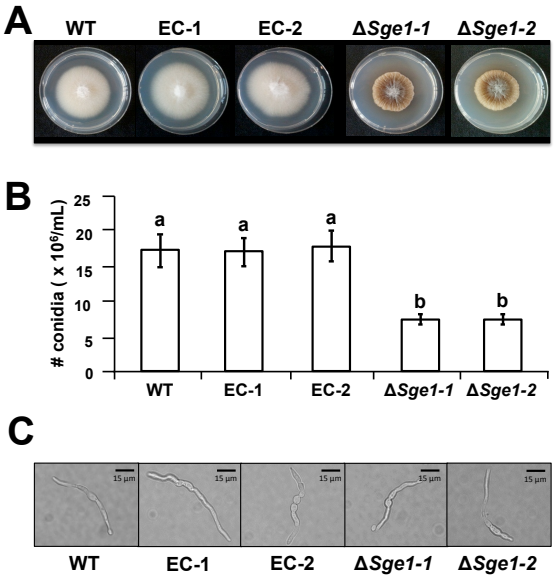


Figure 2. Targeted deletion of *VdSge1* results in altered colony morphology and reduced conidiospore production. **A)** Colony morphology of wild type *Verticillium dahliae* (WT), two *VdSge1* deletion strains (Δ*Sge1-1* and Δ*Sge1-2*) and two ectopic transformants (EC-1 and EC-2) after 7 days of incubation on PDA medium at 22°C. **B)** The average number of conidia produced after 7 days of growth on PDA medium based on two independent experiments. Different letters indicate significant differences at $P<0.05$ as calculated with Student's t test. **C)** Representative picture of microscopic observation of conidial germination at 16 hr after incubation in Czapek-dox medium.

Furthermore, also the pigmentation of the mycelium was affected by the *VdSge1* deletion, as the deletion mutants developed brown mycelium whereas the wild-type strain and ectopic transformants developed white mycelium (Figure 2A). The effect of *VdSge1* deletion on pigmentation is furthermore evident from the agar medium on which the fungal strains were grown, as the medium surrounding the *VdSge1* deletion mutants turned brown whereas the medium surrounding the wild-type strain and ectopic transformants did not change color (Figure 2A). Furthermore, the amount of conidia produced by the *VdSge1* deletion was significantly reduced when compared with the conidial production by the wild-type strain and ectopic transformants, although the appearance of conidia was phenotypically indistinguishable (Figure 2B). Conidial germination was found to be comparable in all strains, indicating that the conidia that are produced are fully viable (Figure 2C). The altered colony morphology as well as the reduced conidia production was restored upon complementation of the *VdSge1* deletion strains with a genomic construct containing the wild-type *VdSge1* locus (Supplemental Figure 2). Thus, *VdSge1* is involved in vegetative growth, mycelium pigmentation and conidiation.

VdSge1 is required for pathogenicity

We subsequently assessed the role of *VdSge1* in pathogenicity on tomato plants. To this end, it was determined that expression of *VdSge1* is induced in wild-type *V. dahliae* during infection of tomato plants in a time course harvested at regular intervals after inoculation (Supplemental Figure 3). *VdSge1* deletion strains appeared to be non-pathogenic on tomato upon root-dip inoculation with conidiospores, whereas ectopic transformants were found to be as virulent as the wild-type strain (Figure 3A). The plants that were inoculated with the *VdSge1* deletion strains remained devoid of any *Verticillium* wilt symptoms throughout the assay up to 21 days post inoculation, while the ectopic transformants and the wild-type strain induced severe wilting symptoms. Fungal outgrowth assays upon plating of stem sections harvested from the hypocotyls of the inoculated plants demonstrated that, while the ectopic transformants and the wild-type strain had extensively colonized the stems of the tomato plants, the *VdSge1* deletion strains were not able to colonize the xylem vessels of the inoculated tomato plants (Figure 3B). Pathogenicity was restored upon complementation of the *VdSge1* deletion strains with a genomic construct containing the wild-type *VdSge1* locus (Supplemental Figure 4). Thus, it can be concluded that *VdSge1* is required for pathogenicity on tomato.

VdSge1 differentially regulates expression of effector genes

Whereas the *H. capsulatum* and *C. albicans* transcriptional regulators Ryp1 and Wor1 were previously demonstrated to regulate expression of phase-specific genes, *F. oxysporum* Sge1 was found to regulate the expression of small *in planta* secreted Six effector proteins during infection of the host. We similarly wanted to test whether VdSge1 regulates the expression of effector genes in *V. dahliae*, using *Ave1* as target (de Jonge et al., 2012). Since the *VdSge1* deletion mutant is not able to infect and colonize tomato plants, assessment of *Ave1* gene expression during plant infection is impossible. Therefore, we tested whether the incubation of *V. dahliae* in a tomato cell suspension culture would induce *Ave1* expression.

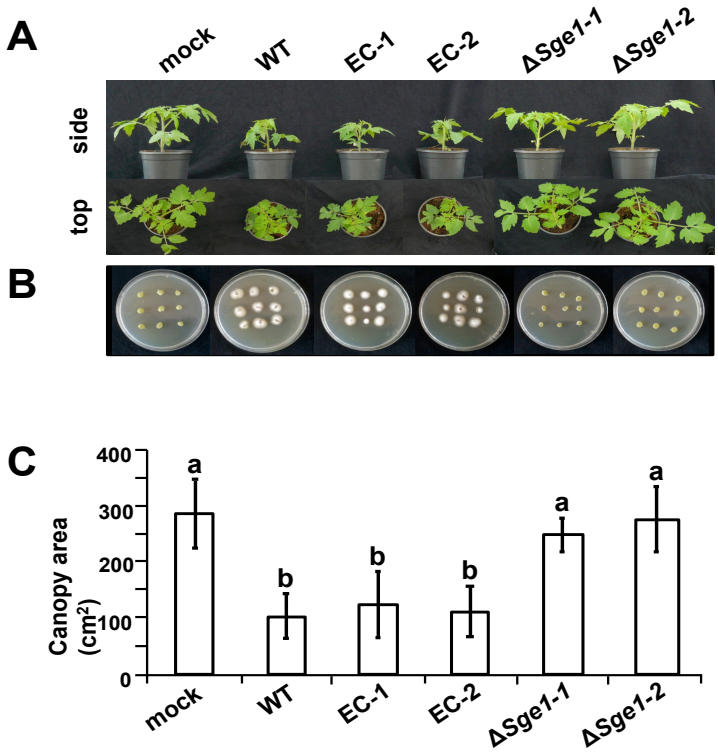


Figure 3. *VdSge1* is required for pathogenicity of *Verticillium dahliae* on tomato. **A)** Side and top view of tomato cultivar MoneyMaker plants inoculated with wild type *Verticillium dahliae* (WT), two *VdSge1* deletion strains (Δ*Sge1-1* and Δ*Sge1-2*) and two ectopic transformants (EC-1 and EC-2), or mock-inoculated at 21 days post inoculation. **B)** Fungal outgrowth at 7 days after plating of stem sections harvested at 21 days post inoculation. **C)** Average canopy area of 6 plants 21 days after inoculation with *V. dahliae* genotypes described above or mock-inoculation. Different letters indicate significant differences at $P<0.05$ as calculated with Student's t test.

A suspension of MSK8 tomato cells was inoculated with *V. dahliae* conidia, and after 96 h the cells were harvested. Firstly, expression of *VdSge1* was assessed in wild type *V. dahliae*, two *VdSge1* deletion lines, and two complementation lines. Real-time PCR analysis confirmed that *VdSge1* is induced in a suspension of MSK8 tomato cells, and no expression was observed in *VdSge1* deletion lines while expression was restored in the complementation lines (Figure 4A). Next, expression of the *Ave1* gene was determined and compared to transcript levels of *V. dahliae* grown in MS medium.

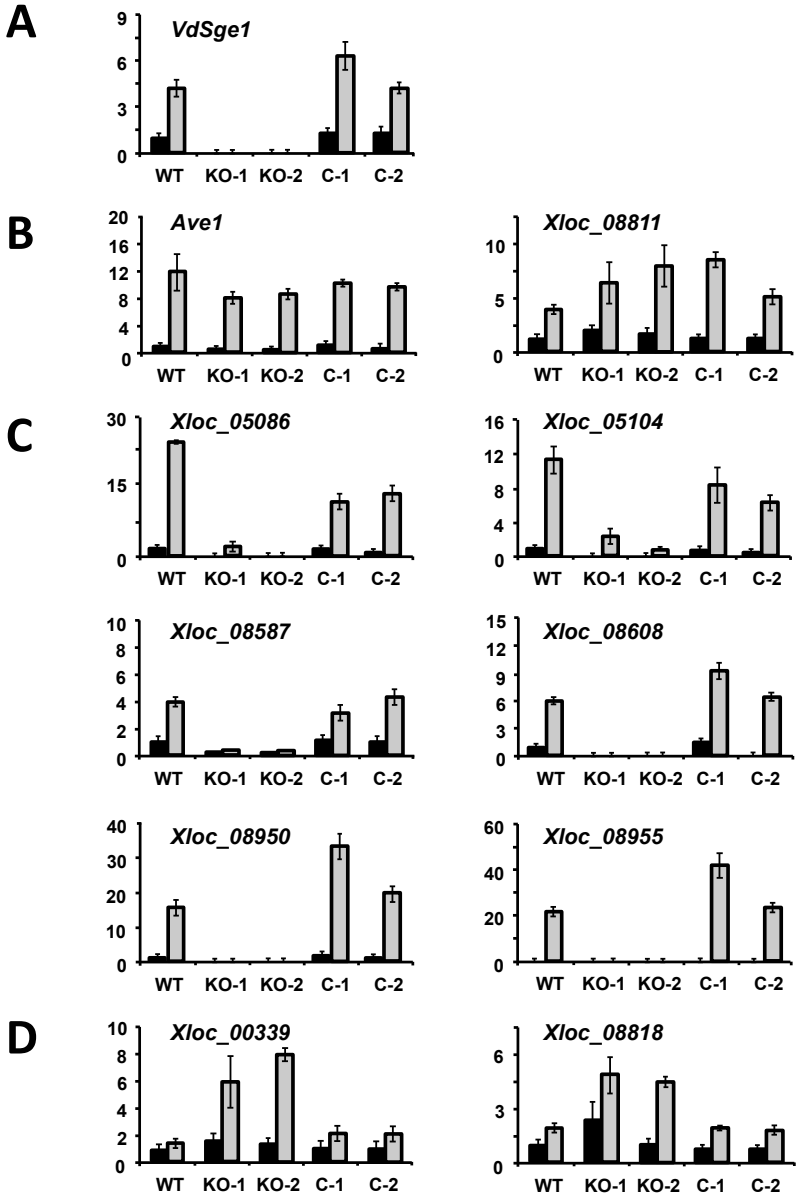


Figure 4. Expression of *VdSge1* and putative *Verticillium dahliae* effector genes in *VdSge1* deletion mutants. Real time PCR was used to measure gene expression levels in wild type *V. dahliae* (WT), two *VdSge1* deletion mutants (KO-1 and -2) and two complementation strains (C-1 and -2) were grown for 4 days in MS medium (black bars) and a tomato cell suspension (MSK8; grey bars) using the elongation factor 1-alpha gene as a reference. Expression in wild type *V. dahliae* in MS medium was set to one, and bars represents averages with stranded deviation of two biological experiments.

As expected, *Ave1* expression was strongly induced upon incubation of the fungus with MSK8 cells (Figure 4B). Surprisingly, although the expression level did not reach the level obtained in the wild type, *Ave1* expression was still also strongly (8- to 10-fold) induced in the *VdSge1* deletion lines, demonstrating that *VdSge1* is not required for *Ave1* induction *in planta*.

To further investigate the role of *VdSge1* in the control of *in planta* expression of effector genes, we evaluated the role of *VdSge1* in the expression of the nine *V. dahliae* genes that, together with *Ave1*, belong to the most highly induced putative effector genes *in planta* (de Jonge et al. 2012; Faino et al. 2012). In addition to *Ave1*, VdSge1 was also not required for induction of *Xloc_08811* (Figure 4B). In contrast, *VdSge1* appeared to be required for the expression of 6 putative effector genes of which the expression was (nearly) completely abolished in the two *VdSge1* deletion lines, while it was restored in the complementation lines (Figure 4C). Interestingly, two of the effectors genes appeared to be negatively regulated by *VdSge1*, as induction was enhanced in the two *VdSge1* deletion lines, while it was restored in the complementation lines (Figure 4D). These data strongly suggest that *VdSge1* differentially regulates effector gene expression in *V. dahliae*.

DISCUSSION

Research on pathogen effectors that are secreted during colonization has dominated research in the plant-microbe interactions over the recent years, as it has been recognized that effectors are crucial for the establishment of microbial infections (Stassen and van der Ackerveken, 2011; de Jonge et al., 2011; Lindeberg et al., 2012). Effectors are typically not produced *in vitro*, but their production is specifically induced upon host colonization. The identification of environmental cues that trigger effector gene expression, and the identification of corresponding microbial regulators of effector gene expression, remains a major goal in phytopathological research, as it may lead to novel strategies to combat microbial infections.

Since some effector genes are induced by nitrogen starvation *in vitro*, nitrogen limitation was proposed as an *in planta* trigger of their induction (van den Ackerveken et al., 1993). However, since nitrogen availability may not be limited *in*

planta, and many effector genes are not induced by nitrogen deprivation, nitrogen starvation cannot be considered as a general trigger for effector gene induction *in planta*, and thus nitrogen response factors likely are not general regulators of effector gene expression (Thomma et al., 2006; Bolton and Thomma, 2008). More recently, the transcriptional regulator Sge1 was identified in the root invading tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*, and found to be localized in the nucleus, required for pathogenicity, and required for the *in planta* expression of various *SIX* effector genes during conditions that mimic *in planta* growth (Michielse et al. 2009). Several of these effector genes were demonstrated to contribute to *F. oxysporum* virulence (Rep et al., 2005; Houterman et al., 2009; Takken and Rep, 2010; Thatcher et al., 2012). Interestingly, Sge1 orthologs occur widely in fungi, and presently two homologs from other plant pathogens have similarly been characterized as regulators of pathogenicity; Fgp1 from the related pathogen *F. graminearum*, and BcReg1 from *Botrytis cinerea* (Michielse et al., 2011; Jonkers et al. 2012). However, a role on effector gene induction in these pathogens is unclear, as no effector proteins have been found to date that are required for infection by either of these pathogens (Amselm et al., 2011; Cuomo et al., 2007). Rather, these studies focused on the role of the Sge1 homologs in the production of secondary metabolites that play a crucial role in pathogenicity (Michielse et al., 2011; Jonkers et al. 2012).

Similar to *Fusarium oxysporum* f. sp. *lycopersici*, the ascomycete fungus *Verticillium dahliae* causes vascular wilt disease in tomato, and both pathogens share important traits of their life styles (Klosterman *et al*. 2011). Although for *F. oxysporum* f. sp. *lycopersici* a considerable number of SIX effector proteins have been identified, until recently no *V. dahliae* effectors were characterized and no homologs of the SIX effectors were found (Klosterman et al. 2011). Recently, the *V. dahliae* effector that is recognized by the Ve1 immune receptor of tomato, and that contributes to fungal virulence on susceptible plant genotypes, was identified as Ave1 (de Jonge et al., 2012). Interestingly, Ave1 homologs were identified in a number of fungal pathogens, including *F. oxysporum* f. sp. *lycopersici* (de Jonge et al., 2012). Surprisingly, we found that induction of *V. dahliae Ave1* under conditions that mimic *in planta* growth is not regulated by Sge1, despite the observation that *VdSge1* deletion strains are not pathogenic on tomato. To further assess the role of *VdSge1* on effector gene induction, we evaluated the induction of additional effector gene candidates under conditions that mimic *in planta* growth. These candidates are derived from a comparative population genomics study in *V. dahliae*, and have been selected based on patterns of selection pressure (R. de Jonge and B.P.H.J. Thomma, unpublished data) and their strong induction *in planta* (de Jonge et al., 2012). Although another effector candidate was identified of which the induction, like that of *Ave1*, is not regulated by VdSge1, induction of six other candidate effector genes was

abolished in the *VdSge1* deletion strain. These data indicate a role of VdSge1 as a positive regulator of at least a subset of candidate effector genes *in planta*, some of which were recently demonstrated to act as genuine virulence factors (R. de Jonge, G.C.M. van den Berg and B.P.H.J. Thomma, unpublished data). Remarkably, however, deletion of *VdSge1* resulted in enhanced induction of two candidate effector genes, suggesting that VdSge1 may also act as a negative regulator of particular effector genes *in planta*. Thus, we conclude that VdSge1 differentially regulates *V. dahliae* candidate effector genes.

The question whether the differential effect of *VdSge1* deletion on candidate effector gene regulation is responsible for the lack of pathogenicity in the *VdSge1* deletion strains presently remains unanswered. Not only was significantly reduced vegetative growth observed for the *VdSge1* deletion strains *in vitro*, also reduced conidiospore production was monitored under these conditions. Interestingly, whereas reduced spore production is found upon the deletion of the *Sge1* homologs in other plant pathogenic species as well, effects on vegetative growth have not been observed previously (Michielse et al., 2009; 2011; Jonkers et al. 2012). Nevertheless, the effects of *VdSge1* deletion on vegetative growth as well as on conidiospore production may significantly affect pathogenicity, as the production and release of conidiospores is a crucial step in the colonization of xylem vessels of the host (Fradin and Thomma, 2006). Furthermore, as previously mentioned, deletion of the *Sge1* homologs was found to affect secondary metabolite production in the other plant pathogenic species (Michielse et al., 2009; 2011; Jonkers et al. 2012). The role of mycotoxins and other secondary metabolites in pathogenicity is particularly well documented for *F. graminearum* (Maier et al., 2006), but also for *B. cinerea* a role of such products in pathogenicity has been established (van Kan, 2006). Although the role of secondary metabolites in pathogenicity of the vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici* and *V. dahliae* is less clear, a significant effect on pathogenicity cannot be excluded. Moreover, although we have not characterized changes in metabolite profiles upon targeted deletion of *VdSge1*, the significant change in pigmentation of the mycelium from white to brown and the secretion of brown pigment into the medium upon *VdSge1* deletion implies an altered metabolite production profile.

In summary, we conclude that *VdSge1* is crucial for pathogenicity of *V. dahliae*, and affects vegetative growth, conidiospore production as well as secondary metabolite production. Furthermore, since *VdSge1* differentially regulates the expression of a collection of candidate effector genes, it is unlikely the central regulator of *V. dahliae* effector gene expression *in planta*.

MATERIALS AND METHODS

Phylogenetic analyses

Phylogenetic analyses of VdSge1 with homologs from other fungal species were conducted using the Phylogeny.fr web-service. Sequences were aligned with MUSCLE, curated with Gblocks and the phylogenetic tree was reconstructed using the *PhyML* program (maximum likelihood method). Statistical reliability of the tree was tested using bootstrap with 500 replications.

Fungal transformations

To generate a *VdSge1* deletion construct, sequences flanking the *VdSge1* coding sequence were amplified from the genomic DNA of *V. dahliae* strain JR2 using the primers KO-VdSge1-LF with KO-VdSge1-LR to amplify the left border, and KO-VdSge1-RF with KO-VdSge1-RR to amplify the right border (Supplemental Table 1). The resulting amplicons were cloned into pRF-HU2 as described previously (Frandsen et al 2008).
To generate a *VdSge1* complementation construct, a 2699 bp *EcoR*I/*Pac*I fragment containing the *VdSge1* coding sequence with 1100 bp upstream and 66 bp downstream sequence was amplified from *V. dahliae* strain JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al 2008).
A. tumefaciens-mediated transformation of *V. dahliae* was performed as described previously (Santhanam 2012), and transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime (Duchefa, Haarlem, The Netherlands), 50 µg/mL of hygromycin (Duchefa, Haarlem, The Netherlands), or 50 µg/mL of nourseothricin (Werner Bioagents, Jena, Germany). Homologous recombination was verified by PCR. Complementation transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime and 100 µg/mL phleomycin (InvivoGen, San Diego, USA).

Pathogenicity assays

Pathogenicity assays were performed on 10-day-old tomato (cv. MoneyMaker) seedlings using root dip inoculation as described previously (Fradin et al., 2009). Disease symptoms were scored up to 14 dpi, pictures were taken, and ImageJ was used to determine the canopy area. To determine *in planta* colonization, stem sections at the height of the first internode were taken, surface sterilized, sliced, placed on PDA supplemented with 50 µg/mL of chloramphenicol, and incubated at 22°C.

Fungal outgrowth was monitored after seven days. All pathogenicity assays were performed three times independently with six plants for each genotype, and plate assays were performed for two biological experiments in triplicate.

Growth, conidiogenesis and germination assays

Radial growth was monitored by placing a 2 µL droplet of a conidial suspension of 10^6 conidia/mL in the center of a PDA or Czapek-dox agar plate, incubated at 22°C and measuring the colony diameter after 10 days. For quantification of conidia production, agar plugs were taken from the fungal colony using a cork borer, shaken in water, and conidia were counted in a haemocytometer. Two biological experiments were performed in triplicate for each genotype. To determine the germination rate, 10^6 conidia/mL were inoculated in Czapek-dox medium at 22°C for 24 h.

Gene expression analysis

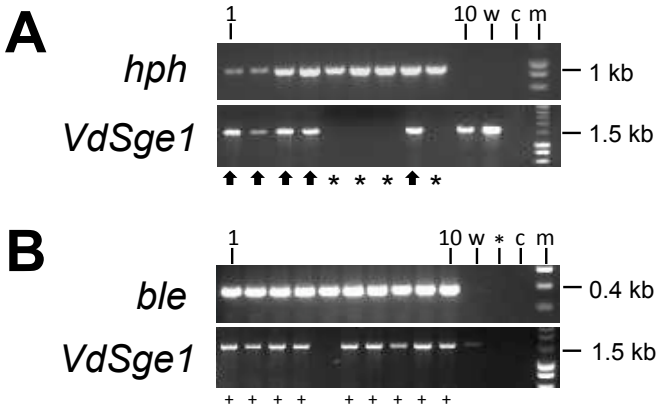
To assess *V. dahliae* gene expression conidia were inoculated into 30 mL of MS medium supplemented with vitamins, 3% sucrose, 1mg/L 2,4-D and 0.1 mg/L kinetin or in 30 mL of a five times diluted five-day-old tomato cell culture (MSK8), such that the final concentration was 10^7 conidia/mL. After 4 days of incubation at 25°C, cultures were harvested by centrifugation and freeze-dried. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands), including on-column DNase treatment, following the manufacturer's instructions. Two µg of total RNA was used for reverse-transcription PCR with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. The resulting cDNA was diluted 10-fold and used as template for expression profiling of *Ave1* and nine other highly *in planta* expressed putative effector genes. Primers used for expression profiling are listed in Supplemental Table 1.

To determine the *in planta* expression of *VdSge1,* ten-day-old tomato seedlings were root inoculated with *V. dahliae*. Whole plants were harvested at 4, 8, 12, 16 dpi and flash-frozen in liquid nitrogen. After grinding, 100 mg of ground material was used for total RNA extraction (Qiagen, Venlo, The Netherlands) and cDNA synthesis (Invitrogen, Carlsbad, USA). Real-time PCR was performed to determine *VdSge1* expression. *VdGAPDH* was used as an endogenous control and reactions were performed in triplicate. Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

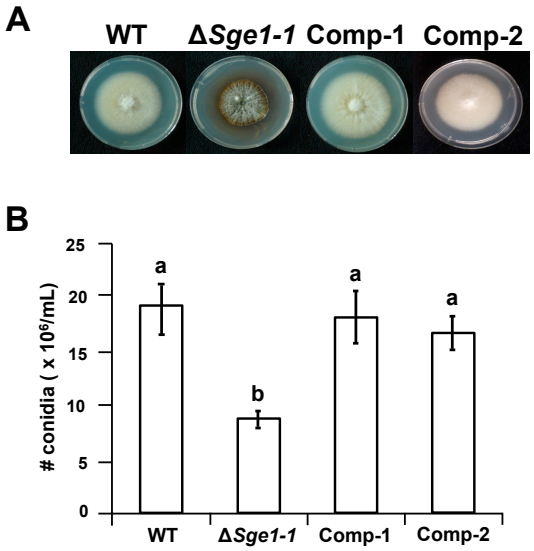
ACKNOWLEDGEMENTS

B.P.H.J.T. is supported by a Vidi grant of the Research Council for Earth and Life sciences (ALW) of the Netherlands Organization for Scientific Research (NWO) and by the Centre for BioSystems Genomics (CBSG) which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research. The authors thank Md. Tohidul Islam for technical assistance and Bert Essenstam and Henk Smid for excellent plant care.

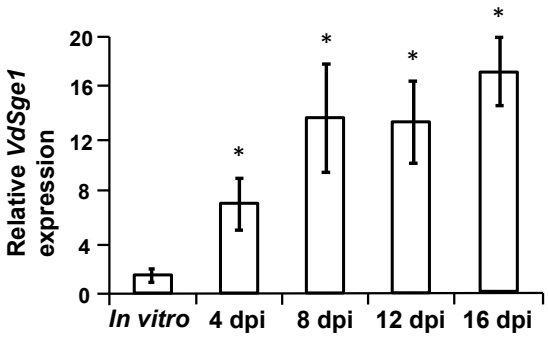
SUPPLEMENTAL FIGURES



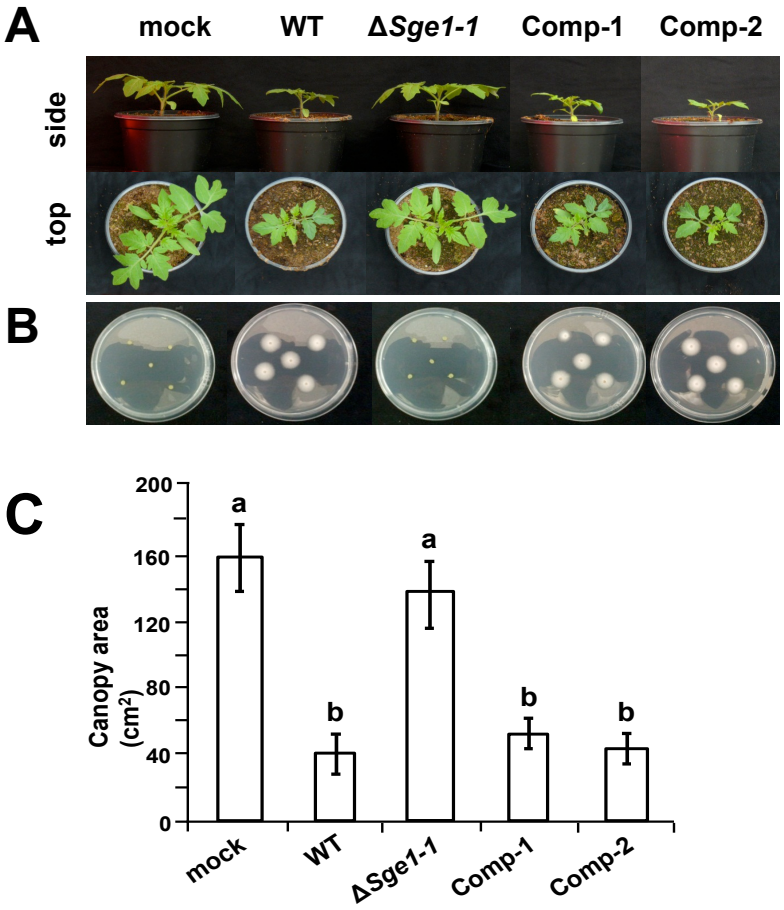
Supplemental Figure 1. Verification of *VdSge1* deletion and complementation strains by PCR. **A)** Amplification of the hygromycin resistance gene (*hph*) and *VdSge1*from wild type *Verticilium dahliae* (w), 10 independent knock-out transformants (lanes 1 to 10), and water control (c) to identify *VdSge1* deletion strains. The marker is indicated (m). True deletion strains are marked with asterisks, while ectopic transformants are marked with arrows. **B)** Amplification of the zeocin resistance gene (*ble*) and *VdSge1* from wild type *V. dahliae* (w), a *VdSge1* deletion strain Δ*Sge1-1* (*), 10 independent complementation strains (lanes 1 to 10), and water control (c). The marker is indicated (m), and true complementation strains are marked (+).



Supplemental Figure 2. Complementation of *VdSge1* deletion strain restores colony morphology and conidiospore production**. A)** Colony morphology of wild type *Verticillium dahliae* (WT), a *VdSge1* deletion strain (Δ*Sge1-1*) and two complementation strains (Comp-1 and Comp-2) after 7 days of incubation on PDA medium at 22°C. **B)** The average number of conidia produced after 7 days of growth on PDA medium based on two independent experiments. Different letters indicate significant differences at P<0.05 as calculated with Student's t test.



Supplemental Figure 3. *VdSge1* is induced during infection of *Verticillium dahliae* on tomato. Ten-day-old tomato cultivar MoneyMaker seedlings were root inoculated with wild type *V. dahliae* and plants were harvested at 4, 8, 12 and 16 days post inoculation (dpi). After RNA isolation and cDNA synthesis, real-time PCR was performed to determine the relative expression level of *VdSge1* using the elongation factor 1-alpha gene as a reference. Expression of *VdSge1 in vitro* (Czapek-dox broth) as set to one. Asterisks indicate significant differences compared to *in vitro* at P<0.05 as calculated with Student's t test.



Supplemental Figure 4. Complementation of *VdSge1* deletion strain restores pathogenicity on tomato. **A)** Side and top view of tomato cultivar MoneyMaker plants inoculated with wild type *Verticillium dahliae* (WT), a *VdSge1* deletion strain (Δ*Sge1-1*) and two complementation strains (comp-1 and comp-2), or mock-inoculated at 14 days post inoculation. **B)** Fungal outgrowth at 7 days after plating of stem sections harvested at 14 days post inoculation. **C)** Average canopy area of 6 plants 14 days after inoculation with *V. dahliae* genotypes described above or mock-inoculation. Different letters indicate significant differences at $P<0.05$ as calculated with Student's t test.

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Chapter 4

Evidence for Functional Diversification within a Fungal NEP1-Like Protein Family

Published as:
Santhanam, P., van Esse, H. P., Küfner, I., Luigi Faino, L., Nürnberger. T., and Thomma, B. P. H. J. 2012. Evidence for functional diversification within a fungal NEP1-like protein family. Mol. Plant-Microbe Interact. 26:278-286.

ABSTRACT

In this study, we functionally analyzed the gene family encoding necrosis- and ethylene-inducing-like proteins (NLPs) of the vascular wilt pathogen *Verticillium dahliae*. We show that the composition of the *NLP* gene family varies little among *V. dahliae* isolates. The cytotoxic activity of NLP family members of a tomato pathogenic *V. dahliae* strain was determined, demonstrating that only two of the seven NLPs induced plant cell death. The genes encoding these cytotoxic NLPs were found to be induced in *V. dahliae* upon colonization of tomato. Interestingly, targeted deletion of either of the two genes in *V. dahliae* significantly compromised virulence on tomato as well as on Arabidopsis plants, whereas deletion of only one of the two genes affected virulence on *N. benthamiana*. This could be attributed to differential induction of the two *NLP* genes in *V. dahliae* upon *N. benthamiana* colonization, revealing that the *in planta* induction of *NLP* genes varies between plant hosts. Intriguingly, one of the *NLP* genes appears to also affect vegetative growth and conidiospore production, as the corresponding deletion strain produced significantly less conidiospores and developed extensive aerial mycelium. In conclusion, we demonstrate that the expanded *V. dahliae NLP* family shows functional diversification, not only revealing differential cytotoxicity between family members, but also that the cytotoxic NLPs play a role in vegetative growth and asexual reproduction in addition to their contribution to virulence.

INTRODUCTION

Microbial pathogens employ secreted effectors to modulate the physiology of their intended host, often through deregulation of host defenses, in order to establish disease (de Jonge et al., 2011). While particular effectors remain extracellular, others are able to enter the host cell cytoplasm (Dodds and Rathjen, 2010). Typically, effectors are species-specific molecules that are often small, cysteine-rich peptides with no homology to previously characterized proteins. However, some effectors seem to be conserved across species (Thomma et al., 2011). An example of such conserved effectors is provided by the fungal LysM effectors, members of which enhance pathogen virulence by suppression of chitin-triggered immunity in a number of plant pathogens (Bolton et al., 2008; de Jonge et al., 2010; Kombrink et al., 2011; Marshall et al., 2011; Mentlak et al., 2012). Another group of conserved effectors are the necrosis- and ethylene-inducing 1 (NEP1)-like proteins (NLPs; Gijzen and Nürnberger, 2006).

The first member of the NLP family has been identified as necrosis- and ethylene-inducing protein (NEP1) found in culture filtrates of the fungus *Fusarium oxysporum* (Bailey, 1995). However, it is presently recognized that NLPs are widely distributed, as they have been identified in many pathogenic bacteria, fungi and oomycetes, and especially in this latter group of organisms the NLP family is significantly expanded (Gijzen and Nürnberger, 2006; Dong et al., 2012; Pemberton and Salmond, 2004). NLPs typically share a conserved NPP1 domain, containing a heptapeptide "GHRHDWE" motif (Fellbrich et al. 2002). NLPs generally induce immune responses and cell death in dicotyledonous plants, and based on crystal structure analysis and mutagenesis it has been proposed that NLPs can function as cytolytic toxins that induce plasma membrane leakage, thus causing cytotoxicity (Qutob et al., 2006; Ottmann et al., 2009). Several reports have shown that NLPs contribute to pathogen virulence (Mattinen et al., 2004; Pemberton et al., 2005), although targeted deletion of *NLP* genes in other species did not appear to affect pathogen virulence (Motteram et al., 2009; Staats et al., 2007). Thus, likely, NLPs play different roles in different pathogens and on different hosts.

Verticillium dahliae is a soil-borne vascular wilt fungus that is characterized by a broad host range of over 200 plant species (Fradin and Thomma, 2006). Little is known about *V. dahliae* pathogenicity, and until recently no effectors of this fungus had been characterized. Recently, the genome sequence of a *V. dahliae* strain has been determined (Klosterman et al., 2011), and subsequently the race 1-specific effector that is recognized by the Ve1 immune receptor of tomato was identified through comparative population genomics of race 1 and race 2 strains (de Jonge et al., 2012).

This effector, named Ave1, is characterized as a small, secreted protein that is required for full virulence on tomato plants lacking the *Ve1* resistance gene (de Jonge et al. 2012). Interestingly, Ave1 is homologous to a widespread family of plant natriuretic peptides, mobile signalling molecules that are secreted in the apoplast, particularly under conditions of biotic and abiotic stress, and play an important role in the regulation of water and ion homeostasis and consequently can affect many downstream processes, including photosynthesis (Gehring and Irvin, 2003; Ruzvidzo et al., 2011). Ave1 homologs are found in a handful of plant pathogenic fungi, but a role in virulence has not yet been demonstrated for these homologs (de Jonge et al., 2012).

Query of the *V. dahliae* genome revealed that 127 (conserved) hypothetical proteins can be designated as small, cysteine-rich candidate effector proteins. However, no orthologs were found of the well-characterized SIX effectors that have been identified in *Fusarium oxysporum* f. sp. *lycopersici*, similar to *V. dahliae* also a vascular wilt pathogen of tomato (Takken and Rep, 2010). Orthologs were only found of effectors that have previously been assigned to large superfamilies, such as the Hce2 (homologs of *C. fulvum* effector Ecp2) family, the LysM effector family (homologs of *C. fulvum* effector Ecp6), and the NLP family (de Jonge and Thomma, 2009; de Jonge et al., 2010; Klosterman et al., 2011; Stergiopoulos et al., 2012).

Already years ago, the first *V. dahliae* NLP, named VdNEP, was identified based on sequencing of expressed sequence tags (ESTs) from cultured mycelium of a cotton-pathogenic *V. dahliae* strain (Wang et al., 2004). The protein was found to induce typical defense responses, including cell death, in various plant species. Although a contribution to fungal aggressiveness was not demonstrated, it was suggested that VdNEP acts a wilt-inducing virulence factor (Wang et al., 2004). Interestingly, compared with other ascomycete plant pathogens, the *NLP* gene family is expanded in the *V. dahliae* genome (Klosterman et al., 2011). While most fungal genomes contain up to three *NLP* genes (Motteram et al., 2009; Staats et al., 2007; Schouten et al., 2008; Dallal et al., 2010; Garcia et al., 2007), *V. dahliae* was found to have eight *NLP* gene homologs (Klosterman et al., 2011). It has been speculated that the expansion of the NLP family, similarly reported for the genome of *Fusarium oxysporum*, may contribute to the broad host range among dicotyledonous plant hosts (Ma et al., 2010; Klosterman et al., 2011). In this manuscript, we describe our study of the *NLP* family of *V. dahliae*. We show that not all of the *V. dahliae* NLPs display cytotoxic activity, and investigate the role of the cytotoxic NLPs in fungal virulence. Finally, we provide evidence for functional diversification not only with the *V. dahliae* NLP family, but even between the cytotoxic NLPs.

RESULTS

Identification of the NLP family in tomato pathogenic strain JR2

Recently the *NLP* family of the defoliating cotton pathogenic isolate V592 was reported to contain nine members (Zhou et al., 2012), while it was previously reported that the genome of the lettuce pathogenic *V. dahliae* strain VdLs.17 contains eight *NLP* gene homologs (Klosterman et al., 2011). The difference in *NLP* gene family size is due to the fact that Zhou et al. (2012) annotate gene VDAG_02984 as an *NLP* family member, whereas this member was not annotated as an *NLP* gene by Klosterman et al. (2011). To re-evaluate the composition of the *NLP* family, an HMMER alignment search was performed on the proteome of the *V. dahliae* strain VdLs.17 with the HMM profile of the NLP-specific NPP1 domain (PF05630). Manual selection resulted in the identification of eight NLP family members, NLP1 to NLP7, and NLP9 (Table 1; Supplemental Figures 1 and 2). A negative bit score for NLP8 disqualified this protein as a bona fide NLP family member due to insufficient support for the validity of the NPP1 domain, although the E-value suggests that the sequence is significantly related (Table 1). The degree of homology between the eight NLP members of *V. dahliae* strain VdLs.17 is rather low (max. 46.6% identity), and four of the NLPs (NLP1, NLP2, NLP3 and NLP6) belong to type I that contains two conserved Cys residues, whereas the remaining four (NLP4, NLP5, NLP7 and NLP9) belong to type II that contains four conserved Cys residues (Supplemental Figures 1 and 2). Only NLP1 to NLP5 have an intact heptapeptide "GHRHDWE" motif, whereas in the remaining NLPs the first amino acid of this motif is changed from Gly to Ala (NLP6 and NLP7) or Asn (NLP9), and the 6th amino acid is changed from Trp into Phe (NLP6) (Supplemental Figure 1).

We subsequently queried the genome sequence of the tomato pathogenic strain JR2 (de Jonge et al., 2012) for its *NLP* gene homologs. This analysis revealed seven *NLP* genes, designated *NLP1* to *NLP5*, *NLP7*, and *NLP9* (Table 1), while a homolog of *NLP6* that is identified in strain VdLs.17 is not present in strain JR2. To further investigate the distribution of *NLP* gene family members in the *Verticillium* genus, we queried the genomes of nine additional *V. dahliae* strains (de Jonge et al., 2012), as well as a *V. tricorpus* strain and of *V. albo-atrum* strain VaMs.102 (Klosterman et al., 2011). To this end, the DNA sequences of the *V. dahliae* strains were mapped to the *V. dahliae* strain VdLs.17 reference genome. The mapping analysis showed that the genomic regions harboring *NLP1* to *NLP5* and *NLP7* to *NLP9* are covered by DNA sequence from the other *V. dahliae* strains, indicating the presence of these genes. However, similar to the JR2 strain, the genomic region

Table 1. Composition of the *NLP* gene family in *V. dahliae*

				V. dahliae											*V. albo-atrum*	*V. tricorpus*
Gene number[a]	Alias[b]	Bit score[c]	E-Value[c]	VdLs.17[d]	V592[e]	JR2[f]	CBS381.66[g]	DVD-3[h]	St14.01[i]	DVD-S94[j]	DVD-S26[k]	DVD-S29[l]	DVD-31[m]	DVD-161[n]	VaMs.102[o]	Mucl9792[p]
VDAG_04701	NLP1	23.9	$9.70E^{-11}$	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_01995	NLP2	338.9	$3.80E^{-98}$	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_06993	NLP3	382.4	$2.90E^{-111}$	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_04550	NLP4	161.4	$9.80E^{-45}$	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_09117	NLP5	107.1	$2.30E^{-28}$	+	+	+	+	+	+	+	+	+	+	+	+	-
VDAG_04834	NLP6	52.7	$1.20E^{-12}$	+	+	-	-	-	-	-	-	-	-	-	-	-
VDAG_03497	NLP7	113.1	$3.30E^{-30}$	+	+	+	+	+	+	+	+	+	+	+	+	+
VDAG_02984	*NLP8*	*-9*	*$1.40E^{-08}$*	+	+	+	+	+	+	+	+	+	+	+	-	+
VDAG_08022	NLP9	64.9	$1.10E^{-15}$	+	+	+	+	+	+	+	+	+	+	+	+	-

[a]Gene numbers according to the reference sequence of *V. dahliae* strain VdLs.17 (Klosterman et al., 2011)
[b]Naming according to Zhou et al. (2012)
[c]Based on HMMER alignment search on *V. dahliae* VdLs.17 proteome with NLP-specific NPP1 domain (PF05630)
[d]Lettuce pathogenic isolate (Bhat and Subbarao, 1996)
[e]Defoliating cotton pathogenic isolate (Zhou et al., 2012)
[f]Tomato pathogenic isolate (Fradin et al., 2009)
[g]Tomato pathogenic isolate (CBS Utrecht, the Netherlands)
[h]Potato pathogenic isolate
[i]Pistacio pathogenic isolate
[j]Soil isolate
[k]Soil isolate
[l]Soil isolate
[m]Tomato pathogenic isolate
[n]Potato pathogenic isolate
[o]Alfalfa pathogenic isolate (Bhat and Subbarao, 1999)
[p]Tomato pathogenic isolate (Lievens et al., 2003)

harboring *NLP6* was not found in any of the other *V. dahliae* strains. *NLP6* was similarly lacking in *V. albo-atrum* strain VaMs.102 (Klosterman et al., 2011), while also *NLP8* is lacking in this strain (Table 1). Interestingly, the *V. tricorpus* genome contains homologs of *NLP3*, *NLP4*, *NLP7* and *NLP8* only.

Only NLP1 and NLP2 display cytotoxic activity

Various reports have shown that NLPs trigger necrosis upon infiltration into leaves of dicotyledenous plants. To test the necrosis-inducing activity of the NLPs encoded by the tomato pathogenic JR2 strain, expression of the corresponding cDNAs in *N. benthamiana* by agroinfiltration was pursued. To this end, the coding sequences of the *NLP* genes were cloned into the Gateway-compatible vector *pFAST-R02* (Shimada et al. 2010) to generate expression constructs driven by the CaMV 35S promoter, and transformed into *A. tumefaciens* strain GV3101. Subsequently, the transgenic *A. tumefaciens* strains were infiltrated into the leaves of *Nicotiana benthamiana* plants. Leaf tissue started to collapse at one to two days post agroinfiltration of *NLP1* and *NLP2*, and the infiltrated leaves developed clear necrosis by three days post agroinfiltration (Figure 1A). In contrast, no tissue collapse or necrosis could be observed upon agroinfiltration of any of the other *NLP* genes. Western analysis performed on total protein extracts of the agroinfiltrated leaf area confirmed the presence of the proteins that did not induce necrosis *in planta* (Figure 1B).

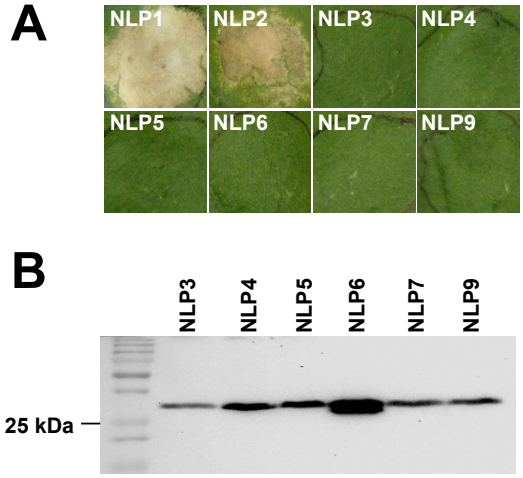


Figure 1. Only expression of *Verticillium dahliae NLP1* and *NLP2* induces cell death in *Nicotiana benthamiana*. (A) Agroinfiltration of constructs for constitutive expression of *Verticillium dahliae NLP* genes reveals necrosis at the injection sites for *NLP1* and *NLP2*, but none of the other *NLP* genes. Pictures were taken at 3 days after agroinfiltration. (B) Western blot analysis on total protein extracts of *N. benthamiana* upon agroinfiltration of constructs for constitutive expression of *Verticillium dahliae NLP* genes that did not induce necrosis in panel A.

To confirm the differential cytotoxic activity, the NLPs were also produced in the yeast *Pichia pastoris* and purified. Whereas production of NLP6 and NLP9 failed, sufficient amounts of protein were obtained from the other NLPs and infiltrated

abaxially into leaves of *Nicotiana tabacum* plants. Also in this experiment, only infiltration of NLP1 and NLP2 resulted in tissue necrosis, while infiltration of NLP3 to NLP5 and NLP7 did not trigger necrosis (Supplemental Figure 3). Collectively, these data show that only NLP1 and NLP2 display cytotoxic activity, while the remaining *V. dahliae* NLPs do not.

NLP1 and *NLP2* are expressed *in planta*, but not under control of VdSge1

It has previously been shown for one of the cytotoxic NLPs from *V. dahliae* that it has the potential to induce necrosis and wilting *in planta*, but a role in virulence on plant hosts has not been demonstrated thus far for any of its cytotoxic NLPs (Wang et al., 2004). To address such role, it was assessed whether both cytotoxic NLPs are expressed during host colonization. To this end, *V. dahliae* strain JR2 was root-inoculated on susceptible plants of the tomato cultivar MoneyMaker, and complete plants were harvested at regular intervals up to 16 days post inoculation (dpi). After RNA isolation and cDNA synthesis, real-time PCR was performed to assess *NLP1* and *NLP2* transcript levels that were calibrated using transcript levels of the elongation factor 1-alpha gene as a reference. This analysis demonstrated that both *NLP1* and *NLP2* are induced *in planta*, with a similar expression pattern that peaks around 12 dpi (Figure 2). By this point in time, major symptoms of disease, including the onset of necrosis and significant stunting, become apparent in susceptible tomato plants.

We recently demonstrated that *V. dahliae Sge1* (*VdSge1*) is required for growth and development as well as for pathogenicity on tomato (Santhanam and Thomma, 2012). In contrast to *F. oxysporum* Sge1, we found that VdSge1 is not a general regulator of effector gene expression, as some candidate effector genes were negatively regulated by *VdSge1*, some positively, and others not (Santhanam and Thomma, 2012). Since the *VdSge1* deletion mutant is not pathogenic on tomato, assessment of *NLP1 and NLP2* gene expression was assessed in a tomato cell suspension culture (Santhanam and Thomma, 2012). To this end, a suspension of MSK8 tomato cells was inoculated with conidia of a *VdSge1* deletion strain and the corresponding wild type, and after 96 h the cells were harvested and examined for *NLP1* and *NLP2* expression with real-time PCR. As expression of *NLP1* and *NLP2* was not abolished in the *VdSge1* deletion strain, we conclude that the expression of the genes encoding cytotoxic NLPs is not governed by VdSge1 (Supplemental Figure 4).

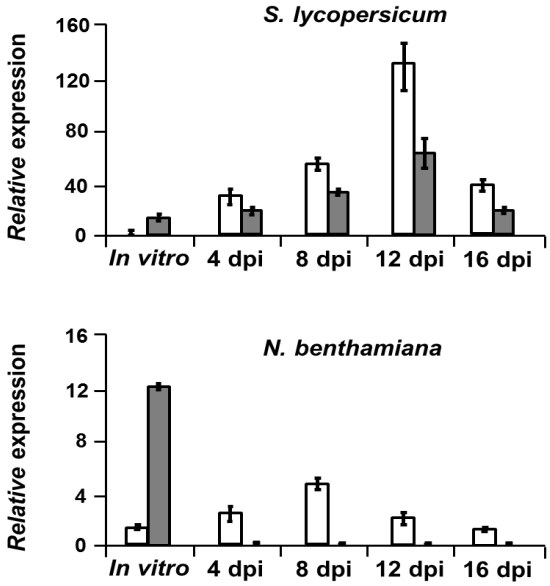


Figure 2. *NLP1* and *NLP2* expression during infection of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. Ten-day-old tomato (*S. lycopersicum*) cultivar MoneyMaker plants (top graph), or 15-day-old *N. benthamiana* plants (bottom graph), were root inoculated with wild type *V. dahliae* and plants were harvested at regular intervals from 4 to 16 days post inoculation (dpi). After RNA isolation and cDNA synthesis, real-time PCR was performed to determine the relative expression levels of *NLP1* (white bars) and *NLP2* (grey bars) using the *V. dahliae* elongation factor 1-alpha gene as a reference, and compared to expression upon *V. dahliae* growth *in vitro* in MS medium. NLP1 and NLP2 expression levels are compared to expression of *NLP1 in vitro*, which is set to 1. No *NLP2* expression was detected in *N. benthamiana*.

NLP1 affects vegetative growth and conidiospore production

Since both *NLP1* and *NLP2* were found to be expressed in *V. dahliae* during colonization of tomato plants, targeted deletion was pursued by replacement of the coding sequence of each of the genes by a hygromycin resistance cassette through homologous recombination. Deletion of the coding sequences was subsequently verified by PCR. Several independent deletion mutants were obtained for each of the genes, of which two were used for further analysis in this study (Supplemental Figure 5). To assess the role of the cytotoxic NLPs on growth and development, various growth characteristics were assessed. Radial growth of the *NLP1* and *NLP2* deletion strains was similar to that of the wild-type strain and of the ectopic transformants (Figure 3A). However, when conidiospores were isolated from one-week-old cultures, *NLP1* deletion strains appeared to have developed approximately 40% less

conidiospores than *NLP2* deletion strains, the wild-type strain and ectopic transformants (Figure 3B). Nevertheless, germination of the conidiospores was similar for all strains, including the *NLP1* deletion strains (Figure 3C). The reduced conidiospore production of the *NLP1* deletion strains was overcome by complementation with a genomic construct containing the wild-type *NLP1* gene, demonstrating that the reduced conidiospore production is a genuine phenotype of *NLP1* deletion (Supplemental Figure 6B). When the fungal cultures got older, *NLP1* deletion strains became morphologically distinct. Whereas the color of the wild-type strain, *NLP2* deletion strains and the ectopic transformants became grey, *NLP1* deletion strains were white (Figure 4A). Closer inspection revealed that the white appearance was due to extensive formation of aerial hyphae that covered the fungal colonies of the *NLP1* deletion strains on the PDA plates (Figure 4B). Also this phenotype was overcome by complementation of the deletion strain with a genomic construct encoding the wild-type *NLP1* gene (Supplemental Figure 6A). All other fungal strains did not show the extensive formation of these aerial hyphae (Supplemental Figure 7). In support for a role in absence of host colonization during growth, we found that NLP1 is expressed *in vitro*, as 228 reads belonging to this *NLP* gene family member were retrieved in the RNAseq data of *V. dahliae* grown in Czapek-dox medium (de Jonge et al., 2012). Collectively, these data support a role for *NLP1* in vegetative growth and asexual reproduction.

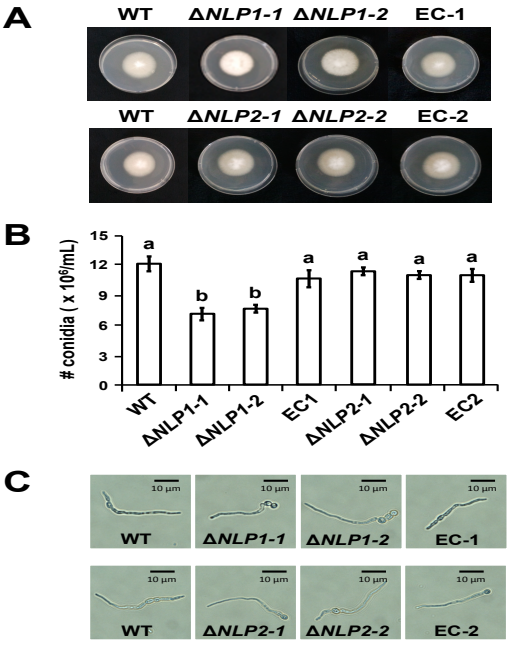


Figure 3. Targeted deletion of *NLP1* affects conidiospore production. A) Radial growth and colony morphology of wild type *Verticillium dahliae* (WT), two *NLP1* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), and ectopic transformants (EC1 and EC2) after 7 days of incubation on PDA medium at 22°C. B) Average number of conidia produced after 7 days of growth on PDA medium based on two independent experiments. Different letters indicate significant differences at $P<0.05$ as calculated with Student's t test. C) Microscopic observation of the germination of conidia at 16 hr after incubation in Czapek-dox medium.

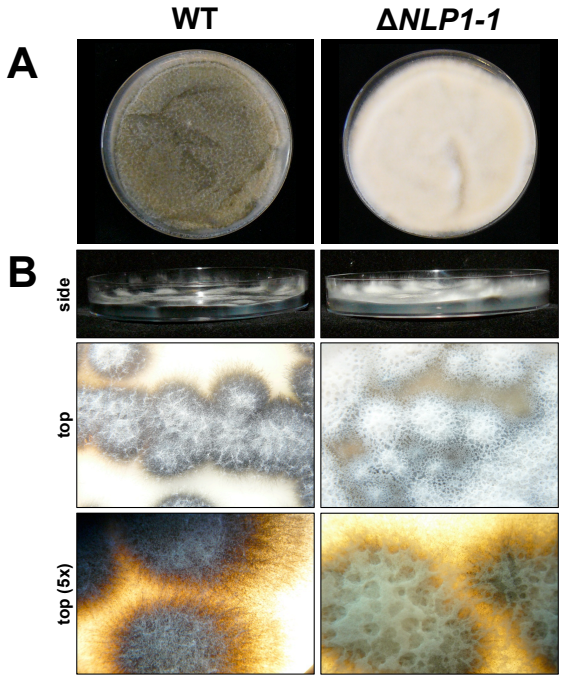


Figure 4. Targeted deletion of *NLP1* induces formation of aerial hyphae. A) Morphological appearance of wild type *Verticillium dahliae* (WT) and an *NLP1* deletion strain (Δ*NLP1-1*) after 14 days of incubation on PDA medium at 22°C. B) Wild type *Verticillium dahliae* (WT), and an *NLP1* deletion strain (Δ*NLP1-1*) after 21 days of incubation on PDA medium at 22°C. Top pictures show side views of petridishes with fungal strains on PDA medium, showing aerial hyphae for the *NLP1* deletion strain. Middle and lower pictures show magnification of fungal colonies.

NLP1 and *NLP2* are required for virulence

To assess the role of *NLP1* and *NLP2* in pathogenicity on tomato plants, the deletion strains were root-inoculated on susceptible MoneyMaker tomato plants along with the wild-type strain and ectopic transformants. Interestingly, whereas ectopic transformants were found to be as virulent as the wild-type strain, *NLP1* as well as *NLP2* deletion strains were found to be significantly less pathogenic (Figure 5). The plants that were inoculated with the *NLP1* and *NLP2* deletion strains still developed *Verticillium* wilt symptoms, but were less stunted and showed less typical disease symptoms (e.g. darkening of the leaves) than plants that were inoculated with ectopic transformants or the wild-type strain (Figure 5). Intriguingly, deletion of *NLP1* seems to have a stronger effect on virulence than deletion of *NLP2*, as plants inoculated with *NLP1* deletion strains remain taller and show fewer symptoms of infection than plants inoculated with *NLP2* deletion strains (Figure 5). This was confirmed upon measuring of the surface area of the foliage of the plants inoculated with the various fungal genotypes, as plants that were inoculated with *NLP1* deletion strains developed as much foliage as mock-inoculated plants, while the amount of foliage developed by plants that were inoculated with *NLP2* deletion strains was significantly reduced (Figure 6A). Nevertheless, plants that were inoculated with *NLP2* deletion strains

developed significantly more foliage than those inoculated with either the wild-type strain or ectopic transformants (Figure 6A). Fungal outgrowth assays upon plating of stem sections harvested from the hypocotyls of the inoculated plants demonstrated that *NLP1* and *NLP2* deletion were still able to colonize tomato plants (Figure 5). However, real-time PCR quantification of fungal biomass demonstrated that *NLP1* and *NLP2* deletion strains developed significantly less fungal biomass *in planta* than the ectopic transformants and the wild-type strain (Figure 6B).

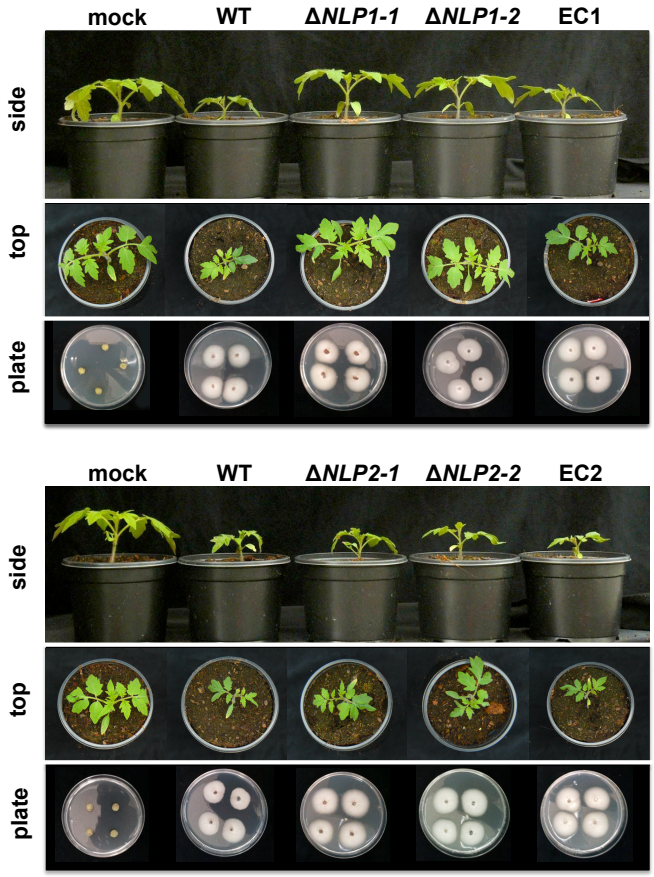


Figure 5. *NLP1* and *NLP2* are required for virulence of *Verticillium dahliae* on tomato**.** Pathogenicity assay to investigate the role of *NLP1* (top panel) and *NLP2* (bottom panel) in *V. dahliae* virulence. Side and top view of tomato cultivar MoneyMaker plants that were mock-inoculated (mock), or inoculated with wild type *Verticillium dahliae* (WT), two *NLP* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), and corresponding ectopic transformants (EC1 and EC2) at 14 days post inoculation are shown. Fungal outgrowth at 7 days after plating of stem sections harvested at 14 days post inoculation are shown at the bottom of each panel.

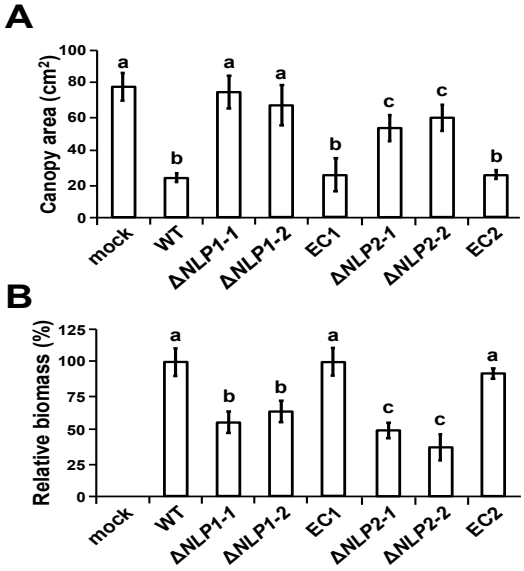


Figure 6. *NLP1* and *NLP2* are required for virulence of *Verticillium dahliae* on tomato**.** A) Average canopy area of 6 tomato cultivar MoneyMaker plants at 14 days after mock-inoculation (mock), or inoculation with wild type *Verticillium dahliae* (WT), two *NLP1* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), and corresponding ectopic transformants (EC1 and EC2). B) Real-time PCR quantification of fungal biomass. Different letter labels indicate significant differences ($P<0.05$).

To investigate whether the observed role of *NLP1* and *NLP2* in pathogenicity is confined only to tomato, or also concerns other host species, we tested the pathogenicity of the *NLP1* and *NLP2* deletion strains on the Brassicaceous and Solanaceous model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*, respectively. Similar to tomato, targeted deletion of *NLP1* as well as of *NLP2* resulted in markedly compromised virulence on Arabidopsis plants, as they were clearly less stunted (Figure 7A). In contrast, a differential contribution of *NLP1* and *NLP2* to *V. dahliae* virulence was monitored on *N. benthamiana*. Whereas targeted deletion of *NLP1* resulted in markedly compromised virulence on *Nicotiana benthamiana*, targeted deletion of *NLP2* did not result in significantly compromised virulence on this host plant (Figure 7B). This was not only confirmed by measurement of the surface area of the foliage of the plants inoculated with the various fungal genotypes, but also by real-time PCR quantification of fungal biomass (Figure 8). Considering the observation that NLP2 displays cytotoxic activity also on *N. benthamiana* (Figure 1), the observation that *NLP2* deletion did not result in significantly compromised virulence on this host plant prompted the question whether this gene is expressed during *N. benthamiana* colonization. Surprisingly, whereas *NLP1* is expressed during colonization of *N. benthamiana* by *V. dahliae* in a similar pattern as observed on tomato (Figure 2), no expression of *NLP2* could be detected at all in this plant species. Nevertheless, overall it can be concluded that *NLP1* and *NLP2* are required for virulence on various host plants.

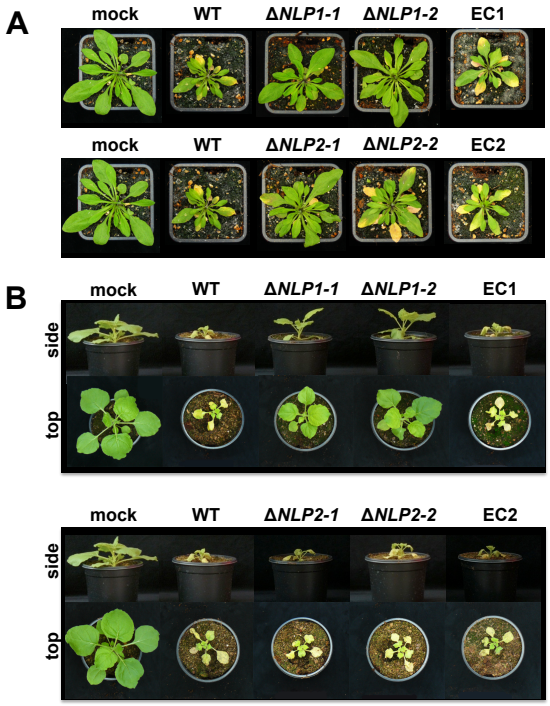


Figure 7. Requirement of *NLP1* and *NLP2* for virulence of *Verticillium dahliae* on Arabidopsis and *Nicotiana benthamiana*. A) Top view of Arabidopsis plants of the Col-0 ecotype, and B) top and side view of *N. benthamiana* plants, that were mock-inoculated (mock), or inoculated with wild type *Verticillium dahliae* (WT), two *NLP1* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), and corresponding ectopic transformants (EC1 and EC2) at 21 dpi (Arabidopsis) and 14 dpi (*N. benthamiana*) are shown.

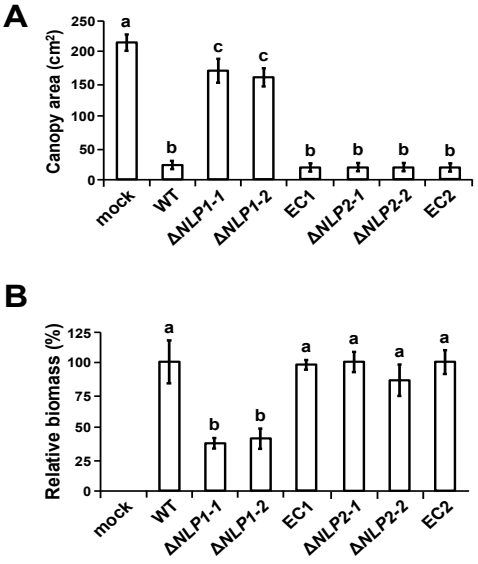


Figure 8. *NLP1*, but not *NLP2*, is required for virulence of *Verticillium dahliae* on *Nicotiana benthamiana*. A) Average canopy area of 6 *N. benthamiana* plants at 14 days after mock-inoculation (mock), or inoculation with wild type *Verticillium dahliae* (WT), two *NLP1* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), and corresponding ectopic transformants (EC1 and EC2). B) Real-time PCR quantification of fungal biomass. Different letter labels indicate significant differences ($P<0.05$).

DISCUSSION

In this study, we provide evidence for significant functional diversification within the NLP family of a fungal pathogen. We have shown that targeted deletion from the *V. dahliae* genome of *NLP1*, encoding a cytotoxic NLP that contributes to virulence on various host plants, directly affects growth and conidiospore production *in vitro*. As this finding was highly unexpected, we carefully checked the veracity of our findings. To generate the *NLP1* deletion construct, genomic sequences flanking the *NLP1* CDS were used, more specifically 1.4 kb upstream and 250 bp downstream sequence. About 2.2 kb upstream of *NLP1* lies a gene encoding a vegetative cell wall protein (VDAG_04700). Although this gene lies well outside the range of sequence that was used to generate the construct for targeted deletion of *NLP1* by homologous recombination, we confirmed that expression of this gene was not affected in the *NLP1* deletion strains when compared with the wild-type *V. dahliae* upon growth *in vitro*. No genes other than *NLP1* were annotated in the region that was used for homologous recombination. Furthermore, mapping of sequence reads obtained with RNAseq from a time course of *V. dahliae* infected *N. benthamiana* resulted in the identification of reads for over 8.000 *V. dahliae* genes (de Jonge et al., 2012), but no reads were mapped in the range of sequence that was used to generate the construct for homologous recombination other than those belonging to *NLP1*. Furthermore, the *in vitro* growth and conidiation phenotype was overcome by complementation of the *NLP1* deletion strain with a genomic construct containing the wild-type *NLP1* gene. And finally, coinciding with a role *in vitro* in absence of the host, our data show that *NLP1* is expressed in *V. dahliae* during growth *in vitro*. Thus, we conclude that *NLP1* unambiguously affects *in vitro* growth and conidiation, and with this observation a novel function for a cytotoxic NLP, outside the host plant, is revealed.

The observed role for a cytotoxic NLP outside the plant and pathogen virulence was unexpected, and obviously leads to the question how such a secreted effector protein affects growth and conidiation. In this respect it is interesting to note that analysis of the crystal structure of a cytotoxic NLP from the phytopathogenic oomycete *Pythium aphanidermatum* not only revealed structural homology to actinoporins, a family of pore-forming toxins produced by sea anemones, but also to particular fungal lectins (Ottmann et al., 2009). All these proteins are small polypeptides containing a central β-sandwich architecture surrounded by helices, and both the actinoporins and lectins are soluble proteins that target cellular surfaces via a surface-exposed cavity (Ottmann et al., 2009; Birck et al., 2004). One of these lectins, XCL from *Xerocomus chrysenteron*, is an insecticidal protein that binds to N-acetyl galactosamine at the cell surface of target cells after which it is internalized and

changes the actin cytoskeleton (Birck et al., 2004). It is tempting to speculate that NLP1 will bind to particular fungal cell wall carbohydrates, after which internalization occurs to influence developmental processes.

Further evidence for functional diversification within the *V. dahliae* NLP family comes from the identification of non-cytotoxic family members. Most fungal genomes contain up to three *NLP* genes (Motteram et al., 2009; Staats et al., 2007; Schouten et al., 2008; Dallal et al., 2010; Garcia et al., 2007). Nevertheless, although the *V. dahliae NLP* family is significantly expanded, also this fungus contains only two genes that encode cytotoxic NLPs. Possibly, the non-cytotoxic NLPs contribute to pathogen virulence through other mechanisms than inducing cellular leakage. However, our data for NLP1 suggest that diversification towards other functions than virulence may be likely as well. Thus far, non-cytolytic NLPs have only been described for oomycete species that are generally characterized by significantly expanded NLP families, containing up to 70 potential *NLP* genes (Dong et al., 2012). Whereas some oomycetes contain cytolytic and non-cytolytic NLPs (Kanneganti et al. 2006; Dong et al., 2012), others contain only non-cytolytic NLPs, such as the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (Cabral et al., 2012). The finding that the non-cytotoxic *H. arabidopsidis* NLPs are expressed during early stages of infection has led to the hypothesis that these NLPs still exert functions related to host colonization (Cabral et al., 2012). However, also for *H. arabidopsidis* NLPs alternative roles that are not related to virulence may occur as well. A role in growth and development outside the host plant may at the same time explain why *NLP* genes also occur in non-pathogenic micro-organisms.

Our study confirms the findings of recent study on a virulent cotton-infecting *V. dahliae* isolate, for which nine potential NLPs were identified of which two displayed cytotoxic activity (Zhou et al., 2012). However, in contrast to our findings, targeted deletion of the genes encoding the cytotoxic NLPs did not affect *V. dahliae* virulence on cotton (Zhou et al., 2012). In our study, we found that targeted deletion of *NLP1* as well as of *NLP2* significantly compromised *V. dahliae* virulence on tomato and Arabidopsis, while deletion of *NLP1*, but not of *NLP2*, compromised virulence on *N. benthamiana*. However, the differential role of *NLP2* we observed in virulence on different hosts could be attributed to the observation that *NLP2* is not expressed during infection on *N. benthamiana*. This is not the case for the study on cotton, because despite the absence of a virulence phenotype for the corresponding deletion strains, *NLP1* as well as *NLP2* were found to be expressed during infection (Zhou et al., 2012). Moreover, also targeted deletion of both genes simultaneously did not affect fungal virulence, suggesting that the role of the cytotoxic NLPs in infection of cotton is dispensable and further supporting the differential contribution of cytotoxic NLPs to virulence of *V. dahliae*. It is not surprising that a broad host range

pathogen such as *V. dahliae* that is able to infect hundreds of hosts will express effectors that do not contribute to virulence on all of its hosts.

In conclusion, our data demonstrate extensive functional diversification within the expanded NLP family of the fungal plant pathogen *V. dahliae*. We have demonstrated differential cytotoxicity among the family members, a differential contribution to virulence of the cytotoxic family members, and finally also a differential effect of the cytotoxic family members outside the host plant on growth and conidiospore production. Future efforts will be devoted to investigate further functional diversification with the group of non-cytotoxic *V. dahliae* NLPs.

MATERIALS AND METHODS

Identification of *Verticillium* NLP family members

The HMM profile of the NPP1 domain (PF05630) was retrieved from the Pfam database (http://pfam.sanger.ac.uk/) and an HMMER alignment search (http://hmmer.janelia.org/) was performed against the *V. dahliae* strain VdLs.17 proteome (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/) using default parameters. The resulting hits were manually selected for positive bit scores and E-values <1e-10. Presence of signal peptides was confirmed using SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) with default parameters. Homologous *NLP* genes were identified in other *V. dahliae* strains by mapping the Illumina DNA sequence reads of these strains (de Jonge et al 2012) with the gsnap/gmap software to the reference genome of *V. dahliae* strain VdLs.17 using default settings (Wu and Watanabe, 2005; Wu and Nacu, 2010). Similarly, *NLP* homologs were identified using Illumina DNA sequence reads of *V. tricorpus* strain MUCL9792 (Faino and Thomma, unpublished data), and reciprocal BLAST searches to the *V. dahliae* VdLs.17 genome were used to confirm the identities of the *NLP* homologs.

Phylogenetic analyses

Phylogenetic analyses of the *V. dahliae* NLP family was conducted using the Phylogeny.fr web-service. Sequences were aligned with MUSCLE and curated with Gblocks. The phylogenetic tree was reconstructed using the PhyML program (maximum likelihood method) and statistical reliability was tested using bootstrap with 500 replications.

Fungal transformations

To generate *NLP1* and *NLP2* deletion constructs, flanking sequences of the *NLP1* and *NLP2* coding sequences were amplified from genomic DNA of *V. dahliae* strain JR2 using the primers mentioned in Supplemental Table 1. The amplified products were cloned into pRF-HU2 as described previously (Frandsen et al 2008).

To generate the *NLP1* complementation construct, a 1871 bp *Eco*RI/*Pac*I fragment containing the *NLP1* coding sequence with 998 bp upstream and 170 bp downstream sequence was amplified from *V. dahliae* strain JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al 2008).

A. tumefaciens-mediated transformation of *V. dahliae* was performed as described previously (Santhanam 2012), and transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime (Duchefa, Haarlem, The Netherlands) and 50 µg/mL of hygromycin (Duchefa, Haarlem, The Netherlands). Homologous recombination was verified by PCR. Complementation transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime and 100 µg/mL phleomycin (InvivoGen, San Diego, USA).

Pathogenicity assays

Pathogenicity assays were performed on ten-day-old tomato seedlings (cv. MoneyMaker), two-week-old *Arabidopsis thaliana* (Col-0) and two-week-old *Nicotiana benthamiana* plants using root dip inoculation as previously described (Fradin et al., 2009). Disease symptoms were scored up to 14 dpi (tomato and *N. benthamiana*) or 21 dpi (Arabidopsis), pictures were taken, and ImageJ was used to determine the canopy area. To determine *in planta* colonization, stem sections at the height of the first internode were taken, surface sterilized, sliced, placed on PDA supplemented with 50 µg/mL of chloramphenicol, and incubated at 22°C. Fungal outgrowth was monitored after seven days. For *in planta* biomass quantification, roots of three inoculated plants were harvested at 14 dpi. The samples were ground to powder and genomic DNA was isolated. Real-time PCR on genomic DNA was carried out using an ABI7300 PCR machine (Applied Biosystems) in combination with qPCR core kit for SYBR Green I (Eurogentec) and analyzed using the 7300 SDS software (Applied Biosystems). *Verticillium* elongation factor 1-alpha was used to quantify fungal colonization. Tomato, Arabidopsis and *N. benthamiana* actin primers were used as endogenous plant control (Supplemental Table 1).

Growth, conidiogenesis and germination assays

Radial growth was monitored by placing a 2 µL droplet of a conidial suspension of 10^6 conidia/mL in the center of a PDA plate and incubated at 22°C. For quantification of conidia production, 5 mL of water was added to the culture and conidial suspension were prepared. Ten times diluted conidial suspension were counted in a haemocytometer. To determine the germination of conidia, 10^6 conidia/mL were inoculated in Czapek-dox medium at 22°C for 16 h and observed under a microscope.

Cytotoxic activity determination

For transient expression of *NLP* genes in *N. benthamiana*, the full length coding sequences containing a 3' FLAG tag were amplified (Supplemental Table 1) and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). After sequence verification the inserts were subcloned in the destination vector pFAST-R02 (Shimanda et al 2010) and transformed to *A. tumefaciens* strain GV3101by electroporation. *A. tumefaciens* strains were subsequently grown in YEB at 28°C overnight. The overnight cultures were diluted to an OD600 of 0.5 with MMA containing 1mL/L of acetosyringone and incubated at room temperature for 3-4 hours. The cultures were infiltrated on abaxial side of the youngest, fully expanded leaves of four-week-old *N. benthamiana*.

To verify protein production, leaves without mid veins were harvested at 3 days after agroinfiltration and flash frozen in liquid nitrogen. Total proteins were extracted using P-PER® Plant Protein Extraction Kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer instructions. The proteins were separated using 15% SDS-PAGE gels. Immunoblotting was performed using ANTI-FLAG M2 antibody and the proteins were visualized by enhanced chemiluminenscence (Pierce Biotechnology, Rockford, IL, USA).

NLP production in *Pichia pastoris* was performed essentially as described previously (Ottmann et al., 2009).

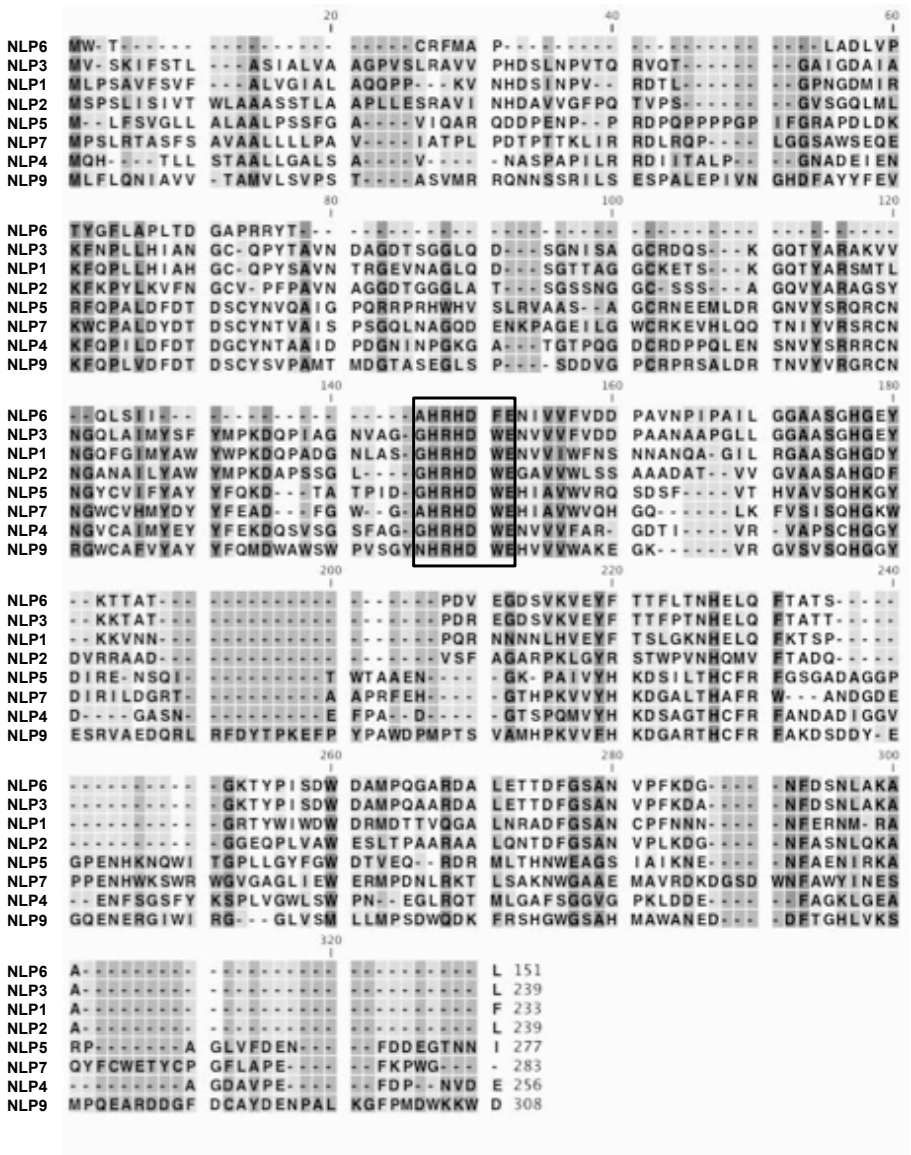
Gene expression analysis

To determine the *in planta* expression of *NLP1* and *NLP2,* ten-day-old tomato seedlings and two-week-old *N. benthamiana* plantlets were root inoculated with *V. dahliae*. Whole plants were harvested at 4, 8, 12, 16 dpi and flash-frozen in liquid nitrogen. After grinding, 100 mg of ground material was used for total RNA

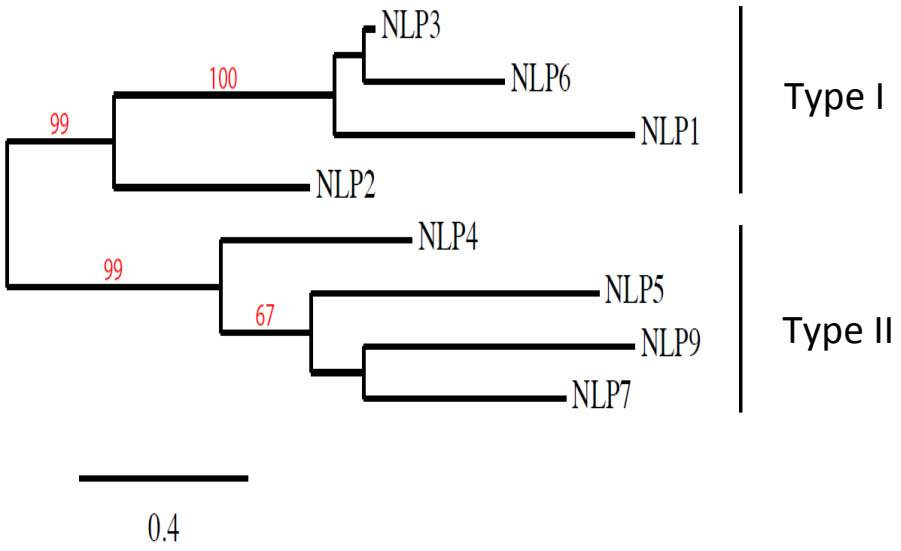
extraction (Qiagen, Venlo, The Netherlands) and cDNA synthesis (Invitrogen, Carlsbad, USA). Real-time PCR was performed to determine *NLP1* and *NLP2* expression. *Verticillium* elongation factor 1-alpha was used as an endogenous control and reactions were performed in triplicate. Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers used in this study are listed in Supplemental Table 1.

ACKNOWLEDGEMENTS

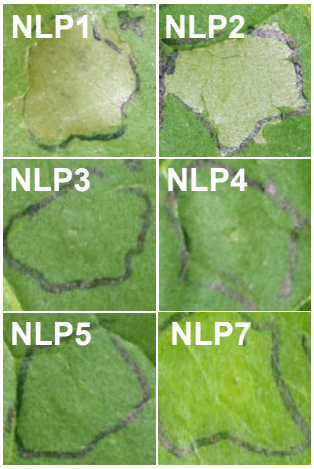
B.P.H.J.T. and H.P.v.E. are supported by a Vidi and a Veni grant, respectively, of the Research Council for Earth and Life sciences (ALW) of the Netherlands Organization for Scientific Research (NWO). This research was supported in part by the Centre for BioSystems Genomics (CBSG) which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research. The authors thank Anja Kombrink for technical assistance, Henk Franssen for access to the fluorescence microscope, Bert Essenstam and Henk Smid for excellent plant care, and Katherine Dobinson and Bart Lievens for the kind gift of *Verticillium* isolates.



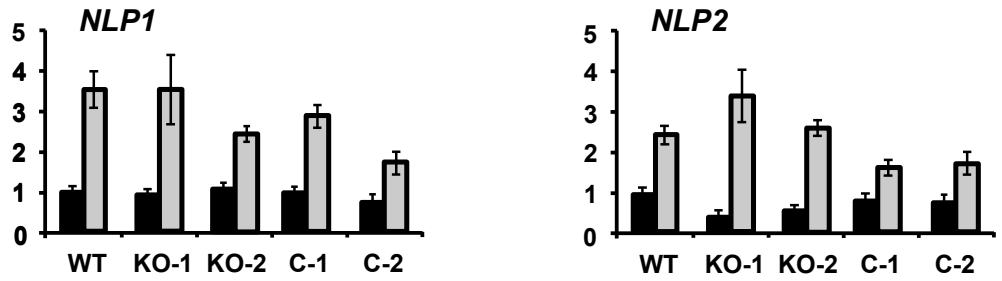
Supplemental Figure 1. Alignment of the *V. dahliae* NLP family. Amino acid sequences of *V. dahliae* NLPs were aligned using ClustalW and conserved residues are shaded in grey. The box indicates the position of the conserved heptapetide motif.



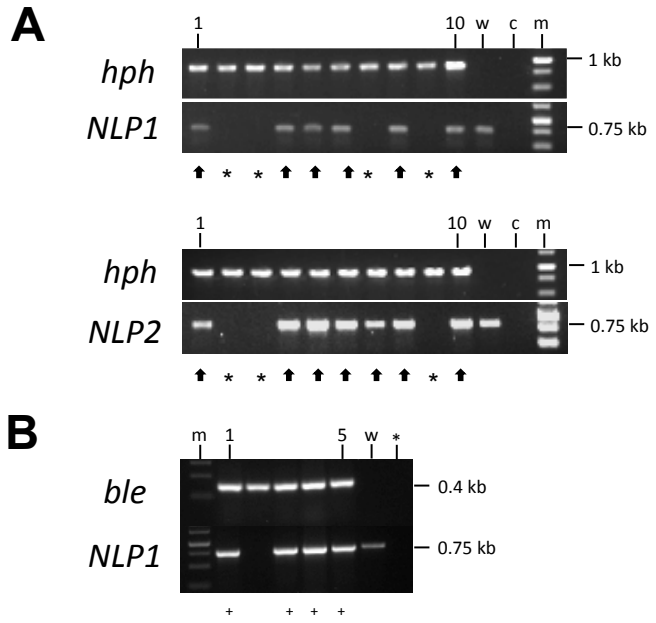
Supplemental Figure 2. Phylogenetic analysis of the *V. dahliae* NLP family**.** The phylogentic tree was constructed using PhyML (maximum likehood method) and bootstrap percentages >60% are indicated at the nodes.



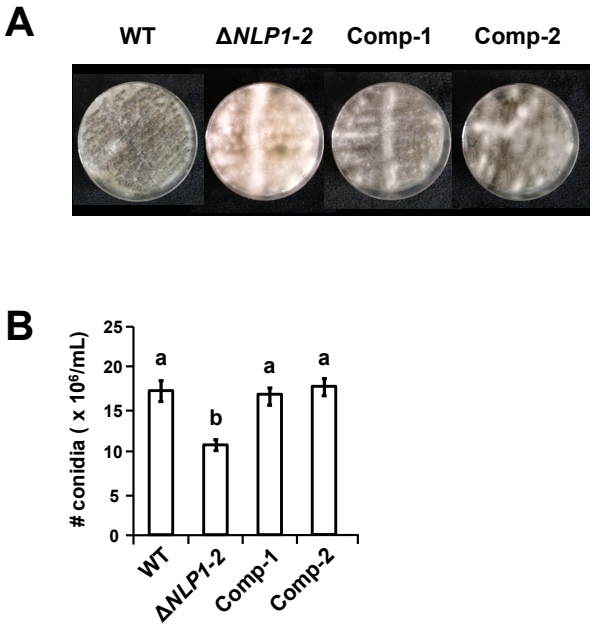
Supplemental Figure 3. Infiltration of heterologously expressed *NLP1* and *NLP2* induces cell death in *Nicotiana tabacum*. Infiltration of *Pichia pastoris*-produced NLP1 and NLP2, but not NLP3, NLP4, NLP5 or NLP7 induces necrosis. Pictures were taken at 3 days post infiltration.



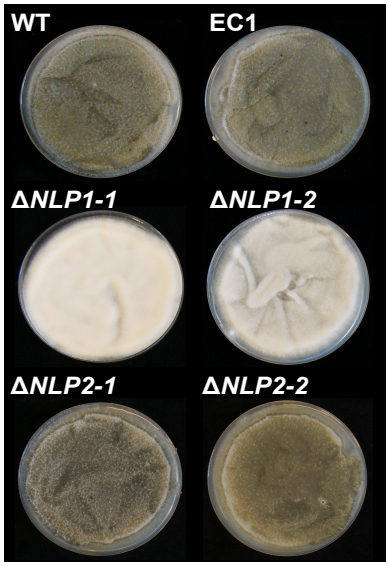
Supplemental Figure 4. Expression of *NLP1* and *NLP2* in *VdSge1* deletion strains. Real time PCR was used to measure gene expression levels in wild type *V. dahliae* (WT), two *VdSge1* deletion strains (KO-1 and -2) and two complementation strains (C-1 and -2) were grown for 4 days in MS medium (black bars) and a tomato cell suspension (MSK8; grey bars) using the *V. dahliae* elongation factor 1-alpha gene as a reference. Expression in wild type *V. dahliae* in MS medium was set to one, and bars represents averages with stranded deviation of two biological experiments.



Supplemental Figure 5. Verification of *NLP1* and *NLP2* deletion strains by PCR. A) Amplification of the hygromycin resistance gene (*hph*) and *NLP1* or *NLP2* from wild type *Verticilium dahliae* (w), 10 independent knock-out transformants (lanes 1 to 10), and water control (c) to identify deletion strains. The marker is indicated (m). True deletion strains are marked with asterisks, while ectopic transformants are marked with arrows. B) Amplification of the zeocin resistance gene (*ble*) and *NLP1* from wild type *V. dahliae* (w), a *NLP1* deletion strain (*), 5 independent complementation strains (lanes 1 to 5), and wild type control (c). The marker is indicated (m), and true complementation strains are marked (+).



Supplemental Figure 6. Complementation of a *NLP1* deletion strain restores colony morphology and conidiospore production. A) Colony morphology of wild type *Verticillium dahliae* (WT), a *NLP1* deletion strain (Δ*NLP1-2*) and two complementation strains (Comp-1 and Comp-2) after 7 days of incubation on PDA medium at 22°C. B) The average number of conidia produced after 10 days of growth on PDA medium based on two independent experiments. Different letters indicate significant differences at $P<0.05$ as calculated with Student's t test.



Supplemental Figure 7. Targeted deletion of *NLP1* affects morphological appearance of the fungal growth. Wild type *Verticillium dahliae* (WT), two *NLP1* deletion strains (Δ*NLP1-1* and Δ*NLP1-2*), an ectopic transformant (EC1), and two *NLP2* deletion strains (Δ*NLP2-1* and Δ*NLP2-2*), after 14 days of incubation on PDA medium at 22°C.

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Supplemental Table 1. Primers used in this study.

Primer	Sequence (5'- 3')	Remarks
KO-NLP1-LF	GGTCTTAAUCGAAGCTTCGTATCATCGTAGC	Left border; forward
KO-NLP1-LR	GGCATTAAUCCGACTTCATTCTGTCTTGG	Left border, reverse
KO-NLP1-RF	GGACTTAAUGAAGTACGATTATAATAGTCG	Right border, forward
KO-NLP1-RR	GGGTTTAAUGAGCCTCCTAAGTTATATAT	Right border, reverse
KO-NLP2-LF	GGTCTTAAUCCAGGCTTTCAACCCGGACTTTAGC	Left border; forward
KO-NLP2-LR	GGCATTAAUATCTTCCCTCCCACCTCCTCCATCC	Left border, reverse
KO-NLP2-RF	GGACTTAAUGTCTGGGTTGTGAGGAAGAGC	Right border, forward
KO-NLP2-RR	GGGTTTAAUGAAGGGTATGGCTCTGAGGG	Right border, reverse
NLP1-comp-F	AAGAATTCATCACCAGAATGTGCGACAG	Complementation, forward
NLP1-comp-R	AATTAATTAACCTAGACCTCCACAACAAGAACA	Complementation, reverse
NLP1-F	ATGCTTCCCTCCGCAGTCTTCT	Full length, forward
NLP1-R	TTAAAACGCGGCGCGCAT	Full length, reverse
NLP2-F	CACCATGTCGCCGTCTCTCATCAGC	Full length, forward
NLP2-R	CTACAGCGCCGCCTTCTG	Full length, reverse
NLP3-F	CACCATGGTTTCCAAGATCTTCTCCA	Full length, forward
NLP3-R	CTAGAGCGCTGCCTTGGC	Full length, reverse
NLP4-F	CACCATGCAGCATACTCTCCTCTC	Full length, forward
NLP4-R	CTACTCGTCAACATTCGG	Full length, reverse
NLP5-F	CACCATGCTTTTCAGTGTCGGA	Full length, forward
NLP5-R	TCAGATGTTGTTGGTGCCCT	Full length, reverse
NLP6-F	CACCATGTGGACTTGTCGCTTT	Full length, forward
NLP6-R	CTACAACGCCGCCTTGGCGA	Full length, reverse
NLP7-F	CACCATGCCTTCTCTCAGAACGG	Full length, forward
NLP7-R	TCAACCCCACGGCTTGAACTCG	Full length, reverse
NLP9-F	CACCATGCTTTTCCTGCAAAAC	Full length, forward
NLP9-R	TCAGTCCCACTTTCTTCCAGT	Full length, reverse
NLP1-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCAAACGCGGCGCGCAT	FLAG tag, reverse
NLP2-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCCAGCGCCGCCTTCTG	FLAG tag, reverse
NLP3-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCGAGCGCTGCCTTGGC	FLAG tag, reverse
NLP4-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCCTCGTCAACATTCGGGTC	FLAG tag, reverse
NLP5-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCGATGTTGTTGGTGCCCT	FLAG tag, reverse
NLP6-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCCAACGCCGCCTTGG	FLAG tag, reverse
NLP7-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCACCCCACGGCTTG	FLAG tag, reverse
NLP9-tag-R	TTAATGATGATGATGATGATGCTTGTCATCGTCATC CTTGTAGTCGTCCCACTTCTTCCAGTCC	FLAG tag, reverse
qPCR-NLP1-F	CTGCAAGGAAACCAGCAAG	Real-time PCR, forward
qPCR-NLP1-R	GTTTGCGTTGTTCGAGTTGA	Real-time PCR, reverse
qPCR-NLP2-F	GCTCAAGTTCAAGCCGTACC	Real-time PCR, forward
qPCR-NLP2-R	GTACAGAAACAAATCGCGGC	Real-time PCR, reverse
VdELF1-a-F	CCATTGATATCGCACTGTGG	Real-time PCR, forward
VdELF1-a-F	TGGAGATACCAGCCTCGAAC	Real-time PCR, reverse

Chapter 5

General discussion

Plant diseases can be devastating, particularly in monoculture crops that have a narrow genetic basis. Successful plant pathogens have the ability to manipulate the host, evade host recognition and suppress plant defenses. Verticillium wilt diseases cause tremendous losses of crops grown in greenhouses and open fields. The importance of the fungus comes from its ability to infect a wide range of dicotyledonous hosts and the lack of natural resistance in many plant species. Thus *Verticillium* causes financial loss that amount to millions of dollars annually. Although *Verticillium* resistance has been described in several plant species (Schaible *et al*., 1951; Bolek *et al*., 2005; Simko *et al*., 2004; Hayes *et al*., 2011), until recently only from tomato a *Verticillium* resistance gene (*Ve1*) had been cloned that mediates resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* (Fradin *et al*., 2009). Putative *Ve1* orthologs have been identified within (Chai *et al*., 2003; Fei *et al*., 2004; Simko *et al*., 2004; Zhang et al., 2013) and outside the Solanaceae family (Vining and Davis, 2009; Hayes *et al*., 2011; Zhang *et al*., 2012), suggesting that Ve1-mediated *Verticillium* resistance may be relatively widespread in plants (Thomma et al., 2011). Moreover, it was recently demonstrated that transfer of tomato *Ve1* mediates resistance against race 1 *Verticillium* strains in Arabidopsis (Fradin *et al*., 2011). Recently, through comparative population genome sequencing, the *Ave1* elicitor was identified that activates Ve1-mediated resistance (de Jonge *et al*., 2012). Interestingly, homologs of *V. dahliae* Ave1 were mainly found in plants, although a few homologs were found in plant pathogenic fungi and a plant pathogenic bacterium (de Jonge *et al*., 2012). Some of these fungal homologs, like the ones from the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*, and the sugar beet pathogen *Cercospora beticola*, are recognized by tomato Ve1. However, other homologs are not, such as the ones from *Colletotrichum higginsianum* and *Xanthomonas axonopodis* (de Jonge *et al*., 2012). Nevertheless, *Ve1* homologs in other plant species may be, or have been, involved in recognition of various plant pathogens.

In spite of the high economic importance of Verticillium wilt, relatively few pathogenicity and virulence factors of *V. dahliae* are known. A number of cell wall degrading enzymes including pectinases, polysaccharidases and pectinases were identified as virulence factors for *V. dahliae* (Bidochka et al., 1999; Dobinson et al., 1997). Furthermore, a homolog of a class II hydrophobin gene (*VDHI*) was found to be required for pathogenicity and microsclerotia formation (Klimes and Dobinson, 2006). More recently, Ave1 was identified as a virulence factor on susceptible tomato plants that lack *Ve1* (de Jonge *et al*., 2012). Finally, with comparative genomics the genetic diversity in a population of ten *V. dahliae* strains that are tomato pathogenic was assessed (de Jonge et al. 2013). This analysis identified a ~30 Mb core genome that is shared by all strains. Furthermore, all strains carried up to ~4 Mb of genome sequence that was unique, or shared by only a subset of strains, labelled as lineage-

specific (LS) regions. Within the core genome, the ratio of nonsynonymous (dN) substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions (dS) per synonymous site (Ks) was calculated within the coding regions for each of the 9471 conserved genes as a measure for selection strength, revealing that only 29 genes are under positive selection ($Ka/Ks > 1$; $P < 0.01$; de Jonge et al., 2013). About half of these encode proteins with a known function, including cellulose binding and regulation of transcription, whereas the other half encodes proteins of unknown function, of which only three encode secreted proteins that are candidate effectors (de Jonge et al. 2013). No remarkable differences were observed in the number of potential effector proteins between the core and LS genomic regions, nor in the number of protein domains classified in the Pfam database. Intriguingly, however, expression of genes residing in the LS regions is overrepresented during plant infection when compared with genes residing in the core genome. Subsequent functional analysis has identified three effector genes, two of unknown function and a LysM effector gene, which significantly contribute to virulence of *V. dahliae* (de Jonge et al., 2013). Nevertheless, it remains to be determined how these effectors contribute to *V. dahliae* virulence.

The aim of the research described in this thesis was to contribute to the understanding of the molecular mechanisms that underlie pathogenicity and virulence of *V. dahliae*, using tomato as model host. Functional genomic approaches, such as random mutagenesis, transcriptomics, RNA interference (RNAi), proteomics, metabolomics, comparative genomics and targeted mutagenesis, were used to unravel mechanisms involved in pathogenicity of *V. dahliae*. This research resulted in the identification of several novel pathogenicity and virulence factors of *V. dahliae*.

Forward and reverse genetic approaches to identify pathogenicity and virulence factors of *V. dahliae*

Since protocols to transform filamentous fungi using *Agrobacterium tumefaciens*-mediated transformation (ATMT) have been developed (de Groot *et al*., 1998), ATMT has been widely used to generate random transformants in a number of plant pathogenic fungi. The advantages of ATMT over conventional transformation techniques such as polyethylene glycol (PEG)-mediated transformation and restriction enzyme-mediated integration (REMI) are the higher transformation efficiency, the higher percentage of single copy insertions, and an increased chance to identify the mutated genes (Meyer *et al*., 2003; Michielse *et al*., 2005). In Chapter 2, a collection of 900 T-DNA transformants of *V. dahliae* was generated using ATMT and tested for reduced virulence on susceptible tomato plants. In total, 80 candidate transformants

with reduced virulence were selected and T-DNA insertion sites were identified. We could identify the insertional site for 43 transformants, while for the remaining 37 transformants the T-DNA insertion sites presently remain unknown due to the inability to amplify the T-DNA flanking sequence, tandem T-DNA insertions, or redundancy of the flanking sequence due to T-DNA insertion in a repeat rich region. Even though ATMT has advantages over conventional transformation techniques, with the quick advancement of sequencing technologies and the associated drop in sequencing prices, ATMT may no longer be the preferred method to identify novel pathogenicity and virulence genes. The generation and maintenance of large numbers of transformants, screening for reduced virulence and identification of the T-DNA insertion sites is challenging, time consuming, labour intensive and expensive. Alternatively, genomics strategies may be exploited to uncover pathogenicity and virulence genes. For instance, comparative genome analysis of pathogenic and non-pathogenic *V. dahliae* strains may be used to unravel novel pathogenicity and virulence genes. Similarly, comparative genomics of multiple pathogenic *V. dahliae* strains may be used, aiming at uncovering allelic variation that may indicate signs of positive selection pressure associated with pathogenicity. Both these comparative genomics strategies may be further advanced by combining them with *in planta* transcriptomics (RNAseq) to identify which of the potential target genes is induced during infection. Such comparative genomic analyses have been demonstrated to provide new insights into genome evolution, speciation and origin of pathogenicity (Stukenbrock and Bataillon, 2012; Stukenbrock et al., 2013). Recently, comparative population genomics in combination with *in planta* expression profiling was used to identify pathogenicity and virulence genes in *V. dahliae* (also discussed above) and in *Colletotrichum* spp. (de Jonge et al., 2012; O'Connell et al., 2012; Gan et al., 2013). Comparative genomics and transcriptome analyses of the Arabidopsis pathogen *Colletotrichum higginsianum* and the maize pathogen *Colletotrichum graminicola* revealed that pathogenicity-related genes are transcribed in consecutive waves that are linked to a pathogenic life style transition (O'Connell et al., 2012). Moreover, by comparative genomics in *F. oxysporum,* Ma et al. (2010) identified the presence of supernumerary chromosomes which are present only in the pathogenic strains, and all known effector genes were found to be localized on one of these supernumerary chromosomes. Thus, comparative genomics strategies can be used for the selection of candidate pathogenicity and virulence genes, which can subsequently be subjected to functional analysis to confirm such role.

Targeted gene deletion is one of the forward genetic approaches to explore gene function. With targeted gene deletion, the gene of interest is replaced by an antibiotic resistance cassette through homologous recombination. A previously described method for targeted gene disruption in *V. dahliae* requires obtaining the

cDNA of the target gene (Rauyaree et al., 2005), the technique. The targeted gene deletion method employed in this thesis does not involve cDNA amplification of the target gene; longer flanking sequences of the target gene can be amplified from genomic DNA and fewer cloning steps are required. The development of the targeted gene deletion protocol for *V. dahliae* as described in this thesis (adapted from Frandsen et al., 2008) made the generation of targeted gene deletion mutants more efficient and less time consuming. A more efficient protocol to generate targeted gene deletion constructs enabled us to more quickly generate several targeted deletion mutants in *V. dahliae* (*VdNRS/ER*, *VdSge1* and *NLP* gene family members).

Differential regulation of effector gene expression

Plant pathogens secrete effector molecules to successfully colonize host plants (Stassen and van der Ackerveken, 2011; de Jonge et al., 2011; Lindeberg et al., 2012). Effector gene expression is typically induced upon host colonization. Earlier it was proposed that nitrogen starvation acts as trigger of effector gene induction (van den Ackerveken et al. 1993), but by now we know that many effector genes do not respond to nitrogen starvation (Bolton and Thomma 2008). Comparative genomics and *in planta* expression profiling in maize pathogen *Colletotrichum graminicola* revealed that pathogenicity and virulence genes are transcribed in successive waves that are linked to pathogenic transitions. During penetration and the biotrophic phase effectors and secondary metabolism enzymes are induced, while hydrolases and transporters are upregulated during the necrotrophic phase (Connell et al., 201l). In the rice pathogen *Magnaporthey oryzae* more than half of the highly upregulated genes encoded proteins that are putatively secreted during initial infection stages (Kawahara et al., 2012). Differential expression of *NLP* genes has been shown in number of plant pathogens. In *Phytophthora sojae,* it was shown that *PsojNIP* is expressed during the transition from the biotrophic to the necrotrophic phase (Qutob et al, 2002). In *Colletotrichum higginsianum* it was shown that out of six NLPs, only four are expressed *in planta* (*ChNLP1, ChNLP2, ChNLP3, ChNLP5*), of which *ChNLP1* and *ChNLP2* are exclusively expressed during the transition from biotrophy to necrotrophy and *ChNLP3* and *ChNLP5* are expressed only during appressorium development (Kleemann et al., 2012).

Identification of microbial regulators of effector gene expression *in planta* could lead to the identification of valuable targets for the development of novel disease control strategies. Studies on transcriptional regulators of effector gene expression are still at an early stage. In the human pathogenic fungi *Candida albicans* and *Histoplasma*

capsulatum transcriptional regulators Wor1 and Ryp1 regulate dimorphic switches, a transition required to cause disease (Huang et al. 2006; Nguyen and Sil 2008). Orthologs of Wor1 and Ryp1 are widely found in fungi. In the tomato vascular wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici,* the ortholog of Wor1 and Ryp1 was identified and named as Sge1. Sge1 was demonstrated to be required for pathogenicity and conidiospore production, and regulate effector gene expression (Michielse et al. 2009). Similarly, in the necrotrophic pathogen *Botrytis cinerea* the ortholog Bcreg1 was shown to be required for conidiation, pathogenicity on bean leaves and toxin production (Michielse et al. 2011). Recently, another ortholog was identified in the wheat pathogen *Fusarium graminearum* as Fgp1 and shown to be required for pathogenicity and mycotoxin synthesis (Jonkers et al., 2012). In Chapter 3 we have demonstrated that VdSge1, the *V. dahliae* ortholog of *F. oxysporum* Sge1 is required for vegetative growth, conidiospore production, pathogenicity and differentially regulates expression of effector genes. These results show that the family of Wor1-like proteins regulates pathogenicity in many fungi through transcriptional regulation of effector genes and toxins productions. Moreover, the function of Wor1-like proteins clearly extends beyond a role in pathogenicity, as several studies have demonstrated regulatory control over functions such as conidiospore production, vegetative growth and secondary metabolism.

Functional diversification of conserved effectors.

Typically, effectors are species-specific molecules, although some effectors seem to be conserved across species (Thomma et al., 2011). For example, the fungal LysM effectors widely occur in fungi, including plant and animal pathogens (de Jonge et al., 2009). Blast analysis of the LysM motif against the protein database from 70 pathogenic and non-pathogenic fungi resulted in identification of 403 proteins that could be grouped into five different types (A-E) based on their domain architecture (de Jonge and Thomma 2009). The tomato pathogenic fungus *C. fulvum* secretes the LysM containing effector protein Ecp6 during colonization that was shown to be required for virulence (Bolton et al., 2008). Ecp6 was shown to bind chitin and suppress chitin triggered immunity (de Jonge et al., 2010). The rice pathogen *Magnaporthe oryzae* secretes the LysM containing effector Slp1that shows similarity to *C. fulvum* Ecp6. Slp1 was shown to accumulate at the plant-fungal interface and, like Ecp6, to bind chitin and suppress chitin-triggered immunity in rice suspension cells (Mentlak et al., 2012).

Similar to LysM effectors, also NLPs are widely conserved across fungal species (de Jonge et al., 2010; Gijzen and Nürnberger, 2006). Moreover, NLPs have

also been identified in other kingdoms of life, including bacteria and oomycetes (Gijzen and Nürnberger, 2006). Upon injection into dicotyledonous plants, NLPs can induce plant defense responses and cause cell death. So far, a number of NLPs has been shown to possess cytotoxic activity and were found to be required for virulence. Analysis of the crystal structure of a cytotoxic NLP from *Pythium aphanidermatum* revealed structural homology to actinoporins and fungal lectins (Ottmann et al., 2009). Apart from cytotoxic activity and a role in virulence, we showed that *V. dahliae* NLP1 affects growth and conidiation (Chapter 3). More efforts should be devoted to identify additional functions of NLPs across various types of microbial species.

So far, non-cytolytic NLPs have been described only for oomycete species. *Phytophthora infestans* and *Phytophthora sojae* were shown to contain cytolytic and non-cytolytic NLPs, while the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* contains only non-cytolytic NLPs (Kanneganti et al. 2006; Dong et al., 2012; Cabral et al., 2012). For *H. arabidopsidis* it was demonstrated that non-cytolytic NLPs are expressed during early infection stages, suggesting that these NLPs may still play a role related to host colonization. In Chapter 3, we have uncovered functional diversification within the *V. dahliae* NLP family by identifying non-cytolytic NLPs. Only two out of eight NLPs display cytolytic activity, while the remaining six are non-cytolytic. Possibly, the non-cytotoxic NLPs contribute to pathogen virulence through other mechanisms than inducing cellular leakage, or have diversified towards completely different functions that are not related to the establishment of wilt diseases. In Chapter 3, we show functional diversification within cytolytic NLPs, as only NLP1 was shown to affect vegetative growth and conidiation in addition to virulence. The role of NLPs in growth and development of microbes may explain why *NLP* genes also occur in non-pathogenic micro-organisms. Functional diversification of conserved effectors is also seen in LysM effector families. The LysM effector proteins Ecp6 and Slp1 were shown to suppress immunity by sequestration of chitin oligomers (de Jonge et al., 2010 and Mentlak et al., 2012). The LysM effectors Mg3LysM and Mg1LysM of the wheat pathogen *Mycosphaerella graminicola* are able to bind chtin but only Mg3LysM is able to suppress chtin induced immunity. Unlike Ecp6 and Slp1, both Mg3LysM and Mg1LysM are able to protect fungal hyphae against chitinases (Marshall et al., 2011). Comparative genomics revealed the presence of an expanded LysM effector gene family in *V. dahliae*. All sequenced *V. dahliae* strains contain six genes that encode LysM effectors in their core genome and none of them appear to be induced *in planta* or required for virulence (de Jonge et al., 2013). Remarkably, *V. dahliae* strain Vdls.17 contains one additional LysM gene in a lineage-specific region which is

significantly expressed *in planta* and shown to be required for full virulence of this strain on tomato (de Jonge et al., 2013).

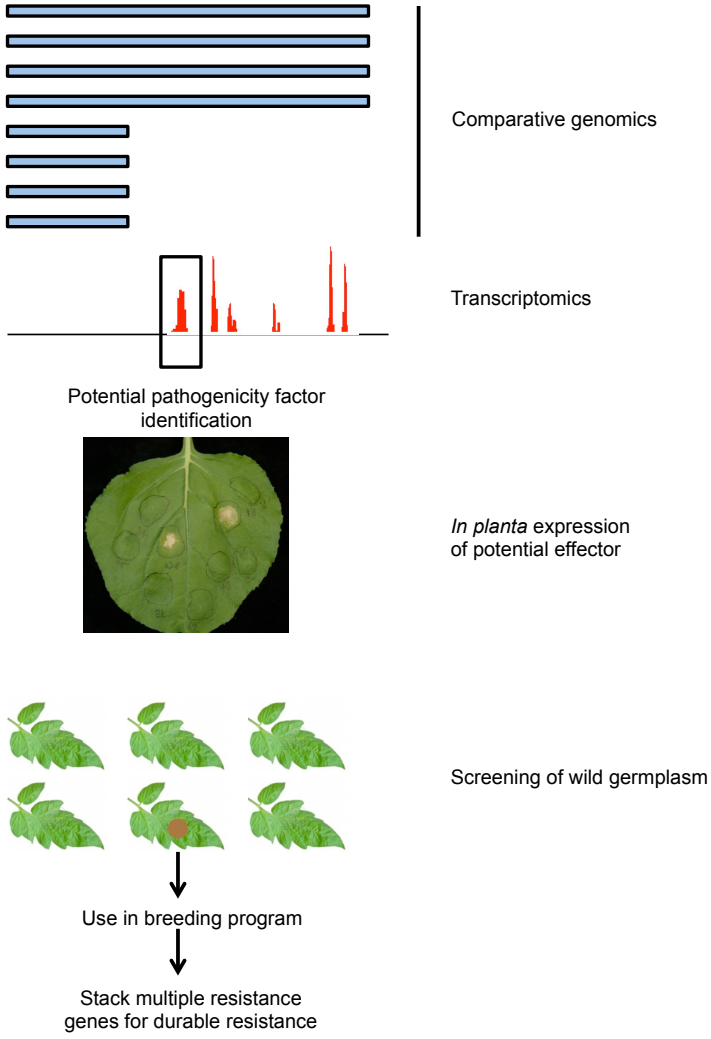

Figure 1. Proposed methodology for obtaining durable crop resistance. Comparative genomics in combination with transcriptomics is a powerful tool to quickly identify potential pathogenicity and virulence factors. The identified patthogenicity and virulence factors are expressed *in planta* using model plant species and responsiveness is scored to establish a proof of concept. Wild germplasm is screened to identify resistance scources and responsive germplasm is used in breeding programs. Multiple resistance genes should be stacked in commercial varieties for durable resistance.

From pathogenicity genes to effective crop protection

Understanding the basic molecular mechanism of pathogenicity and virulence of a pathogen will help plant breeders to generate disease resistant crop genotypes. Deployment of disease resistance mechanisms into crops may provide durable resistance. Genetic resistance is the preferred method for disease control. As most of the resistance genes identified so far are either race- or species-specific, it is required to stack multiple resistance genes into a cultivar to provide durable resistance. Resistance which is effective against a large number of pathogen genotypes is known as non-race-specific resistance, sometimes also referred to as partial resistance. It is thought that partial resistance may be more durable than race-specific resistance because pathogens are less likely to evolve the ability to overcome partial resistance. Wild germplasm is a source of genetic variation that can be used to improve crop yield and disease resistance. The challenge ahead is to translate the fundamental information about the molecular mechanisms of pathogenesis into effective and durable disease control. Discovery of genes that are involved in host-pathogen interactions will help breeders to identify resistant germplasm. In potato, a rapid screening method for *P. infestans* effectors was used to identify new resistance genes in related *Solanum* species (Song et al., 2003; van der Vossen et al., 2003, 2005; Huang et al., 2005; Vleeshouwers et al., 2008; Wang et al., 2008; Oh et al., 2009). Resistance genes that recognize highly conserved, non-redundant, effectors that are essential for pathogenicity represent potentially durable sources of resistance (Whisson et al., 2011). Bioinformatics and (functional) genomics have changed the way the research is conducted in plant pathology and provide a wealth of novel opportunities.

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Summaries

Summary

Vascular wilt diseases caused by soil-borne pathogens are among the most devastating plant diseases worldwide. The ascomycete fungus *Verticillium dahliae* causes vascular wilt diseases in hundreds of dicotyledonous plant species, including important crops such as eggplant, lettuce, olive, spinach and tomato. The resting structures, microsclerotia, are triggered by root exudates to germinate and penetrate the roots after which the fungus grows into the xylem vessels. The fungus colonizes these vessels and interferes with the transportation of water and nutrients, resulting in the development of symptoms such as stunting, wilting, chlorosis and vascular browning. Verticillium wilt diseases are difficult to control due to the longevity of the microsclerotia, the broad host range of the pathogen, the inability of fungicides to kill the fungus once it has colonized the xylem vessels and the lack of natural resistance in many plant species.

Chapter 1 is the introduction to this thesis that describes the identified pathogenicity and virulence factors of *V. dahliae* and strategies to identify these components. In spite of the economic importance of *V. dahliae*, relatively few pathogenicity genes have been identified in this species. With the availability of whole genome sequences and the development of functional genomics tools such as random mutagenesis, targeted mutagenesis, transcriptomics, RNA interference (RNAi) and comparative genomics, more strategies have become available to identify novel pathogenicity and virulence genes.

Chapter 2 focuses on the identification of virulence and pathogenicity genes of *V. dahliae* by screening of a library of random T-DNA insertion mutants. Using *Agrobacterium tumefaciens*-mediated transformation, 900 T-DNA transformants with random insertions were generated and screened for altered virulence on susceptible tomato plants. This screening, followed by inverse PCR on selected transformants, resulted in the identification of 55 potential pathogenicity and virulence genes. One of the potential pathogenicity genes, *VdNRS/ER*, is a homolog of a nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER), which is presumably involved in the biosynthesis of UDP-rhamnose. Using targeted mutagenesis, *VdNRS/ER* was deleted from wild-type *V. dahliae* and the resulting deletion mutants were characterized. *VdNRS/ER* deletion mutants exhibit unaltered vegetative growth and sporulation, but the deletion mutants were no longer pathogenic on tomato and *N. benthamiana* and showed impaired root attachment on tomato seedlings. These data suggest that UDP-rhamnose is required for pathogenesis of *V. dahliae.*

Chapter 3 describes the role of the *V. dahliae* homolog of Sge1, a transcriptional regulator that was shown to play a role in pathogenicity and regulate

effector gene expression in *Fusarium oxysporum*. In this chapter it is demonstrated that *V. dahliae Sge1* (*VdSge1*) is required for radial growth and production of asexual conidiospores. It is furthermore shown that *VdSge1* deletion strains have lost pathogenicity on tomato. Since the *VdSge1* deletion mutants are not able to infect and colonize tomato plants, a tomato cell suspension culture was used to the study the expression of *Ave1*, as well as nine other genes of which the expression is highly induced *in planta*. This assay revealed that VdSge1 is not required for the induction of the Ave1 effector that activates resistance mediated by Ve1 in tomato. Furthermore, the expression of one other putative effector gene was not affected by *VdSge1* deletion. However, *VdSge1* was shown to be required for the expression of six putative effector genes, whereas expression of the remaining two putative effectors genes was negatively regulated. Thus, the data show that *VdSge1* is required for *V. dahliae* pathogenicity and differentially regulates effector gene expression.

Chapter 4 describes the functional characterization of the gene family encoding necrosis- and ethylene-inducing-like proteins (NLPs) of *V. dahliae*. The cytotoxic activity of NLP family members was determined using agroinfiltration into tobacco leaves. This resulted in the identification of two out of the seven NLPs, *VdNLP1* and *VdNLP2*, that induced plant cell death. The genes encoding these cytotoxic NLPs were found to be induced in *V. dahliae* upon colonization of tomato. Targeted deletion of *VdNLP1* and *VdNLP2* significantly reduced the virulence of *V. dahliae* on tomato and Arabidopsis plants. In contrast, only deletion of *VdNLP1* affected virulence on *N. benthamiana* whereas deletion of *NLP2* did not. However, subsequent transcriptional analysis revealed that *VdNLP2* was not expressed in *V. dahliae* during colonization of *N. benthamiana*. Moreover, *VdNLP2* also affects vegetative growth and conidiospore production. In conclusion, the expanded *V. dahliae NLP* family shows differential cytotoxic activity between family members and *in planta* induction of the cytotoxic NLP genes varies between plant hosts. In addition, VdNLP2 plays a role in vegetative growth and conidiospore production in addition to its contribution to virulence. Thus, evidence is provided for functional diversification within the *V. dahliae* NLP family.

Finally in **Chapter 5**, the major findings of this thesis are discussed and placed in a broader perspective.

Samenvatting

Verwelkingsziekten veroorzaakt door bodempathogenen behoren wereldwijd tot de meest verwoestende plantenziekten. De ascomycete schimmel *Verticillium* veroorzaakt verwelkingsziekte in honderden dicotyle plantensoorten, waaronder belangrijke gewassen zoals aubergine, sla, olijf, spinazie en tomaat. De ruststructuren (microsclerotia) ontkiemen wanneer deze geactiveerd worden door wortelexudaten en de schimmel dringt de wortels van vatbare planten binnen waarna ze de houtvaten binnendringt. De schimmel koloniseert deze vaten en verstoort het transport van water en voedingsstoffen, waarop symptomen zoals dwerggroei, verwelking, chlorose en verkleuring van het vaatweefsel ontstaan. Verwelkingsziekten veroorzaakt door *Verticillium* zijn moeilijk te bestrijden door de lange levensduur van de microsclerotia, het brede gastheerbereik van de ziekteverwekker, het onvermogen van fungiciden om de schimmel in de houtvaten te doden en het gebrek aan natuurlijke resistentie in veel plantensoorten.

Hoofdstuk 1 is de inleiding tot dit proefschrift en beschrijft eerder gekarakteriseerde pathogeniteits- en virulentiefactoren van *V. dahliae* naast strategieën om deze componenten te identificeren. Ondanks het economische belang van *V. dahliae* zijn relatief weinig pathogeniteitsfactoren van deze soort geïdentificeerd. Met de beschikbaarheid van de volledige genoomsequentie en de ontwikkeling van gereedscahppen voor functionele genomica, zoals mutagenese, transcriptomics, RNA-interferentie (RNAi) en vergelijkende genomica zijn meer strategieën beschikbaar gekomen om nieuwe pathogeniteits- en virulentiefactoren te identificeren.

Hoofdstuk 2 beschrijft de identificatie van pathogeniteits- en virulentiegenen van *V. dahliae* door middel van de screening van een bank met willekeurig geïntegreerde T-DNA insertiemutanten voor veranderde aggressiviteit. Door middel van *Agrobacterium tumefaciens* werden 900 T-DNA transformanten gegenereerd die werden gescreend op vatbare tomatenplanten. Deze screening resulteerde in de identificatie van 55 mogelijke pathogeniteits- en virulentiegenen. Een van de mogelijke pathogeniteitsgenen codeert voor VdNRS/ER, een homoloog van een nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER) dat vermoedelijk betrokken is bij de biosynthese van UDP-rhamnose . Met behulp van gerichte mutagenese werd het gen dat codeert voor VdNRS/ER verwijderd uit wild-type *V. dahliae* en de deletiemutanten werden bestudeerd. VdNRS/ER deletiemutanten vertoonden ongewijzigde groei en sporulatie, en de mutanten waren niet meer pathogeen op tomaat en *Nicotiana benthamiana*. Daarnaast vertoonden de mutanten

een verminderde aanhechting aan de wortels van tomatenplanten. Deze bevindingen suggereren dat UDP-rhamnose vereist is voor pathogeniteit van *V. dahliae*.

Hoofdstuk 3 beschrijft de rol van de *V. dahliae* homoloog van Sge1, een transcriptionele regulator die een rol speelt bij pathogeniteit en de regulering van de expressie van effectoren in *Fusarium oxysporum*. In dit hoofdstuk wordt aangetoond dat *V. dahliae* Sge1 (VdSge1) vereist is voor radiale groei en de productie van conidiosporen. Verder wordt aangetoond dat *VdSge1* deletiestammen geen ziekte meer veroorzaken op tomaat. Aangezien de *VdSge1* deletiemutanten niet in staat zijn om tomatenplanten te infecteren werd een celsuspensiecultuur van tomaat gebruikt om de de expressie van *Ave1* te bestuderen samen met negen andere genen waarvan de expressie sterk geïnduceerd is tijdens infectie. Zo bleek dat *VdSge1* niet vereist is voor de inductie van de Ave1 effector die Ve1-resistentie activeert in tomaat. Bovendien werd de expressie van een ander effectorgen ook niet beïnvloed door *VdSge1*. Echter, VdSge1 bleek vereist voor de expressie van zes mogelijke effectorgenen, terwijl expressie van de resterende twee mogelijke effectorengenen negatief werd gereguleerd. VdSge1 is dus vereist voor pathogeniteit van *V. dahliae* en reguleert de expressie van effectorgenen differentieel.

Hoofdstuk 4 beschrijft de functionele karakterisering van de genen die coderen voor de familie van necrose - en ethyleen-inducerende eiwitten (NLPs) van *V. dahliae*. De cytotoxische activiteit van de NLPs werd bepaald door middel van agroinfiltratie in tabaksbladeren. Dit resulteerde in de identificatie van twee van de zeven NLPs, VdNLP1 en VdNLP2, die geïnduceerde celdood veroorzaakten. De genen die coderen voor deze cytotoxische NLPs bleken geïnduceerd in *V. dahliae* tijdens de kolonisatie van tomaat. Gerichte uitschakeling van *VdNLP1* en *VdNLP2* verminderde de virulentie van *V. dahliae* op tomaat en Arabidopsis. Echter, alleen de uitschakeling van *VdNLP1* verminderde de virulentie op *N. benthamiana* terwijl uitschakeling van *NLP2* geen effect had. *VdNLP2* bleek dan ook niet tot expressie te komen in *V. dahliae* tijdens de kolonisatie van *N. benthamiana*. Daarnaast heeft VdNLP2 ook invloed op de vegetatieve groei en productie van conidiosporen. Kortom, de leden van de NLP familie van *V. dahliae* vertonen differentieel cytotoxische activiteit, en de inductie van de cytotoxische *NLP* genen varieert tussen waardplanten. VdNLP2 speelt naast een rol in virulentie tevens een rol tijdens vegetatieve groei en productie van conidiosporen.

Tenslotte worden in **hoofdstuk 5** de belangrijkste bevindingen van dit proefschrift besproken en in een breder perspectief geplaatst.

Acknowledgements

I thank my daily supervisor and promoter **Prof. Bart Thomma**. I thank you for giving me an opportunity to work in the Verticillium lab. I really appreciate the independence you gave me while designing and executing my experiments and the support you provided during result analysis was immense. Whenever I approached you with problems you guided me towards the path of solutions. Thanks for the encouragement and productive suggestions you gave me, particularly with respect to making choices, it helped me to shape my character. This thesis would not have taken this shape without your patience, commitment and efforts, particularly when I was writing in India. You are a smart worker, efficient and good in execution; you are the inspiration for my sixth proposition. Thank you ☺

I am thankful to my promoter **Prof. Pierre de Wit** for his valuable comments and suggestions for my propositions.

I am thankful to my external supervisor **Dr. Fons Debets**. Thanks for the annual discussions on my research progress and for appreciating my work and boosting my confidence.

I would like to thank **Prof. Francine Govers** and **Dr. Jan van Kan** for giving me an opportunity to be a student assistant for the genomics course, which I enjoyed.

I would to like to thank **Ali Ormel** for the paper works related to my travels and administration related to my studies.

Grardy van den Berg, I thank you for helping me in my day-to-day lab activities. You have helped in solving problems with PCR, cloning and transformation. Your planning and efficiency is great. During the last year you helped me to clone and transform a couple of *NLP* genes. Thanks a lot. ☺

I had a memorable time in the Nederlands, mostly because of **Anja Kombrink, Emilie Fradin, Ronnie de Jonge and Koste Yadeta**. **Emilie**, I was glad to be your bench mate. Your frequent interactions on my projects helped me to discuss freely and thanks for the motivation you have given. I especially thank you for directing me towards Suzan Gabriëls (Monsanto). Thanks Emilie. **Anja**, I thank you for answering frequent questions (I know I have bugged you a lot), making the lab environment lighter, sharing media plates ;) and wonderful dinners. Your technical assistance for NLP IP experiments is highly appreciated. Thank you. **Ronnie**, thanks for the bioinformatics stuff you taught me. Dude, the level of excitement you showed at each stage of your comparative genomics project was good. Not everybody can show excitement and share their new discoveries like you did. Keep it going. Thank you **Manon** for your hospitality. **Koste**, thank you for the technical help you have

provided (qPCR and Arabidopsis inoculations) and all fun we had as a group. You are very patient and calm in nature. During the initial days of my studies I have constantly bugged you, so thanks for patiently answering my queries. Thanks man. I wish all of you my best wishes, and I will cherish our friendship for a long time.

I thank **Peter van Esse** for the fruitful collaboration, which resulted in a publication. I thank **Andrea Sánchez-Vallet** and **Mireille van Damme** for intriguing scientific discussions and making me believe that I am one of the best ☺, Andrea, thanks for all the evening chats and dinners.

I thank **Daniele Sueldo** and **Bilal Ökmen** for being my friend and nice company. I thank **Mojtoba Keykha Sabe**r for the collaboration to identify T-DNA insertion sites in a number of random transformants. I wish you good luck. I thank **Klaas Bouwmeester** for providing me tomato suspension cultures, photoshop tips and kindness that you showed, **Patrick Smit** for discussions on basic science and sharing your interview experience.

I made lot of wonderful friends during my stay in the Laboratory of Phytopathology, which has helped me in several ways during my day-to-day activities; I thank **Zhao Zhang, Jordi Boshoven, Chunxu Song, Xu Cheng, Lisha Zhang, Thomas Liebrand, Yan Wang, Yu Du,** and **Miao Han**.

All this work benefited from the helping hands from students: **Md. Tohidul Islam**, **Hugo de Vries**, and **Jelle Postma**. Thank you guys.

I would like to thank **Aundy Kumar**, **Viswanath and Rajeesh** for sharing the light moments and nice Indian dinners we enjoyed together.

I would also like to express my sincere gratitude to **Bert Essenstam** and **Henk Smid** for taking great care of my experiments in the greenhouse. Bert, I like your efficient way of working and also the way you pay attention to minute details. Thanks for the friendly chats and smiles. ☺

I would like to extend my gratitude to **Rolf Folkertsma** and **V. K. Kishore** from Monsanto Holding Pvt. Ltd. for hiring me even before I completed my thesis. **Suzan Gabriëls**, thank you for introducing me to the organization.

I would like to thank my friends **Dalton Carmel**, **Jaymurthy**, **Suresh Kumar vasa** and **Ram Prasath** for being with me during both fun and hard times.

Finally, I thank my mother and father for understanding my passion and letting me go overseas. I thank my brother (**Sriraman** and **Srinivasan**), my sister (**Sudhamani**), my bother in law (**Venkatesh**) and my sister in law (**Saritha**) for their moral and financial support.

Curriculum vitae

Parthasarathy Santhanam was born on 8th November 1979 in Pondicherry, India. After completing his B.Sc degree in Botany, he obtained his M.Sc degree in Plant Biotechnology in 2003. In February 2005, he received MASHAV fellowship offered by the Government of Israel for a period of six months to study the role of saponins in salt stress. In April 2006 he received a DELTA fellowship offered by the Dutch Government to study the branching pattern and root formation in Stoloniferous *Petunia* under the supervision of Dr. Janny L. Peters and Dr. Josef F. Stuefer in the department of Plant Genetics of the Radboud University in Nijmegen, The Netherlands. He performed his PhD study from October 2008 to September 2012 in the Laboratory of Phytopathology of the Wageningen University on the project entitled "Insertional mutagenesis in the vascular wilt pathogen *Verticillium dahliae*" under the supervision of Prof. Bart Thomma. Since October 2012, Partha is working as a Plant Pathologist in Monsanto Holdings Pvt. Ltd. in Bangalore, India.

Publications

Klosterman, S. J., Subbarao, K. V., Kang, S., Veronese, P., Gold, S. E., Thomma, B. P.H. J., Chen, Z., Henrissat, B., Lee, Y-H., Park, J., Garcia-Pedrajas, M. D., Barbara, D. J., Anchieta, A., de Jonge, R., Santhanam, P., Maruthachalam, K., Atallah, Z., Amyotte, S. G., Paz, Z., Inderbitzin, P., Hayes, R. J., Heiman, D. I., Young, S., Zeng, Q., Engels, R., Galagan, J., Cuomo, C., Dobinson, K. F., and Ma, L. J. 2011. Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathog. 7:e1002137.

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Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Parthasarathy Santhanam
Date: 20 January 2014
Group: Phytopathology, Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Insertional mutagenesis in the vascular wilt pathogen Verticillium dahliae.	Oct 11, 2008
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Random insertional mutagenesis in fungal genomes to identify virulence factors, Methods Mol Biol. 2012;835:509-17	2012
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
Joint European PhD retreat ,Cologne, Germany	Apr 15-17, 2010
EPS PhD student day, Wageningen University	May 20, 2011
ExPectationS (EPS Career Day)	Nov 18, 2011
► **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic Agents, Utrecht University	Jan 22, 2009
EPS Theme 2: Interactions between Plants and Biotic Agents, University of Amsterdam	Feb 03, 2011
EPS Theme 2: Interactions between Plants and Biotic Agents, Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03 2012
► **Seminars (series), workshops and symposia**	
NBIC Bioinformatics symposium, Maastricht	Dec 15-16, 2008
Seminar Dr. Rays H.Y Jiang	Jun 10, 2009
Seminar Dr Ton Bisseling "From epigenetics to novel organelles and organs"	Sep 09, 2009
Seminar Prof. Richard Oliver	Nov 20, 2009
Galaxy workshop	Sep 02, 2010
Seminar Prof. Naoto Sibuya "Chitin perception and signaling in rice and arabidopsis"	Sep 09, 2010
Seminar Prof. Regine Kahmann "Effectors of the plant-pathogen fungus Ustilago maydis"	Oct 29, 2010
Joint CBS-PRI-Phyto Symposium	Nov 12, 2010
► **Seminar plus**	
► **International symposia and congresses**	
10th international Verticillium symposium, Corfu (Greece)	Nov 15-21, 2009
2nd International VIB Ph.D. Student Symposium VIBes in Biosciences 2010	Oct 14-16, 2010
From Foliar to Root-Interacting Pathogens and Symbionts, Göttingen, Germany.	Feb 16-18, 2011
26th Fungal Genetics Conference, Asilomar, USA	Mar 15-20, 2011
► **Presentations**	
Oral: 10th international Verticillium symposium, Greece	Nov 15-21, 2009
Poster: PhD retreat ,Cologne	Apr 15-17, 2010
Oral: PhD Summer School Rhizosphere Signaling, Wageningen	Aug 23-25, 2010
Poster: 26th Fungal genetics conference, Asilomar, USA.	Mar 15-20, 2011
Poster: From Foliar to Root-Interacting Pathogens and Symbionts, Göttingen, Germany.	Feb 16-18, 2011
► **IAB interview**	2011
► **Excursions**	
Subtotal Scientific Exposure	*16.6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD Summer School Rhizosphere Signaling, Wageningen	Aug 23-25, 2010
PhD Autumn School Host-Microbe Interactomics, Wageningen	Nov 01-03, 2011
► **Journal club**	
Participation in literature discussion every Monday in our group	2009-2012
► **Individual research training**	
Subtotal In-Depth Studies	*4.8 credits**

4) Personal development	date
► **Skill training courses**	
Interpersonal Communication	Oct 26-27, 2010
Competence Assessment	Nov 09, 2010
Advanced Course Guide to Scientific Artwork	Nov 04-05, 2010
► **Organisation of PhD students day, course or conference**	
Participated in organizing PhD autumn school 2011	Nov 01-03, 2011
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

This research was conducted at the laboratory of Phytopathology of Wageningen University and was financially supported by the Netherlands Organization for Scientific Research (NWO).

Cover:
Front and back: *Verticilium dahliae* transformatnts generated by random mutagenesis using *Agrobacterium tumefaciens*-mediated transformation**.**

Printed at Wohrman Print Service, Zutphen, The Netherlands