Identification of host-specific effectors mediating pathogenicity of the vascular wilt pathogen *Verticillium dahliae*

Jinling Li

Propositions

1. A single *Verticillium dahliae* effector of defoliating pathotype strains is responsible for pathogenicity on various host plants and for defoliation on cotton and olive. (this thesis)

2. LysM effectors can be used to engineer chimeric immune receptors with improved pathogen recognition capacities in plants. (this thesis)

3. Artificial intelligence will revolutionize all aspects of biology.

4. Looking at our body as a holobiome that comprises an entire microbial ecosystem will change medicinal practices.

5. Leadership training should be an integral part of any PhD program.

6. A good team player is someone who can always find a "niche" in a team.

Propositions belonging to the thesis, entitled

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Jinling Li

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Jinling Li

Thesis Committee

Promotor
Prof. Dr B.P.H.J. Thomma
Professor of Phytopathology
Wageningen University & Research

Co-promotor
Dr L. Faino
Researcher in Plant Pathology
Dept. Environmental Biology
Università Sapienza, Italy

Other members
Prof. Dr Y. Bai, Wageningen University & Research
Prof. Dr G. Doehlemann, University of Cologne, Germany
Prof. Dr M. Rep, University of Amsterdam
Dr R. Geurts, Wageningen University & Research

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Thesis

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Jinling Li
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Chapter 1

General introduction

Introduction

Plant diseases that are caused by pathogenic microorganisms have long been known as major biotic constraints to crop production worldwide (Strange and Scott, 2005; Fisher et al., 2012). For example, persistent and epidemic outbreaks of fungal and oomycete infections on important staple crops such as rice (blast caused by *Magnaporthe oryzae*), wheat (rust caused by *Puccinia graminis*), potato (late blight caused by *Phytophthora infestans*) and soybean (rust caused by *Phakospora pachyrizi*) result in enormous yield losses and pose a threat to global food security (Fisher et al., 2012). Current efforts to control plant diseases include the deployment of resistant varieties and the application of agrochemicals (Zhan et al., 2015). However, excessive agrochemical applications do not only cause environmental problems but also lead to the selection for resistance to the chemical agents in pathogen populations. Resistant plant varieties can lose their disease control efficacy due to rapid evolutionary changes in pathogen populations that give rise to novel genotypes that defeat host resistance (Zhan et al., 2015). A striking example is the outbreak of wheat stem rust in Africa caused by the highly aggressive *P. graminis* f. sp. *tritici* race Ug99 that has broken widely deployed resistance genes and is virulent on most of the wheat varieties grown worldwide (Pretorius et al., 2000). Due to its resistance-breaking capacities, this novel, virulent race of the pathogen represents a major threat to global wheat production (Singh et al., 2011) and emphasizes the importance of developing effective strategies to protect agricultural production systems from disease threats (Dodds, 2010). To develop innovative, effective disease management strategies, a thorough understanding of the mechanisms by which plant pathogens cause disease in their host plants is of vital importance (Gibriel et al., 2016; Gawehns et al., 2013).

Effectors: versatile molecules employed by microbial symbionts with diverse lifestyles

In nature, plants and microbes engage in an array of symbiotic relationships, ranging from pathogenic to mutualistic. Pathogenic interactions between plants and microbial pathogens are frequently described as ongoing arms races in which plants try to halt microbial ingress while pathogens attempt to continue the symbiosis (Jones and Dangl, 2006; Thomma et al., 2011; Cook et al., 2015). In such arms races, plants develop immune receptors of various types to detect pathogen invasion through sensing pathogen-derived or pathogen-induced molecular patterns of various nature that betray microbial invasion, also called invasion patterns, to activate appropriate immune responses (Cook et al., 2015; Rodriguez-Moreno et al., 2017). In turn, successful pathogens secrete so-called effector molecules to deregulate host immune responses, and thus support host colonization (Jones and Dangl, 2006; Rovenich et al., 2014; Cook et al., 2015). These effector molecules act either in the apoplastic space of the plant or inside plant cells to facilitate the infection process (De Wit, 2016; Büttner, 2016; Giraldo and Valent, 2013). Whereas most extensively studied effectors are proteinaceous molecules, typically small cysteine-rich proteins, other types of microbially-secreted molecules, such as secondary metabolites and small RNAs, have been assigned typical effector activities as well (Rodriguez-Moreno et al., 2018).

Many pathogens establish their initial phase of infection in the apoplast, which represents a hostile environment that contains various plant-secreted hydrolytic enzymes, including chitinases and glucanases, (Sánchez-Vallet et al., 2015; Rovenich et al., 2016). Host-secreted chitinases target fungal cell walls to release chitin fragments that activate host immune receptors on the one hand, and degrade fungal cell walls to induce cellular collapse, and thus inhibition of fungal proliferation, on the other hand (Sánchez-Vallet et al., 2015). To prevent this, the tomato leaf mould fungus *Cladosporium fulvum* secretes the apoplastic effector Avr4 that binds fungal cell walls through an invertebrate chitin-binding domain such that the cell wall chitin is inaccessible to chitinases and consequently protects it against hydrolysis (van den Burg et al., 2006; van Esse et al., 2007). Besides Avr4, *C. fulvum* also secretes the LysM domain-containing effector protein Ecp6 (extracellular protein 6) to interfere with chitin recognition and prevent the activation of chitin-induced plant immunity during host colonization (de Jonge et al., 2010). Structural analysis of Ecp6 revealed that the concerted action of two LysM domains (LysM1 and LysM3) results in the formation of a groove with ultra-high chitin-binding affinity that permits it outcompeting host immune receptors for chitin binding (Sánchez-Vallet et al., 2013). Additionally, the remaining singular LysM domain (LysM2) of Ecp6 binds chitin with lower affinity but can still perturb chitin-triggered immunity (Sánchez-Vallet et al., 2013). Because of its lower affinity, it has been hypothesized that, instead of scavenging of chitin fragments, LysM2 may suppress chitin-triggered immunity by interfering with the host chitin receptor complex formation that is needed to activate immune signalling (Sánchez-Vallet et al., 2013, 2015). Interestingly, in contrast to most fungal effectors that are lineage-specific or only occur in a limited set of related fungal species (Stergiopoulos and de Wit, 2009; de Jonge et al., 2011), conserved Ecp6 orthologs, termed LysM effectors, widely occur in the fungal kingdom (de Jonge and Thomma, 2009). Various fungal plant pathogens, such as the Septoria tritici blotch fungus *Zymoseptoria tritici*, the Brassicaceae anthracnose fungus *Colletotrichum higginsianum*, the rice blast fungus *M. oryzae* and the vascular wilt fungus *Verticillium dahliae* have also been shown to secrete LysM effectors to perturb chitin-triggered immunity and promote fungal host colonization (Marshall et al., 2011; Mentlak et al., 2012; Takahara et al., 2016; Kombrink et al., 2017). Particular LysM effectors, such as Mg1LysM and Mg3LysM from *Z. tritici* and Vd2LysM from *V. dahliae*, were found to additionally protect fungal hyphae against plant hydrolytic enzymes (Marshall et al., 2011; Kombrink et al., 2017).

Besides various hydrolytic enzymes, the host apoplast also contains a diverse group of proteases that contribute to basal defence (van Esse et al., 2008). Hence, some microbial effectors target and inhibit such proteases in order to promote host colonization (van Esse et al., 2008; Jashni et al., 2015). For example, *C. fulvum* secretes the cysteine protease inhibitor Avr2 that inhibits a set of tomato proteases in order to promote virulence (van Esse et al., 2008). The smut fungus *Ustilago maydis* inhibits apoplastic proteases of its maize host via the secreted effector Pit2 (Mueller et al., 2013). In addition, the oomycete pathogen *P. infestans* effector AVRblb2 targets the host papain-like cysteine protease C14 and specifically prevents its secretion (Bozkurt et al., 2011). Therefore, the apoplast is a dynamic battlefield for various microbial effectors to modulate apoplastic immunity and thus support successful infection (Du et al., 2016).

In addition to apoplastic effectors, various plant pathogens are able to translocate their effectors into the host cytoplasm where they modulate diverse cellular processes to favour pathogen infection (Giraldo and Valent, 2013; Grant et al., 2006). The mechanism of effector delivery to host cells has been well characterized in plant pathogenic bacteria (Galán et al., 2014). It has been shown that many bacterial plant pathogens (e.g. *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas* spp. and Erwinia spp.) have evolved a "syringe-like" type III secretion system (T3SS) to directly deliver effectors into the host cytosol (Grant et al., 2006; Büttner and He, 2009). Although various fungal and oomycete plant pathogens also secrete effectors that operate inside host cells (Djamei et al., 2011; Yaeno et al., 2011), the mechanisms by which effectors are delivered into host cells remain poorly understood (Petre and Kamoun, 2014). Many highly divergent oomycete effectors contain an N-terminal RxLR motif (Tyler et al., 2006; Jiang et al., 2008), which has been implicated in effector uptake (Whisson et al., 2007; Dou et al., 2008). Kale and colleagues (2010) proposed that the RxLR motif mediates effector uptake by binding to host cell surface phosphatidylinositol-3-phosphate (PI3P). Upon binding to PI3P, oomycete RxLR effectors are presumed to enter host cells via vesicle-mediated endocytosis (Kale et al., 2010; Kale and Tyler, 2011). However, this model remains controversial as several studies challenge reproducibility of the assays (Wawra et al., 2013; Petre and Kamoun, 2014). A recent study showed that the RxLR motif of the oomycete effector AVR3a is cleaved off prior to secretion, which suggests a role for the RxLR motif in the effector secretion from the pathogen, rather than a direct role in mediating host cell entry (Wawra et al., 2017). So far, no conserved motifs involved in effector uptake have been identified in fungal effectors (Rafiqi et al., 2012). Direct evidence for fungal effector translocation into plant cells comes from live fluorescence microscopy studies in the rice blast pathogen *M. oryzae* (Giraldo and Valent, 2013). Live-cell imaging of transgenic *M. oryzae* strains expressing fluorescently-tagged effector proteins has allowed tracking of secreted effectors during pathogen invasion (Yi and Valent, 2013). Several fluorescently-labelled effectors have been shown to preferentially accumulate in a unique host-pathogen interfacial structure, called the biotrophic interfacial complex (BIC), before being translocated into the rice cytoplasm (Khang et al., 2010; Giraldo et al., 2013). Interestingly, *M. oryzae* has evolved a distinct, Golgi-independent secretion system to deliver effectors to BICs (Giraldo et al., 2013). Unravelling the mechanisms how filamentous pathogens deliver their effectors into plant cells remains an important challenge in the field of molecular plant pathology.

Plant cell surface-localized immune receptors that comprise receptor-like proteins (RLPs) and receptor kinases (RKs) monitor the apoplastic space and sense various pathogen-derived ligands, such as fungal chitin, bacterial flagellin, elongation factor Tu, to activate immune responses and prevent microbial ingress (Jones and Dangl, 2006; Thomma et al., 2011). In order to establish themselves, adapted pathogens deliver cytoplasmic effectors to not only target cell surface-localized immune receptors but also interfere with important downstream immune signalling activated by immune receptors (de Jonge et al., 2011; Rovenich et al., 2014; Macho and Zipfel, 2015; Toruño et al., 2016). Some bacterial effectors have been shown to directly target immune receptors for degradation (Macho and Zipfel, 2015). For example, the bacterial effector protein AvrPtoB functions as an E3 ubiquitin ligase that promotes proteasome-mediated degradation of the Arabidopsis RK-type immune receptors FLS2 (flagellin-sensing 2) and CERK1 (chitin elicitor

receptor kinase 1) (Gohre et al., 2008; Gimenez-Ibanez et al., 2009). Upon ligand perception, RK-type immune receptors directly associate with members of the receptor-like cytoplasmic kinases (RLCKs), which are thought to be responsible for transducing signals from extracellular ligand perception into intracellular downstream signalling via phosphorylation events (Couto and Zipfel, 2016; Tang et al., 2017). Several studies have shown that multiple bacterial cytoplasmic effectors specifically target RLCKs to interfere with immune signalling (Lee et al., 2013). For instance, the *P. syringae* cysteine protease effector AvrPphB proteolytically cleaves RLCK VII subfamily proteins BIK1, PBL1, and other RLCKs (Zhang et al., 2010). Additionally, the cytoplasmic effector AvrAC from *X. campestris* pv. *campestris* is an uridylyl transferase that uridylylates and inhibits kinase activity of two closely related RLCKs, BIK1 and RIPK, thereby blocking downstream immune signalling (Feng et al., 2012). In addition to interacting with RLCK members, a number of microbial effectors target and inactivate the mitogen-activated protein kinases (MAPKs) cascades that transduce and amplify the pathogen-derived signals from upstream immune receptors (Bi and Zhou, 2017; Meng and Zhang, 2013). For instance, the *P. syringae* cytoplasmic effector HopAI1 directly dephosphorylates MAPKs, MPK3 and MPK6 to inhibit MPK activation (Zhang et al., 2007). Another *P. syringae* effector, HopF2, can target Arabidopsis MAPK kinase 5 (MKK5) and suppresses MKK5 phosphorylation through its ADP-ribosyltransferase enzymatic activity (Wang et al., 2010). Perturbation of plant MAPK cascades is not limited to bacterial pathogens (Bi and Zhou, 2017), as the *P. infestans* cytoplasmic RxLR effector PexRD2 was found to interact with host MAPKKKε to perturb immune signalling (King et al., 2014).

Plant hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have been shown to play important roles in plant defence, and several cytoplasmic effectors were shown to target and modify the biosynthesis of, and signalling pathways activated by, these defence hormones (Kazan and Lyons, 2014). For example, *U. maydis* secretes the chorismate mutase Cmu1 into the cytosol of maize plants to interfere with SA biosynthesis (Djamei et al., 2011). Cmu1 presumably acts together with the maize cytosolic chorismate mutase ZmCm2 to alter the flow of chorismate from the plastid to the cytosol, consequently diminishing the available chorismate for SA biosynthesis in plastids (Djamei et al., 2011). Interestingly, secreted chorismate mutases have been found in many plant-associated microbes, pointing towards a common strategy for host manipulation (Djamei et al., 2011). In addition, the effectors Pslsc1 and Vdlscl that are secreted by the two taxonomically distinct filamentous pathogens *P. sojae* and *V. dahliae*, respectively, are isochorismatases that can hydrolyze the SA precursor isochorismate to actively suppress SA accumulation (Liu et al., 2014). Besides targeting SA signalling, cytoplasmic effectors that target JA or ET signalling have been described as well (Patkar et al., 2015; Kim et al., 2013; Ma and Ma, 2016). For instance, *M. oryzae* secretes a JA monooxygenase effector to convert host-derived JA to 12-hydroxyjasmonic acid to perturb JA signalling and attenuate host innate immunity (Patkar et al., 2015). XopD, a cytoplasmic effector from the bacterial tomato pathogen *X. euvesicatoria*, directly targets and desumoylates the tomato ethylene responsive transcription factor SlERF4 to suppress ethylene-induced immunity and promote pathogen infection (Kim et al., 2013). These examples demonstrate that the manipulation of plant hormone signalling by effectors is an important strategy for plant pathogens to establish infection.

Effectors are not uniquely employed by plant pathogens, as endophytes and mutualistic microbes similarly employ effectors to establish their association with plants (Rovenich et al., 2014; Cook et al., 2015). For instance, the symbiotic arbuscular mycorrhizal fungus *Glomus intraradices* secretes effector protein SP7 to interact with pathogenesis-related transcription factor ERF19 and attenuates ethylene-mediated plant defence, thereby promoting fungal colonization in host roots (Kloppholz et al., 2011). The mutualistic fungus *Laccaria bicolor* genome was found to contain hundreds of secreted proteins (Martin et al., 2008). Of these, the effector MiSSP7 has been shown to perturb JA-mediated host immune responses and facilitate symbiosis through its interaction with the host plant JA signalling repressor PtJAZ6 (Plett et al., 2014). These findings show that, like their pathogenic counterparts, mutualistic fungi similarly secrete effectors to subvert host immunity for establishing successful symbioses.

All microbial symbionts establish themselves in environments that also comprise other microbes, including antagonists (Rovenich et al., 2014). Likely, microbes may secrete effector molecules that act in self-defense and competition with other microbiome co-inhabitants (Rovenich et al., 2014; Snelders et al., 2018). For instance, the effector Zt6, secreted by the wheat pathogen *Z. tritici*, was found to possess highly potent cytotoxic activity against various prokaryotic and eukaryotic microbes but not against *Z. tritici* itself (Kettles et al., 2017). Therefore, Zt6 may act in microbial competition and niche protection (Kettles et al., 2017; Snelders et al., 2018).

In conclusion, effectors are highly versatile molecules that play essential roles in niche establishment of symbionts with diverse lifestyles. Clearly, functional characterization of effector molecules and deciphering their role could provide novel insight into how microbes establish their associations with host plants (Rovenich et al., 2014).

Plant pathogen effector identification

As effector molecules are pivotal for the infection process of plant pathogens, research on deciphering microbial pathogenesis has focused on the identification and characterization of effectors from various plant pathogens (de Jonge et al., 2011; Gibriel et al., 2016). Prior to the advent of genomic sequencing, map-based cloning approaches and biochemical analyses of pathogen secretomes during infection have been pursued to identify novel effectors (Stergiopoulos and de Wit, 2009). For example, proteins AvrPi-ta and ACE1 from the rice blast fungus *M. oryzae*, as well as effector proteins AvrLm1 and AvrLm6 from the oilseed rape pathogen *Leptosphaeria maculans*, were identified by map-based cloning (Orbach et al., 2000; Böhnert et al., 2004; Gout et al., 2006; Fudal et al., 2007). Proteomic analysis of apoplastic fluids that were isolated from *C. fulvum*-infected tomato leaves by using polyacrylamide gel electrophoresis and mass spectrometry led to identification of several effector proteins of *C. fulvum* (Joosten and de Wit, 1988; Wubben et al., 1994; Laugé et al., 1998, 2000; Bolton et al., 2008). Using a similar approach, a number of SIX (secreted in xylem) effector proteins from the tomato wilt fungus *Fusarium oxysporum* f.sp. *lycopersici* were identified in the xylem sap of infected tomato plants (Rep et al., 2004; Houterman et al., 2007, 2008).

Recent advances in genome sequencing technologies and rapid development of new bioinformatics algorithms enable genomics-based discovery of effector candidates (Gibriel et al., 2016). The majority of microbial effector proteins are secreted via the classical endoplasmic reticulum/Golgi-dependent secretion route, which requires the N-terminal signal peptide for extracellular secretion (Von Heijne, 1990). Therefore, effector candidates can be identified by computational prediction from pathogen genomes through querying for the presence of N-terminal signal peptides (de Jonge et al., 2011; Gibriel et al., 2016). In addition, conserved amino acid motifs that are specific to particular groups of effectors have been used for effector identification as well (Gibriel et al., 2016). For instance, in several oomycete pathogens, consensus N-terminal RxLR and LxLFLAK motifs that have been implicated in effector uptake can be queried to identify RxLR and Crinker (CRN) effector genes (Tyler et al., 2006; Schornack et al., 2010; Haas et al., 2009). In contrast, prediction of effector genes from fungal genomes is less straightforward due to the lack of conserved motifs (Sperschneider et al., 2015). Recently, the machine learning program 'EffectorP' was developed by using sequences of experimentally validated fungal effectors to improve effector prediction from secretomes (Sperschneider et al., 2016). Sperschneider et al. (2016) showed that in addition to presence of N-terminal secretion signal peptide, low molecular weight, overall protein charge, as well as serine and tryptophan content were important features that can be used for more precise fungal effector prediction.

The relatively low cost of present-day genome sequencing (Faino and Thomma 2014) has made it possible to sequence (populations of) plant pathogen species that can be leveraged for effector discovery through comparative genomics (Thomma et al., 2015; Jones et al., 2018). In comparative genomics, genome sequences are compared in order to reveal the similarities and differences between selected genomes and subsequently link these to phenotypic differences (Jones et al., 2018). For example, genome comparisons of *U. maydis* and *Sporisorium reilianum*, two smut fungi that infect maize but cause different symptoms, revealed 43 distinct genomic regions that primarily contain species-specific genes encoding putative effectors (Schirawski et al., 2010). Functional analyses confirmed that some of these effectors in *U. maydis* contribute to virulence on maize (Schirawski et al., 2010). In another study, comparative analysis of the genome sequences of three Fusarium species with different host ranges, namely *F. oxysporum* f.sp. *lycopersici* (a tomato pathogen), *F. graminearum* (a cereal pathogen) and *F. verticillioides* (another cereal pathogen), revealed that four dispensable chromosomes are specific to the tomato-infecting *F. oxysporum* f. sp. *lycopersici* (Ma et al., 2010). Interestingly, pathogenicity of *F. oxysporum* f. sp. *lycopersici* towards tomato can be specifically attributed to one dispensable chromosome, which contains a number of effector genes (Ma et al., 2010). These studies highlight the power of comparative genomics for the identification of virulence effectors from various plant pathogens.

Comparative genomics can also be used to identify effectors that are recognized by host immune receptors and thus act as avirulence factors (Gibriel et al., 2016). The tomato immune receptor Ve1 mediates resistance to race 1 strains of *V. dahliae*, while strains that are not contained by Ve1 are assigned to race 2 (Schaible et al., 1951; Kawchuk et al., 2001; Fradin et al., 2009). Comparative analyses of race 1 and race 2 strains revealed 50 kb of sequence that is specifically present in race 1 strains, containing only a single effector gene that is highly expressed *in planta*, named

Ave1 (for Avirulence on Ve1 tomato). Functional analyses confirmed that recognition of effector Ave1 by the immune receptor Ve1 mediates resistance to race 1 strains of *V. dahliae* (de Jonge et al., 2012). Using a similar approach, the effector protein AVRFOM2 that is recognized by melon immune receptor Fom-2 was identified by comparative genome analysis of strains of the melon wilt fungus *F. oxysporum* f. sp. *melonis* (Schmidt et al., 2016). More recently, Salcedo et al. (2017) used comparative whole-genome analysis of chemically mutagenized and natural *P. graminis* f. sp. *tritici* isolates to identify the effector protein AvrSr35 that is recognized by the corresponding wheat resistance protein Sr35.

The vascular wilt pathogen *V. dahliae*

The *Verticillium* genus comprises ten soil-borne asexual species of Ascomycete fungi that differ significantly in their lifestyles, ranging from saprophytic to pathogenic (Fradin and Thomma, 2006; Klosterman et al., 2009; Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014; Klimes et al., 2015). Among these, *V. isaacii*, *V. klebahnii*, *V. zaregamsianum*, *V. tricorpus*, and *V. nubilum* are mostly considered saprophytes that occasionally cause opportunistic infections of plants (Inderbitzin et al., 2011; Gurung et al., 2015; Fradin and Thomma, 2006; Seidl et al., 2015). The remaining species *V. dahliae*, *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae*, and *V. longisporum* are plant pathogens that mostly cause vascular wilt diseases on crops and lead to significant crop losses (Pegg and Brady, 2002; Fradin and Thomma, 2006; Inderbitzin et al., 2011; Depotter et al., 2016). However, despite the fact that the latter five species are all pathogenic, they differ significantly in host range. *V. dahliae* is the most notorious plant pathogen with an extremely wide host range that comprises hundreds of plant species, including economically important crops like tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), olive (*Olea europaea*), sunflower (*Helianthus annuus*) and tobacco (*Nicotiana tabacum*) (Figure 1) (Pegg and Brady, 2002; Fradin and Thomma, 2006). In contrast, other pathogenic *Verticillium* species have more restricted host ranges as, for instance, *V. albo-atrum* mainly infects hop, soybean, tomato and potato (Fradin and Thomma, 2006), while *V. longisporum* generally causes disease on brassicaceous plant hosts such as oilseed rape and cauliflower (Depotter et al., 2016), and *V. alfalfae* only infects lucerne (Inderbitzin et al., 2011). The economic losses caused by Verticillium wilts on the 20 most affected hosts collectively amount up to €3 billion worldwide (Depotter et al., 2016).

V. dahliae typically colonizes plant water-conducting xylem vessels, thus disrupting transportation of water and minerals to the foliage which may cause typical Verticillium wilt symptoms in infected plants (Fradin and Thomma, 2006; Yadeta and Thomma, 2013). Verticillium wilt disease control is particularly challenging since traditional fungicides cannot reach the pathogens once they have entered xylem vessels (Fradin and Thomma, 2006; Klosterman et al., 2011). Moreover, *V. dahliae* can survive in the soil for long periods of time due to the formation of resilient resting structures, called microsclerotia, and therefore agricultural practices such as crop-rotation do not result in effective crop protection. Although genetic resistance is the preferred strategy for disease management, only a few Verticillium wilt resistance genes have been identified (Schaible et al., 1951; Simko et al., 2004; Hayes et al., 2011; Song et al., 2017). The substantial economic impact of Verticillium wilt disease combined with the lack of effective disease control treatments substantiates the need for developing novel disease control strategies (Fradin and Thomma, 2006).

Figure 1. Disease symptoms caused by *V. dahliae* on various host plants. Verticillium wilt symptoms caused by *V. dahliae* on cotton (**A**), tomato (**B**), tobacco (**C**), olive (**D**) and sunflower (**E**). Photos are courtesy of Longfu Zhu (panel **A**), Yin Song (panel **B-C**), Jesús Mercado-Blanco (panel **D**), Shuqing Yang (panel **E**).

To design novel control strategies to combat Verticillium wilt disease, a thorough understanding of the molecular mechanisms underlying *V. dahliae* pathogenesis is of fundamental importance. A number of pathogenicity and virulence factors of *V. dahliae* have been identified (Zhou et al., 2012; de Jonge et al., 2012; Santhanam and Thomma, 2013; Santhanam et al., 2017; Kombrink et al., 2017; Liu et al., 2014; Klimes et al., 2015). For instance, the transcriptional regulator Sge1 has been shown to differentially regulate expression of a number of putative effector genes *in planta* and is required for radial growth, conidiospore production, and pathogenicity (Santhanam and Thomma, 2013). Also the transcriptional regulator Vta2 was found to be required for pathogenicity (Tran et al., 2014). Vta2 suppresses the formation of microsclerotia and controls the expression of effector genes that may be involved in *V. dahliae* pathogenicity (Tran et al., 2014). In addition, a rhamnose synthase of *V. dahliae*, which functions in the formation rhamnose-containing glycans in the fungal cell wall, was found to be essential for pathogenicity on tomato and *Nicotiana benthamiana* (Santhanam et al., 2017). Necrosis and ethylene-inducing-like proteins (NLPs) are a group of conserved effectors that are widely spread in bacteria, fungi, as well as oomycetes (Gijzen

and Nürnberger, 2006; Oome and Van den Ackerveken, 2014). Interestingly, most ascomycete plant pathogens contain up to three *NLP* genes, while the *V. dahliae* genome was predicted to have eight *NLP* genes (Klosterman et al., 2011). Members of the expanded NLP family have been shown to be associated with functional diversification, as they display differential cytotoxicity (Zhou et al., 2012; Santhanam et al., 2013). Surprisingly, only NLP1 and NLP2 show cytotoxic activity, while the remaining NLP family members are non-cytotoxic (Zhou et al., 2012; Santhanam et al., 2013). Further evidence for functional diversification within the *V. dahliae* NLP family comes from the differential contributions of NLP1 and NLP2 to fungal virulence on distinct host plants. Although both NLP1 and NLP2 contribute to virulence of *V. dahliae* strain JR2 on tomato and Arabidopsis (Santhanam et al., 2013), neither is required for virulence of strain V592 on cotton (Zhou et al., 2012). Moreover NLP1, but not NLP2, is required for virulence of *V. dahliae* strain JR2 on *N. benthamiana*, owing to the fact that *NLP1*, but not *NLP2*, is expressed on this plant species (Santhanam et al., 2013). Thus, it has been proposed that the functional diversification of *V. dahliae* NLP effectors may contribute to the broad host range of the pathogen (Zhou et al., 2012; Santhanam et al., 2013; Klimes et al., 2015).

Through comparative genomics, highly variable lineage-specific (LS) genomic regions that are only found in subsets of the *V. dahliae* population were identified (de Jonge et al., 2013; Faino et al., 2016). Interestingly, these LS genomic regions are greatly enriched in *in planta*-induced effector genes, targeted deletion of which typically results in significantly compromised fungal aggressiveness, suggesting that these effectors contribute to host colonization (de Jonge et al., 2012, 2013; Kombrink et al., 2017). Remarkably, *V. dahliae* LS regions are also enriched in transposable elements (TEs) (Faino et al., 2016). TEs can change their genomic position, which may induce gene disruption, or cause double-strand DNA breaks by TE excision (Seidl and Thomma, 2014). Therefore, TEs may actively contribute to the genetic plasticity of LS regions (Faino et al., 2016; Seidl and Thomma, 2017). In addition to active role in promoting genomic plasticity, TEs can contribute to genome evolution of *V. dahliae* in a passive manner as well (Faino et al., 2016). It has been shown that highly variable LS regions evolved by genomic rearrangements that are mediated by erroneous double-strand repair, frequently utilizing TEs as substrate due to their abundance and high degree of sequence similarity (Faino et al., 2016). Thus, it has been proposed that TEs are important drivers for adaptive genome evolution in *V. dahliae* (Faino et al., 2016; Seidl and Thomma, 2014; Seidl and Thomma, 2017).

Thesis outline

Upon infection of its hosts, *V. dahliae* secretes effectors to enable host colonization. The aim of the research described in this thesis is to gain more insight into molecular mechanisms of *V. dahliae* pathogenesis, with a specific focus on the discovery of novel effectors that contribute to the establishment of *V. dahliae* infections on diverse host plants.

On particular host plants, *V. dahliae* strains are assigned to so-called "pathotypes". For example, *V. dahliae* strains that are highly virulent and cause rapid and severe defoliation on cotton, olive, okra (*Hibiscus esculentus*) and pistachio (*Pistacia vera*) are referred to as strains of the defoliating (D) pathotype, whereas strains that are moderately virulent and induce wilting symptoms

without defoliation on these hosts are assigned to the non-defoliating (ND) pathotype. However, the molecular basis that underlies differential virulence between D and ND pathotype strains remained elusive. In **Chapter 2**, we employed comparative genomics and transcriptomics to identify *V. dahliae* genes that are responsible for defoliation symptoms. To this end, genome sequences of multiple D pathotype and ND pathotype strains were determined. Subsequently, candidate genes were subjected to functional analyses to assess their role in defoliation of cotton and olive.

Chapter 3 focussed on elucidating the molecular basis of *V. dahliae* pathogenicity on tomato. For this purpose, comparative genomics of pathogenic and non-pathogenic *V. dahliae* strains was performed. Targeted deletion of candidate genes was pursued to identify pathogenicity factors. Moreover, candidate genes were introduced into a non-pathogenic *V. dahliae* strain and in the saprophytes *V. tricorpus* and *V. nubilum* to test whether these recipient strains gain pathogenicity on tomato.

In **Chapter 4**, genome comparisons of a sunflower pathogenic strain with several non-pathogenic strains were carried out to identify *V. dahliae* genes that contribute to virulence on sunflower. Subsequently, we examined the role of candidate genes in virulence through gene deletion analysis.

Despite the notion that most *V. dahliae* strains have a broad host range, the pathogenic potential and the severity of symptoms that are induced on a particular host plant may vary considerably between *V. dahliae* strains. In **Chapter 5**, we set out to determine whether *V. dahliae* strains that infect the same host plant also have similar effector profiles. To this end, we identified the core and lineage-specific (LS) effector repertoires in a collection of *V. dahliae* strains for which we also test the ability to cause disease on tomato, cotton, sunflower, Arabidopsis, and *N. benthamiana*.

Previous studies have shown that the *C. fulvum* effector Ecp6 outcompetes host immune receptors for chitin binding (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). In **Chapter 6**, I generated chimeric chitin receptors by replacing the LysM domain-containing ectodomain of the Arabidopsis chitin receptors with the LysM domains of Ecp6. I further investigated whether expression of the chimeric chitin receptors in Arabidopsis results in enhanced chitin-induced immunity to fungal infection.

Finally, in **Chapter 7**, the results described in this thesis are summarized and discussed in a broader perspective to illustrate the role of *V. dahliae* effectors in fungal pathogenicity.

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Chapter 2

A single *Verticillium dahliae* effector is responsible for the highly aggressive defoliating pathotype on cotton and olive

Jinling Li[1,#], Luigi Faino[1,#], Leonie Weber[2], Tingli Liu[3], Mojtaba Keykha Saber[4], Carmen Gómez-Lama Cabanás[5], Antonio Valverde-Corredor[5], Grardy C.M. van den Berg[1], Hui Tian[1], Jelle Hiemstra[4], Jesús Mercado-Blanco[5], Volker Lipka[2], Baolong Zhang[3,$], Longfu Zhu[6,$], and Bart P.H.J. Thomma[1,$,*]

[1]Laboratory of Phytopathology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands
[2]Department of Plant Cell Biology, Albrecht-von-Haller-Institute, Georg-August-University Göttingen, Julia-Lermontowa-Weg 3, 37077, Göttingen, Germany
[3]Provincial Key Laboratory of Agrobiology, Jiangsu Academy of Agricultural Sciences, Nanjing 210095, China
[4]Wageningen Plant Research, Wageningen University & Research, Zetten, Netherlands
[5]Department of Crop Protection, Institute for Sustainable Agriculture (CSIC), Avenida Menéndez Pidal s/n, Campus 'Alameda del Obispo', 14004, Córdoba, Spain.
[6]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, Hubei 430070, China

[#] These authors contributed equally
[$] These authors contributed equally

[*]**Author for correspondence**: Bart P.H.J. Thomma, Laboratory of Phytopathology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands. Tel. 0031-317-484536, Fax 0031-317-483412, e-mail: bart.thomma@wur.nl

Abstract

Plant pathogens from diverse taxonomic origins have been shown to secrete effector molecules to manipulate host physiology and support successful infection. *Verticillium dahliae* is a soil-borne fungal pathogen that causes Verticillium wilt disease in a wide variety of economically important plant species. On particular host plants, *V. dahliae* strains are assigned to so-called pathotypes. For example, *V. dahliae* strains that are highly virulent and cause rapid, severe defoliation on cotton (*Gossypium hirsutum*), olive (*Olea europaea*), okra (*Hibiscus esculentus*) and pistachio (*Pistacia vera*) are referred to as strains of the defoliating (D) pathotype, whereas strains that are moderately virulent and only induce wilting symptoms without defoliation on these hosts are assigned to the non-defoliating (ND) pathotype. In this study, we used comparative genomics and transcriptomics to elucidate the molecular basis that are responsible for the ability of D pathotype strains to cause defoliation symptoms. Comparative genomics revealed two D pathotype-specific candidate effector genes that are highly expressed during host colonization. Intriguingly, these two genes appeared to be identical copies that arose by a segmental duplication. We demonstrate that this duplicated effector gene is a pathogenicity factor on cotton and olive. Furthermore, application of heterologously produced effector protein to cotton seedling induces defoliation, showing that the effector protein is directly responsible for these symptoms.

Introduction

Verticillium dahliae is a soil-borne fungal pathogen that colonizes the xylem vessels of a wide range of dicotyledonous host plants, including economically important crops such as tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*) and olive (*Olea europaea*), resulting in Verticillium wilt disease (Fradin and Thomma, 2006). Infection typically starts by penetration of the root, after which the fungus enters and colonizes xylem vessels, affecting water transport, and causing characteristic symptoms that include wilting, stunting, chlorosis and early senescence (Fradin and Thomma, 2006; Klosterman et al., 2009). Due to the broad host range of the pathogen, the scarcity of disease resistance in crop germplasm and the long-term prevalence of its resilient survival structures in the soil, agricultural practices such as crop rotation do not result in efficient disease management (Fradin and Thomma, 2006). Disease control is particularly difficult as fungicides are generally ineffective to eliminate the fungus from infected plants once it has entered xylem vessels (Fradin and Thomma, 2006; Inderbitzin and Subbarao, 2014).

Although *V. dahliae* causes wilt disease in a broad range of host plants, virulence capacities and the severity of symptoms that are induced on host plants can vary considerably between individual strains. For instance, particular strains of *V. dahliae* can cause severe symptoms that include defoliation on cotton, olive, okra (*Hibiscus esculentus*) and pistachio (*Pistacia vera*), while other strains cause milder wilting symptoms and do not cause defoliation (Schnathorst and Mathre, 1966; Schnathorst and Sibbett, 1971a; Jiménez-Díaz et al., 2011; Korolev et al., 2008; Hadizadeh and Banihashemi, 2007). Consequently, on particular host plants, *V. dahliae* strains are assigned to "pathotypes"; groups of *V. dahliae* strains that show differential virulence capacities and disease symptoms (Schnathorst and Mathre, 1966; Schnathorst and Sibbett, 1971b). As such, *V. dahliae* strains that are highly virulent and cause rapid and severe defoliation on cotton, olive, okra and pistachio are referred to as strains of the defoliating (D) pathotype, whereas strains that are moderately virulent and only induce mild wilting symptoms without defoliation are assigned to the non-defoliating (ND) pathotype (Schnathorst and Mathre, 1966). The currently increasing prevalence of the highly virulent D pathotype strains poses a significant threat to cotton and olive plantations worldwide (Dervis et al., 2010; López-Escudero et al., 2004; Milgroom et al., 2016; Leyva-Pérez et al., 2017). Recent studies suggest that D pathotype strains originated once in North America and subsequently spread to other continents by dispersal of contaminated plant commodities (Milgroom et al., 2016; Jiménez-Díaz et al., 2017). Despite many efforts to develop molecular markers to robustly and accurately differentiate D and ND pathotype strains (Pérez-Artés et al., 2000; Mercado-Blanco et al., 2002, 2003; Collins et al., 2005), the molecular basis for the difference in aggressiveness between D and ND pathotype strains remains unknown so far.

In order to establish disease on their host plants, adapted pathogens secrete so-called effector molecules to modulate plant immunity and promote successful infection (Jones and Dangl, 2006; de Jonge et al., 2011; Rovenich et al., 2014; Cook et al., 2015). It is becoming increasingly apparent that secreted effector molecules play crucial roles in the virulence of diverse plant pathogens, including bacteria, fungi, oomycetes and nematodes (de Jonge et al., 2011; Rovenich et al., 2014; Rodriguez-Moreno et al., 2018; Mitchum et al., 2013). For example, chitin-binding lysin

motif (LysM) effectors occur in a wide variety of fungal plant pathogens (de Jonge and Thomma, 2009), and have been demonstrated to contribute to virulence of various fungal pathogens by suppressing chitin-induced plant immunity during host colonization (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Takahara et al., 2016; Kombrink et al., 2017). Thus, it is well established that comprehensive identification of effector repertoires and determination of their modes of action is key to decipher virulence mechanisms of plant pathogens, and ultimately to design effective disease management strategies (Gibriel et al., 2016).

In the past, various methods, including proteomic and genetic approaches, have been used to identify effectors from plant pathogens (Laugé et al., 1998, 2000; Bolton et al., 2008; Böhnert et al., 2004; Fudal et al., 2007). Recently, genomics strategies have facilitated large-scale and comprehensive effector discovery in various plant pathogens (Hu et al., 2011; Thomma et al., 2016). Moreover, comparative genomics of closely related pathogens or different strains of the same pathogenic species have led to the identification of core and lineage-specific effector repertoires (Gibriel et al., 2016). For example, genome comparisons of *Ustilago maydis* and *Sporisorium reilianum*, two smut fungi that infect maize but cause different symptoms, revealed 43 distinct genomic regions that primarily contain species-specific genes encoding putative effectors. Functional analyses confirmed that some of these effectors in *U. maydis* contribute to virulence on maize (Schirawski et al., 2010). Similarly, comparative genomics studies on *V. dahliae* have facilitated the identification of key virulence factors that mediate successful host infection (Klosterman et al., 2011; de Jonge et al., 2012, 2013; Kombrink et al., 2017). We previously identified extensive, large scale chromosomal rearrangements, likely mediated by erroneous double-stranded break repair mechanisms, that gave rise to highly variable lineage-specific (LS) genomic regions that are only present in a part of the *V. dahliae* population (de Jonge et al. 2013; Faino et al., 2016). Interestingly, these LS genomic regions are greatly enriched for *in planta*-induced effector genes that contribute to host colonization (de Jonge et al., 2013). One of best characterized LS effector is the *V. dahliae* effector Ave1 that is important for fungal aggressiveness on tomato plants that lack the race-specific resistance gene *Ve1*, as well as on Arabidopsis (de Jonge et al., 2012), tobacco and cotton (Song et al., 2018). Another LS effector, Vd2LysM, was found to contribute to virulence of strain VdLs17 on tomato (Kombrink et al., 2017).

The aim of this study was to elucidate the molecular basis for the difference in aggressiveness between *V. dahliae* strains that belong to the D and ND pathotype, respectively. To this end, comparative genomics was performed to identify genomic features in strains of the D pathotype that confer the ability to cause defoliation.

Materials and methods

Disease assays

Cotton (*Gossypium hirsutum* cv. Xinluza063), *Nicotiana benthamiana*, and *Arabidopsis thaliana* (Col-0) plants were grown under controlled greenhouse conditions (Unifarm, Wageningen, the Netherlands). Olive (*Olea europaea* cv. Picual) plants were grown in a greenhouse under natural lighting and day/night temperature of 27/21°C (Córdoba, Spain). Two-week-old cotton and *A. thaliana* seedlings, three-week-old *N. benthamiana* seedlings and eight-month-old olive plants were used for inoculation assays.

Verticillium dahliae strains (Table S1) were grown on potato dextrose agar (PDA) at 22°C for 7-10 days. Conidiospores were collected from PDA plates and washed with tap water for inoculation assays. *V. dahliae* disease assays on cotton, *N. benthamiana* and *A. thaliana* plants were performed using the root-dip inoculation method as previously described (Fradin et al., 2009; Song et al., 2018). Disease assays on olive plants were performed as previously described (Leyva-Pérez et al., 2017; Gómez-Lama Cabanás et al., 2018).

Disease symptoms were scored up to 21 (*A. thaliana* and *N. benthamiana*), 28 (cotton), or 132 (olive) days post inoculation (dpi). To this end, *A. thaliana* and *N. benthamiana* plants were photographed, and Image J was used to determine the canopy area of *N. benthamiana*, and the total rosette area of *A. thaliana*. A rectilinear scale was used to measure the height of cotton plant. The cotton defoliation symptoms classified as 0 (0% leaf drop off), 1 (<25% leaf drop off), 2 (<50% leaf drop off), 3 (<75% leaf drop off) and 4 (<100% leaf drop off) (Liu et al., 2014; Zhang et al., 2017). Fungal biomass in *N. benthamiana*, *A. thaliana* and cotton were determined with real-time PCR as previously described (Fradin et al., 2009; Song et al., 2018). Disease severity in inoculated olive plants were monitored by assessing disease symptoms twice a week based on the percentage of leaves affected by chlorosis, or defoliation: 0 (plant without Verticillium wilt symptoms), 1 (overall 1-33% leaves affected), 2 (overall 34-66% leaves affected), 3 (overall 67-100% leaves affected), 4 (the plant was dead) (González et al., 2015; Gómez-Lama et al., 2018). Disease parameters such as disease incidence (DI), mortality (M), and disease intensity index (DII) were also calculated for each treatment. Specifically, the disease intensity index (DII) was defined as DII = (Σ$Si \times Ni$)/(4 × Nt) (Si = severity of symptoms; Ni = the number of plants with Si symptoms severity; Nt = the total number of plants). A final DI was also established as the percentage of affected plants at the end of the bioassay. Finally, the area under the disease progress curve (AUDPC) of DII plotted over time (days; Campbell and Madden, 1990) and the final severity were calculated. Analysis of variance (ANOVA) analyses were performed, and mean values were compared by the Fisher's protected LSD at P = 0.05 using the Statistix program (Version 10.0 for Windows. Analytical software 1985-2013).

Genome sequencing and deep transcriptome sequencing

The genome of *V. dahliae* strain CQ2 (Table S1) was sequenced using long-read PacBio Single-Molecule Real-Time (SMRT) sequencing technology. The PacBio library for sequencing was constructed as described previously (Faino et al., 2015). *V. dahliae* strains Vd39, V4, BP2, V574,

V700, V117, V76, ST.100, T9 and V991 (Table S1) were sequenced using the Illumina HiSeq 2000. Library preparation (500-bp insert size) and Illumina sequencing (100-bp paired-end reads) were performed at the Beijing Genome Institute (BGI, China).

For deep transcriptome sequencing, 12-day-old cotton seedlings were root-inoculated with conidiospores of *V. dahliae* strain V991 that belongs to D pathotype as described previously (Gao et al., 2013). Stems of inoculated cotton plants were harvested at 6, 9, 12, 15 days post inoculation (dpi) for total RNA extraction. RNA extraction was performed following the procedure of the Spectrum Plant Total RNA Kit of Sigma-Aldrich (USA). cDNA synthesis, library preparation (200 bp insert size), and Illumina sequencing (90 bp paired-end reads) were performed at the Beijing Genome Institute (BGI, China).

Phylogenetic analysis of sequenced *V. dahliae* strains

A phylogenetic tree of the *V. dahliae* strains was generated by REALPHY (version 1.12) (Bertels et al., 2014) using Bowtie2 (Langmead and Salzberg, 2012) to map genomic reads against the reference *V. dahliae* strain JR2, for which a gapless genome assembly is available (Faino et al., 2015). A maximum likelihood phylogenetic tree was inferred using RAxML (version 8.2.8) with the GTRGAMMA model and 500 bootstrap replicates (Stamatakis, 2014). *V. alfalfae* MS 102 was used to root the tree.

V. dahliae comparative genomics

The *V. dahliae* strain CQ2 genome was assembled using HGAP (v3.0) with default parameters (Chin et al., 2013). The genome of CQ2 was annotated using MAKER2 (v2) software (Holt and Yandell, 2011) followed by manual curation of gene models present at the regions of interest. The manual curation included the identification of gene models which were predicted by the MAKER2 annotation and the adjustment of exon/intron boundaries using transcriptome sequencing evidence. In order to identify *V. dahliae* gene(s) that are responsible for cotton defoliation, short DNA sequences (reads) from Illumina sequencing of D and ND pathotype strains (Table S1) were mapped onto the genome of CQ2 using BWA software (Li and Durbin, 2010) with default options. Additionally, we used sequence information from 72 *V. dahliae* strains which were deposited at the NCBI database under project number PRJNA171348. The presence/absence analysis was performed using BEDtool bedtools software (Quinlan and Hall, 2010) and R software as follows: the CQ2 genome was sliced in bins of 100 bp and a sliding window of 50 bp was checked for presence of reads derived from other D pathotype strains and for the absence of coverage of ND pathotype strains. The coverage was normalized using # reads in a bin x 10,000,000 / total amount of mapped reads. The coverage distribution for each sample was evaluated and the cut off set to 5. Lineage specific (LS) regions, here defined as genomic regions that are only present in D pathotype strains, were determined and genes localized within these regions were extracted using BEDtools intersect (v2.25) (Quinlan and Hall 2010). To validate the bioinformatic gene prediction in these LS regions, RNA reads that obtained from transcriptome sequencing of cotton plants infected by D pathotype strain V991 were mapped to LS regions using software Tophat v1.4.0 (Trapnell et al., 2010).

Generation of *D* gene deletion strains

To generate a *D* single gene deletion construct, sequence stretches of approximately 1.2 kb upstream and 1.3 kb downstream of the *D* coding sequence were amplified from genomic DNA of D pathotype strain CQ2, using primer pairs SKO-D-LBF/LBR and SKO-D-RBF/RBR (Table S3). The amplicons were cloned into vector pRF-HU2 as described previously (Frandsen et al, 2008), and the resulting deletion construct was transformed into CQ2 via *Agrobacterium tumefaciens*-mediated transformation (Santhanam, 2012). Putative deletion transformants were selected on PDA containing 200 µg/mL cefotaxime and 50 µg/mL hygromycin B (Duchefa, Haarlem, The Netherlands) and homologous gene replacement was verified with PCR analysis using outside primer-F and outside primer-R (Table S3). To generate *D* double gene deletion mutants, sequence stretches of approximately 1.1kb upstream and 1.2 kb downstream of the *D* coding sequence were amplified using primer pairs DKO-D-LBF/LBR and DKO-D-RBF/RBR (Table S3). The amplified products were cloned into vector pRF-NU2. Next, the gene replacement construct was transformed into a *D* gene single deletion mutant. Putative double deletion transformants were selected on PDA containing 50 µg/mL hygromycin B and 15 µg/mL nourseothricin (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), and were subjected to PCR to confirm genuine double *D* gene deletions using outside primer-F and outside primer-R (Table S3). Reverse transcription-PCR (RT-PCR) was used to confirm no *D* gene transcripts in *D* double deletion mutants using primers D-RT-F) and D-RT-R), and *V. dahliae GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene as an endogenous control (Table S3). Meanwhile, the same *D* single deletion construct and double deletion construct were used to generate *D* single and *D* double deletion mutants in *V. dahliae* strain V150I, which causes defoliation on olive (Collado-Romero et al., 2006).

D gene complementation was performed by using a genomic construct consisting of the *D* gene coding sequence with 1.1 kb upstream and 1.2 kb downstream sequences (*pD::D*) using primer pairs D-com-F and D-com-R (Table S3). The amplified products were cloned into GatewayTM compatible vector PCG using a standard BP reaction (Zhou et al., 2013). The gene complementation construct was further transformed into a *D* single deletion mutant, a *D* double deletion mutant, a ND pathotype strain JR2, respectively. Putative *D* gene complementation transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime and 25 µg/mL geneticin (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Reverse transcription-PCR (RT-PCR) was used to examine *D* gene transcription in these transformants.

Gene expression analysis

To assess *D* gene expression in *D* deletion strains and complementation strains, various *V. dahliae* strains were grown at 22°C in liquid Czapek-Dox medium for one week while shaking at 150 rpm and the resulting mycelium and conidia were harvested for RNA extraction. First-strand cDNA synthesis was performed by using MMLV reverse transcriptase system (Promega, Wisconsin, USA). Reverse transcription-PCR (RT-PCR) was conducted with primers CQ2D-F and CQ2D-R (Table S3) in a total volume of 25 µl with 17.9 µl sterilized water, 5 µl 5x PCR buffer, 0.5 µl dNTPs, 0.5 µl of each primer, 0.1 µl GoTag DNA polymerase (Promega, Wisconsin, USA) and 1.0 µl of first-strand cDNA (100 ng/ µl). The *V. dahliae GAPDH* gene was used as endogenous loading control. The resulting PCR products were subjected to agarose gel electrophoresis.

Heterologous production of D effector protein in *Pichia pastoris* and bioassays on cotton seedlings

D effector protein was produced in *P. pastoris* following previously described procedures (Sánchez-Vallet et al., 2013; Kombrink et al., 2017). Briefly, the *D* gene was cloned into *P. pastoris* expression vector pPIC9 (Invitrogen) after performing PCR using primers to add the N-terminal His- and FLAG-tag (Table S3) (Kombrink et al., 2017). A pPIC9 vector containing the His-FLAG tagged *D* gene was transformed into *P. pastoris* strain GS115. Putative *P. pastoris* transformants were cultured in a small volume of BMM medium to check D effector protein expression and one clone was selected for culturing in a BioFlo 120 fermenter (Eppendorf AG, Hamburg, Germany) (Kombrink et al., 2017). The His-tagged D effector protein was purified using a Ni^{2+}-NTA Superflow column (Qiagen). After elution from the column, the protein was dialysed against 100 mM NaCl and the concentration was determined using absorbance at 280 nm. The purified protein was analysed by SDS-PAGE and Western blotting using primary Mouse-anti-His mAb antibody (Sánchez-Vallet et al., 2013; Kombrink et al., 2017).

To assess the effect of treatment with the D effector protein, three-week-old cotton seedlings were uprooted from soil and carefully rinsed with tap water. Next, the roots were placed into 10 mL D protein solution (8.96 μM). Treatments with water and *V. dahliae* chitin binding Vd2LysM effector protein (8.96 μM) (Kombrink et al., 2017) were used as negative controls. The cotton seedlings were incubated in a growth chamber at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity for 16 days and regularly inspected for the occurrence of symptoms.

Results

V. dahliae inoculation on cotton

Various strains of *V. dahliae* have been sequenced during the last years, including several previously characterized D pathotype strains (V991, T9, V117 and TM6), as well as ND pathotype strains (BP2, V4, cd3 and HN) (Schnathorst and Mathre 1966; López-Escudero et al., 2010; Zhang et al., 2012; Xu et al., 2012; Liu et al., 2014). In order to select additional D and ND strains to increase the robustness of the comparative genome analysis, we generated a phylogenetic tree of all in house-sequenced *V. dahliae* strains (Table S1) (Figure S1). This phylogenetic analysis revealed that six additional strains clustered with previously characterized D pathotype strains, while other strains were more related to previously characterized ND strains (Figure S1). Interestingly, four strains (V574, V700, v679, and Vd39) are relatively closely related to the D pathotype strains, yet phylogenetically distinct. In order to assign these four strains to the correct pathotype and use them in our comparative genomics study, we performed disease assays to evaluate their ability to cause defoliation on cotton. Additionally, we included the presumed D strains V76, V117, CQ2 and ST100 and the presumed ND strains JR2 and VdLs17. The disease assay confirmed that V76, V117, CQ2 and ST100 belong to the D pathotype (Figure 1), while all other strains (JR2, VdLs17, V574, V700 and Vd39) classified as ND pathotype strains (Figure 1).

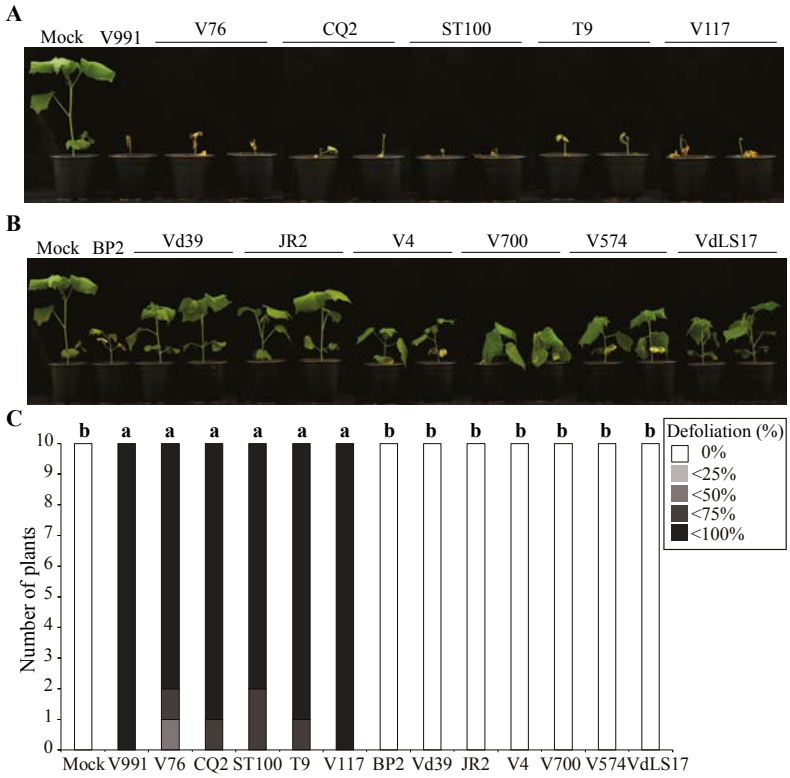

Figure 1. Phenotype of cotton plants inoculated with various sequenced *Verticillium dahliae* strains. (A) Typical phenotype of cotton plants (cv. Xinluza063) upon mock-inoculation or inoculation with *V. dahliae* strains V991, V76, CQ2, ST100, T9 and V117 at 28 days post inoculation (dpi). Previously characterized D pathotype strain V991 used as inoculation control. **(B)** Typical phenotype of cotton plants upon mock-inoculation or inoculation with *V. dahliae* strains BP2, Vd39, JR2, V4, V574, V700, VdLS17 at 28 (dpi). Previously characterized ND pathotype strain BP2 used as inoculation control. **(C)** Defoliation were classified as 0 (0% leaf drop off), 1 (<25% leaf drop off), 2 (<50% leaf drop off), 3 (<75% leaf drop off) and 4 (<100% leaf drop off) at 28 dpi. Significance levels were calculated based on 5,000 bootstrap replicates of the median difference between random scores of two treatments and different letter labels indicate statistically significant differences ($P < 0.05$). Inoculation experiments were performed with ten plants for each fungal strain and repeated twice independently with similar results.

Comparative genomics identifies D pathotype-specific effector genes

The genome of the D pathotype strain CQ2 was sequenced with PacBio technology and used as a reference in a comparative genomics approach that used eight D pathotype strains (V991, T9, V117, TM6, V76, 463, CQ2 and ST100) and nine ND pathotype strains (BP2, V4, HN, cd3, JR2, VdLs17, V574, V700 and Vd39) to identify ~200 kb of D pathotype-specific sequence, encoding ~30 genes. In order to reduce the interval of interest, and thus the number of candidate genes, sequences of seventy-two additional *V. dahliae* strains were mined. Strain BGI_32 caught our special attention as it clustered neither with the D pathotype strains nor with the ND pathotype strains (Figure S2). As subsequent disease assays assigned this isolate to the ND pathotype (Figure S3), we were able to limit the D-specific region to ~24 kb with only seven predicted genes (Table S2).

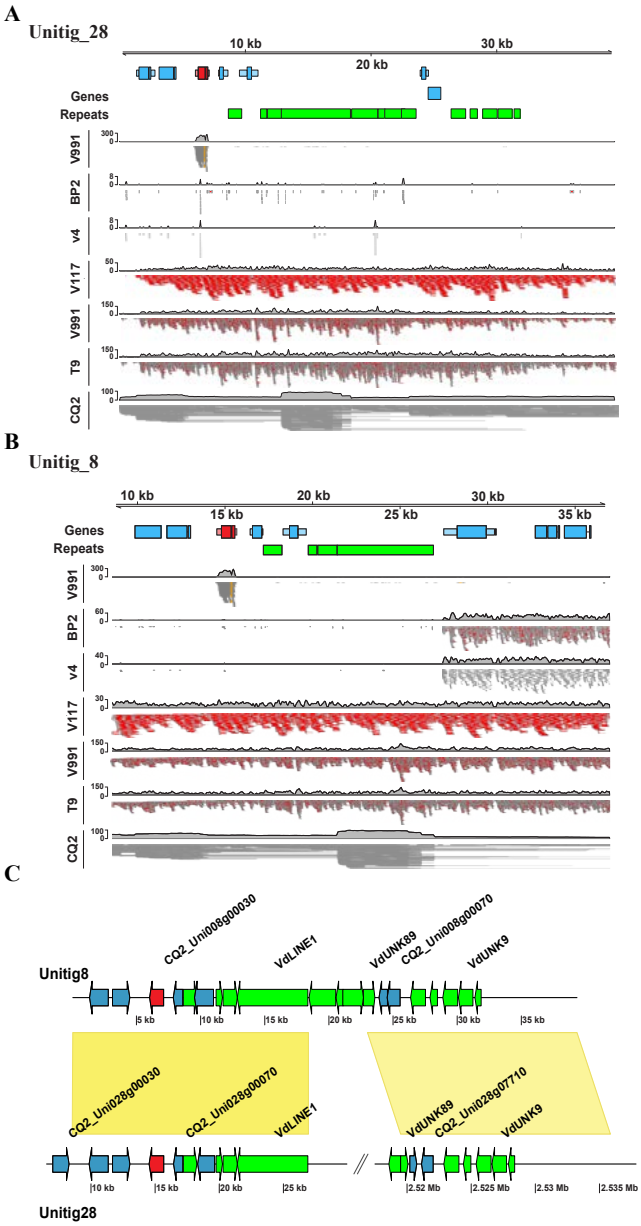

Figure 2. Two identical copies of effector gene generated by segmental duplication. (A-B) Schematic representation of genomic region (Unitig_28 and Unitig_8) where two candidate effector genes located. Gene models shown in blue, the candidate effector genes displayed in red, while repetitive elements displayed in green. RNA reads from cotton plants infected by a D pathotype strain (V991) that only mapped to candidate gene are showed in dark grey. Reads from DNA-seq of ND pathotype strains (V4 and BP2) and D pathotype strains (V117, V991 and T9) mapped onto CQ2 genome. The reads are showed as coverage plot and the depth of DNA reads coverage indicated in red. **(C)** Syntenic assignment of Unitig_28 and Unitig_8, the extensive synteny (yellow blocks indicate 100% sequence identity) points towards a segmental duplication. Gene models shown in blue, the candidate effector gene displayed in red, while repetitive elements displayed in green.

In order to characterize the seven D-specific genes, interproscan analysis (Finn et al., 2017) was conducted, showing that two genes (CQ2_Uni028g00060 and CQ2_Uni008g00030) are predicted to be secreted. Expression analysis using deep transcriptome sequencing of a time course of cotton plants inoculated with D pathotype strain V991 showed that these two genes are highly expressed (Figure S4). Surprisingly, the two genes share 100% sequence identity, despite being located on separate contigs, namely Unitig_28 and Unitig_8 (Figure 2A-B). Moreover, alignment of contigs Unitig_28 and Unitig_8 revealed that genomic regions surrounding the two genes are completely identical as well (Figure 2C), suggesting that these arose by a segmental duplication. We tentatively named these two candidates *D* gene, for potentially mediating defoliation (D) on cotton.

The D effector is responsible for cotton defoliation.

To further examine the role of the D effector in pathogenicity on cotton plants, targeted gene deletions of the two gene copies were performed in D pathotype strain CQ2 by homologous recombination (Figure S5A). To this end, we first generated single gene copy deletion mutants followed by another round of transformation to remove the second gene copy. Single (ΔD) and double (ΔΔD) *D* gene deletions were confirmed by PCR (Figure S5B).

We inoculated cotton seedlings with wild type strain CQ2, single *D* gene deletion mutants (ΔD#1 and ΔD#2), double *D* gene deletion mutants (ΔΔD#1 and ΔΔD#2) and monitored disease progression. While the wild type strain CQ2 caused severe defoliation symptoms on cotton plants at 28 days post inoculation (dpi) (Figure 3A-B), ΔD mutants displayed significantly compromised aggressiveness with clearly less defoliation symptoms (Figure 3A-B). Importantly, ΔΔD mutants appeared to be unable to cause disease on cotton, as plants inoculated with ΔΔD mutants showed no defoliation (Figure 3A-B). Moreover, no fungal biomass could be recorded in ΔΔD mutants-inoculated plants with real-time PCR, confirming that the D effector acts as pathogenicity factor (Figure 3C). Complementation of single and double deletion mutants with a genomic fragment comprising the *D* gene coding sequence (*pD::D*) restored the aggressiveness, leading to defoliation symptoms on cotton (Figure 3). Interestingly, introduction of the genomic fragment comprising the *D* gene coding sequence (*pD::D*) into the ND pathotype strain JR2 enhanced aggressiveness on cotton plants, resulting in severe stunting and the induction of defoliation (Figure 4). These results demonstrate that the D effector is a pathogenicity factor of *V. dahliae* D pathotype strain and responsible for defoliation symptoms on cotton.

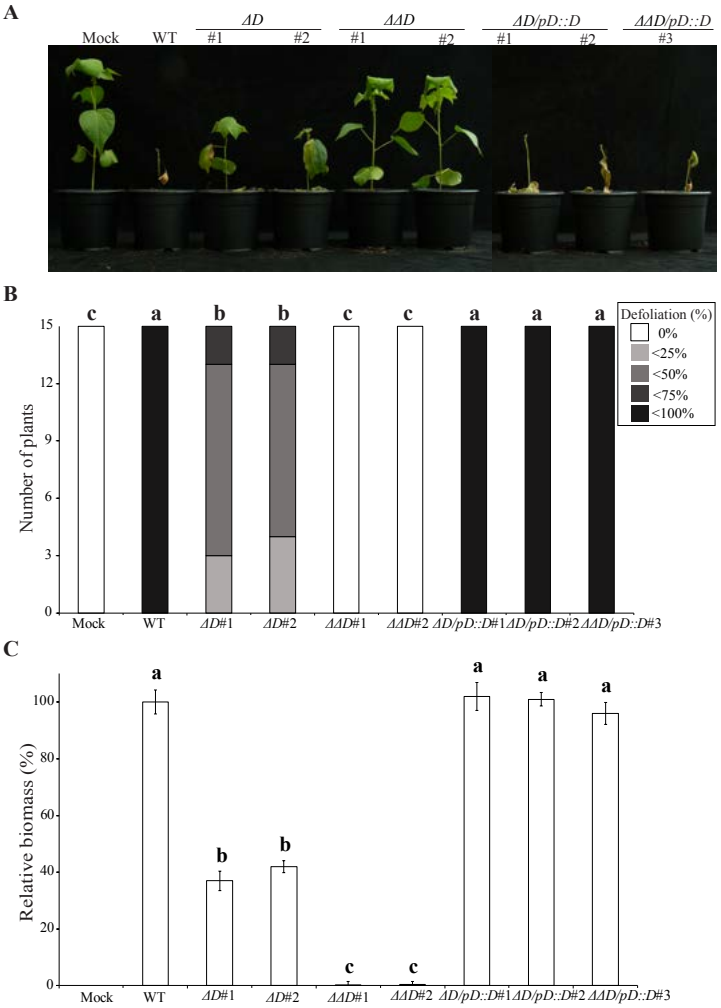

Figure 3. D effector is responsible for cotton defoliation. (A) Typical phenotype of cotton (cv. Xinluza063) upon mock-inoculation or inoculation with wild type strain CQ2 (WT), two ΔD mutants (ΔD #1 and ΔD #2), two ΔΔD mutants (ΔΔD #1 and ΔΔD #2), two Δ*D* complementary strains (ΔD/*pD::D* #1 and ΔD/*pD::D* #2) and one ΔΔD complementary strain (ΔΔD/*pD::D* #3) at 28 days post inoculation (dpi). **(B)** Defoliation were classified as 0 (0% leaves drop off), 1 (<25% leaves drop off), 2 (<50% leaves drop off), 3 (<75% leaves drop off) and 4 (<100% leaves drop off) at 28 dpi. In total, 15 plants were assessed for every treatment and the data represent one of two experiments. Significance levels were calculated based on 5,000 bootstrap replicates of the median difference between random scores of two treatments and different letter labels indicate statistically significant differences ($P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 28 dpi. Bars represent *V. dahliae ITS* levels relative to cotton *ubiquitin* (*GhUB*) levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in cotton plants upon inoculation with the wild type CQ2 is set to 100%. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). Experiments were repeated twice independently with similar results.

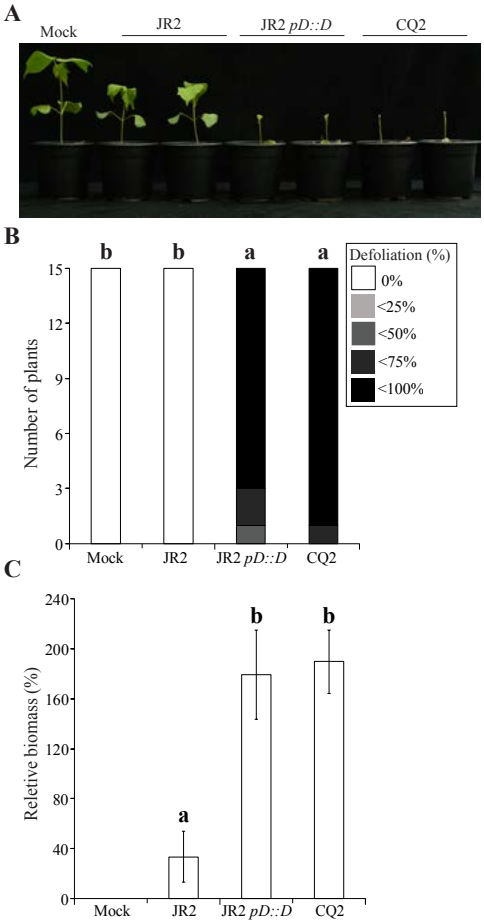

Figure 4. Introduction of *D* gene in ND pathotype strain resulted in defoliation symptoms. (A) Typical phenotype of cotton (cv. Xinluza063) plants that were mock-inoculated or inoculated with ND pathotype strain JR2, one *D* expression transformant of JR2 (JR2 *pD::D*) and D pathotype strain CQ2 at 28 days post inoculation (dpi). **(B)** Defoliation were classified as 0 (0% leaves drop off), 1 (<25% leaves drop off), 2 (<50% leaves drop off), 3 (<75% leaves drop off) and 4 (<100% leaves drop off) at 28 dpi. In total, 15 plants were assessed for every treatment and the data represent one of two experiments. Significance levels were calculated based on 5,000 bootstrap replicates of the median difference between random scores of two treatments and different letter labels indicate statistically significant differences ($P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 28 dpi. Bars indicate the *V. dahliae* biomass relatively to the cotton biomass. Significant differences were calculated with the Mann-Whitney U test ($P < 0.05$) and depicted by different letter labels. Error flags represent the standard error.

The D effector is responsible for olive defoliation

Since we observed that the D effector is responsible for defoliation symptoms on cotton, we further examined whether *V. dahliae* strains that cause olive defoliation carry the *D* gene. We confirmed the presence of the *D* gene in the previously characterized D pathotype strains V150I, V641I, V356I and V403II (Collado-Romero et al., 2006; Maldonado-González et al., 2015), while olive-pathogenic strain 812I that belongs to ND pathotype (Maldonado-González et al., 2015) does not carry the *D* gene (Figure S8).

Table 1. Infection assay of *D* gene deletion strains on olive.

Treatments	Disease parameters				
	AUDPC[a]	Final DI (%)[b]	Final DII[c]	M (%)[d]	S[e]
V-150I (WT)	70.5 a	44.4	0.33	11.1	1.31
ΔΔD #5	1.6 b	22.2	0.02	0	0.08
ΔΔD #7	0 b	0	0	0	0
ΔΔD #8	0 b	0	0	0	0
Control	0 b	0	0	0	0

[a]AUDPC, area under the disease progress curve over time up to 132 days post inoculation (dpi). [b]Final DI, final disease incidence (%). [c]Final DII, disease intensity index was calculated from data on incidence and severity of symptoms recorded at 132 (dpi). [d]M, dead plants (%) at 132 (dpi). [e]S, mean of disease severity symptoms at 132 (dpi). Different letter labels indicate statistically significant differences ($P < 0.05$). Inoculation experiments were repeated twice independently with similar results.

To further investigate whether the D effector is also responsible for olive defoliation, targeted gene deletions were generated by deletion of the two *D* gene copies in strainV150I (Figure S5A, C). Subsequently, we inoculated olive plants with three double *D* gene deletion mutants (ΔΔD#5, ΔΔD#7 and ΔΔD#8) along with the corresponding wild type strain V150I and evaluated disease symptom development. While wild type strain V150I caused severe disease symptoms, including severe defoliation and eventually plant death, ΔΔD mutants displayed severely compromised pathogenicity (Table 1). Thus, our results demonstrate that the D effector, besides cotton defoliation, is also responsible for olive defoliation.

The D effector is a pathogenicity factor on *Nicotiana benthamiana* and *Arabidopsis thaliana*

To investigate whether the observed role of D effector in pathogenicity is confined to cotton and olive, or also extends to other host species that do not normally respond to *V. dahliae* infection with defoliation, we inoculated the *D* deletion mutants (ΔD and ΔΔD) on the model plants *N. benthamiana* and *A. thaliana*. Compared with the wild-type strain, ΔD mutants showed markedly reduced aggressiveness on *N. benthamiana*, demonstrated by increased canopy (foliage) area and a significant reduction of fungal biomass *in planta* (Figure 5A, C, D).

Furthermore, ΔΔD mutants failed to cause disease on *N. benthamiana*, as the inoculated plants did not show any disease symptoms throughout the assay (Figure 5A, C). As no fungal biomass could be detected in ΔΔD mutants-inoculated plants (Figure 5D), we conclude that the D effector acts as a pathogenicity factor on *N. benthamiana*. Similarly, ΔD mutants exhibited clearly compromised aggressiveness on *A. thaliana* plants when compared with wild type strain, evidenced by increased *A. thaliana* rosette leaves area and the reduction in fungal biomass (Figure 5B, E, F). Moreover, ΔΔD mutants appeared to be non-pathogenic on *A. thaliana* plants as well, as plants inoculated with ΔΔD mutants did not show disease symptoms (Figure 5B, E) or accumulation of fungal biomass (Figure 5F). Collectively, it can be concluded that, besides for pathogenicity on cotton and olive, the D effector is required for pathogenicity on *N. benthamiana* and *A. thaliana*.

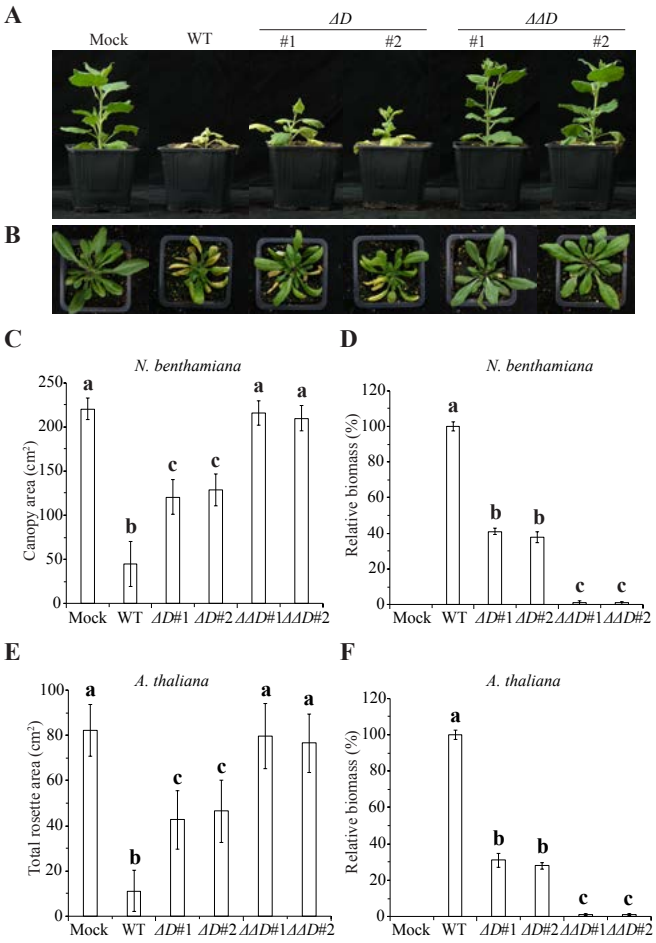

Figure 5. D effector is a pathogenicity factor on *Nicotiana benthamiana* and *Arabidopsis thaliana*. **(A)** Typical phenotype of *N. benthamiana* plants that were mock-inoculated or inoculated with wild type strain CQ2 (WT), two ΔD mutants (#1 and #2), two ΔΔD mutants (#1 and #2) at 21 days post inoculation (dpi). **(B)** Typical phenotype of *A. thaliana* (Col-0) plants that were mock-inoculated or inoculated with indicated fungal strains in panel A at 21 (dpi). **(C)** Quantification of the canopy area of *N. benthamiana* at 21 dpi. Bars represent the average canopy area of five plants with standard deviation. **(D)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *V. dahliae ITS* levels relative to *NbRuBisCo* (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in *N. benthamiana* plants upon inoculation with the wild type CQ2 is set to 100%. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(E)** Quantification of the rosette area of five *A. thaliana* plants at 21 dpi. Bars represent the average rosette area of five plants with standard deviation. **(F)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *V. dahliae ITS* levels relative to *AtRuBisCo* (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in *A. thaliana* plants upon inoculation with the wild type CQ2 is set to 100%. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). *N. benthamiana* and *A. thaliana* inoculation experiments were performed with five plants for each fungal strain and repeated twice independently with similar results.

The D effector protein induces defoliation

To test whether the D effector protein is able to induce symptoms of Verticillium wilt disease, cotton seedlings were treated with the D effector protein (8.96 μM) and monitored for symptom development, while treatments with water and the *V. dahliae* chitin-binding effector protein Vd2LysM (8.96 μM) were used as negative controls (Kombrink et al., 2017). Intriguingly, application of the D effector protein induced wilting, slight chlorosis and the first detachment of cotton cotyledons after 10 days (Figure 6A-B), while severe chlorosis and detachment of more than half of the leaves (55±6.45%) were observed after 16 days (Figure 6C-E). In contrast, treatment with water or Vd2LysM effector protein induced no visible effect on cotton plants throughout the assay. These results suggest that the effector protein itself, rather than extensive fungal proliferation in the xylem vessels, is responsible for the defoliation symptoms.

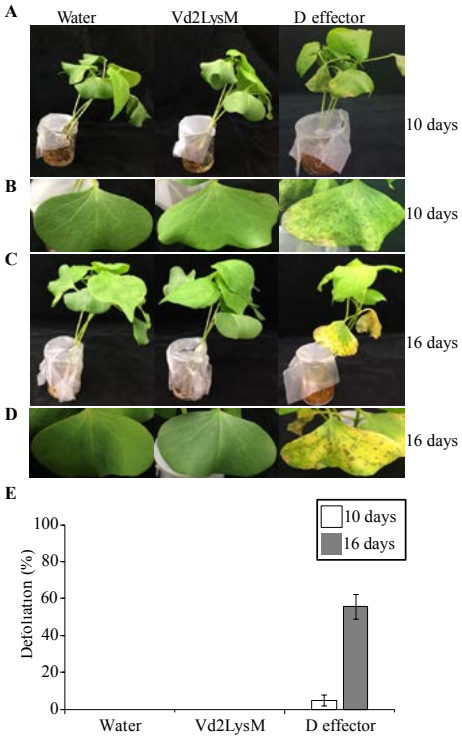

Figure 6. D effector protein induces cotton defoliation *in vitro*. (**A-B**) Typical appearance of cotton seedlings (cv. Xinluza063) applied with D effector protein at 10 days. Note, marginal chlorosis and wilting symptoms occurred on cotyledons. (**C-D**) Typical appearance of cotton seedlings applied with D effector protein at 16 days. Cotyledons became severe chlorosis, meanwhile more cotyledons and true leaves dropped off. (**E**) The bar graph showed the defoliation (%) at 10 and 16 days. Bars represent the average defoliation of two biological replicates with standard deviation. Experiments were repeated twice independently with similar results.

Discussion

The ability of a plant pathogen to cause disease on plants is often linked to the presence of specific pathogenicity genes within its genome (van der Does and Rep, 2007). There is mounting evidence that pathogenicity of a plant pathogens is mediated by so-called effector molecules that are secreted by the pathogen during host infection (Jones and Dangl, 2006; de Jonge et al., 2011; Rovenich et al., 2014; Rodriguez-Moreno et al., 2018). It is generally observed that effector activities are redundant and that the contribution of a single effector to disease establishment is dispensable. However, a number of studies have shown that a single effector is able to govern pathogenicity and therefore enable to cause disease on particular host plants. For example, the transcriptional activator-like effector protein PthA has been shown to be essential for pathogenicity of the bacterial citrus pathogen *Xanthomonas citri*, as transfer of the *pthA* gene into non-pathogenic strains confers the ability to cause citrus disease (Swarup et al., 1991, 1992; Duan et al., 1999). Similarly, the necrotrophic effector SnTox1 is sufficient to enable the wheat pathogen *Parastagonospora nodorum* to establish disease on wheat lines that carry the corresponding susceptibility gene *Snn1* (Liu et al., 2012, 2016).

Despite the observation that *V. dahliae* strains are collectively characterized by their broad host range, pathogenicity and the severity of symptoms that are induced can vary considerably between individual strains (Bhat and Subbarao, 1999; Fradin and Thomma, 2006). Defoliating (D) and non-defoliating (ND) pathotypes have been described already decades ago for olive- and cotton-infecting *V. dahliae* strains (Schnathorst and Mathre, 1966; Schnathorst and Sibbett, 1971a), but the genetic factor(s) that are responsible for the ability of D pathotype strains to cause defoliation symptoms remained elusive. In this study, we show that a single effector, named D effector, governs pathogenicity of D pathotype strains on cotton and olive, as deletion of both copies of the *D* effector gene resulted in loss of the ability to cause defoliation symptoms. Moreover, introduction of this effector gene into a ND pathotype isolate was accompanied by an acquisition of the ability to cause defoliation. Furthermore, application of heterologously produced effector protein to cotton seedlings induced defoliation, showing that the effector protein is directly responsible for defoliation symptoms. Thus, we demonstrate that a single effector is sufficient to cause disease establishment.

While mating type-related genes have been found in the *V. dahliae* genome, *V. dahliae* is considered to be a strictly asexual species that propagates clonally since a sexual cycle has never been observed and the mating type distribution is skewed towards one of the two (Usami et al., 2008, 2009; Atallah et al., 2010). Such asexual reproduction results in offspring that is genetically highly identical to each other and to the parent, and thus *V. dahliae* has a highly clonal population structure (Klosterman et al., 2009; Milgroom et al., 2014, 2016; Rafiei et al., 2017). The clonal population structure in *V. dahliae* was first described as vegetative compatibility groups (VCGs) (Joaquim and Rowe, 1990; Strausbaugh, 1993), as vegetatively compatible *V. dahliae* isolates that form stable heterokaryons are assigned to the same VCG (Rowe, 1995; Katan, 2000). Consequently, isolates within the same VCG are genetically similar to each other, while isolates between different VCGs were thought to be genetically different populations that may vary in

many traits, including those related to pathogenicity (Katan, 2000). Clonal lineages defined by VCGs were later well supported by molecular genetic markers (Collado-Romero et al., 2006; Milgroom et al., 2014). Interestingly, clonal lineages that are characterized by VCGs and molecular markers are also well associated with particular *V. dahliae* pathotypes (Collado-Romero et al., 2006; Collins et al., 2005 Jiménez et al., 2011). For instance, isolates in clonal lineage VCG1A have been shown to belong to the highly virulent D pathotype, whereas the ND pathotype strains were found in all other lineages (Daayf et al., 1995; Hiemstra and Rataj-Guranowska, 2003; Collado-Romero et al., 2006; Dervis et al., 2007; Korolev et al., 2008). It has been proposed that D pathotype strains arose from North America and subsequently dispersed clonally to widespread geographic locations (Bell, 1992). A single nucleotide polymorphism (SNP) genotyping study of a worldwide collection of *V. dahliae* from diverse hosts revealed that D pathotype strains from Europe, North America and China all belong to VCG1A group and have nearly identical SNP haplotypes, which further supports the hypothesis that *V. dahliae* D pathotype strains arose from a single origin and subsequently dispersed to other continents (Milgroom et al., 2014, 2016). Since our study showed that the D effector is responsible for defoliation symptoms, the *D* effector gene does not only serve as an ideal molecular marker to accurately differentiate D and ND pathotype strains, but can also be used as a probe to monitor the spread of highly virulent D pathotype strains worldwide.

Plant pathogens have to continuously adapt their effector repertoire to escape host immunity and maintain aggressiveness (Rovenich et al., 2014; Rodriguez-Moreno et al., 2018). The emergence of novel effector genes can increase virulence or lead to host range expansion (Friesen et al., 2006; Raffaele et al., 2010). Various mechanisms, such as genome hybridization (Stukenbrock et al., 2012), gene duplication (Dutheil et al., 2016), and horizontal gene transfer (HGT) (Friesen et al., 2006; de Jonge et al., 2012) have been described that can facilitate the development of effector gene repertoires in pathogenic microbes. It has been suggested that the well-characterized *V. dahliae* LS effector Ave1 has been acquired from plants via HGT (de Jonge et al., 2012). Comparisons of gapless genome assemblies of two *V. dahliae* strains have revealed that segmental genomic duplications are enhanced in highly variable lineage-specific (LS) genomic regions (Faino et al., 2016). Interestingly, these regions are enriched for *in planta*-expressed effector genes that contribute to fungal virulence, suggesting that gene duplications contribute to evolution of pathogen virulence (de Jonge et al., 2013; Faino et al., 2016). In the present study, we found that two identical copies of the *D* effector gene arose by a segmental duplication, and the high level of similarity between flanking sequences of the *D* effector gene suggests that this duplication occurred rather recently (Figure 2C). The relevance of the occurrence of two copies of the *D* effector gene and their impact on fungal adaption remains unknown at this point. Possibly, the emergence of two copies of the *D* effector gene is relevant to maintain highly aggressiveness of D pathotype strains on diverse host plants. In filamentous pathogens, effector genes that arose from gene duplication events typically evolve in a so called "duplication-divergence" pattern: following a gene duplication event, one gene copy diverges to some extent due to functional redundancy and evolves a distinct function (Plissonneau et al., 2017). For example, a large number of Crinklers (CRN) effector genes of oomycete pathogen *Phytophthora sojae* underwent sequence diversification after gene duplication (Shen et al., 2013). Similarly, gene duplications followed by sequence divergence were proposed to be responsible for the generation of novel effector genes

in the smut fungus *Ustilago maydis* (Dutheil et al., 2016). In addition to experiencing functional diversification, the recent duplications of effector genes may also be subject to differential loss of the duplicated gene copies (Dong et al., 2015; Pedersen et al., 2012). Frequent effector gene losses after segmental duplications have been proposed to occur in the powdery mildew fungus *Blumeria graminis*, which contribute to the diversity of the effector repertoires of the pathogen (Wicker et al., 2013; Menardo et al., 2017). Possibly, selective forces from host immune systems contribute to this process. However, it is unclear whether the two copies of the *D* effector gene will experience sequence divergence or gene loss over time.

To date, a number of *V. dahliae* effectors have been shown to facilitate fungal aggressiveness and promote host colonization. For example, the *V. dahliae* LysM-containing-effector Vd2LysM is a chitin-binding protein that can suppress chitin-induced immune responses and protects fungal hyphae against hydrolysis by plant hydrolytic enzymes (Kombrink et al., 2017). In addition, *V. dahliae* effector protein VdSCP41 has been shown to interact with Arabidopsis transcription factors CBP60g and SARD1, two master immune regulators, to modulate plant immunity (Qin et al., 2018). Moreover, the race 1 strain-specific effector Ave1 contributes to *V. dahliae* virulence not only on tomato plants that lack resistance gene *Ve1*, but also on *A. thaliana*, tobacco as well as on cotton (de Jonge et al., 2012; Song et al., 2018). However, to date the exact function of Ave1 during plant colonization remains unknown. In the present study, we show that the D effector acts as a pathogenicity determinant not only on cotton, olive, but also on *N. benthamiana* and *A. thaliana* (Figure 3; Table 1; Figure 5). Although the intrinsic function of the D effector remains unknown at this point, the finding that the D effector mediates pathogenicity on various plant hosts implies that the molecular target(s) of this effector might be broadly conserved in the plant kingdom.

Plant hormones such as salicylic acid (SA), abscisic acid (ABA), and ethylene (ET) are key signalling molecules that regulate multiple aspects of plant growth, development, and defense (Kazan and Lyons, 2014; Pieterse et al., 2012; Fu and Dong, 2013). Various pathogens independently evolved effectors to target the these hormones pathway to promote disease (Kazan and Lyons, 2014; Ma and Ma, 2016). Accumulating evidence has shown that *V. dahliae* can secrete effectors to interfere with plant hormone homeostasis or signalling pathways to modulate plant physiological processes and ultimately facilitate pathogen dissemination (Liu et al., 2014; Tanaka et al., 2015). For instance, the *V. dahliae* effector VdIsc1 suppresses SA accumulation to facilitate host infection (Liu et al., 2014). Wiese and DeVay (1970) previously reported that the concentration of ABA in cotton plants infected by a D pathotype strain was twice as high as in healthy plants, whereas the ABA concentration in ND pathotype strain-infected cotton plants was unaffected. While ET production in ND strain-infected plants was consistently higher than in healthy plants, ET production in D pathotype strain infected-cotton plants was two times higher than in ND strain-infected plants (Wiese and DeVay, 1970). Therefore, it is tempting to speculate that D pathotype strains may employ the D effector to manipulate plant hormone levels (e.g. ABA or ET) and their signalling pathways to enable disease establishment, but this remains to be demonstrated.

Currently, there is no effective fungicide commercially available to control Verticillium wilt once crops have been infected (Fradin and Thomma, 2006). Although biocontrol measures and cultivation practices have been applied to combat Verticillium wilt diseases, the control efficiency

largely depends on field conditions (Fradin and Thomma, 2006; Inderbitzin and Subbarao, 2014). Thus, development of resistance cultivars has been considered the most desirable strategy for the control of Verticillium wilt in various crops (Fradin and Thomma, 2006; Klosterman et al., 2009). Despite the devastating impact of the highly virulent D pathotype strains on cotton and olive plantations, there is presently no resistance (*R*) gene against D pathotype strains in either of these plant hosts (López-Escudero and Mercado-Blanco, 2011; Shaban et al., 2018). Since effectors are important virulence molecules of pathogens that can be potentially recognized by host plants, microbial effectors have been exploited as probes to identify *R* genes in resistance breeding (Laugé et al., 1998; Vleeshouwers and Oliver, 2014). Plant germplasm is screened for *R* genes that trigger robust responses, including a hypersensitive response, upon transient expression of the effector gene *in planta* or infiltration of purified effector protein (Takken et al., 1999; Vleeshouwers et al., 2011). After the detection of effector-responding genotypes, the corresponding *R* genes can be genetically mapped in populations derived from crosses between responding and non-responding genotypes (Laugé et al., 1998; Vleeshouwers et al., 2011). This effector-assisted breeding has been successfully used for identifying a number of *R* genes in wild tomato species against the leaf mould pathogen *Cladosporium fulvum* (Laugé et al., 1998; Takken et al., 1999; de Wit, 2016), as well as against the late blight pathogen *Phytophthora infestans* in potato (Du et al., 2015; Vleeshouwers and Oliver, 2014). Thus, we propose that the D effector can be used as a probe to screen cotton and olive germplasm, but also the germplasm of other plant species, for the occurrence of *R* gene-mediated recognition.

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Supplemental information

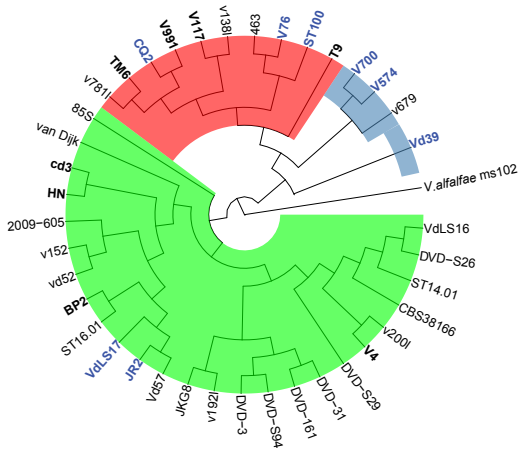

Figure S1. Phylogenetic tree of *in house*-sequenced *V. dahliae* strains. *V. dahliae* strains clustering with known D pathotype strains (TM6, V991, V117 and T9) are showed in light-red, while strains clustering with known ND pathotype strains (V4, BP2, cd3 and HN) are showed in light-green. Strains that distinct from the D and ND pathotype groups are showed in light-blue. Strains that used for phenotypic characterization in this work are display in bold with blue color. Phylogenetic relationship between sequenced *V. dahliae* strains was inferred using RealPhy (Bertels et al., 2014) and *V. dahliae* strain JR2 used as reference. *V. alfalfae* ms102 was used as root of the tree.

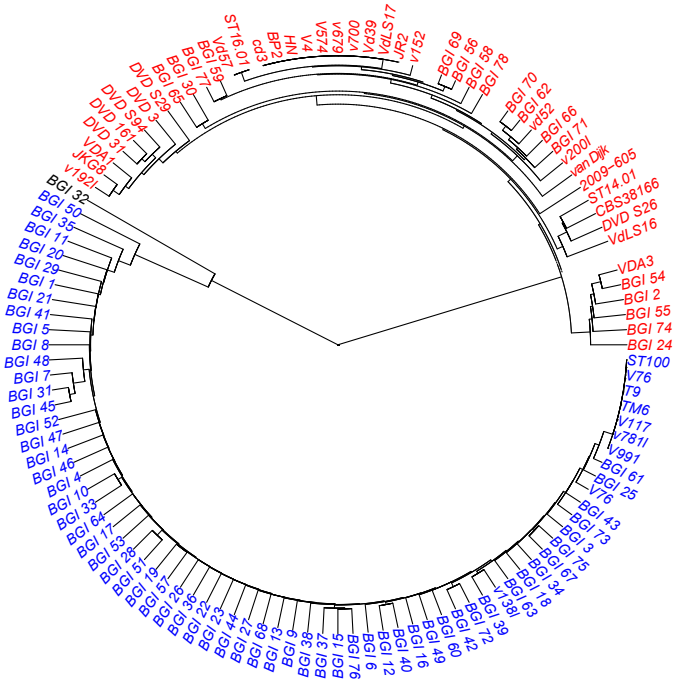

Figure S2. Clustering analysis of seventy-two sequenced *V. dahliae* strains. Sequences of seventy-two *V. dahliae* strains were aligned on the assembled genome of CQ2 and clustered in three groups based on presence/absence polymorphism. Strains clustering with known D pathotype strains are displayed in blue, while strains clustering with ND pathotype strains are showed in red. Strain BGI_32 that is the most divergent from the two groups showed in black.

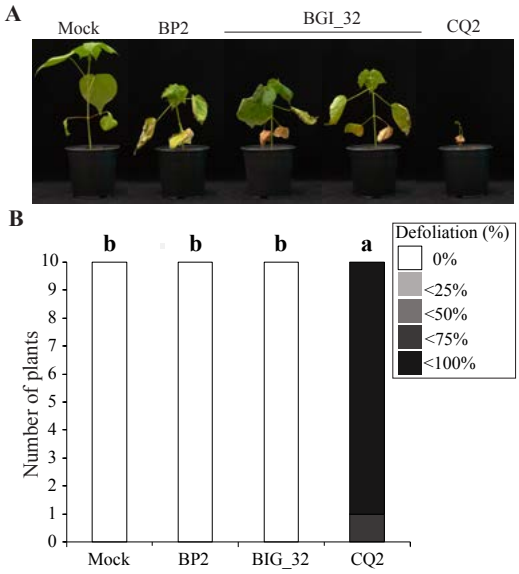

Figure S3. Phenotype of cotton plants inoculated with *V. dahliae* strain BGI_32. (A) Typical phenotype of cotton plants(cv. Xinluzao63) upon mock-inoculation or inoculation with BP2, BGI_32 and CQ2 at 28 days post inoculation (dpi). ND pathotype strain BP2 and D pathotype strain CQ2 were used as inoculation control. **(B)** Defoliation were classified as 0 (0% leaves drop off), 1 (<25% leaf drop off), 2 (<50% leaf drop off), 3 (<75% leaf drop off) and 4 (<100% leaf drop off) at 28 dpi. Significance levels were calculated based on 5,000 bootstrap replicates of the median difference between random scores of two treatments and different letter labels indicate statistically significant differences ($P < 0.05$). Inoculation experiments were performed with ten plants for each fungal strain and repeated twice independently with similar results.

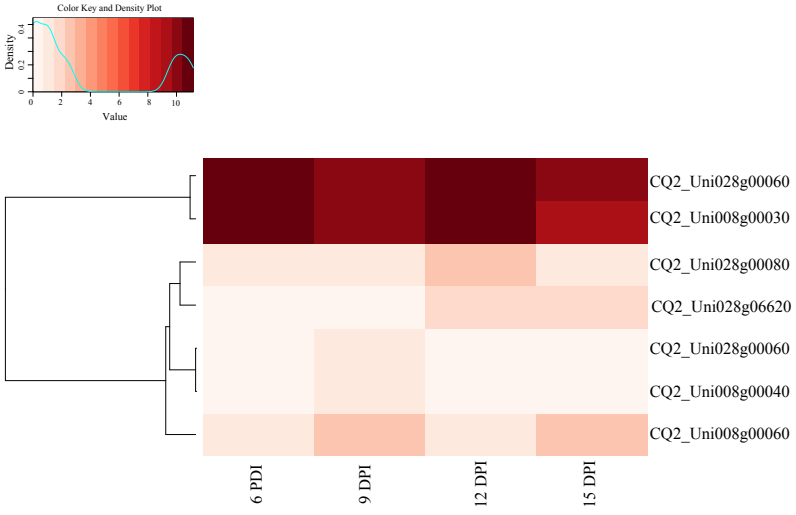

Figure S4. Expression analysis of the seven candidate genes. The heatmap showed the expression level of each gene during a time course of cotton infected by D pathotype strain V991 at 6, 9, 12 and 15 days post inoculation (DPI). The scaled expression values are color-coded according to scale bar at left corner.

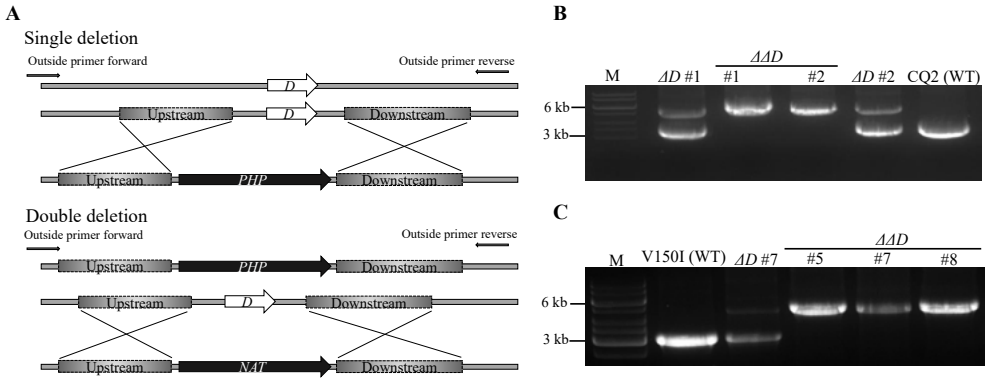

Figure S5. Construction and verification of *D* single deletion and double deletion mutants. (A) Schematic representation of the homologous recombination events to establish targeted replacement of *D* gene with phosphotransferase *(HPH)* and the nourseothricin resistance gene cassette (*NAT*). **(B)** Verification of *D* single deletion (ΔD) and double deletion mutants (ΔΔD) in *V. dahliae* cotton defoliating strain CQ2 by PCR. Amplicons generated with outside primers indicated in panel A are shown for wild type strain CQ2 (WT), two ΔD mutants (#1 and #2) and two ΔΔD mutants (#1 and #2). **(C)** Verification of *D* single deletion (ΔD) and double deletion mutants (ΔΔD) in *V. dahliae* olive defoliating strain V150I by PCR. Amplicons generated with outside primers indicated in panel A are shown for wild-type strain V150I (WT), one Δ*D* mutant (#7) and three ΔΔD mutants (#5, #7 and #8).

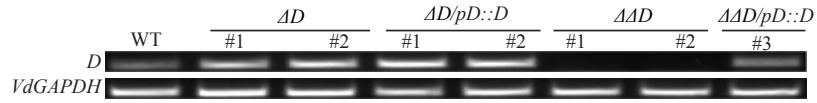

Figure S6. Detection of *D* gene transcripts in various *D* deletion and complementation strains. Amplification of *D* gene fragment (from left to right) from cDNA in wild type strain CQ2 (WT), two ΔD strains (#1 and #2), two ΔD complementation strains (ΔD/pD::D #1 and #2), two ΔΔD strains (#1 and #2) and one ΔΔD complementation strain (ΔΔD/pD::D #3). *V. dahliae GAPDH* gene used as endogenous control.

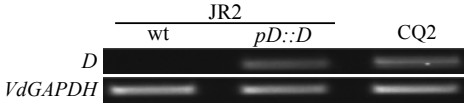

Figure S7. Expression of *D* gene (*pD::D*) in ND pathotype strain JR2. Amplification of *D* gene fragment (from left to right) from cDNA in wild type strain JR2, one *D* expression (*pD::D*) transformant of JR2 and CQ2 (used as positive control). *V. dahliae GAPDH* gene used as endogenous control.

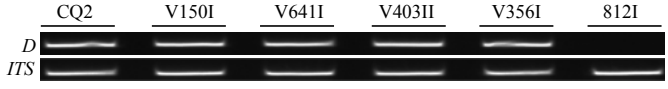

Figure S8. PCR detection of *D* gene in *V. dahliae* strains. Amplification of *D* gene fragment (from left to right) from genomic DNA in CQ2, V150I, V641I, V403I, V356I, 812I. As an endogenous control, a fragment of the Verticillium *ITS* region was amplified.

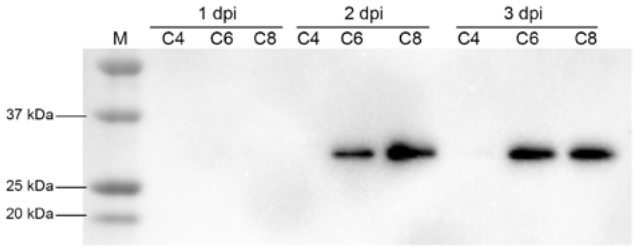

Figure S9. Detection of D effector protein expressed in the *Pichia pastoris*. Protein samples were collected from three putative D-expression *P. pastoris* transformants (named as C4, C6 and C8) at 1, 2 and 3 days post inoculation (dpi) in BMM medium, and were subjected to immunoblotted. Monoclonal antibody of His_6 epitope-tag was used to detect His- tagged D effector proteins (predicted molecular weight of 32 kDa). Note that *P. pastoris* transformants C6, C8 expressed D effector protein properly while C4 failed to produce D effector protein.

Table S1. *V. dahliae* strains used in this study.

Strain	Sequencing platform	Reference	Origin	Geographical location
HN	Illumina	Xu et al., 2012	Cotton	China
cd3	Illumina	Xu et al., 2012	Cotton	China
VdLs17	PacBio	Faino et al., 2015	Lettuce	USA
JR2	PacBio	Faino et al., 2015	Tomato	Canada
Vd57	Illumina	This study	Strawberry	Germany
V152	Illumina	Kombrink et al., 2017	Oak	Hungary
Vd52	Illumina	Kombrink et al., 2017	Pepper	Austria
van Dijk	Illumina	Kombrink et al., 2017	Chrysanthemum	The Netherlands
BP2	Illumina	Zhang et al., 2012	Cotton	China
ST16.01	Illumina	This study	Cotton	Syria
2009-605	Illumina	This study	Bell pepper	Ukraine
V4	Illumina	Keykhasaber, 2017	Olive	Spain
V200I	Illumina	This study	Strawberry	Germany
CBS38166	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S26	Illumina	de Jonge et al., 2012	Soil	Canada
VdLS16	Illumina	de Jonge et al., 2012	Lettuce	USA
ST14.01	Illumina	de Jonge et al., 2012	Pistachio	USA
DVD-S29	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-31	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-161	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S94	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-3	Illumina	de Jonge et al., 2012	Potato	Canada
V192I	Illumina	This study	Cotton	Spain
JKG8	Illumina	Kombrink et al., 2017	Potato	The Netherlands
85S	PacBio	This study	Sunflower	France
Vd39	Illumina	This study	Sunflower	Germany
V574	Illumina	Milgroom et al., 2014	Artichoke	Spain
v700	Illumina	Milgroom et al., 2014	Artichoke	Spain
v679	Illumina	Milgroom et al., 2014	Artichoke	Spain
T9	Illumina	Keykhasaber, 2017	Cotton	USA
V781I	Illumina	This study	Olive	Spain
V138I	Illumina	This study	Cotton	Spain
TM6	Illumina	Keykhasaber, 2017	Cotton	China
V117	Illumina	Keykhasaber, 2017	Olive	Spain
V991	Illumina	Zhang et al., 2012	Cotton	China
CQ2	PacBio	This study	Cotton	China
ST100	Illumina	de Jonge et al., 2012	Soil	Belgium
V76	Illumina	This study	Cotton	Mexico
463	Illumina	This study	Cotton	Mexico

Table S2. D pathotype-specific (D-LS) regions identified by comparative genomics.

Contig Names	Start (bp)	End (bp)	Size (bp)
unitig_2_complete	1,030,380	1,031,982	1,602
unitig_2_complete	1,052,304	1,053,244	940
unitig_8_Part_Chr	6,022	7,117	1,095
unitig_8_Part_Chr	7,869	8,611	742
unitig_8_Part_Chr	9,525	11,004	1,479
unitig_26_Part_Chr	52,257	54,090	1,833
unitig_26_Part_Chr	54,284	55,934	1,650
unitig_26_Part_Chr	151,504	151,944	440
unitig_26_Part_Chr	152,148	153,466	1,318
unitig_26_Part_Chr	154,179	154,941	762
unitig_26_Part_Chr	154,815	156,521	1,706
unitig_26_Part_Chr	156,881	159,404	2,523
unitig_26_Part_Chr	159,772	161,414	1,642
unitig_26_Part_Chr	162,174	163,225	1,051
unitig_26_Part_Chr	165,628	166,710	1,082
unitig_26_Part_Chr	166,846	167,777	931
unitig_26_Part_Chr	191,563	192,205	642
unitig_26_Part_Chr	198,885	200,078	1,193
unitig_26_Part_Chr	199,987	200,815	828
unitig_26_Part_Chr	203,274	203,828	554
unitig_26_Part_Chr	204,157	204,774	617
unitig_26_Part_Chr	212,513	213,631	1,118
unitig_26_Part_Chr	216,642	219,899	3,257
unitig_26_Part_Chr	317,134	317,938	804
unitig_26_Part_Chr	318,784	319,771	987
unitig_28_Part_Chr	14,530	15,671	1,141
unitig_28_Part_Chr	16,435	17,160	725
unitig_28_Part_Chr	18,315	19,638	1,323
unitig_28_Part_Chr	2,218,774	2,220,207	1,433
unitig_28_Part_Chr	2,220,360	2,221,215	855
unitig_28_Part_Chr	2,229,134	2,229,758	624
unitig_28_Part_Chr	2,504,628	2,505,000	372

Table S3. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description
SKO-D-LBF	GGTCTTAAUAATCCCATAAAAGCGCTGAA	For single *D* deletion generation, left border, forward
SKO-D-LBR	GGCATTAAUCTGTCGTTTGCTCAGTTGGA	For single *D* deletion generation, left border, reverse
SKO-D-RBF	GGACTTAAUGATGG-TAGGGGGAAGGAGAG	For single *D* deletion generation, right border, forward
SKO-D-RBR	GGGTTTAAUGCACCATGCATAAAACGATG	For single *D* deletion generation, right border, reverse
DKO-D-LBF	GGTCTTAAUCGACAGACAGGAGGATGTCA	For double *D* deletion generation, left border, forward
DKO-D-LBR	GGCATTAAUGCGGCTCGAACTCTCTAAAC	For double *D* deletion generation, left border, reverse
DKO-D-RBF	GGACTTAAUCTATTCGCATTTTCGCGACT	For double *D* deletion generation, right border, forward
DKO-D-RBR	GGGTTTAAUTGAACAGCAGACCAACAGGA	For double *D* deletion generation, right border, reverse
outside primer-F	CTTCGATTGCTGTCACTGGA	Verification of *D* deletion mutants
outside primer-R	TGAACAGCAGACCAACAGGA	Verification of *D* deletion mutants
D-com-F	ggggacagctttcttgtacaaagtg-gaaAAAATCCCATAAAAGCGCTGAA	Complementation of *D* gene, forward
D-com-R	ggggacaactttgtataataaagttgtTTCCGCG-CATAATGAACTCG	Complementation of *D* gene, reverse
D-F (RT)	CGACTTGACGCATTTGGTTA	*V. dahliae D*, RT-PCR
D-R (RT)	CTGAGAACGACTTTCTCAT	*V. dahliae D*, RT-PCR
D-*Pich*-F	CGGTATGAATTCATTGCCATCCCGCAATC-CGATAC	D effector protein expression in *pichia pastoris*
D-*Pich*-R	CGGTATGCGGCCGCTTATTATCAAAGCTG-GCCTGCGTCAAAAGA	D effector protein expression in *pichia pastoris*
ITS-F	AAAGTTTTAATGGTTCGCTAAGA	Verticillium ribosomal internal transcribed spacer region (ITS), fungal biomass quantification
ITS-R	CTTGGTCATTTAGAGGAAGTAA	Verticillium ribosomal internal transcribed spacer region (ITS), fungal biomass quantification
VdGAPDH-F	CGAGTCCACTGGTGTCTTCA	*V. dahliae GAPDH*, RT PCR
VdGAPDH-R	CCCTCAACGATGGTGAACTT	*V. dahliae GAPDH*, RT PCR
GhUb-F	GAAGGCATTCCACCTGACCAAC	Cotton *ubiquitin* gene, fungal biomass quantification
GhUb-R	CAAAACTCCAAAATCATACCCAAAG	Cotton *ubiquitin* gene, fungal biomass quantification
AtRubisco-F	GCAAGTGTTGGGTTCAAAGCTGGTG	Arabidopsis *Rubisco* gene, fungal biomass quantification
AtRubisco-R	CCAGGTTGAGGAGTTACTCGGAATGCTG	Arabidopsis *Rubisco* gene, fungal biomass quantification

Primer name	Oligonucleotide sequence (5'→3')	Description
NbRubisco-F	TCCGGGTATTAGGAAAAGCGT	*N. benthamiana Rubisco* gene, fungal biomass quantification
NbRubisco-R	CCCAAGATCTGGGTCAGAGC	*N. benthamiana Rubisco* gene, fungal biomass quantification

[a] USER cloning sites present in primer sequence are underlined; Gateway cloning sites present in primer sequence are indicated in lower case letter. RT-PCR: Reverse Transcription-PCR.

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Chapter 3

A single effector mediates pathogenicity of *Verticillium dahliae* on tomato

Jinling Li[1$], Luigi Faino[1$] and Bart P.H.J. Thomma[1*]

[1]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands.
[$]These authors contributed equally to this work

*For correspondence: Bart P.H.J. Thomma, Laboratory of Phytopathology, WageningenUniversity & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands. Tel. 0031-317-484536, e-mail: bart.thomma@wur.nl

Abstract

In order to establish disease, many plant pathogens secrete so-called effector molecules to support host colonization, frequently through the modulation of host physiology. Accordingly, many effector molecules have been shown to be pivotal for microbial pathogenesis. *Verticillium dahliae* is a xylem-invading fungal pathogen that causes devastating vascular wilt disease on hundreds of plant species, including the economically important crop tomato (*Solanum lycopersicum*). Although individual strains are typically characterized by their broad host range, differential pathogenicity occurs on nearly all hosts. Currently, the molecular basis underlying differences in pathogenicity between *V. dahliae* strains remains unknown. In this study, we used comparative genomics to identify *V. dahliae* genes that confer pathogenicity on tomato plants. While genome analysis of tomato-pathogenic and non-pathogenic *V. dahliae* strains revealed five effector candidate genes that specifically occur in pathogenic strains, only one of them was expressed during host colonization. Functional analyses showed that this single effector governs pathogenicity of *V. dahliae* on tomato, as deletion of the effector gene resulted in loss of the ability to colonize tomato plants. Furthermore, transfer of this effector gene into a non-pathogenic *V. dahliae* isolate resulted in the ability to cause disease on tomato. Moreover, introduction of the gene in the non-pathogenic, saprophytic, sister species *V. tricorpus* and *V. nubilum* similarly resulted in tomato disease. Thus, we demonstrate that a single effector, named Tom1, mediates pathogenicity of *V. dahliae* on tomato.

Introduction

Verticillium is a relatively small genus of Ascomycete fungi that consists of ten soil-borne, asexual species with different lifestyles that range from saprophytic to pathogenic (Klosterman et al., 2009; Inderbitzin and Subbarao, 2014; Klimes et al., 2015). Among these species, *V. isaacii*, *V. klebahnii*, *V. zaregamsianum*, *V. tricorpus* and *V. nubilum* are considered to be saprophytes that occasionally cause opportunistic infections on plants that have been weakened by other stresses (Inderbitzin et al., 2011; Gurung et al., 2015; Seidl et al., 2015; Shi-Kunne et al., 2018). The remaining *Verticillium* species, *V. dahliae*, *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae* and *V. longisporum*, can cause vascular wilt diseases on economically important crops (Pegg and Brady, 2002; Fradin and Thomma, 2006; Inderbitzin et al., 2011; Depotter et al., 2016). However, despite the fact that the latter five species are all pathogenic, they differ significantly in host range. *V. dahliae* is the most notorious plant pathogen that can infect over 200 plant species (Fradin and Thomma, 2006). In contrast, other pathogenic *Verticillium* species have more restricted host ranges as, for instance, *V. albo-atrum* mainly infects hop, soybean, tomato and potato (Fradin and Thomma, 2006), while *V. longisporum* mainly causes diseases on brassicaceous plant hosts such as oilseed rape and cauliflower (Depotter et al., 2016), and *V. alfalfae* only infects lucerne (Inderbitzin et al., 2011). It has been estimated that the economic losses caused by Verticillium wilt diseases on the 20 most affected hosts collectively amount up to €3 billion worldwide (Depotter et al., 2016).

Verticillium wilt diseases are difficult to control due to the long viability of the resting structures, the scarcity of disease resistance in host germplasms, and the inability of fungicides to eliminate the pathogens once they have entered host plant xylem tissues (Fradin and Thomma, 2006; Klosterman et al., 2011). The high economic impact of Verticillium wilt diseases, combined with the absence of curative treatments, substantiates the need for developing novel disease control strategies.

Unravelling the mechanisms by which microbial pathogens cause diseases on plants is of fundamental importance to combat pathogen infections and control crop losses. Over the years, accumulating evidence has shown that the pathogenicity of a plant pathogen is largely mediated by so-called effector molecules that are secreted by the pathogen during host infection (Jones and Dangl, 2006; de Jonge et al., 2011; Rovenich et al., 2014; Rodriguez-Moreno et al., 2018). Most of secreted effector molecules are involved in suppression of plant immune responses or manipulation of host physiology to enable successful host colonization (Rovenich et al., 2014; Rodriguez-Moreno et al., 2018). It is generally observed that effector activities are redundant and single effectors are dispensable for virulence of the pathogens. As effectors are crucial for establishing parasitic symbiosis, the identification and functional characterization of effectors is important for a mechanistic understanding of microbial pathogenesis that ultimately provides valuable knowledge to develop effective disease management strategies (Gibriel et al., 2016).

Recent advances in genomic sequencing technologies and the rapid development of new bioinformatics algorithms, pipelines, and effector identification methods enable genomics-based discovery of effector candidates from various plant pathogens (Faino and Thomma, 2014; Thomma et al., 2015; Gibriel et al., 2016). Moreover, facilitated by the wealth of publically available genome sequences of multiple plant pathogens, comparative genomics approaches

have been applied to discover effectors by inferring differences in gene content or gene presence/absence polymorphisms between closely related pathogens, or different strains of the same species (Thomma et al., 2015; Gibriel et al., 2016). For instance, genome analysis of the cereal pathogens *Fusarium graminearum* and *F. verticillioides*, and the tomato pathogen *F. oxysporum* f. sp. *lycopersici* permitted the identification of four dispensable chromosomes that are specific to tomato-infecting *F. oxysporum* f. sp. *lycopersici* (Ma et al., 2010). Interestingly, pathogenicity of *F. oxysporum* f. sp. *lycopersici* on tomato could be specifically attributed to one dispensable chromosome that contains a number of effector genes (Ma et al., 2010). Comparative genome studies of *V. dahliae* isolates revealed that extensive genome rearrangements established highly variable lineage-specific (LS) genomic regions that only occur in part of the *V. dahliae* population (de Jonge et al., 2013; Faino et al., 2016). Interestingly, these LS genomic regions are greatly enriched for *in planta*-expressed effector genes, some of which have shown to be important for fungal aggressiveness (de Jonge et al., 2012, 2013; Kombrink et al., 2017). For instance, the most well-characterized LS effector is the race-specific effector Ave1 that is recognized by the tomato immune receptor Ve1 (Fradin et al., 2009; de Jonge et al., 2012). Interestingly, Ave1 is a potent virulence factor on tomato plants that lack resistance gene *Ve1*, Arabidopsis, tobacco and cotton (de Jonge et al., 2012; Song et al., 2018). Comparative genomics has further identified LS effectors that are important for virulence (de Jonge et al., 2013), including Vd2LysM, a chitin-binding effector that contributes to virulence on tomato through suppression of chitin-triggered host immunity (Kombrink et al., 2017). More recently, the *V. dahliae* D (for defoliating) effector that is responsible for causing defoliation symptoms on cotton and olive was identified through comparative genomics of strains that belong to the defoliating (D) and the non-defoliating (ND) pathotype *V. dahliae* (**Chapter 2**).

Tomato is considered one of the most important vegetable crops worldwide, with a net economic value exceeding 58 billion dollars (Vincent et al., 2013). Genetic resistance against *V. dahliae* has been characterized in tomato, as the tomato immune receptor Ve1 mediates resistance to race 1 strains of *V. dahliae* by recognition of the race1-specific effector Ave1 (Fradin et al., 2009; de Jonge et al., 2012). However, race 2 isolates that have overcome Ve1-mediated recognition by omission of the Ave1 effector gene arose (Dobinson et al., 1996; de Jonge et al., 2012). Besides race 1 and race 2 strains that have the ability to cause disease on particular tomato genotypes, strains that are unable to cause disease on tomato occur as well. In this study, we aimed to identify the molecular basis underlying *V. dahliae* pathogenicity on tomato by using comparative genomics.

Materials and methods

Pathogenicity assays

Pathogenicity assays were performed on twelve-day-old tomato seedlings of susceptible tomato (cv. Moneymaker) using the root-dipping inoculation method as previously described (Fradin et al., 2009). The disease symptoms were scored up to 21 days post inoculation (dpi). *V. dahliae* stem section outgrowth assays, canopy area measurements and fungal biomass quantifications were performed as previously described (Fradin et al., 2009; Santhanam et al., 2013; Song et al., 2018).

Phylogenetic analysis of sequenced *V. dahliae* strains

A phylogenetic tree of the *V. dahliae* strains was generated by REALPHY (version 1.12) (Bertels et al., 2014) using Bowtie2 (Langmead and Salzberg, 2012) to map genomic reads against the reference genome of *V. dahliae* strain JR2 for which a gapless genome assembly is available (Faino et al., 2015). A maximum likelihood phylogenetic tree was inferred using RAxML (version 8.2.8) with the GTRGAMMA model and 500 bootstrap replicates (Stamatakis, 2014). The genome sequence of *V. alfalfae* strain ms102 was used to root the tree.

V. dahliae comparative genomics

In order to identify *V. dahliae* genes that mediate pathogenicity on tomato, the whole-genome assembly of *V. dahliae* strain JR2 (Faino et al., 2015) was used as a reference. Next, short reads from pathogenic and non-pathogenic *V. dahliae* strains were mapped onto the reference using BWA software (Li and Durbin, 2010) with default options. The presence/absence analysis was performed using R scripts, and genomic regions were considered present if the breadth of coverage was ≥5x, while those with breadth of coverage <5x were considered absent. Genomic regions that are only present in tomato-pathogenic strains were determined and genes localized within these regions were extracted using an R script. To further characterize potential effector genes, SignalP (version 4.1) (Petersen et al., 2011) software was used to identify secretion signal peptides at the N-termini of the encoded proteins. Subsequently, the machine-learning approach applied in EffectorP (version 1.0) (default parameters) was used to identify potential effector genes (Sperschneider et al., 2016).

Gene expression analysis

To determine expression profiles of effector candidate genes during *V. dahliae* infection of tomato, two week-old tomato (cv. Moneymaker) seedlings were inoculated with *V. dahliae* strain JR2 and stems were harvested at 4, 8, 12, 16 and 18 days post inoculation (dpi). Total RNA extraction and cDNA synthesis were performed as previously described (Santhanam et al., 2013). Quantitative real time-PCR (qRT-PCR) was performed with primers listed in Table S3, using the *V. dahliae GAPDH* (GAPDH, glyceraldehyde-3-phosphate dehydrogenase) gene as an endogenous control (Table S3).

Generation of *Tom* gene deletion strains and complementation strains

To generate a *Tom1* gene deletion construct, sequences that flank the *Tom1* coding sequence were amplified from genomic DNA of *V. dahliae* strain JR2 using the primers KO-Tom1-LBF and KO-Tom1-LBR, and primers KO-Tom1-RBF and KO-Tom1-RBR (Table S3), and cloned into the binary vector pRF-HU2 (Frandsen et al, 2008). The resulting *Tom1* gene deletion construct was used to transform *V. dahliae* strain JR2 as previously described (Santhanam et al., 2013). The putative deletion transformants were selected on PDA containing cefotaxime (200 µg/mL) and hygromycin B (50 µg/mL) (Duchefa, Haarlem, the Netherlands), and the absence of the *Tom* gene was verified with PCR.

To generate the *Tom1* complementation construct, a genomic fragment consisting of the *Tom1* coding sequence plus 1.2 kb upstream and 1.1 kb downstream (*pTom1::Tom1*) were amplified using primers Tom1-com-F and Tom1-com-R (Table S3) and cloned into the Gateway™ compatible vector PCG using a standard BP reaction (Zhou et al., 2013). The resulting complementation construct was transformed into the *Tom1* deletion mutant, *V. dahliae* strain ST100, *V. tricorpus* strain MUCL9792, and *V. nubilum* strain PD397, respectively. Putative *Tom1* complementation transformants were selected on PDA supplemented with cefotaxime (200 µg/mL) and geneticin (25 µg/mL) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and successful transformation was confirmed with PCR.

Results

Verticillium dahliae inoculations on susceptible tomato plants

Previously, various *V. dahliae* strains have been sequenced in our lab, of which strains JR2, VdLs17, 2009-605, DVD3, DVD-S161, DVD-31, DVD-S26, DVD-S29, DVD-S94, V4 and CBS38166 were known to be able to cause disease on tomato, while strain ST100 lacks the ability to infect tomato (de Jonge et al., 2012, 2013; Faino et al., 2015). In order to identify additional tomato non-pathogenic strains, we generated a phylogenetic tree of all sequenced *V. dahliae* strains (Table S1), revealing that *V. dahliae* strains v781I, TM6, CQ2, V991, V117, V1381I, 463, V76 and T9 clustered with the tomato non-pathogenic strain ST100, while the remaining strains were found to be more related to previously characterized tomato-pathogenic strains (Figure 1).

Of particular interest are strains V574, V700, v679 and Vd39 that are phylogenetically the most distinct under the close relatives of ST100 (Figure 1). Thus, we selected V991, V117, T9, V574 and Vd39 to evaluate their capacity to cause disease on tomato. In contrast to JR2, which induced clear stunting and significant reduction in canopy area development on inoculated tomato plants, all of these strains failed to cause any visible tomato disease symptoms just like ST100 (Figure 2A-B). This finding was further corroborated by fungal recovery assays, as plating stem sections of the inoculated plants on agar medium resulted in fungal outgrowth from all sections of JR2-inoculated plants, while no fungal growth was recovered from stem sections of plants inoculated with any of the other strains (Figure 2C). Collectively, these data demonstrate that stains ST100, V991, V117, T9, Vd39 and V574 are non-pathogenic on tomato.

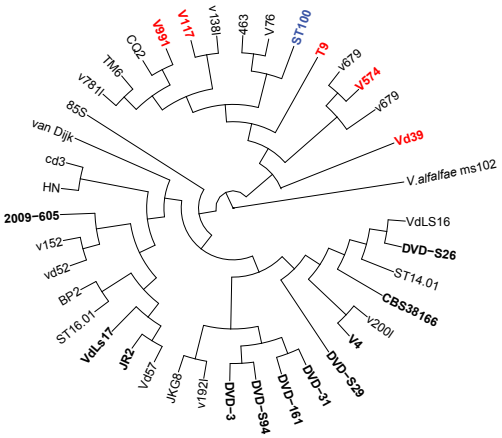

Figure 1. Phylogenetic tree of sequenced *V. dahliae* strains. Previously characterized tomato-pathogenic and non-pathogenic strains are shown in bold black and blue font, respectively. Strains that were selected for phenotypic characterization in this study are shown in red font. Phylogenetic relationships between sequenced *V. dahliae* strains are inferred using RealPhy (Bertels et al., 2014). *V. alfalfae* strain ms102 was used to root the tree.

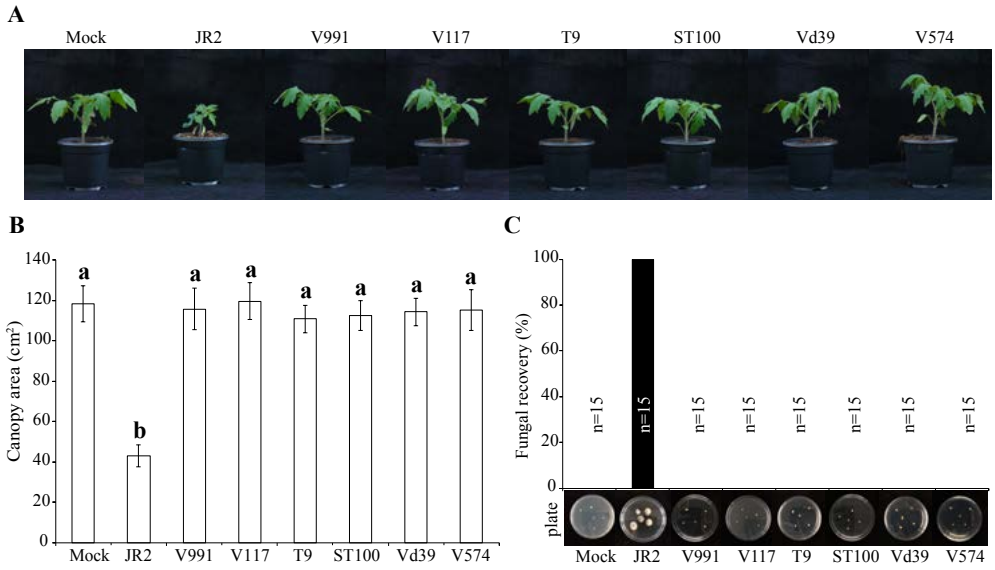

Figure 2. Disease assays of selected *V. dahliae* strains on tomato. (A) Typical appearance of tomato (cv. Moneymaker) plants upon mock-inoculation or inoculation with *V. dahliae* strain JR2, V991, V117, T9, ST100, Vd39 and V574 at 21 days post inoculation (dpi). **(B)** Average canopy area with standard deviations of five tomato plants inoculated with the various *V. dahliae* strains or upon mock-inoculation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of 15 stem sections on PDA medium. All experiments have been repeated three times and similar results were observed.

Comparative genomics identifies pathogenicity effector candidates

Recently, a gapless, complete whole-genome assembly of tomato-pathogenic strain JR2 was generated (Faino et al., 2015) and used for the reference genome. Here, we used the genome sequences of eleven pathogenic strains (JR2, VdLs17, 2009-605, DVD3, DVD-S161, DVD-31, DVD-S26, DVD-S29, DVD-S94, V4 and CBS38166) and six non-pathogenic strains (ST100, V991, V117, T9, Vd39 and V574) for comparative genome analysis and identified ~133 kb of sequence that is shared by all pathogenic strains and absent from all non-pathogenic strains, collectively encoding thirty-four genes. Five of these thirty-four genes encode putative effectors based on EffectorP (Sperschneider et al., 2016) (Table S2). Real-time PCR revealed that one of the five effector candidate genes (VDAG_JR2_Chr3g13460) is expressed during host colonization, with a peak in expression at 12 days post inoculation, while the remaining four effector candidates are not expressed (Figure 3).

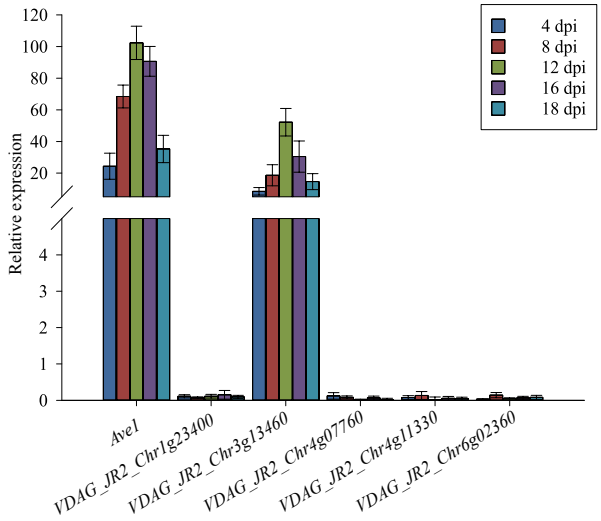

Figure 3. Expression of five candidate genes during *V. dahliae* tomato colonization. Twelve day-old tomato (cv. Moneymaker) seedlings were root-inoculated with *V. dahliae* strain JR2 and plants were harvested at intervals between 4 and 18 days post inoculation (dpi). Quantitative real-time PCR was performed to determine the relative expression of the five candidate genes using the *V. dahliae GAPDH* gene as a reference. The previously characterized effector gene *Ave1* (de Jonge et al., 2012) was used as a positive control. Bars represent averages with standard deviation of two biological repeats.

Thus, we tentatively named this *in planta*-expressed effector gene as *Tom1* gene, for potentially mediating *V. dahliae* pathogenicity on **tom**ato. The *Tom1* gene encodes a secreted protein of 127 amino acids, with no predicted functional domains as determined with InterPro analysis (Finn et al., 2017). To analyse potential *Tom1* gene diversity, we mined the genomes of 39 sequenced *V. dahliae* strains for *Tom1* gene sequence variation. Intriguingly, only one single nucleotide polymorphism (SNP) was identified in one pathogenic strain, which results in a synonymous substitution that does not affect the sequence of the encoded protein.

The Tom1 effector is essential for *V. dahliae* pathogenicity on tomato

To investigate the role of *Tom1* in pathogenicity of *V. dahliae* on tomato, we generated *Tom1* deletion mutants (Figure S1A) in *V. dahliae* strain JR2 and inoculated these on susceptible tomato plants. Compared to the wild type strain, *Tom1* deletion strains (ΔTom1#1 and ΔTom1#2) appeared to have lost their ability to infect tomato, as no disease symptoms were observed (Figure 4A). This finding was corroborated by measurements of canopy area development, as ΔTom1-inoculated plants developed a similar canopy area as mock-inoculated plants, while JR2-inoculated plants displayed significantly less canopy area development (Figure 4B). Moreover, fungal recovery assays showed that no fungus could be recovered from stem sections of ΔTom1-inoculated plants, in contrast to the abundant fungal outgrowth that was monitored from JR2-inoculated plants (Figure 4C). Importantly, the loss of pathogenicity displayed by *Tom1* deletion strains can be restored upon introduction of a genomic construct that encodes the *Tom1* gene (Figure 4). Collectively, these data show that *Tom1* encodes a pathogenicity effector that is crucial for colonization of tomato plants.

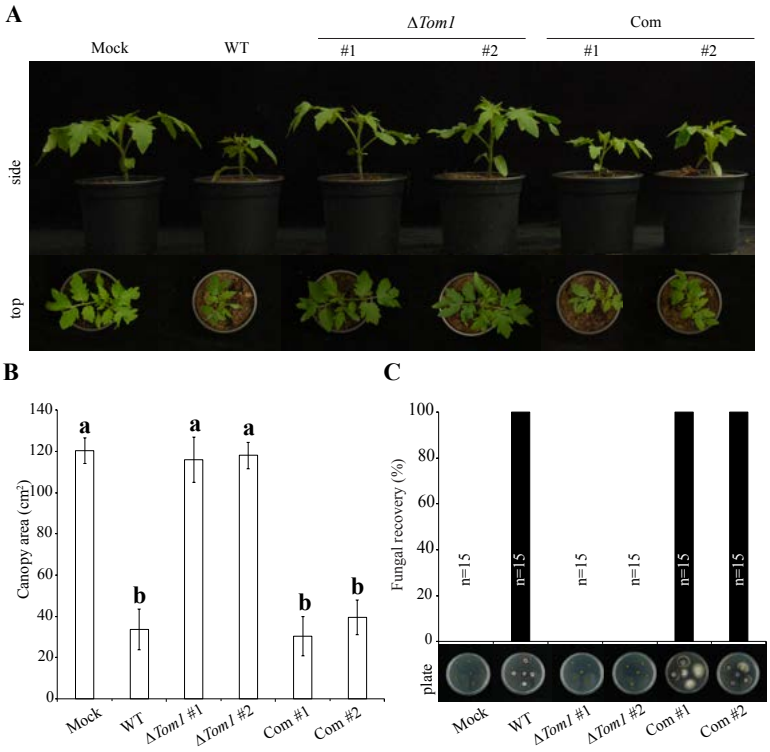

Figure 4. The *Tom1* gene is required for pathogenicity on tomato. (A) Typical appearance of tomato (cv. Moneymaker) plants upon mock-inoculation or inoculation with wild type strain JR2 (WT), two independent *Tom1* deletion strains (ΔTom1 #1 and ΔTom1 #2) and two independent *Tom1* complementation strains (Com #1 and Com #2) at 21 days post inoculation (dpi). **(B)** Average canopy area with standard deviations of five tomato plants inoculated with the different *V. dahliae* strains or upon mock-inoculation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of 15 stem sections on PDA medium. All experiments have been repeated three times and similar results were observed.

Tom1 introduction is sufficient to cause pathogenicity on tomato

Whilst not pathogenic on tomato, *V. dahliae* strain ST100 is pathogenic on a diversity of other plant hosts, and thus likely possesses a complement of effector genes to subvert plant immunity (**Chapter 2**). To investigate whether the *Tom1* gene is not only required, but perhaps even sufficient to mediate pathogenicity on tomato, we introduced the gene into the tomato non-pathogenic *V. dahliae* strain ST100 (Figure S1B).

Interestingly, *Tom1*-expressing *V. dahliae* ST100 strains gained the ability to infect tomato, as tomato plants inoculated with the *Tom1* expression strains showed a clear reduction in canopy area development when compared with plants that were inoculated with the wild-type ST100 strain (Figure 5A-B). Fungal outgrowth assays confirmed that the *Tom1* expression strains colonized tomato plants while the wild-type ST100 strain was not able to do so, confirming that the transformants gained tomato pathogenicity (Figure 5C).

Previous studies have shown that the *Verticillium* species *V. tricorpus* and *V. nubilum* have a saprophytic, rather than a pathogenic, lifestyle (Inderbitzin et al., 2011; Seidl et al., 2015). Thus, we tested whether *V. tricorpus* strain MUCL9792 and *V. nubilum* strain PD397 can gain tomato pathogenicity upon introduction of the *Tom1* gene (Figure S1C, D). As anticipated, tomato plants inoculated with the wild type *V. tricorpus* and *V. nubilum* strains showed no disease symptoms (Figure 6A, 7A). In contrast, *Tom1*-expressing transformants of both species gained the ability to infect tomato, as evidenced by significant reductions in canopy area development (Figure 6B, 7B), as well as by fungal recovery assays (Figure 6C, 7C). Taken together, our data show that the *Tom1* effector is necessary and sufficient to cause *Verticillium* wilt disease on tomato.

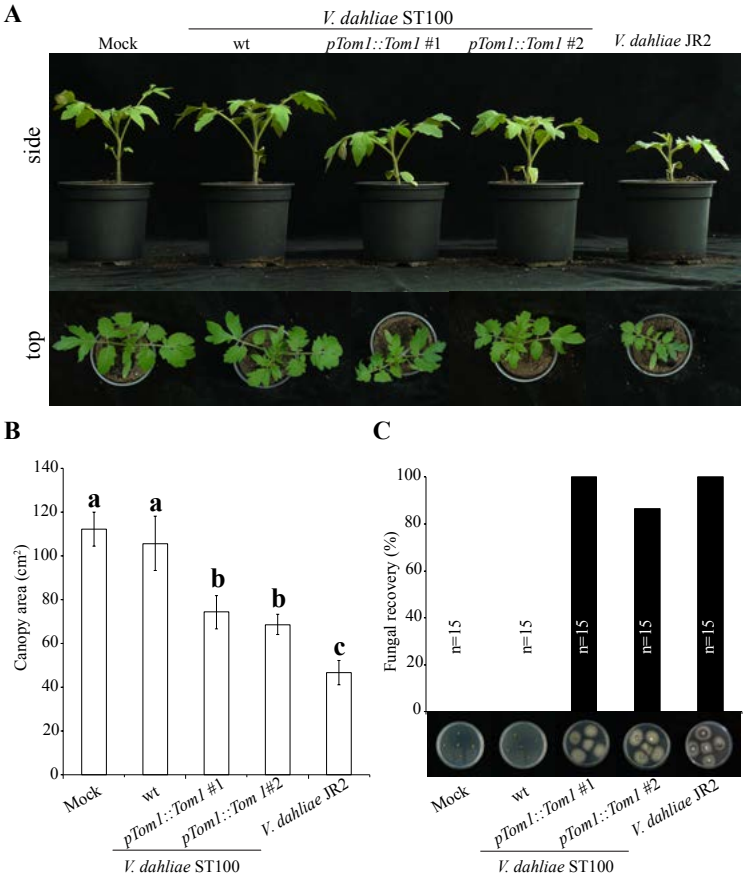

Figure 5. Introduction of *Tom1* into the non-pathogenic *V. dahliae* strain ST100 results pathogenicity on tomato. **(A)** Typical appearance of tomato plants (cv. Moneymaker) upon mock-inoculation or inoculation with wild type strain ST100 (wt), two independent *Tom1* expression transformants (ΔTom1 #1 and ΔTom1 #2) and *V. dahliae* strain JR2 at 21 days post inoculation (dpi). **(B)** Average canopy area with standard deviations of five tomato plants inoculated with the various *V. dahliae* strains or upon mock-inoculation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of 15 stem sections on PDA medium. All experiments have been repeated three times and similar results were observed.

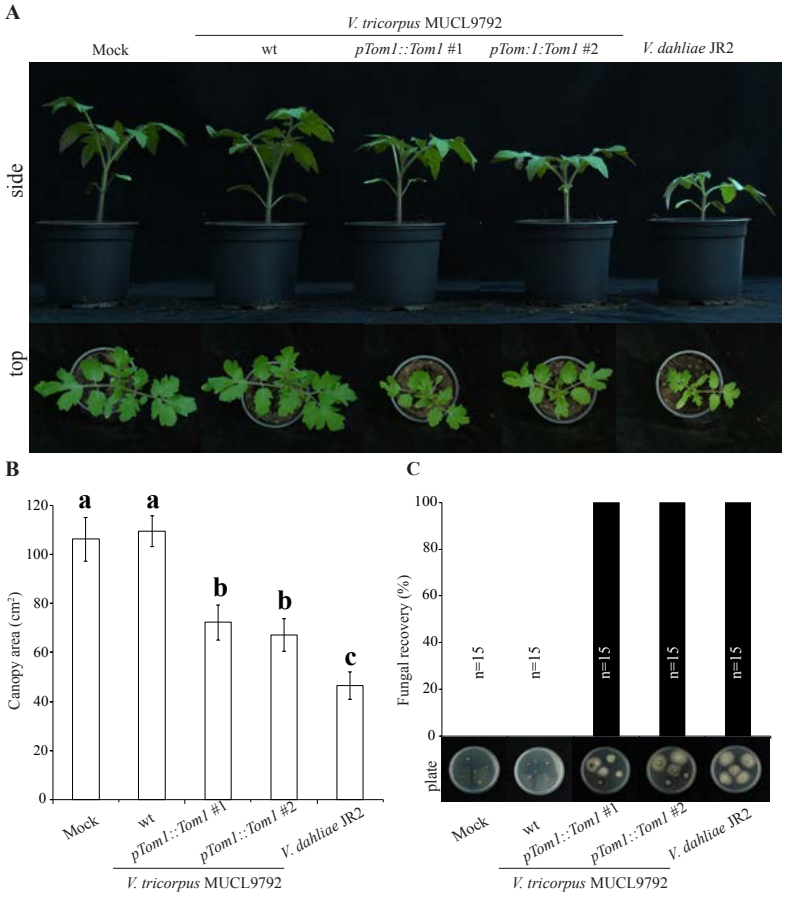

Figure 6. Introduction of *Tom1* into the saprophytic sister species *V. tricorpus* incites the ability to cause disease on tomato. (A) Typical appearance of tomato plants cv. Moneymaker upon mock-inoculation or inoculation with wild type *V. tricorpus* strain MUCL9792 (wt), two independent *Tom1* expression transformants (*pTom1::Tom1* #1 and *pTom1::Tom1* #2) and *V. dahliae* strain JR2 at 21 days post inoculation (dpi). **(B)** Average canopy area with standard deviations of five tomato plants inoculated with the various fungal strains or upon mock-inoculation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of 15 stem sections on PDA medium.

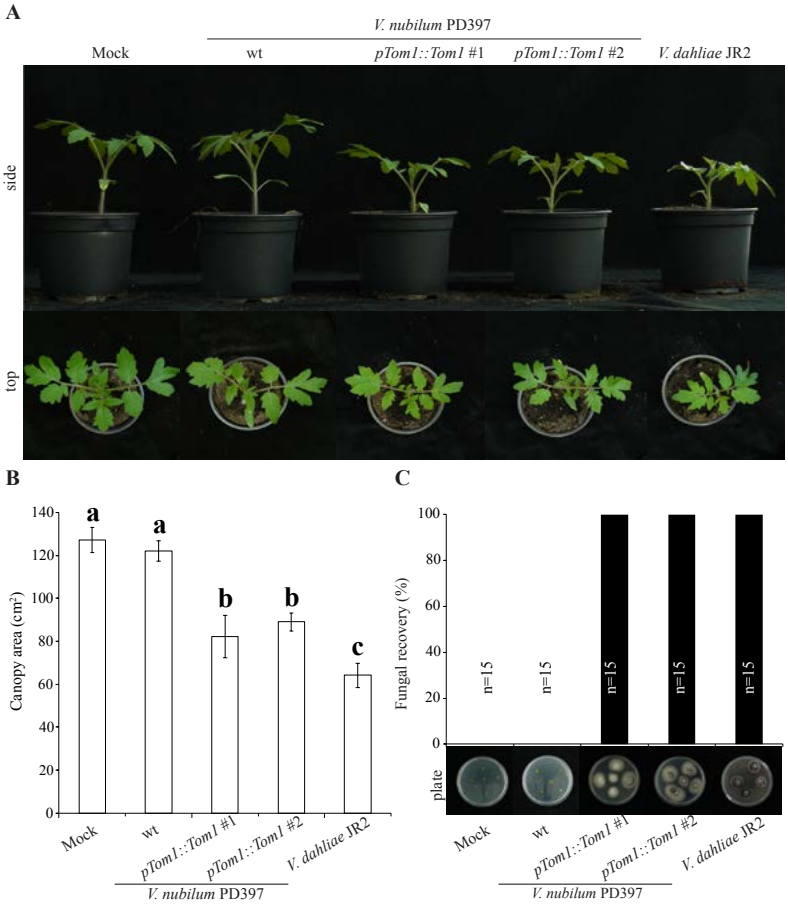

Figure 7. Introduction of *Tom1* into the saprophytic sister species *V. nubilum* enable to cause disease on tomato. **(A)** Typical appearance of tomato cv. Moneymaker upon mock-inoculation or inoculation with wild type *V. nubilum* strain PD397 (wt), two independent *Tom1* expression transformants (*pTom1::Tom1* #1 and *pTom1::Tom1* #2) and *V. dahliae* strain JR2 at 21 days post inoculation (dpi). **(B)** Average canopy area with standard deviations of five tomato plants inoculated with the various fungal strains or upon mock-inoculation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of 15 stem sections on PDA medium. All experiments have been repeated three times and similar results were observed.

Discussion

Plant pathogens employ a multitude of effectors to modulate host immunity and promote disease (Rovenich et al., 2014, Toruño et al., 2016). As such, effectors of plant pathogens have been shown to display functional redundancy as mutation or deletion of single effector typically does not result in significant loss of virulence in host plants (de Jonge et al., 2011; Tan et al., 2015; Win et al., 2012). Therefore, individual contributions of single effector to host colonization are often considered to be dispensable. Nevertheless, a number of studies have shown that a single effector molecule is able to govern the pathogenicity of pathogen and enables disease

establishment. For example, the transcriptional activator-like effector protein PthA has been shown to mediate pathogenicity of bacterial pathogen *Xanthomonas citri* on citrus (Swarup et al., 1991, 1992; Duan et al., 1999), as introduction of pthA into non-pathogenic strains confers the ability to cause disease symptoms on citrus. Additionally, the necrotrophic effector ToxA confers pathogenicity on wheat lines harbouring the corresponding susceptibility gene *Tsn1* in the fungal wheat pathogens *Pyrenophora tritici-repentis* and in *Parastagonospora nodorum* (Friesen et al., 2006). We recently showed that the D effector confers pathogenicity on cotton, olive, Arabidopsis and *Nicotiana benthamiana* to *V. dahliae* strains of the defoliating pathotype (**Chapter 2**). In the present study, we show that a single effector, named Tom1, is required for pathogenicity of *V. dahliae* on tomato, as deletion of the effector gene leads to loss of the ability to colonize tomato plants (Figure 4). Moreover, introduction of this effector gene into a non-pathogenic *V. dahliae* isolate is sufficient to cause wilt disease on tomato (Figure 5). Thus, our data further reinforce the notion that a single effector can be sufficient to enable disease establishment.

Host specificity refers to the capability of pathogenic microbial species, or members of a species, to cause disease only on a particular range of defined plant hosts (Borah et al., 2018). The basis of host specificity, i.e. the molecular factors that determine the taxonomic range of hosts that a specific pathogenic microbe infects, is largely unknown (Nissan et al., 2006). Over the years, a number of studies have suggested that the host range of the pathogen is governed by the presence of host-specific virulence genes that contribute to disease establishment (van der Does and Rep, 2007). For instance, comparative analysis of the genome sequence of *Fusarium oxysporum* f.sp. *lycopersici*, a vascular wilt pathogen of tomato, with those of phylogenetically related *Fusarium* pathogens revealed that a mobile pathogenicity chromosome that contains various effector genes is responsible for host specificity on tomato, as transfer of this chromosome to non-pathogenic strains can render these pathogenic on tomato (Ma et al., 2010).

Several studies have also shown that even a single pathogenicity effector can act as a pathogen host-specificity determinant. For instance, the necrotrophic toxin effector ToxA not only mediates pathogenicity of the fungal wheat pathogens *P. tritici-repentis* and *P. nodorum*, but also defines host specificity on particular wheat genotypes (Friesen et al., 2006, 2008). In addition, HsvG, a type III effector of the gall-forming bacterial pathogen *Pantoea agglomerans* pv. *gypsophilae*, functions as a pathogenicity effector and determines host specificity on the ornamental plant *Gypsophila paniculata* L. Gypsophila. Mutations of *HsvG* abolished the ability of the pathogen to cause disease symptoms on *G. paniculata* L. Gypsophila, but still retained full pathogenicity on beet (Valinsky et al., 1998; Nissan et al., 2006). While *V. dahliae* strains are collectively characterized by their broad host range, pathogenic isolates display various levels of host specificity as individual isolates only infect a limited number of plant species (Bhat and Subbarao, 1999). So far, little is known about the genetic basis for host-specificity of *V. dahliae* strains. In this study, we show that the Tom1 effector enables *V. dahliae* strains to cause wilt disease on tomato. Interestingly, no *Tom1* gene expression was observed at any of time points based on RNA-Seq of a time course of *V. dahliae*-infected Arabidopsis plants (data not shown). RNA-Seq of a time course of *V. dahliae*-inoculated *N. benthamiana* plants (Faino et al., 2012) showed that *Tom1* is only expressed at 12 days post inoculation (data not shown), when major symptoms of disease, including wilting and

significant stunting were already. Therefore, it is unlikely that the Tom1 effector plays a role on Arabidopsis and *N. benthamiana* that is as determinant as it is on tomato. Moreover, *V. dahliae* strains of the D pathotype that lack the *Tom1* effector gene are unable to cause disease on tomato, but can still induce severe disease on Arabidopsis and *N. benthamiana* as shown in **Chapter 2**. Thus, we reason that the Tom1 effector may act as a pathogenicity effector that determines host specificity of *V. dahliae* on tomato, but future experiments need to confirm this hypothesis.

So far, the most sustainable strategy to combat Verticillium wilt disease is the deployment of resistant cultivars (Fradin and Thomma, 2006). Although the tomato resistance (*R*) gene *Ve1* has been introduced into tomato cultivars to confer resistance to *V. dahliae* race 1 strains (Schaible et al., 1951; Kawchuk et al., 2001; Fradin et al, 2009), race 2 isolates that escape Ve1-mediated recognition through omission of the *Ave1* effector gene arose and have steadily become a threat to tomato production (Pegg and Brady, 2002; Yadeta, 2012). Unfortunately, there is presently no *R* gene against race 2 isolates on tomato (Yadeta, 2012). As effectors are important virulence molecules of pathogens that can be potentially recognized by plant hosts (Rodriguez-Moreno et al., 2018), they have been used as tools to probe plant germplasm to identify *R* genes in resistance breeding (Laugé et al., 1998; Vleeshouwers and Oliver, 2014). This can be done by screening plant germplasm for *R* genes that trigger robust immune responses, such as a hypersensitive response, upon transient expression of effector gene *in planta* or purified effector protein infiltration (Vleeshouwers et al., 2011). This effector-assisted breeding has been successfully used for identifying a number of *R* genes in wild tomato species against the leaf mould pathogen *Cladosporium fulvum* (Laugé et al., 1998; Takken et al., 1999; de Wit, 2016) as well as against the late blight pathogen *Phytophthora infestans* in potato (Du et al., 2015; Vleeshouwers and Oliver, 2014). It is thus advisable that the Tom1 effector is used for screening tomato germplasm for *R* gene-mediated recognition. Alternatively, host-induced gene silencing (HIGS), which involves host plants expressing double-stranded RNAs to silence essential pathogen genes, can be exploited. HIGS has been recently developed for controlling Verticillium wilt disease in cotton as well as in tomato (Zhang et al., 2016; Song and Thomma, 2016). Song and Thomma (2016) showed that HIGS can be utilized to suppress Verticillium wilt disease by silencing virulence genes of *V. dahliae* in tomato, which can offer an alternative to *R* genes against *V. dahliae*. In addition, spray-induced gene silencing (SIGS) in which sRNAs are used to treat plants may be pursued as well (Wang et al., 2016; Wang et al., 2017; Wang and Jin, 2017). Since we show that the Tom1 effector mediates *V. dahliae* pathogenicity on tomato, we expect that the *Tom1* gene is an ideal silencing target for HIGS or SIGS, and thus establishment of Verticillium wilt resistance.

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Supplemental information

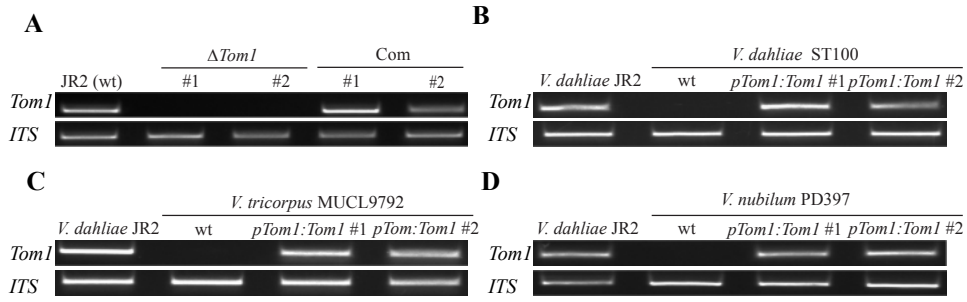

Figure S1. Verification of *Tom1* deletion and complementation strains. (A) Amplification of *Tom1* from genomic DNA of *V. dahliae* strain JR2, two independent *Tom1* deletion strains (ΔTom1 #1 and ΔTom1 #2) and two *Tom1* complementation strains (Com #1 and Com #2). As an endogenous control, a fragment of the *Verticillium* ITS region was amplified. **(B)** Amplification of *Tom1* from genomic DNA of *V. dahliae* strain JR2, wild type *V. dahliae* strain ST100 (wt), and two independent *Tom1* expression transformants (*pTom1::Tom1* #1 and *pTom1::Tom1* #2). As an endogenous control, a fragment of the *Verticillium* ITS region was amplified. **(C)** Amplification of *Tom1* from genomic DNA of *V. dahliae* strain JR2, wild type *V. tricorpus* strain MUCL9792 (wt), and two independent *Tom1* expression transformants (*pTom1::Tom1* #1 and *pTom1::Tom1* #2). As an endogenous control, a fragment of the *Verticillium* ITS region was amplified. **(D)** Amplification of *Tom1* from genomic DNA in *V. dahliae* strain JR2, wild type *V. nubilum* strain PD397 (wt), and two independent *Tom1* expression transformants (*pTom1::Tom1* #1 and *pTom1::Tom1* #2). As an endogenous control, a fragment of the *Verticillium* ITS region was amplified.

Table S1. *V. dahliae* strains used in this study.

Strain	Sequencing platform	Reference	Origin	Geographical location
HN	Illumina	Xu et al., 2012	Cotton	China
cd3	Illumina	Xu et al., 2012	Cotton	China
VdLs17	PacBio	Faino et al., 2015	Lettuce	USA
JR2	PacBio	Faino et al., 2015	Tomato	Canada
Vd57	Illumina	Chapter 2 of this thesis	Strawberry	Germany
V152	Illumina	Kombrink et al., 2017	Oak	Hungary
Vd52	Illumina	Kombrink et al., 2017	Pepper	Austria
van Dijk	Illumina	Kombrink et al., 2017	Chrysanthemum	The Netherlands
BP2	Illumina	Zhang et al., 2012	Cotton	China
ST16.01	Illumina	Chapter 2 of this thesis	Cotton	Syria
2009-605	Illumina	Chapter 2 of this thesis	Bell pepper	Ukraine
V4	Illumina	Keykhasaber, 2017	Olive	Spain
V200I	Illumina	Chapter 2 of this thesis	Strawberry	Germany
CBS38166	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S26	Illumina	de Jonge et al., 2012	Soil	Canada
VdLS16	Illumina	de Jonge et al., 2012	Lettuce	USA
ST14.01	Illumina	de Jonge et al., 2012	Pistachio	USA
DVD-S29	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-31	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-161	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S94	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-3	Illumina	de Jonge et al., 2012	Potato	Canada
V192I	Illumina	Chapter 2 of this thesis	Cotton	Spain
JKG8	Illumina	Kombrink et al., 2017	Potato	The Netherlands
85S	PacBio	Chapter 2 of this thesis	Sunflower	France
Vd39	Illumina	Chapter 2 of this thesis	Sunflower	Germany
V574	Illumina	Milgroom et al., 2014	Artichoke	Spain
v700	Illumina	Milgroom et al., 2014	Artichoke	Spain
v679	Illumina	Milgroom et al., 2014	Artichoke	Spain
T9	Illumina	Keykhasaber, 2017	Cotton	USA
V781I	Illumina	Chapter 2 of this thesis	Olive	Spain
V138I	Illumina	Chapter 2 of this thesis	Cotton	Spain
TM6	Illumina	Keykhasaber, 2017	Cotton	China
V117	Illumina	Keykhasaber, 2017	Olive	Spain
V991	Illumina	Zhang et al., 2012	Cotton	China
CQ2	PacBio	Chapter 2 of this thesis	Cotton	China
ST100	Illumina	de Jonge et al., 2012	Soil	Belgium
V76	Illumina	Chapter 2 of this thesis	Cotton	Mexico
463	Illumina	Chapter 2 of this thesis	Cotton	Mexico

Table S2. Tomato-pathogenic strain-specific genes identified by comparative genomics.

Gene ID	Effector P (Yes/No)
VDAG_JR2_Chr1g01610	No
VDAG_JR2_Chr1g01620	No
VDAG_JR2_Chr1g07050	No
VDAG_JR2_Chr1g07060	No
VDAG_JR2_Chr1g12270	No
VDAG_JR2_Chr1g13280	No
VDAG_JR2_Chr1g23400	Yes
VDAG_JR2_Chr2g00380	No
VDAG_JR2_Chr2g00780	No
VDAG_JR2_Chr2g03850	No
VDAG_JR2_Chr2g03860	No
VDAG_JR2_Chr2g12810	No
VDAG_JR2_Chr3g06060	No
VDAG_JR2_Chr3g09230	No
VDAG_JR2_Chr3g09240	No
VDAG_JR2_Chr3g09250	No
VDAG_JR2_Chr3g13460	Yes
VDAG_JR2_Chr4g02130	No
VDAG_JR2_Chr4g07760	Yes
VDAG_JR2_Chr4g11330	Yes
VDAG_JR2_Chr5g02370	No
VDAG_JR2_Chr5g08390	No
VDAG_JR2_Chr6g02360	Yes
VDAG_JR2_Chr6g02370	No
VDAG_JR2_Chr6g03370	No
VDAG_JR2_Chr6g03410	No
VDAG_JR2_Chr7g03300	No
VDAG_JR2_Chr7g05290	No
VDAG_JR2_Chr8g06110	No
VDAG_JR2_Chr8g06120	No
VDAG_JR2_Chr8g08950	No
VDAG_JR2_Chr8g09620	No
VDAG_JR2_Chr8g09630	No
VDAG_JR2_Chr8g09640	No

Table S3. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
VdAve1-F	AGCTTTCTACGCTTGGA	qRT-PCR
VdAve1-R	TTGGCTGGGATTGCT	qRT-PCR
VDAG_JR2_Chr1g23400-F	ACTGGAAGGTGACGATGTCC	qRT-PCR
VDAG_JR2_Chr1g23400-R	GGGGTACGCTACCTTCTTCC	qRT-PCR
VDAG_JR2_Chr3g13460-F	GCTTCACTGTGTCCGCTGTA	qRT-PCR
VDAG_JR2_Chr3g13460-R	TCCATATCTTCCCAGGTTGG	qRT-PCR
VDAG_JR2_Chr4g07760-F	ACCATGTAAGACTTGAGGCCA	qRT-PCR
VDAG_JR2_Chr4g07760-R	AGCGCTCCTGACTGTTATGA	qRT-PCR
VDAG_JR2_Chr4g11330-F	GCGTACCTCGATTCTCTTCG	qRT-PCR
VDAG_JR2_Chr4g11330-R	TTCCGTAAGGTGGCTGAGAT	qRT-PCR
VDAG_JR2_Chr6g02360-F	TTAACATGACGGACCACGAA	qRT-PCR
VDAG_JR2_Chr6g02360-R	ACCCTGAAGCACAGCTTCTC	qRT-PCR
Tom1-LBF	GGTCTTAAUTGTAATCGGCGATAGGAAGG	For *Tom1* deletion, left border, forward
Tom1-LBR	GGCATTAAUGACTTTGGCAACATGGTGAA	For *Tom1* deletion, left border, reverse
Tom1-RBF	GGACTTAAUAGATCACCCACCTGACCTTG	For *Tom1* deletion, right border, forward
Tom1-RBR	GGGTTTAAUCGCGACTCTGCCTTCTTAAC	For *Tom1* deletion, right border, reverse
Tom1-com-F	ggggacagctttcttgtacaaagtggaaTGTAATCG-GCGATAGGAAGG	For *Tom1* complementation, forward
Tom1-com-R	ggggacaactttgtataataaagttgtCGCGACTCT-GCCTTCTTAAC	For *Tom1* complementation, reverse
ITS-F	AAAGTTTTAATGGTTCGCTAAGA	Fungal biomass quantification
ITS-R	CTTGGTCATTTAGAGGAAGTAA	Fungal biomass quantification
SI-RUB-F	GAACAGTTTCTCACTGTTGAC	Tomato *rubisco* gene
SI-RUB-R	CGTGAGAACCATAAGTCACC	Tomato *rubisco* gene
VdGAPDH-F	CGAGTCCACTGGTGTCTTCA	*V. dahliae GAPDH*, qRT-PCR
VdGAPDH-R	CCCTCAACGATGGTGAACTT	*V. dahliae GAPDH*, qRT-PCR

[a] USER cloning sites in primer sequences are underlined; Gateway cloning sites in primer sequences are in lower cases. QRT-PCR: Quantitative Real Time-PCR.

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Chapter 4

The *Verticillium dahliae* effector Sun1 quantitatively contributes to virulence on sunflower

Jinling Li[1$], Hesham Gibriel[1$], Hélène Missonnier[2], Luigi Faino[1], Xiaoqian Shi-Kunne[1], Michael F. Seidl[1], Alban Jacques[2#], and Bart P.H.J. Thomma[1#*]

[1]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands.
[2]Department of Physiologie, Pathologie et Génétique Végétales (PPGV), Université de Toulouse, INP- PURPAN, Toulouse, France, Syngenta France S.A.S., Saint-Sauveur, France

[$]These authors contributed equally to this work
[#]These authors contributed equally to this work

[*]For correspondence: Bart P.H.J. Thomma, Laboratory of Phytopathology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands. Tel. 0031-317-484536, e-mail: bart.thomma@wur.nl

Abstract

Plant pathogens employ effector molecules to manipulate host physiology and establish themselves within their hosts. *Verticillium dahliae* is a highly destructive xylem-colonizing fungal pathogen that causes vascular wilt disease on diverse crops, such as tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), and sunflower (*Helianthus annuus*). In this study, we show that only particular *V. dahliae* strains cause vascular wilt disease on sunflower. Based on comparative genomics of a sunflower pathogenic strain with a diversity of non-pathogenic strains, we identified two candidate effector genes that occur in the sunflower-pathogenic strain 85S and that are highly expressed during host colonization. Intriguingly, these two candidate effector genes appeared to be identical copies that arose by a segmental duplication. Moreover, we show that this duplicated effector gene quantitatively contributes to *V. dahliae* virulence on sunflower but not on *Nicotiana benthamiana* or *Arabidopsis thaliana*.

Introduction

Plants and microbes engage in a diverse array of symbiotic relationships, ranging from pathogenic to mutualistic. Pathogenic interactions between plants and microbial pathogens have been described as ongoing arms races in which plants try to halt microbial ingress while pathogens attempt to continue symbiosis (Jones and Dangl, 2006; Thomma et al., 2011; Cook et al., 2015; Rovenich et al., 2014). In these arms races, plants have developed various types of immune receptors to detect invading pathogens through sensing various pathogen-derived or pathogen-induced molecular patterns, so-called invasion patterns, that betray microbial invasion to activate appropriate immune responses (Cook et al., 2015; Rodriguez-Moreno et al., 2017). In turn, to enable a parasitic life on their hosts, microbial pathogens secrete so-called effector molecules to deregulate host immune responses and support successful host infection (Jones and Dangl, 2006; Rovenich et al., 2014; Cook et al., 2015). Whereas most extensively studied effectors are proteinaceous molecules, other types of microbially secreted molecules, such as secondary metabolites and small RNAs, have also been described as non-canonical effectors (Wang et al., 2015; Rodriguez-Moreno et al., 2018). Interestingly, ongoing research has revealed that effectors are not exclusively secreted by plant pathogens but other types of symbionts such as endophytes and mutualists similarly employ effectors to establish host interactions (Rovenich et al., 2014). For instance, the mutualistic fungus *Laccaria bicolor* secretes the effector protein MiSSP7 to interact with jasmonic acid (JA) signalling components of the host and facilitate symbiosis by suppressing host immunity (Plett et al., 2014). Moreover, as all symbionts establish themselves in environments that comprise other microbes including antagonists, effector molecules may also act in self-defense and competition with microbiome co-inhabitants (Rovenich et al., 2014; Snelders et al., 2018).

Vascular wilts caused by soil-borne fungal species of the *Verticillium* genus are among the most devastating plant diseases worldwide with an estimated annual loss of €3 billion in the 20 most affected hosts (Depotter et al., 2016). Within the *Verticillium* genus, *V. dahliae* is the most notorious pathogenic species that has the ability to infect over 200 plant species including high-value crops such as tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), and sunflower (*Helianthus annuus*) (Inderbitzin and Subbarao, 2014). *V. dahliae* invades plants via roots and then colonizes the water-conducting xylem vessels, which disrupts water transport and causes the characteristic wilting symptoms (Fradin and Thomma, 2006; Klosterman et al., 2011). Verticillium wilt disease management is notoriously challenging since conventional fungicides are generally ineffective to eradicate the pathogen once it has entered the xylem tissues (Fradin and Thomma, 2006; Klosterman et al., 2009). Moreover, due to the broad host range of the pathogen and long viability of the resting structures in the soil, cultivation practices such as crop rotation do not result in efficient disease management (Pegg and Brad, 2002; Fradin and Thomma, 2006). Although genetic resistance has been considered as a preferred strategy for disease control, only few Verticillium wilt resistance genes have been identified so far (Schaible et al., 1951; Simko et al., 2004; Fradin and Thomma, 2006). For example, in tomato, immune receptor Ve1 confers resistance to race 1 *V. dahliae* and *Verticillium albo-atrum* strains through recognition of the race 1-specific effector protein Ave1 (Fradin et al., 2009; de Jonge et al., 2012). Transfer of tomato Ve1

into other crop species like tobacco and cotton can provide resistance against race 1 *Verticillium* strains (Song et al., 2018). The high economic impact of Verticillium wilt disease, combined with the absence of curative treatments, substantiates the need for developing novel disease control strategies. Recently, host-induced gene silencing (HIGS), which involves host expression of double-stranded RNAs to target and silence essential pathogen genes to confer host resistance, has been developed for controlling Verticillium wilt disease in cotton and tomato (Song and Thomma, 2016; Zhang et al., 2016).

To design novel control strategies to combat Verticillium wilt disease, a thorough understanding of molecular mechanisms underlying pathogenesis of *V. dahliae* is of fundamental importance (Fradin and Thomma, 2006). Over the past years, forward genetic approaches such as random mutagenesis and proteomic techniques have been performed to identify a number of pathogenicity and virulence factors of *V. dahliae* (El-Bebany et al., 2010; Santhanam, 2014; Chen et al., 2016; Zhang et al., 2017). The ease and low cost of present-day genome sequencing (Faino and Thomma, 2014) have made it possible to sequence multiple *V. dahliae* isolates from various host niches. Using comparative population genomics, we previously identified the *V. dahliae* race-specific effector Ave1 that is crucial for fungal aggressiveness during tomato colonization (de Jonge et al., 2012). Subsequently, Ave1 was demonstrated to contribute to fungal virulence not only on tomato plants that lack the *Ve1* resistance gene, but also on tobacco, *A. thaliana*, as well as on cotton (de Jonge et al., 2012; Song et al., 2018). More recently, we applied comparative genomics to identify the *V. dahliae* defoliating (D) pathotype-specific effector (named D effector) that is responsible for defoliation symptoms on cotton and olive (**Chapter 2**). Interestingly, we found that the D effector acts as a pathogenicity determinant of *V. dahliae* on diverse host species (**Chapter 2**). Using a similar approach, we uncovered a *V. dahliae* effector (named Tom1) that mediates pathogenicity of *V. dahliae* on tomato (**Chapter 3**).

Sunflower is a worldwide planted oil crop that is known to maintain stable yields across a wide variety of environmental conditions (Kane and Rieseberg, 2007; Badouin et al., 2017). However, Verticillium wilt disease constitutes an important constraint for the production of sunflower (Yao et al., 2011; Guo et al., 2017). Despite huge yield losses caused by *V. dahliae*, so far relatively little is known about how the pathogen causes disease on sunflower. In this study, comparative genome analysis of a sunflower pathogenic strain with multiple non-pathogenic strains was performed to identify *V. dahliae* effector gene candidates that are essential for virulence on sunflower.

Materials and methods

Fungal strains and plant materials

Verticillium dahliae strains (Table S1) were grown on potato dextrose agar (PDA) at 22°C. Sunflower (*H. annuus* cv. Tutti), *Nicotiana benthamiana*, cotton (*G. hirsutum* cv. Simian3), tomato (*S. lycopersicum* cv. Moneymaker), and *Arabidopsis thaliana* (Col-0) plants were grown under controlled greenhouse conditions (Unifarm, Wageningen, The Netherlands) at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity[2].

V. dahliae genome sequences and phylogenetic tree construction

Genomes of the four *V. dahliae* strains (JR2, VdLs17, CQ2, and 85S) were sequenced using Single-Molecule Real-Time (SMRT) sequencing technology (Faino et al., 2015; Depotter et al., 2018) (Table S1). Additionally, genomes of *V. dahliae* strains V574 and BP2 were sequenced using the Illumina HiSeq 2000 (**Chapter 2** of this thesis) (Table S1). A phylogenetic tree of the *V. dahliae* strains was generated with REALPHY (version 1.12) (Bertels et al., 2014) using Bowtie2 (Langmead and Salzberg, 2012) to map genomic reads against the reference *V. dahliae* strain JR2. A maximum likelihood phylogenetic tree was inferred using RAxML (version 8.2.8) with the GTRGAMMA model and 500 bootstrap replicates (Stamatakis, 2014).

V. dahliae strain 85S effector identification

In order to identify effector genes of *V. dahliae* strain 85S that mediate aggressiveness on sunflower plants, we mapped DNA reads from non-pathogenic strains to the genome assembly of *V. dahliae* strain 85S using BWA (BWA-mem algorithm) (v0.7.12) (Li and Durbin, 2010). Read coverage mapping of all *V. dahliae* strains over the 85S reference genome was calculated in 100 bp windows using BEDTools coverage (v2.25) (Quinlan and Hall, 2010). Genomic regions were considered present if the breadth of coverage ≥50%, while those with breadth of coverage <50% were considered absent. lineage-specific (LS) genomic regions, here defined as genomic regions that are only present in *V. dahliae* strain 85S and absent in all other non-pathogenic *V. dahliae* strains, were determined and genes localized within these LS regions were extracted using BEDtools intersect (v2.25) (Quinlan and Hall, 2010).

A gene annotation for *V. dahliae* strain 85S was generated using the Maker2 pipeline (Holt and Yandell, 2011). To identify potential effector genes of strain 85S, N-terminal signal peptides were first predicted with SignalP (version 4.1) (Petersen et al., 2011). Subsequently, the machine-learning approach applied in EffectorP (version 1.0) (default parameters) was used (Sperschneider et al., 2016). Effector genes localized within LS regions were extracted using BEDtools intersect (Quinlan and Hall, 2010). Syntenies between effector gene copies was analysed using NUCmer (version 3.1) (-- maxmatch), which is part of the MUMer package (Kurtz et al., 2004), and visualized using R package genoPlotR (v0.8.6) (Guy et al., 2010).

Gene expression analysis

To determine expression of *Sun1 in planta*, 12-day-old sunflower seedlings were root-inoculated with conidiospores of *V. dahliae* strain 85S as described previously (Fradin et al., 2009). Stems of inoculated sunflower plants were harvested at 9, 16, 24, 32 and 40 days post inoculation (dpi), flash frozen in liquid nitrogen and stored at -80°C for total RNA extraction. Total RNA extraction and cDNA synthesis were performed as described previously (Song et al., 2018). Quantitative real time PCR was preformed to detect the expression of *Sun1* using primers Sun1-F(RT-PCR) and Sun1-R(RT-PCR), whereas the *V. dahliae GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene served as an endogenous control (Table S2).

Generation of *Sun1* deletion strains

To generate single *Sun1* gene deletion constructs, sequence stretches of approximately 1.1 kb upstream and 1.2 kb downstream of the *Sun1* coding sequence were amplified from genomic DNA of *V. dahliae* strain 85S using primer pairs SKO-Sun1-LBF/LBR and SKO-Sun1-RBF/RBR (Table S2). The amplicons were cloned into the pRF-HU2 vector as described previously (Frandsen et al., 2008) and the resulting deletion construct was transformed into strain 85S via *Agrobacterium tumefaciens*-mediated transformation as described earlier (Santhanam, 2012). Putative deletion transformants were selected on PDA supplemented with cefotaxime (200 µg/mL) and hygromycin B (50 µg/mL) and homologous gene replacement was verified by PCR analysis using outsider primer F and outsider primer R (Table S2).

To generate *Sun1* double deletion mutants, sequence stretches of approximately 1.3 kb upstream and 1.1 kb downstream of the *Sun1* coding sequence were amplified using primer pairs DKO-Sun1-LBF/LBR and DKO-Sun1-RBF/RBR (Table S2). The amplified products were cloned into vector pRF-NU2. Next, the gene replacement construct was transformed into a *Sun1* single deletion mutant. Putative double deletion transformants were selected on PDA supplemented with nourseothricin (15 µg/mL) and hygromycin B (50 µg/mL) and confirmed by PCR analysis using outsider primer F and outsider primer R (Table S2).

Pathogen inoculations and fungal recovery assays

Inoculations were performed on sunflower, cotton, tomato, *N. benthamiana*, and *A. thaliana* plants as previously described (Fradin et al., 2009). Disease symptoms were scored up to 21 (tomato, *A. thaliana*, and *N. benthamiana*), 28 (cotton), or 45 (sunflower) days post inoculation (dpi). Plants were photographed and Image J was used to determine the canopy areas of tomato while a rectilinear scale was used to measure plant height of sunflower. Fungal biomass in *A. thaliana*, *N. benthamiana*, and sunflower were determined as previously described (Song et al., 2018). Real-time quantitative PCR (qPCR) was conducted to quantify fungal colonization (Ellendorff et al., 2009) using the fungus-specific primers ITS-F and ITS-R (Table S2). Primers targeting the sunflower *elongation factor 1-alpha* (*EF-1α*) gene, *A. thaliana RuBisCo*, and *N. benthamiana RuBisCo* gene were used as endogenous plant controls as described earlier (Table S2) (Song et al., 2018).

Fungal recovery assays were conducted to detect *V. dahliae* strains *in planta* as previously described (Fradin et al., 2009). Stem sections were harvested at 45 days post inoculation (dpi), surface-sterilized and sliced into small discs. For each *V. dahliae* strain, 12 to 15 stem slices from five pooled plants transferred onto PDA supplemented with chloramphenicol (35 µg/mL) and incubated at 22°C for 7 days.

Results

Verticillium dahliae inoculations on sunflower

Various strains of *V. dahliae* have been sequenced in our laboratory but their capacity to infect sunflower remained unknown (Klosterman et al., 2011; de Jonge et al., 2013; Thomma unpublished data). Therefore, in addition to the sunflower-pathogenic *V. dahliae* strain 85S, five strains of which the genome has been sequenced were tested for their ability to infect sunflower. These comprise JR2 and VdLs17, for which a gapless genome assembly has previously been generated (Faino et al., 2015) and that are known to cause Verticillium wilt on tomato and lettuce, respectively, and three *V. dahliae* strains that are phylogenetically distant from JR2 and VdLs17, namely the cotton-pathogenic strains CQ2, BP2 and V574 (Figure S1). Interestingly, in contrast to strain 85S that induced clear stunting, chlorosis and wilting on inoculated sunflower plants, JR2, CQ2, VdLs17, BP2 and V574 failed to cause visible disease symptoms, suggesting that these strains do not have the capacity to infect sunflower (Figure 1A-B).

This finding was further corroborated by fungal recovery assays, as plating of stem sections of the inoculated plants on agar medium resulted in fungal outgrowth from all sections of 85S-inoculated plants while no fungal growth was recovered from stem sections of plants inoculated with any of the other strains (Figure 1C). Thus, except for *V. dahliae* strain 85S that can be classified as a sunflower pathogenic strain, all other strains (JR2, CQ2, VdLs17, BP2 and V574) were classified as non-pathogenic on sunflower.

Comparative genomics identifies the effector gene *Sun1*

We aimed to identify effectors that mediate aggressiveness on sunflower plants by comparative genomics of pathogenic strain 85S and the non-pathogenic strains JR2, VdLs17, CQ2, BP2, and V574. To this end, the genomes of *V. dahliae* strains 85S, CQ2, VdLs17, as well as JR2 were sequenced with PacBio technology (Faino et al., 2015; Depotter et al., 2018). In addition, the genomes of BP2 and V574 were sequenced by using the Illumina HiSeq 2000 (**Chapter 2** of this thesis). Gene annotation for *V. dahliae* strain 85S using the Maker2 pipeline (Holt and Yandell, 2011) yielded a total of 10,580 protein-coding genes. Of these genes, 1,062 genes were predicted to encode putative secreted proteins. Subsequently, we used the machine-learning approach applied in EffectorP (Sperschneider et al., 2016) to identify 174 candidate effectors for *V. dahliae* strain 85S. To identify 85S-specific effector genes, a reference-based alignment strategy was used. All reads of the non-pathogenic *V. dahliae* strains were mapped onto the genome assembly of strain 85S and lineage specific (LS) regions for which no synteny occurred in any of the non-pathogenic strains were extracted. This comparative analysis revealed 6,924 LS sequences of 100 bp in size, collectively comprising 159 genes including five that encode secreted proteins. Of these five genes, two were classified as effector genes based on EffectorP. Remarkably, both effector genes were found to encode a putative effector of 125 amino acids and share 100% sequence identity, despite the observation that they are located on two different contigs, namely unitig_14 and unitig_4 (Figure 2A). Moreover, alignment of unitig_14 and unitig_4 revealed that genomic regions surrounding the two genes are completely identical as well (Figure 2B), suggesting that these

arose by a segmental duplication. We named the putative effector genes *Sun1*, as candidates for mediating virulence on **sun**flower. Similar to most fungal effectors reported so far (Stergiopoulos and de Wit, 2009; de Jonge et al., 2011), no Sun1 homologs were found in other species.

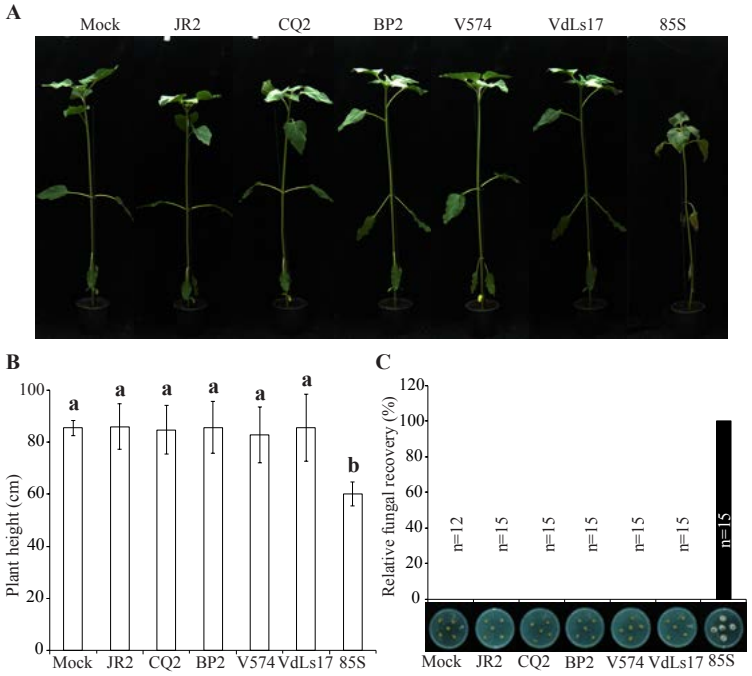

Figure 1. Phenotypes of sunflower plants inoculated with sequenced *Verticillium dahliae* strains. (A) Side view of sunflower (cv. Tutti) plants upon mock-inoculation or inoculation with *V. dahliae* strains JR2, CQ2, BP2, V574, VdLs17 and 85S at 45 days post inoculation (dpi). Besides the stunting, the plant inoculated with strain 85S also displays chlorosis and wilting symptoms. **(B)** Quantification of *V. dahliae*-induced plant stunting at 45 days post inoculation (dpi). Bars represent the average height of five plants with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal outgrowth at 10 days after plating of sunflower stem sections harvested at 45 (dpi) with various *V. dahliae* strains. The bar graph shows the percentage of stem section slices from which fungal outgrowth was observed. Inoculation experiments were performed with five plants for each fungal strain and independently repeated twice with similar results.

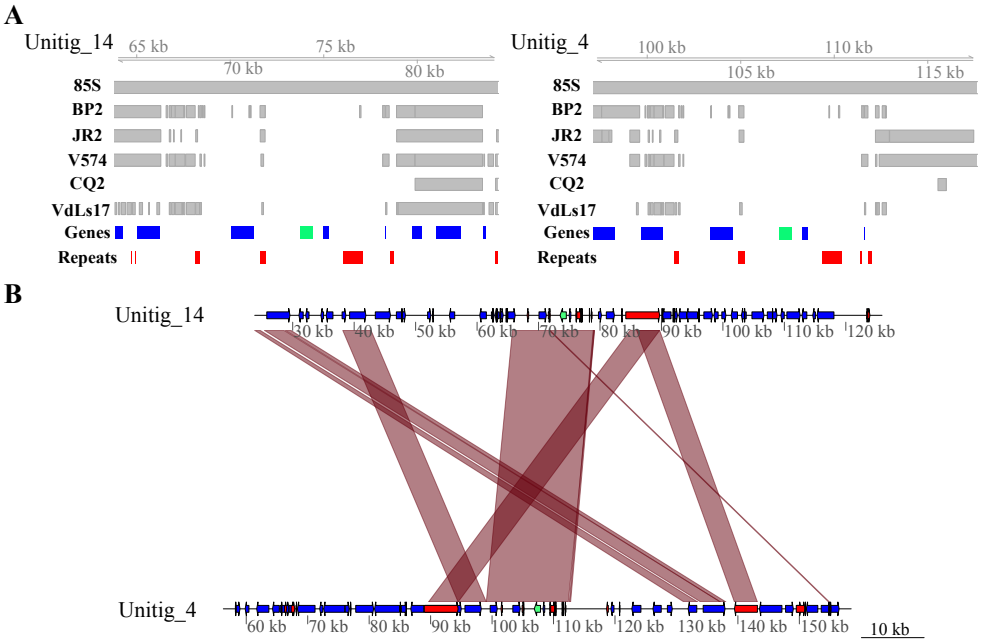

Figure 2. Comparative genomics of *V. dahliae* strains identifies the *Sun1* effector gene. (A) The alignment of reads derived from non-pathogenic *V. dahliae* strains to the sunflower-pathogenic *V. dahliae* strain 85S reveals two LS regions of ~4 kb on contigs unitig_14 and unitig_4. Note, besides other genes (blue), these two LS regions harbor the effector gene *Sun1* (green) that is flanked by repeats (red), and solely present in the strain 85S. **(B)** Synteny analyses of unitig_4 and unitig_14 harboring the *Sun1* effector gene. A region encompassing 50 kb upstream and downstream of *Sun1* effector gene is shown. Highly syntenic regions (>xx% identity) upstream and downstream of this effector gene on unitig_14 and unitig_4 are indicated with red ribbons.

To further assess the potential of Sun1 as a virulence effector on sunflower, the transcript level of *Sun1* during infection of *V. dahliae* strain 85S on sunflower was investigated using real-time PCR in a time course experiment up to 40 days post inoculation (dpi). This analysis revealed that *Sun1* increased significantly at 9 days post-inoculation (dpi), with a peak in expression at 16 dpi and remained relatively high at later time points (Figure 3).

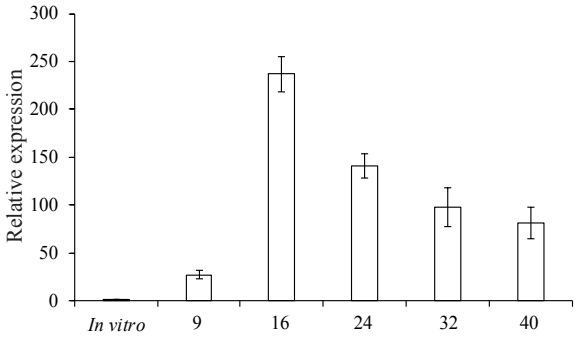

Figure 3. *The Sun1* is highly induced during infection of *Verticillium dahliae* on sunflower. Twelve day-old sunflower (cv. Tutti) seedlings were root-inoculated with *V. dahliae* strain 85S and stems were harvested at 9, 16, 24, 32 and 40 days post inoculation (dpi). Real-time PCR was performed to determine the relative expression level of *Sun1* using the *V. dahliae GAPDH* gene as a reference. Expression of *Sun1 in vitro* (potato dextrose agar) was set to 1.

The Sun1 effector quantitatively contributes to virulence on sunflower

To further assess the contribution of the Sun1 effector to virulence of *V. dahliae* strain 85S on sunflower, we generated deletion mutants for both effector gene copies through homologous recombination (Figure S2A). To this end, we first generated single gene copy deletion mutants, followed by deletion of the second *Sun1* gene copy. Putative single and double gene deletion mutants were confirmed by PCR (Figure S2B). Thereby, we also confirmed that the predicted presence of the two gene copies is genuine and not the consequence of an assembly artefact (Figure S2B). Two independent single (ΔSun1#1 and ΔSun1#2) and two independent double (ΔΔSun1#1 and ΔΔSun1#2) gene deletion mutants were selected for subsequent inoculation assays.

Single deletion (ΔSun1) mutants exhibited markedly reduced virulence on sunflower when compared with wild-type strain 85S at 45 days post inoculation (dpi) (Figure 4A), demonstrated by significantly reduced stunting and compromised fungal colonization of the host plants (Figure 4B-C). Interestingly, sunflower plants that were inoculated with double deletion (ΔΔSun1) mutants showed a similar disease phenotype as plants upon inoculation with ΔSun1 mutants during relatively early stages of infection up until 24 dpi, as no significant difference in disease symptoms and plant height reduction was observed. However, from 32 dpi onwards, sunflower plants that were inoculated with ΔΔSun1 mutants showed significantly less wilting symptoms and stunting than plants inoculated with ΔSun1 mutants (Figure 4B).

Moreover, real-time PCR quantification of fungal biomass demonstrated that ΔΔSun1 mutants accumulated significantly less fungal biomass during infection than *ΔSun1* mutants and the wild type strain (Figure 4C). Taken together, these data demonstrate that the Sun1 effector quantitatively contributes to virulence on sunflower plants.

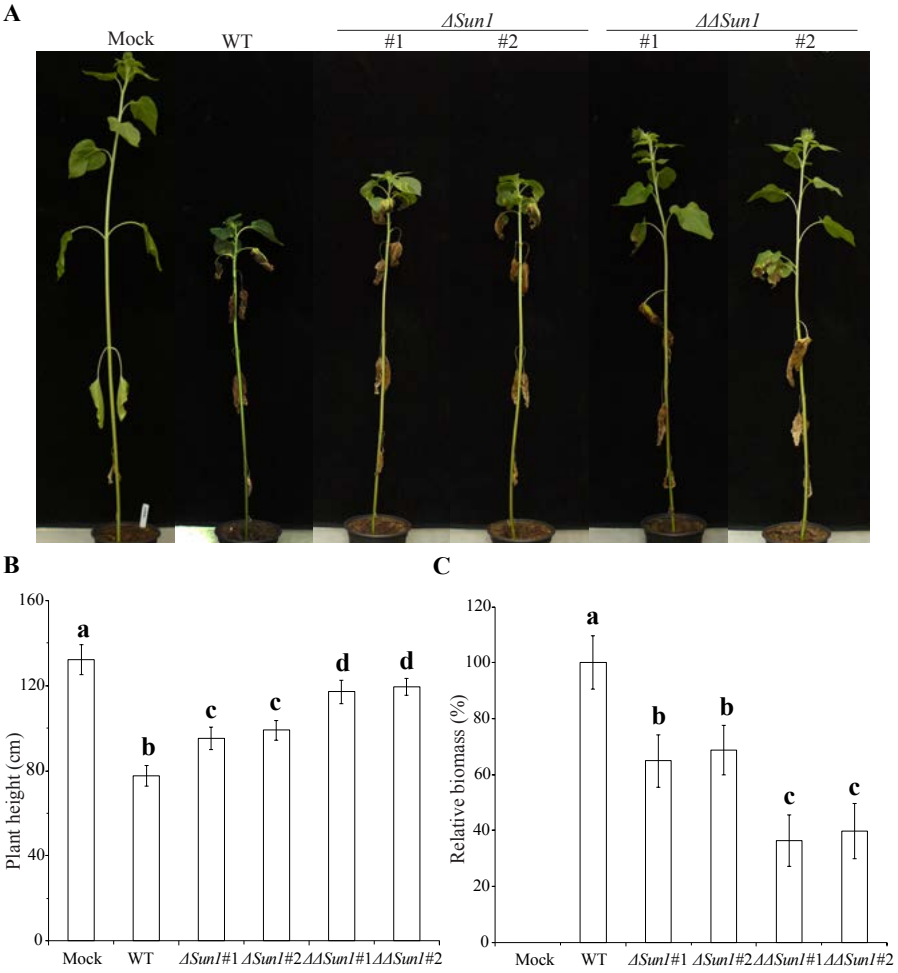

Figure 4. *Sun1* is required for full virulence on sunflower. (A) Typical phenotype of sunflower (cv. Tutti) upon mock-inoculation or inoculation with wild-type strain 85S (WT), two *Sun1* single deletion strains (ΔSun1#1 and ΔSun1#2) and two *Sun1* double deletion strains (ΔΔSun1#1 and ΔΔSun1#2). Photographs were taken at 45 days post inoculation (dpi). **(B)** Quantification of *V. dahliae*-induced plant stunting at 45 dpi. Bars represent the average height of five plants with standard deviation. **(C)** Fungal biomass as determined with real-time PCR at 45 dpi. Bars represent *V. dahliae ITS* levels relative to sunflower *elongation factor 1-α* levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in sunflower plants upon inoculation with the wild-type strain 85S is set to 100%. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). Inoculation experiments were performed with five plants for each fungal strain and independently repeated twice with similar results.

The Sun1 effector is dispensable for virulence on *Nicotiana benthamiana* and *Arabidopsis thaliana*

To investigate whether the observed role of *Sun1* in virulence is confined to sunflower only or also extends to other host species, we first tested the virulence of the *V. dahliae* strain 85S on the Solanaceous crop tomato, the model plant *N. benthamiana*, the Malvaceae crop cotton and the Brassicaceous model plant *A. thaliana*. While 85S failed to cause wilt disease on tomato (Figure S3) and caused only mild wilt disease symptom on cotton (Figure S4), clear symptoms of disease were observed on *A. thaliana* and *N. benthamiana* upon inoculation. Next, we examined the virulence of *Sun1* deletion mutants (ΔSun1 and ΔΔSun1) on *A. thaliana* and *N. benthamiana* to evaluate the contribution of this effector to virulence on these two hosts. However, unlike our observations on sunflower, deletion of *Sun1* did not result in significantly compromised virulence on *N. benthamiana*, as similar Verticillium wilt symptoms, including wilting and stunting, were observed for ΔSun1 and ΔΔSun1 mutants as for the wild-type strain 85S at 3 weeks post inoculation (Figure 5A, C). Real time PCR quantification of fungal biomass confirmed that all strains colonized *N. benthamiana* plants to a similar extent (Figure 5D). Similarly, Sun1 effector was also not found to contribute to virulence on *A. thaliana* (Figure 5B, E-F). Therefore, we conclude that the Sun1 effector does not play a general role in virulence, but its contribution appears to be confined to virulence on sunflower.

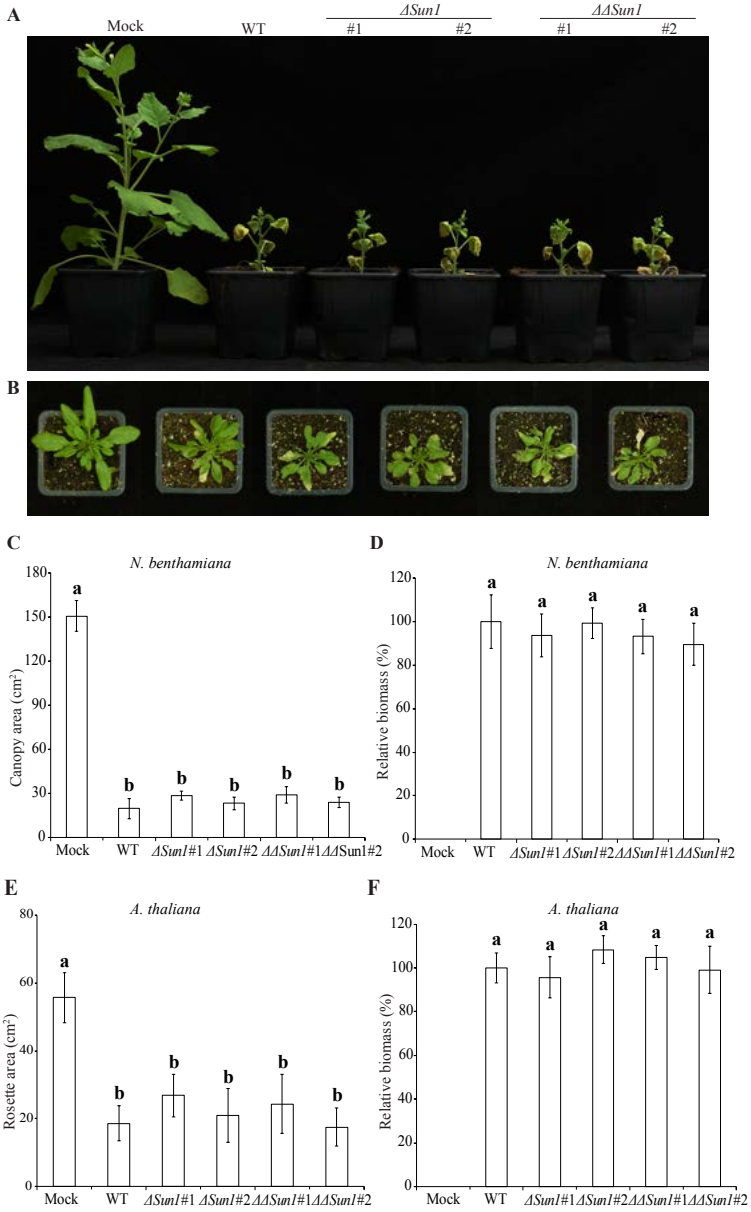

Figure 5. *Sun1* is dispensable for virulence on *Nicotiana benthamiana* and *Arabidopsis thaliana*. (A) Typical phenotype of *N. benthamiana* plants that were mock-inoculated or inoculated with wild type strain 85S (WT), two *Sun1* single deletion strains (ΔSun1#1 and ΔSun1#2) and two *Sun1* double deletion strains (ΔΔSun1#1 and ΔΔSun1#2). **(B)** Typical phenotype of *A. thaliana* (Col-0) plants that were mock-inoculated or inoculated with indicated fungal strains in panel **A** at 21 days post inoculation (dpi). **(C)** Quantification of the canopy area of *N. benthamiana* at 21 dpi. Bars represent the average canopy area of five plants with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(D)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *V. dahliae ITS* levels relative to *N. benthamiana RuBisCo* levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in *N. benthamiana* plants upon

inoculation with the wild-type strain 85S is set to 100%. The same letter labels indicate no statistically significant differences (Student's *t*-test; $P < 0.05$). **(E)** Quantification of the rosette area of five *A. thaliana* plants at 21 dpi. Bars represent the average rosette area of five plants with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(F)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *V. dahliae ITS* levels relative to *A. thaliana RuBisCo* levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in *A. thaliana* plants upon inoculation with the wild-type strain 85S is set to 100%. The same letter labels indicate no statistically significant differences (Student's *t*-test; $P < 0.05$). Photographs were taken at 21 days post inoculation (dpi). Inoculation experiments were performed with five plants for each fungal strain and independently repeated twice with similar results.

Discussion

While *V. dahliae* strains are typically characterized by their broad host range, pathogenic isolates still display differential host specificity as individual isolates only infect a limited number of plant species (Bhat and Subbarao, 1999). Like many filamentous pathogens, *V. dahliae* also employs effector molecules as virulence factors to establish disease (Klimes et al., 2015). To date, a number of *V. dahliae* effectors have been reported to contribute to disease establishment on various hosts. For example, the effector proteins Ave1 and NLP1 (necrosis-and ethylene-inducing-like protein 1) have been shown to function as virulence factors on multiple host plants, such as tomato, *N. benthamiana*, as well as *A. thaliana* (de Jonge et al., 2012; Song et al., 2018; Santhanam et al., 2013). The recently identified D effector was shown to act as a pathogenicity factor of *V. dahliae* defoliating (D) pathotype strains on cotton, olive, *N. benthamiana* and *A. thaliana* (**Chapter 2**). In contrast, other *V. dahliae* effectors may promote fungal virulence only on particular host species or genotypes. For instance, the chitin-binding effector Vd2LyM was reported to contribute to fungal virulence on tomato, but not on *N. benthamiana* or *A. thaliana* (Kombrink et al., 2017). Similarly, the effector NLP-2 is required for full virulence of *V. dahliae* strain JR2 on tomato and *A. thaliana*, but not on *N. benthamiana* (Santhanam et al., 2013). In addition, the Tom1 effector was found to function as a pathogenicity effector that determines host specificity of *V. dahliae* on tomato only (**Chapter 3**). In this study we observed that the Sun1 effector quantitatively contributes to fungal aggressiveness on sunflower (Figure 4), but not on *N. benthamiana* or *A. thaliana* (Figure 5). Thus, we anticipate that the Sun1 effector may specifically facilitate fungal virulence on sunflower, but future experiments on other hosts are needed to confirm this hypothesis.

Various mechanisms have been described that can facilitate the development of effector gene repertoires in pathogenic microbes, such as genome hybridization (Stukenbrock et al., 2012), gene duplication (Dutheil et al., 2016), and horizontal gene transfer (HGT) (Friesen et al., 2006; de Jonge et al., 2012). It has been suggested that the well-characterized *V. dahliae* race-specific effector Ave1 has been acquired from plants via HGT (de Jonge et al., 2012). Genomic comparisons of multiple *V. dahliae* strains revealed that all *V. dahliae* strains carry highly variable LS genomic regions, accounting for 1-5 Mb of their ~35-Mb genome, that are unique or shared by only a subset of *V. dahliae* isolates (de Jonge et al., 2013; Faino et al., 2015, 2016). Interestingly, numerous *in planta*-induced effector genes reside in LS genomic regions that largely consist of segmental genomic duplications, suggesting that gene duplications may play an important role in the emergence of effector genes in *V. dahliae* (Jonge et al., 2013; Faino et al., 2016). In the present

study, we show that two identical *Sun1* effector genes arose by a segmental genomic duplication event and the high level of similarity between flanking sequences of the *Sun1* effector gene suggests that this duplication occurred rather recently (Figure 2). In line with this finding, two exact copies of the *D* effector gene in *V. dahliae* defoliating pathotype strains have emerged by a recent segmental duplication as well (**Chapter 2**). The relevance of the occurrence of two identical effector gene copies and their impact on fungal adaption remains unknown at this point. Possibly, the emergence of two copies of the effector gene is relevant to maintain fungal aggressiveness on host plants. In this light it is interesting to note that the functionality of the two copies is not redundant, as they both quantitatively contribute to virulence and, consequently, deletion of a single copy markedly affects fungal virulence.

Recent duplications of effector genes can be subjected to subsequent evolutionary diversification, leading to novel or altered functionality of one of the two gene copies (Plissonneau et al., 2017; Sanchez-Vallet et al., 2018). Thus, effector genes of filamentous pathogens that arise from gene duplication events typically evolve in a so called "duplication-divergence" pattern: following a gene duplication event, one gene copy diverges to some extent due to functional redundancy and evolves a distinct function (Plissonneau et al., 2017). For example, gene duplications followed by sequence divergence were proposed to be responsible for the generation of novel effector genes in the smut fungus *Ustilago maydis* (Dutheil et al., 2016). Similarly, a large number of effector genes of the oomycete pathogen *Phytophthora sojae* underwent sequence diversification after gene duplication (Shen et al., 2013). In addition to experiencing functional diversification, the recent duplications of effector genes may also be subject to differential loss of the duplicated gene copies (Dong et al., 2015; Pedersen et al., 2012). Frequent effector gene losses after segmental duplications have been proposed to occur in the powdery mildew fungus *Blumeria graminis*, which contribute to the diversity of the effector repertoires of the pathogen (Wicker et al., 2013; Menardo et al., 2017). Possibly, selective forces from host immune systems contribute to this process. However, it is unclear whether either of the two copies of the *Sun1* effector gene will experience sequence divergence or gene loss over time.

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Supplemental information

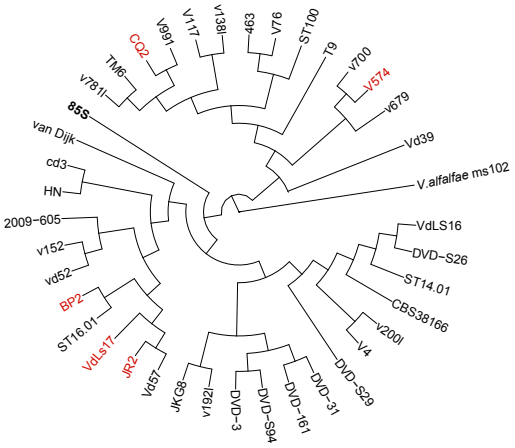

Figure S1. Phylogenetic tree of *V. dahliae* strains. Sunflower-pathogenic strain 85S is shown in bold black. Strains that were selected for phenotypic characterization in this study are shown in red font. Phylogenetic relationships between sequenced *V. dahliae* strains are inferred using RealPhy (Bertels et al., 2014). *V. alfalfae* strain ms102 was used to root the tree.

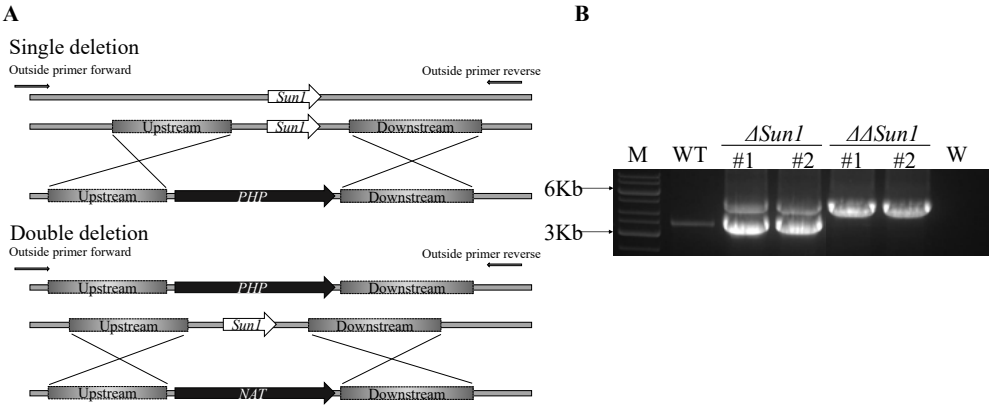

Figure S2. Construction and verification of *Sun1* single deletion and double deletion mutants. (A) Schematic representation of the homologous recombination events to establish targeted replacement of *Sun1* with phosphotransferase *(HPH)* and the nourseothricin resistance gene cassette (NAT). **(B)** Verification of *Sun1* single deletion and double deletion strains by PCR. Amplicons generated with outside primers indicated in panel A are shown for wild-type strain 85S (WT), two *Sun1* single deletion strains (ΔSun1#1 and ΔSun1#2) and two *Sun1* double deletion strains (ΔΔSun1#1 and ΔΔSun1#2). Water was used as negative control (W).

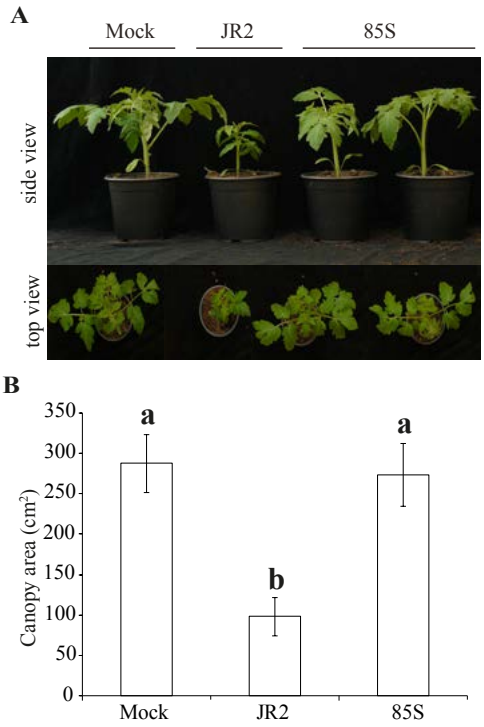

Figure S3. Phenotypes of tomato plants inoculated with *V. dahliae* strain 85S. (A) Typical phenotype of tomato (cv. Moneymaker) plants upon mock-inoculation or inoculation with *V. dahliae* strains 85S and JR2 (as positive inoculation control) at 21 days post inoculation (dpi). **(B)** Quantification of the canopy area of tomato plants at 21 dpi. Bars represent average of canopy area of five plants with standard deviation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). Inoculation experiments were performed with five plants for each fungal strain and independently repeated twice with similar results.

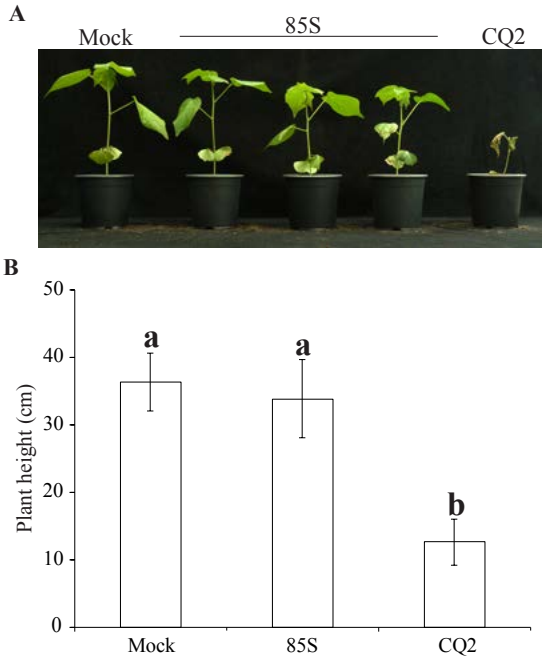

Figure S4. Phenotypes of cotton plants inoculated with *V. dahliae* strain 85S. (A) Typical phenotype of cotton (cv. Simian3) plants upon mock-inoculation or inoculation with *V. dahliae* strains 85S and CQ2 (as positive inoculation control) at 28 days post inoculation (dpi). **(B)** Quantification of the plant height of cotton plants at 28 dpi. Bars represent average of height of five plants with standard deviation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). Inoculation experiments were performed with five plants for each fungal strain and independently repeated twice with similar results.

Table S1. *V. dahliae* strains used in this study.

Strain	Sequencing platform	Reference	Origin	Geographical location
HN	Illumina	Xu et al., 2012	Cotton	China
cd3	Illumina	Xu et al., 2012	Cotton	China
VdLs17	PacBio	Faino et al., 2015	Lettuce	USA
JR2	PacBio	Faino et al., 2015	Tomato	Canada
Vd57	Illumina	Chapter 2 of this thesis	Strawberry	Germany
V152	Illumina	Kombrink et al., 2017	Oak	Hungary
Vd52	Illumina	Kombrink et al., 2017	Pepper	Austria
van Dijk	Illumina	Kombrink et al., 2017	Chrysanthemum	The Netherlands
BP2	Illumina	Zhang et al., 2012	Cotton	China
ST16.01	Illumina	Chapter 2 of this thesis	Cotton	Syria
2009-605	Illumina	Chapter 2 of this thesis	Bell pepper	Ukraine
V4	Illumina	Keykhasaber, 2017	Olive	Spain
V200I	Illumina	Chapter 2 of this thesis	Strawberry	Germany
CBS38166	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S26	Illumina	de Jonge et al., 2012	Soil	Canada
VdLS16	Illumina	de Jonge et al., 2012	Lettuce	USA
ST14.01	Illumina	de Jonge et al., 2012	Pistachio	USA
DVD-S29	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-31	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-161	Illumina	de Jonge et al., 2012	Tomato	Canada
DVD-S94	Illumina	de Jonge et al., 2012	Soil	Canada
DVD-3	Illumina	de Jonge et al., 2012	Potato	Canada
V192I	Illumina	Chapter 2 of this thesis	Cotton	Spain
JKG8	Illumina	Kombrink et al., 2017	Potato	The Netherlands
85S	PacBio	Chapter 2 of this thesis	Sunflower	France
Vd39	Illumina	Chapter 2 of this thesis	Sunflower	Germany
V574	Illumina	Milgroom et al., 2014	Artichoke	Spain
v700	Illumina	Milgroom et al., 2014	Artichoke	Spain
v679	Illumina	Milgroom et al., 2014	Artichoke	Spain
T9	Illumina	Keykhasaber, 2017	Cotton	USA
V781I	Illumina	Chapter 2 of this thesis	Olive	Spain
V138I	Illumina	Chapter 2 of this thesis	Cotton	Spain
TM6	Illumina	Keykhasaber, 2017	Cotton	China
V117	Illumina	Keykhasaber, 2017	Olive	Spain
V991	Illumina	Zhang et al., 2012	Cotton	China
CQ2	PacBio	Chapter 2 of this thesis	Cotton	China
ST100	Illumina	de Jonge et al., 2012	Soil	Belgium
V76	Illumina	Chapter 2 of this thesis	Cotton	Mexico
463	Illumina	Chapter 2 of this thesis	Cotton	Mexico

Table S2. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')[a]	Description
SKO-Sun1-LF	GGTCTTAAUCAGATACCGATTATTGATCCTCGAC	For single *Sun1* deletion generation
SKO-Sun1-LR	GGCATTAAUCGTTAAGAGTTCATAGGCGAAGTTA	For single *Sun1* deletion generation
SKO-Sun1-RF	GGACTTAAUCTCGAAATTACAGAGCTTGCTATGA	For single *Sun1* deletion generation
SKO-Sun1-RB	GGGTTTAAUACTTGGCTATTTCTTCGTCTTTAGG	For single *Sun1* deletion generation
DKO-Sun1-LF	GGTCTTAAUTAGATTGTGTCCTGTGCAAGATATG	For double *Sun1* deletion generation
DKO-Sun1-LR	GGCATTAAUAGGGTTAACGTACATTATCAGCATG	For double *Sun1* deletion generation
DKO-Sun1-RF	GGACTTAAUGATCCCCTTGTCCATTATCTAGTGA	For double *Sun1* deletion generation
DKO-Sun1-RB	GGGTTTAAUCCGGGACAAAGGAAGGTTAATATAC	For double *Sun1* deletion generation
outside primer-F	GCCTCACAACCAATCCACAG	Verification of *Sun1* deletion mutants
outside primer-R	ACATCGCCTCAGAGTCACAA	Verification of *Sun1* deletion mutants
Sun1-F (RT)	CTCATACTCTCCTCCGGTTCAT	*Sun1*, qRT-PCR
Sun1-R (RT)	TTGTACCATCTCCACACGTTAAGTA	*Sun1*, qRT-PCR
VdGAPDH-F	CGAGTCCACTGGTGTCTTCA	*V. dahliae GAPDH*, qRT-PCR
VdGAPDH-F	CCCTCAACGATGGTGAACTT	*V. dahliae GAPDH*, qRT-PCR
Ha-ELF-1α-F	ACCAAATCAATGAGCCCAAG	Sunflower *elongation Factor 1-α* gene
Ha-ELF-1α-R	GAGACTCGTGGTGCATCTCA	Sunflower *elongation Factor 1-α* gene
AtRubisco-F	GCAAGTGTTGGGTTCAAAGCTGGTG	Arabidopsis *Rubisco* gene, fungal biomass quantification
AtRubisco-R	CCAGGTTGAGGAGTTACTCGGAATGCTG	Arabidopsis *Rubisco* gene, fungal biomass quantification
NbRubisco-F	TCCGGGTATTAGGAAAAGCGT	*N. benthamiana Rubisco* gene, fungal biomass quantification
NbRubisco-R	CCCAAGATCTGGGTCAGAGC	*N. benthamiana Rubisco* gene, fungal biomass quantification
ITS-F	AAAGTTTTAATGGTTCGCTAAGA	Fungal biomass quantification
ITS-R	CTTGGTCATTTAGAGGAAGTAA	Fungal biomass quantification

[a] USER cloning sites present in primer sequence are underlined, RT-PCR, real-time PCR.

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Chapter 5

Verticillium dahliae strains that infect the same host plants display highly divergent effector catalogs

Hesham A.Y. Gibriel[1,2,$], Jinling Li[1,$], Longfu Zhu[3], Michael F. Seidl[1#*], Bart P.H.J. Thomma[1#*]

[1]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands.
[2]Current address: School of Agriculture and Food Science, University College Dublin, Dublin, Ireland.
[3]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, Hubei 430070, China.

[$] These authors contributed equally
[#]These authors also contributed equally to this work

[*]Corresponding authors: Bart P.H.J. Thomma (E-mail: bart.thomma@wur.nl), and Michael F. Seidl (E-mail: michael.seidl@wur.nl)

Abstract

Effectors are proteins secreted by pathogens to support colonization of host plants, many of which are thought to deregulate host immunity. Effector genes are often localized within dynamic lineage-specific (LS) genomic regions of pathogen genomes, allowing rapid evolution of effector catalogues. It is thought that such localization permits pathogens to be competitive in the co-evolutionary arms races with their hosts. For a broad host-range pathogen such as *Verticillium dahliae* it is unclear to what extent single members from their total effector repertoires contribute to disease development on multiple hosts. Here, we determined the core and LS effector repertoires of a collection of *V. dahliae* strains, as well as the ability of these strains to infect a range of plant species comprising tomato, cotton, *Nicotiana benthamiana*, Arabidopsis, and sunflower to assess whether the presence of particular LS effectors correlates with the ability to infect particular plant species. Surprisingly, we found that *V. dahliae* strains that are able to infect the same host plant harbour highly divergent LS effector repertoires. Furthermore, we observed differential *V. dahliae* core effector gene expression between host plants. Our data suggest that different *V. dahliae* lineages utilise divergent effector catalogs to colonize the same host plant, suggesting considerable redundancy among the activities of effector catalogs between lineages.

Introduction

Plant pathogens cause devastating diseases on crop plants, threatening food security worldwide (Fisher et al., 2012; Pennisi, 2010). In order to establish their infection, pathogens secrete effector molecules during attempted host ingress many of which modulate host physiology, often by deregulating host immune responses (Cook et al., 2015). However, in turn, effectors may become recognized by plant immune receptors, leading to the activation of immune responses and attempted arrest of pathogen invasion. Thus, pathogens need to continuously evolve their effector catalogues by modifying or purging existing effectors that became recognized, or by acquiring novel effectors to suppress effector-triggered immune responses (Cook et al., 2015).

Genomes of plant pathogens are often thought to have evolved roughly two distinct compartments; one comprising gene-rich, repeat-poor genomic regions that contain core genes that mediate general physiology, and one comprising gene-poor, plastic, repeat-rich genomic regions that contain effector genes and other pathogenicity-related genes (Dong et al., 2015; Raffaele and Kamoun, 2012). The plastic genomic regions are either embedded within core chromosomes, or reside on separate chromosomes that are often referred to as conditionally dispensable chromosomes (CDCs) (Dong et al., 2015; Raffaele and Kamoun, 2012). For instance, effector genes of the tomato-pathogen *Fusarium oxysporum* f.sp. *lycopersici*, known as secreted in xylem (*SIX*) genes, are located on dispensable chromosomes (Ma et al., 2010; Schmidt et al., 2013). In contrast, all effectors of the fungal wheat pathogen *Zymoseptoria tritici* are located on core chromosomes, while no recognizable effector genes reside on dispensable chromosomes (Kema et al., 2018; Marshall et al., 2011; Meile et al., 2018). Core chromosomes also carry effector genes in other fungal plant pathogens, such as the fungal smut pathogen *Ustilago maydis* and the fungal tomato pathogen *Cladosporium fulvum* (Hemetsberger et al., 2015; Stergiopoulos et al., 2010) A genome compartmentalisation with physically separated effector-containing regions is often referred to as a "two-speed" genome organisation since it is thought that gene-rich, repeat-poor genomic regions evolve slowly, while gene-poor, repeat-rich genomic regions evolve quicker (Croll and McDonald, 2012; Raffaele and Kamoun, 2012) Accordingly, the occurrence of effector genes within plastic genomic regions allows rapid evolution of effector catalogues and permits pathogens to be competitive in the co-evolution with hosts and evade their immune systems (Dong et al., 2015; Raffaele and Kamoun, 2012).

Verticillium dahliae is a soil-borne fungal plant pathogen that is able to infect a broad range of plant species, including crops such as tomato, potato, lettuce, and cotton (Fradin and Thomma, 2006; Inderbitzin et al., 2011). The fungus infects plants through their roots and subsequently colonizes the water-conducting xylem vessels, leading to vascular wilt disease (Fradin and Thomma, 2006). Comparative genomics study of *V. dahliae* strains revealed that they carry highly dynamic, repeat rich, lineage-specific (LS) regions that are only present in a subset of *V. dahliae* strains, and that account for up to 4 Mb of the ~35 Mb genome size (de Jonge et al., 2013; Faino et al., 2016). These LS regions are enriched for *in planta*-induced effector genes that contribute to fungal virulence (de Jonge et al., 2013; Faino et al., 2016). However, effector genes are not only found in LS regions, as also the core genome harbours effector genes, such as those encoding

a family of necrosis and ethylene-inducing-like proteins (NLPs) some of which were found to induce cell death in dicotyledonous plants (de Jonge et al., 2011; Santhanam et al., 2013). Similarly, also a family of lysin motif (LysM) effectors is encoded in the core genome, various homologs of which have been reported to enhance virulence by suppression of chitin-triggered immunity in other fungal pathogens (de Jonge et al., 2010; Kombrink et al., 2017; Marshall et al., 2011; Mentlak et al., 2012; Takahara et al., 2016). However, only a single LysM effector that is encoded in an LS region of *V. dahliae* strain VdLs17, and is not found in the genomes of other *V. dahliae* strains, was found to contribute to virulence by suppression of chitin-triggered immunity, whereas no role in virulence could be attributed to any of the core LysM effectors (Kombrink et al., 2017).

Whereas *V. dahliae* is characterized by its generally broad host range, and the observation that individual strains infect a diversity of hosts, differential pathogenicity among hosts occurs for individual strains (Bhat and Subbarao, 1999). In this study, we analysed the genomes of a collection of *V. dahliae* strains and assessed their core and LS effector catalogues in relation to their host ranges. To this end, we selected a set of strains that are well-adapted to cause disease on tomato (*Solanum lycopersicum*), cotton (*Gossypium hirsutum*), Australian tobacco (*Nicotiana benthamiana*), Arabidopsis (*Arabidopsis thaliana*), and sunflower (*Helianthus annuus*) and determined their core and LS effector catalogues as well as their *in-planta* expression profiles.

Materials and methods

V. dahliae strains and plant inoculations

In total, 21 *V. dahliae* strains were used in this study (Table S1). All strains were grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) at 22°C, and conidiospores were collected from 10-day-old plates and washed with tap water. Disease assays were performed on sunflower (*Helianthus annuus* L. cv. Tutti), cotton (*Gossypium hirsutum* cv. Simian 3), tomato (*Solanum lycopersicum* cv. Moneymaker), *Nicotiana benthamiana* and Arabidopsis (Col-0) plants using the root-dipping inoculation method as previously described (Fradin et al., 2009; Song et al., 2017). Briefly, two-week-old (Arabidopsis, tomato, cotton, sunflower) or three-week-old (*N. benthamiana*) seedlings were carefully uprooted and the roots were rinsed in water. Subsequently, the roots were dipped in a suspension of 10^6 conidiospores/mL for 8 min. Control plants were treated similarly by root dipping in tap water without conidiospores. Disease symptoms were scored up to 21 (tomato, *N. benthamiana*, Arabidopsis), 28 (cotton) or 45 (sunflower) days post inoculation (dpi).

Genome sequencing and assembly

The genome sequences of 21 *V. dahliae* strains were previously determined, 17 of which were previously sequenced using Illumina HiSeq 2000 (de Jonge et al., 2013; de Jonge et al., 2012; Kombrink et al., 2017) and four that were sequenced using long-read PacBio Single-Molecule Real-Time (SMRT) sequencing technology (Table S1) (Faino et al., 2015). *V. dahliae* strains that were sequenced with short-read sequencing technology (17 strains) were assembled with the A5 pipeline (default parameters) that automates data cleaning, error correction, assembly,

and quality control (Tritt et al., 2012). Genome assembly statistics for 21 *V. dahliae* strains were calculated using QUAST (Gurevich et al., 2013). Repeats were identified using RepeatModeler (version 1.0.8) (default parameters) (Smit and Hubley, 2010).

Gene prediction and annotation

Previously generated gene annotations of *V. dahliae* strains JR2 (Faino et al., 2015) and CQ2 (Unpublished data) were used in this study. For the remaining 19 *V. dahliae* strains, gene annotation was performed using the Maker2 pipeline (Holt and Yandell, 2011) that combines *ab initio* protein-coding gene evidence from SNAP (Korf, 2004), Augustus (Stanke and Waack, 2003), and GeneMark-HMM (Lukashin and Borodovsky, 1998). Additionally, Maker2 was provided with the previously generated reference gene annotation of *V. dahliae* strain JR2 (Faino et al., 2015), gene annotation of *V. dahliae* strain CQ2 (Unpublished data), and protein homologs of 260 predicted fungal proteomes obtained from the UniProt database (Apweiler et al., 2004).

Effector profiling

We determined core and LS regions of each *V. dahliae* strain. For LS regions, pairwise whole-genome alignments of the 21 *V. dahliae* strains were performed using NUCmer (version 3.1) (--maxmatch), which is part of the MUMer package (Kurtz et al. 2004), and LS regions (here defined as genomic regions that are shared by <19 *V. dahliae* strains) were extracted. Subsequently, core regions (regions shared by ≥19 *V. dahliae* strains) were determined. Genes localized within core and LS regions were extracted using BEDtools intersect (Quinlan and Hall 2010).

To identify candidate effectors, N-terminal signal peptides were first predicted with SignalP (version 4.1) (Petersen et al., 2011). Subsequently, the machine-learning approach applied in EffectorP (version 1.0) (default parameters) was used (Sperschneider et al., 2016). Effector genes localized within core and LS regions were extracted using BEDtools intersect (Quinlan and Hall, 2010). Sequence similarity between predicted LS effectors was established by an all-vs.-all analyses using BLASTp (E-value cutoff 1e-5) (Altschul et al., 1990). Clustering of LS effector sequences into different families was performed using MCL (default options) (Li et al., 2003) and visualized using the function heatmap.2, which is a part of the R package gplots (Warnes et al., 2015).

Assessment of gene expression

To assess gene expression levels, two RNA-seq datasets were used. The first RNA-seq dataset was previously generated from *V. dahliae* strain JR2-infected *Nicotiana benthamiana* plants at 4, 8, 12, and 16 day-post inoculation (dpi) (de Jonge et al., 2012; Faino et al., 2014). The second RNA-seq dataset was obtained from *V. dahliae* strain V991 infecting cotton plants at 6, 9, 12, and 15 dpi (L. Zhu, unpublished data). Mapping of RNA-seq datasets to the corresponding genomes was performed using STAR (version 2.5.3) (--runThreadN 16) (Dobin et al., 2013), and gene expression levels were determined using RSEM (version 1.2.3) (calculate-expression command) (default parameters) (Li and Dewey, 2011), by calculating transcripts per million (TPM) for each gene in each sample.

Results

Verticillium dahliae pathogenicity on a panel of potential host plants

To evaluate the pathogenicity of a collection of *V. dahliae* strains on a panel of potential host plant species, we conducted inoculation experiments with 21 strains on the Solanaceae crop plant tomato and model plant *N. benthamiana*, the Malvaceae crop plant cotton, the Asteraceae crop plant sunflower, and the Brassicaceae model plant Arabidopsis. Despite the fact that *V. dahliae* is generally considered as a broad host range pathogen, there is no individual *V. dahliae* strain in this collection that has the capacity to cause disease on all five plant species (Table 1).

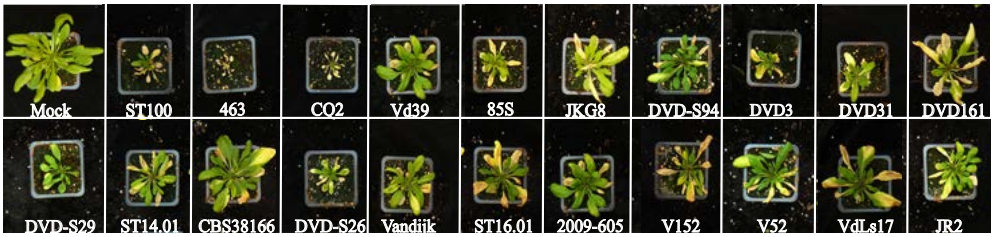

Figure 1. A collection of *V. dahliae* strains all are pathogenic on *Arabidopsis thaliana*. Typical appearance of *A. thaliana* (Col-0) plants upon mock-inoculation or inoculation with a collection of *V. dahliae* strains. Note that disease symptoms ranging from stunting, wilting to severe tissue necrosis. Pictures show representative plants at 21 days after inoculation taken from one of two independent inoculation experiments.

Table 1. Inoculation experiments with *V. dahliae* strains on a collection of potential host plants.

Strain	Arabidopsis	*N. benthamiana*	Cotton	Tomato	Sunflower
ST100	+	+	+	-	-
463	+	+	+	-	-
CQ2	+	+	+	-	-
Vd39	+	+	+	-	-
85S	+	+	+	-	+
JKG8	+	+	+	+	-
DVD-S94	+	+	+	+	-
DVD3	+	+	+	+	-
DVD31	+	+	+	+	-
DVD161	+	+	+	+	-
DVD-S29	+	+	+	+	-
ST14.01	+	+	+	+	-
CBS38166	+	+	+	+	-
DVD-S26	+	+	+	+	-
Vandijk	+	+	+	-	-
ST16.01	+	+	+	-	-
2009-605	+	+	-	+	-
V152	+	+	-	-	-
V52	+	+	+	+	-
VdLs17	+	+	+	+	-
JR2	+	+	+	+	-

"**+**"= pathogenic;"**-**"= non-pathogenic. Disease symptoms were scored up to 21 days post inoculation (dpi) (tomato, *N. benthamiana*, Arabidopsis), 28 dpi (cotton) or 45 dpi (sunflower). The inoculation experiments were executed twice with similar results.

All isolates are pathogenic on Arabidopsis (Figure 1) and on *N. benthamiana* (Figure 2), albeit that the severity of disease symptoms that are induced by different strains vary considerably. Most strains were also found to cause disease on cotton, with the exception of strains 2009-605 and V152 that are non-pathogenic on this species. Interestingly, *V. dahliae* strains CQ2, 463 and ST100 cause severe defoliation, while the other pathogenic strains induce mild to moderate disease symptoms that includes wilting, stunting and chlorosis in the absence of defoliation (Figure 3).

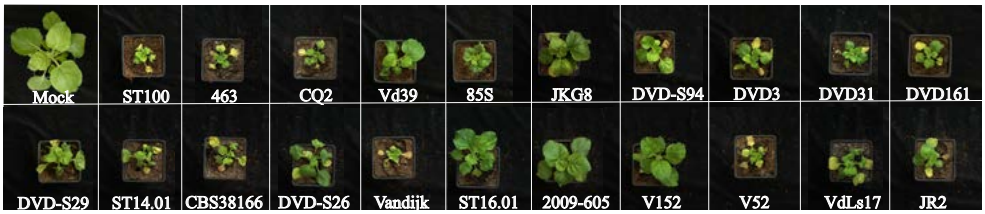

Figure 2. A collection of *V. dahliae* strains all are pathogenic on *Nicotiana benthamiana*. Typical appearance of *N. benthamiana* plants upon mock-inoculation or inoculation with a collection of *V. dahliae* strains. Note that disease symptoms including stunting, wilting and severe tissue necrosis. Pictures show representative plants at 21 days after inoculation taken from one of two independent inoculation experiments.

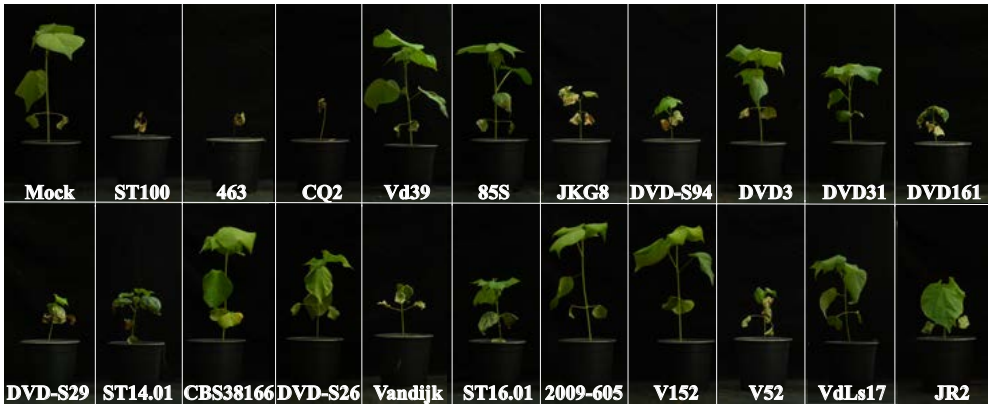

Figure 3. A collection of *V. dahliae* strains display differential pathogenicity on cotton plants. Typical appearance of cotton (cv. Simian 3) plants upon mock-inoculation or inoculation with a collection of *V. dahliae* strains. Note that several *V. dahliae* strains cause defoliation symptoms, while others induce wilting, stunting but not defoliation. Pictures show representative plants at 28 days after inoculation taken from one of two independent inoculation experiments.

Fewer *V. dahliae* strains are able to cause disease on tomato (Figure 4). Besides several strains that cause defoliation on cotton, the tomato non-pathogenic strains also include several non-defoliators on cotton like Vd39 and 85S. Interestingly, whereas strain V152 that is non-pathogenic on cotton also fails to cause disease on tomato, the cotton non-pathogenic strain 2009-605 is able to cause wilt disease on tomato (Figure 4). Strikingly, except for *V. dahliae* strain 85S that induces clear wilt disease symptoms on sunflower plants such as stunting, including stunting, chlorosis and necrosis, all other strains fail to cause sunflower disease (Figure 5). Thus, differential pathogenicity occurs within the collection of *V. dahliae* strains tested here.

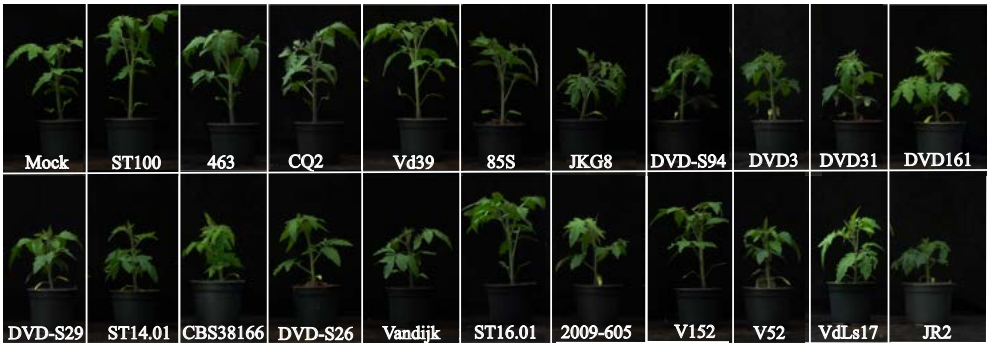

Figure 4. A collection of *V. dahliae* strains display differential pathogenicity on tomato plants. Typical appearance of tomato (cv. Moneymaker) plants upon mock-inoculation or inoculation with a collection of *V. dahliae* strains. Note that pathogenic strains induced clear stunting and significant reduction in canopy area development on inoculated plants. Pictures show representative plants at 21 days after inoculation taken from one of two independent inoculation experiments.

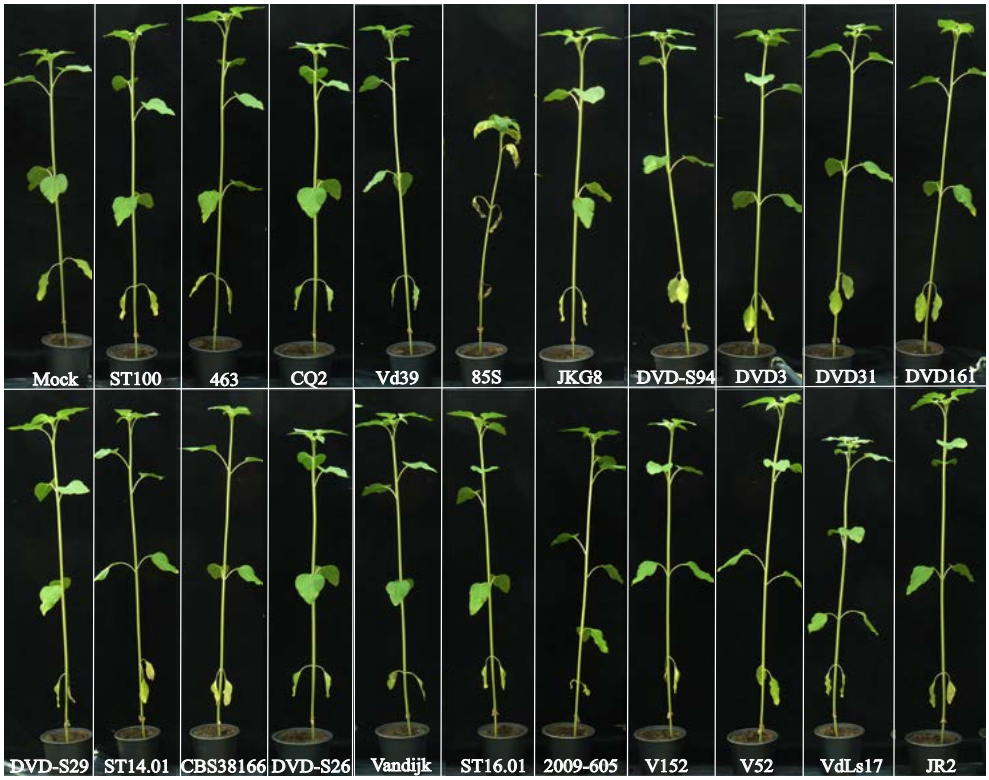

Figure 5. A collection of *V. dahliae* strains display differential pathogenicity on sunflower plants. Typical appearance of sunflower (cv. Tutti) plants upon mock-inoculation or inoculation with a collection of *V. dahliae* strains. Note that besides the stunting, the plant inoculated with pathogenic strain 85S also displays chlorosis and wilting symptoms. Pictures show representative plants at 45 days after inoculation taken from one of two independent inoculation experiments.

Genome assemblies and annotations of a collection of *V. dahliae* strains

The genome sequences of 21 *V. dahliae* strains (Table S1) were obtained from previous studies, 17 of which were determined using the Illumina HiSeq 2000 platform (de Jonge et al., 2013; de Jonge et al., 2012; Kombrink et al., 2017), and four were sequenced using long-read PacBio Single-Molecule Real-Time (SMRT) sequencing technology (Faino et al., 2015). As the genomes of *V. dahliae* strains that were previously sequenced with Illumina technology showed a reduced N50 size of about 35.55 kb (de Jonge et al., 2012), all the Illumina sequenced genomes were assembled in this study. The short reads of the 17 *V. dahliae* strains that were sequenced with Illumina technology were assembled into ~34 Mb, with the largest assembly of 35.90 Mb for *V. dahliae* strain Vd39, and the smallest assembly of 33.14 Mb for *V. dahliae* strain DVD-S29 (Table S2). All assemblies comprised between 1,000 and 4,180 scaffolds with an N50 of approximately 50 kb, except for *V. dahliae* strains 463 and v52 that were assembled in 4,188 and 3,419 scaffolds with an N50 of 17.74 kb and 21.80 kb, respectively (Table S2).

To assess the completeness of the assemblies, the Benchmarking Universal Single-Copy Orthologs (BUSCO) software was used, which uses a set of 1,315 core Ascomycota genes as queries (Simão et al., 2015). The BUSCO scores amounted to ~94% for all the assemblies, except for *V. dahliae* strains 463 and v52 that resulted in 75% and 78.6%, respectively (Table S2). For the PacBio sequenced strains, BUSCO scored 99.30% for 85S and 97.50% for CQ2, while the BUSCO scores for the gapless genome assemblies of JR2 and VdLs17 (Faino et al., 2015) amounted to 99.40% and 98.90%, respectively (Table S2).

Repetitive elements are strong drivers of genome evolution in plant pathogens (Seidl and Thomma, 2017). Thus, the amounts and types of repetitive elements in the genomes of *V. dahliae* strains were predicted by combining *de novo* and known repetitive elements with RepeatMasker (Smit and Hubley, 2010). The repeat content within the *V. dahliae* genomes varied between 6.64% (2.26 Mb) for *V. dahliae* strain 2009-605 and 13.43% (4.83 Mb) for *V. dahliae* strain 85S (Table 2). Out of all the annotated repetitive elements, different repeat families were identified, which included long terminal repeats (LTRs) (2 Mb, ~5.7%), long interspersed nuclear elements (LINEs) (40 kb, ~0.11%), and short interspersed nuclear elements (SINEs) (2.9 kb, ~0.01%) (Table S3).

We subsequently inferred gene annotations for the various *V. dahliae* strains. For *V. dahliae* strains JR2 and CQ2 a previously determined gene annotation was used (Faino et al., 2015). The completeness of gene annotation for both strains was assessed using BUSCO that scored only a low score for CQ2 (72%) compared to JR2 (90.80%). Thus, gene annotations were inferred for all 20 *V. dahliae* strains, except for *V. dahliae* strain JR2. The Maker2 pipeline (Holt and Yandell, 2011) was used that combines *de novo*, homology-based, and previous gene annotations for *V. dahliae* strains CQ2 (B. Thomma and J. Li, unpublished data) and JR (Faino et al., 2015) and protein homologs of 260 predicted fungal proteomes. The number of genes varied from 10,461 for *V. dahliae* strain VanDijk to 11,341 for *V. dahliae* v52 (Table 2).

Table 2. Summary of genome sizes, core and lineage-specific (LS) regions, number of genes, and number of effector genes for each strain.

	Genome size (Mb)							Number of genes						EffectorP		
Strain	Total	Repeats	% Repeats	Core	% Core	LS	% LS	Total	Secreted	Core	LS	Core/ secreted	LS/ secreted	Total	Core	LS
2009-605	34.06	2.26	6.64	32.16	94.43	1.90	5.57	10893	10893	9707	1186	1020	85	190	170	20
463	34.03	3.56	10.46	32.66	95.96	1.38	4.04	11188	11188	10671	517	967	35	204	190	14
85S	35.93	4.83	13.43	34.11	94.93	1.82	5.07	10580	10580	9706	874	995	67	174	168	6
CBS38166	34.03	2.83	8.32	32.59	95.76	1.44	4.24	10676	10676	9823	853	969	55	180	168	12
DVD161	33.47	2.61	7.81	32.31	96.53	1.16	3.47	10624	10624	9872	752	980	55	195	172	23
DVD31	33.58	2.85	8.50	32.48	96.72	1.10	3.28	10642	10642	9941	701	981	53	185	164	21
DVD3	34.42	3.47	10.10	33.04	96.00	1.37	4.00	10741	10741	9934	807	982	53	191	172	19
DVD-S26	34.74	2.82	8.11	32.59	93.82	2.15	6.18	10977	10977	9871	1106	979	78	196	178	18
DVD-S29	33.14	2.55	7.69	32.07	96.77	1.07	3.23	10561	10561	9866	697	978	42	193	178	15
DVD-S94	34.42	3.31	9.61	32.98	95.80	1.44	4.20	10684	10684	9827	857	990	67	185	165	20
JKG8	33.85	2.75	8.13	32.56	96.21	1.28	3.79	10565	10565	9647	918	1006	68	170	156	14
ST100	34.92	3.66	10.49	33.28	95.32	1.64	4.68	10697	10697	9789	908	972	69	189	170	19
ST14	34.48	3.39	9.83	32.92	95.49	1.55	4.51	10660	10660	9716	944	976	63	175	162	13
ST16	34.22	2.31	6.74	31.95	93.37	2.27	6.63	11005	11005	9687	1318	1017	91	195	174	21
v152	33.98	2.79	8.22	32.21	94.82	1.76	5.18	11137	11137	10103	1034	988	71	195	175	20
v52	33.54	2.41	7.19	31.83	94.91	1.71	5.09	11341	11341	10648	693	980	50	207	188	19
VanDijk	33.17	2.34	7.06	31.97	96.39	1.20	3.61	10461	10461	9591	870	999	69	180	163	17
Vd39	35.90	4.45	12.39	33.69	93.84	2.21	6.16	10662	10662	9579	1083	988	86	171	159	12
CQ2	35.82	4.15	11.60	34.03	95.00	1.79	5.00	10528	10528	9628	900	994	70	169	165	4
JR2	36.15	4.26	11.77	33.68	93.15	2.47	6.85	11432	11432	10328	1104	1001	66	212	200	12
VdLs17	35.97	4.20	11.68	33.52	93.19	2.45	6.81	10856	10856	9688	1168	1000	90	179	165	14

Identification of core and lineage-specific (LS) effector catalogs

Initially, the secretomes for each of the *V. dahliae* strains were predicted, identifying between 9,67 secreted proteins for *V. dahliae* strain 463 and 1,017 proteins for *V. dahliae* strain ST16 (Table 2). Subsequently, the machine-learning algorithm of EffectorP (Sperschneider et al., 2016) was used, which identified between 169 effectors for *V. dahliae* strain CQ2 and 212 effectors for *V. dahliae* strain JR2 (Table 2).

Subsequently, we determined the core genome, here defined as regions that are shared by ≥19 *V. dahliae* strains, and LS regions, here defined as regions that are shared by <19 *V. dahliae* strains, for all *V. dahliae* strains. The core regions of all *V. dahliae* strains comprise 32.79 Mb (93-97%) of the genome, while LS regions comprise between 1.07 (strain DVDS-29) and 2.47 Mb (strain JR2) (3-7%) (Table 2). The core regions of *V. dahliae* strains harbor 9,886 genes, comprising 988 genes that encode secreted proteins, of which 171 were classified as effectors based on EffectorP (Table 2). The LS regions of *V. dahliae* strains harbor between 517 genes for *V. dahliae* strain 463 and 1,318 genes for *V. dahliae* strain ST16 (Table 2). Of these LS genes, 35 genes encode secreted proteins for *V. dahliae* strain 463 and 91 for *V. dahliae* strain ST16, of which ~15 genes were classified as effectors for each *V. dahliae* strain (Table 2). We tested these predictions on the previously identified LS effector gene of *V. dahliae* strain JR2, namely *Ave1*, which was successfully identified as an LS effector gene (de Jonge et al., 2012) (Figure S1).

To assess general characteristics of core and LS effector genes, features such as their distance to transposable elements (TEs), gene length, inter-genic length, and expression were determined. For all strains, we observed that LS effector genes are shorter in length than core effector genes (Figure 6A, B; Figure S2). The average inter-genic length of LS effector genes is slightly longer (1476 bp) compared to the average inter-genic length of core effector genes (1232 bp) (Figure 6A, B; Figure S3). Moreover, LS effector genes localize closer to TEs than core effector genes, even though this trend is not significant for *V. dahliae* strain JR2 (Figure 6A, B; Figure S4). Finally, LS effector genes of *V. dahliae* strain JR2 were found to be significantly higher expressed *in planta* on *N. benthamiana* than core effector genes (Figure 7A), although no such difference was found between LS and core effectors of *V. dahliae* strain CQ2 *in planta* on cotton (Figure 7B).

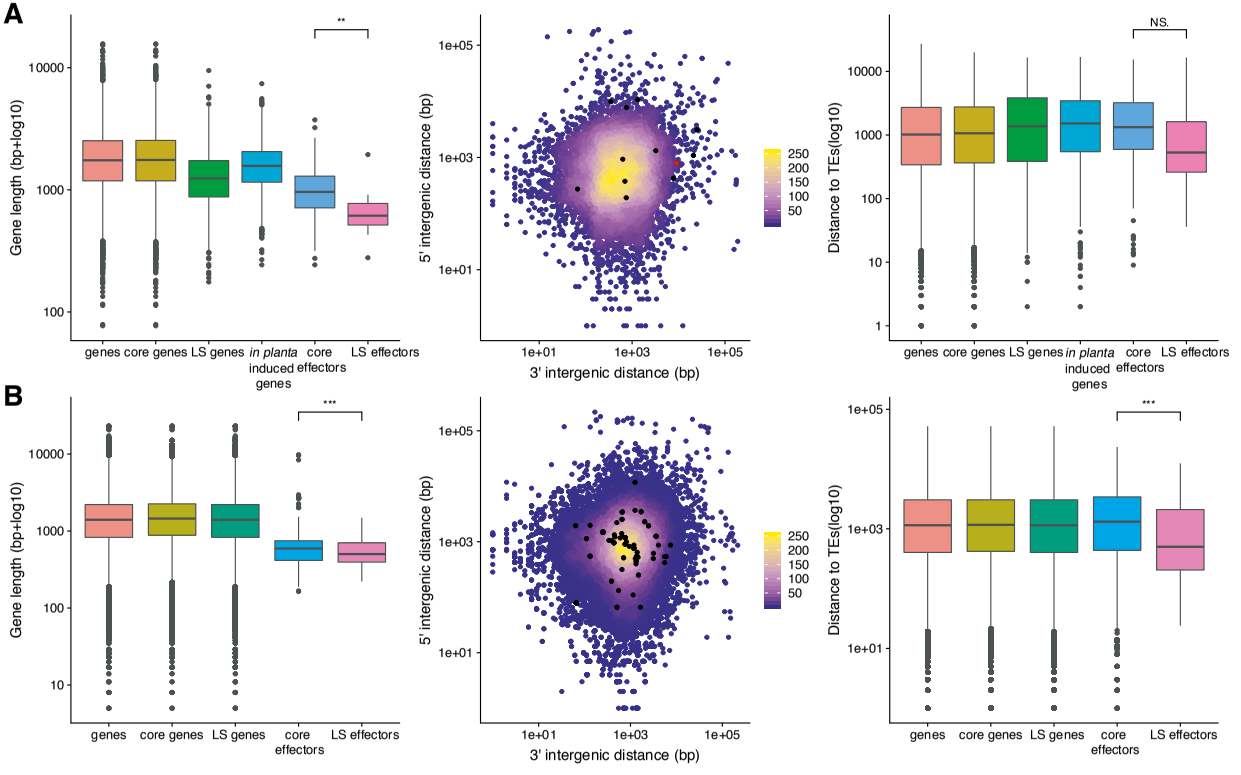

Figure 6. Characteristics of core vs LS effector genes of *V. dahliae* strains. (A) and **(B)** show gene length, two-dimensional density plot of 5'- and 3'-flanking inter-genic regions, where LS effectors are indicated in (black) and Ave1 effector in (red) color, and distance to closest transposable element (TE) for core and LS effector genes of *V. dahliae* strain JR2 **(A)** vs all *V. dahliae* strains **(B)**.

V. dahliae strains that infect the same host plant harbor divergent effector repertoires

It has previously been shown that *V. dahliae* strains display differential capacity to infect particular host plants (Bhat and Subbarao, 1999). We furthermore showed that especially the LS effectors contribute to *V. dahliae* pathogenicity on individual plant hosts (de Jonge et al., 2013; de Jonge et al., 2012; Kombrink et al., 2017). Collectively, this suggests that *V. dahliae* strains may harbour an array of specialized effectors that only function on particular host plants, and thus we assessed whether the presence of particular LS effectors in the various *V. dahliae* strains correlates with the ability to infect particular hosts. In total, we predicted 333 LS effectors over the various strains (Table 2) that were clustered into 110 families that are either shared by sub-groups of *V. dahliae* strains, or are strain-specific (Figure 8). Furthermore, we searched the 333 LS effector genes against *V. dahliae* genomes using BLAST (tblastn) and identified *XLOC_00170*, *XLOC_008951*, and *XLOC_009059* of *V. dahliae* strain JR2, which are shared by a subset of *V. dahliae* strains (Figure 8). Intriguingly, we observed highly dissimilar LS effector catalogs among *V. dahliae* strains that are able to infect the same host plant (Figure 8). Strikingly, *V. dahliae* strains that infect the same host plant do not cluster based on their LS effector repertoires (Figure 8). Thus, *V. dahliae* strains harbor highly divergent LS effector catalogs, the composition of which does not correlate with the host plant they are able to infect.

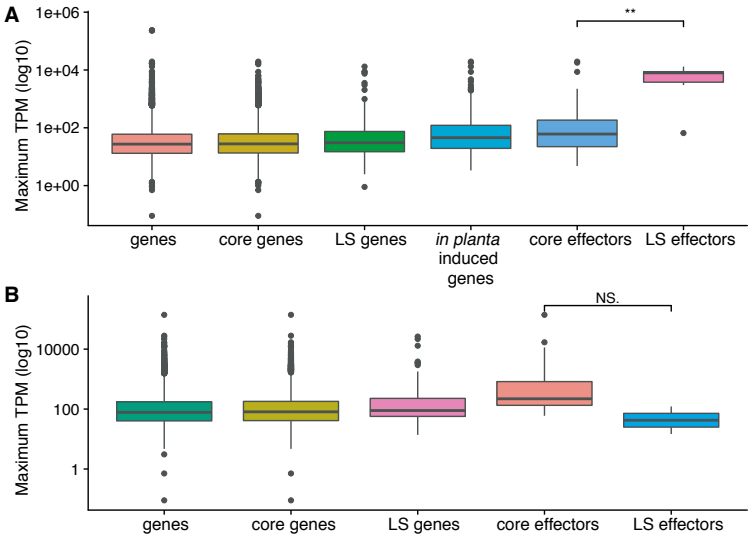

Figure 7. Maximum TPM values of core and LS effector genes over four time points during *N. benthamiana* and cotton infection by *V. dahliae* strains JR2 vs CQ2. (A) Maximum TPM values of core and LS effector genes over 4, 8, 12, and 16 time points during *N. benthamiana* infection by *V. dahliae* strain JR2. **(B)** Maximum TPM values of core and LS effector genes over 6, 9, 12, and 15 time points during cotton infection by *V. dahliae* strain CQ2.

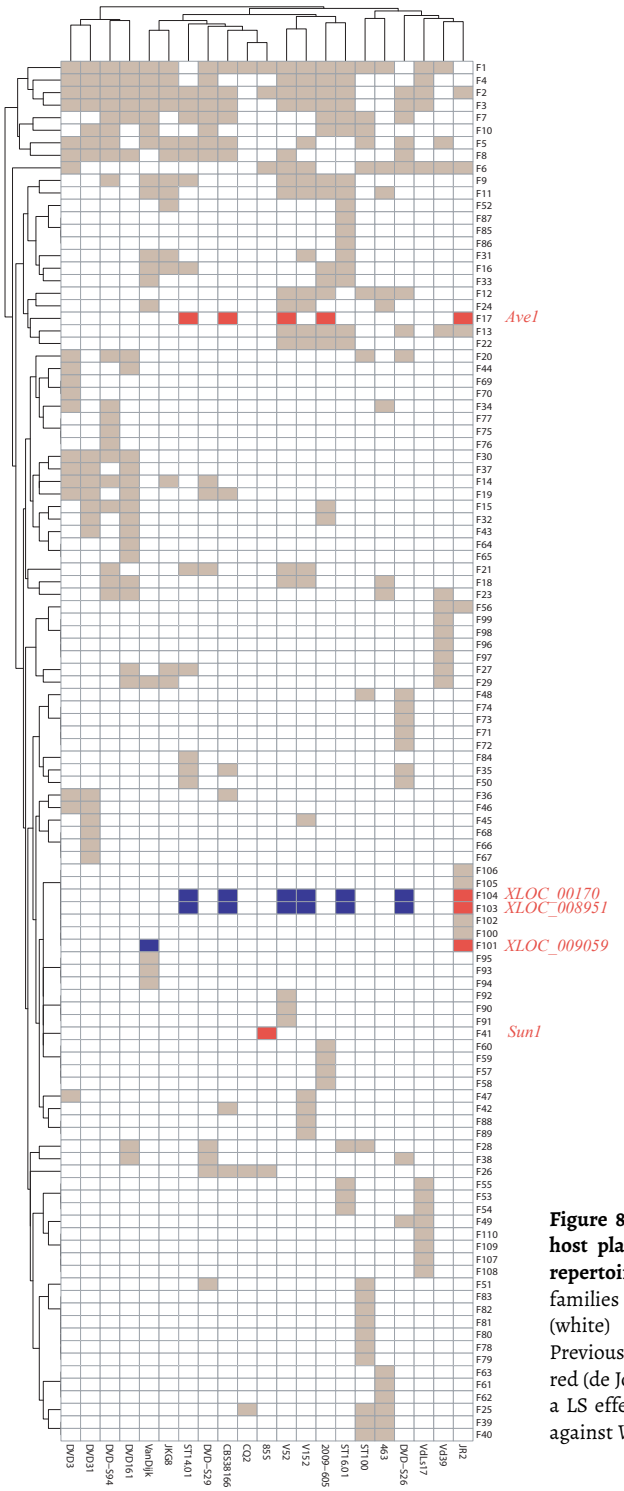

Figure 8. *V. dahliae* strains that infect the same host plant harbor highly divergent LS effector repertoires. LS effectors were clustered into 110 families and their presence (brown) and absence (white) between *V. dahliae* strains is shown. Previously described LS effectors are colored in red (de Jonge et al., 2013). Blue colors indicate that a LS effector is present based on BLAST (tblastn) against *V. dahliae* genomes.

Variability in core effector transcription profiles

In many plant pathogens, core effector genes are highly induced *in planta* and play essential roles on a multitude of hosts (Guyon et al., 2014; Hemetsberger et al., 2015; Santhanam et al., 2013; Yin et al., 2017). Thus, we assessed the expression of *V. dahliae* core effector genes during invasion of different plant species. First, we mapped RNA-seq datasets from *N. benthamiana* plants colonized by *V. dahliae* strain JR2 (de Jonge et al., 2012) against the reference genome sequence of *V. dahliae* strain JR2 (Faino et al., 2015), and cotton plants colonized by *V. dahliae* strain V991 (LF Zhu, unpublished data) against its closely related *V. dahliae* strain CQ2. Subsequently, RNAseq reads overlapping core effector genes of *V. dahliae* JR2 and *V. dahliae* strain CQ2 were quantified and core effector genes that are shared between both strains were determined. We observed that the transcription profiles can be clustered into: 1) transcribed effector genes on both hosts, 2) differentially transcribed effector genes between the two hosts, and 3) non-transcribed effector genes on either hosts (Figure 9A). Of the 165 shared core effector genes between JR2 and CQ2, 61 effector genes were transcribed during cotton as well as *N. benthamiana* colonization, whereas 19 effector genes were only transcribed on cotton, and 41 effector genes were transcribed on *N. benthamiana* (Figure 9B). Additionally, we identified 44 effector genes that were non-transcribed in both strains (Figure 9A). Thus, differential *V. dahliae* core effector gene expression is observed on different host plants. Overall, we are not able to link the composition or the expression of effector gene catalogs to the ability to infect particular host plants.

Figure 9. Variability in transcription profiles of core effector genes of *V. dahliae* strains JR2 and CQ2. (A) Pie chart showing the number of non-transcribed, transcribed, and differentially transcribed core effector genes of *V. dahliae* strain JR2 and CQ2 on *N. benthamiana* and cotton. **(B)** Transcription profile comparisons of core effector genes of JR2 (left) and CQ2 (right) over four time points during infection. The log-scaled color gradient shows TPM values for each core effector at different time points.

Discussion

For broad host-range pathogens such as *V. dahliae* it is unclear to what extent co-evolution of the pathogen with multiple hosts occurs simultaneously, and what implications this has for their effector repertoires. Whereas it is conceivable that broad host-range pathogens employ core effectors target broadly conserved general physiological processes in a multitude of hosts, they may also harbour an array of specialized effectors that only exert activity on particular host species. Presence of such specialized effectors is suggested by the observation that different *V. dahliae* strains, despite their general ability to infect a wide array of host plants, generally display differential capacity to infect particular hosts (Bhat and Subbarao, 1999). Therefore, in this study we investigated whether the absence or presence of particular effectors correlates with the ability to infect particular hosts. Intriguingly, we observed that *V. dahliae* strains that are able to infect the same host plant, in this case focussed on tomato, cotton, *N. benthamiana*, Arabidopsis, and sunflower, harbour highly divergent LS effector catalogs (Figure 8). This strongly suggests that different strains infect the same host plant by utilizing different effector compositions. If one assumes that different strains of *V. dahliae* must target the same host physiological processes in order to establish the infection, this implies that there must be a significant degree of redundancy among the various effectors that occur in the different strains. Interestingly, fungi of the ascomycete species *Fusarium oxysporum* display a similar infection biology as *V. dahliae*, being soil-borne pathogens that colonize the xylem tissues of their host plants to cause vascular wilt disease and with a largely overlapping host range. However, whereas individual strains of *V. dahliae* are characterized by their generally large host range, strains of *F. oxysporum* are generally host-specific and are therefore assigned to *formae specialis*. Comparative genomics revealed that *Fusarium* strains within a *formae specialis* carry highly overlapping LS effector repertoires (Coleman et al., 2009; Ma et al., 2010; van Dam et al., 2016). Moreover, strains within the same *Fusarium formae specialis* cluster based on their LS effector repertoires (van Dam et al., 2016). Thus, in contrast to *F. oxysporum* strains that harbour highly overlapping LS effectors based on their host range, *V. dahliae* strains evolved highly divergent LS effector catalogs, the composition of which does not correlate with the host plant they are able to infect.

Plant pathogens harbour an array of *in planta* highly-induced core effector genes that play essential roles on a multitude of hosts (Guyon et al., 2014; Hemetsberger et al., 2015; Santhanam et al., 2013; Yin et al., 2017). We quantified RNAseq reads (RNAseq reads of *V. dahliae* JR2-infecting *N. benthamiana* plants and RNAseq reads of *V. dahliae* strain V991-infecting cotton) overlapping core effector genes of JR2 and CQ2 (closely related to V991). Subsequently, we categorized the effectors in three groups based on their expression profiles. We identified a group of effector genes that are transcribed in both strains (Figure. 9B), suggesting that this group of effectors contributes to *V. dahliae* colonization of *N. benthamiana* and cotton. Additionally, we observed a group of effector genes that are highly transcribed in *V. dahliae* strain JR2 on *N. benthamiana* but only lowly transcribed in strain CQ2 on cotton, and vice versa (Figure 9B), suggesting that they differentially contribute to virulence on these two host plants. Furthermore, we identified a group of effector genes that are not transcribed in either strains (Figure 9A), suggesting that this group of core effectors do not play a role in virulence on *N. benthamiana* or on cotton.

Nevertheless, it may well be that this group of effector genes is transcribed during *V. dahliae* colonization of other host plants. Surprisingly, we observed that a member of the family of necrosis- and ethylene-inducing-like proteins (NLPs), namely *NLP-1*, is found within the group of non-transcribed effector genes (Figure 9), whereas we previously found based on real-time PCR that this effector gene is transcribed in *V. dahliae* strain JR2 on *N. benthamiana* and on tomato, but also in *V. dahliae* strain V592 on cotton (Santhanam et al., 2013; Zhou et al., 2012). Likely, the lack of *NLP-1* expression based on our RNAseq data may be due to the low amount of fungal RNAseq reads among plant-derived ones, as only 0.05% of the reads could be mapped to the *V. dahliae* genome (Faino et al., 2014). Thus, it needs to be taken into account that only highly expressed fungal genes are identified, and it might be worthwhile to confirm whether the group of non-transcribed effector genes still contains lowly or moderately expressed genes based on real-time PCR.

Localization of pathogen effector genes within dynamic genomic regions allows pathogens to rapidly evolve to evade plant immunity once an effector gets recognized by the host (Dong et al., 2015; Raffaele and Kamoun, 2012). When we assessed the core and LS effector repertoires among the analysed *V. dahliae* strains, we observed no remarkable differences in the number of core and LS effectors (Table 2). Nevertheless, by comparing features of core and LS effectors we observed that LS effectors are typically shorter in length, localize significantly closer to TEs, and have slightly longer inter-genic lengths when compared with core effectors (Figure 6). Consistent with this observation, a recent study that analysed the genomes of nine *Verticillium* spp. showed that species-specific genes displayed significantly shorter gene lengths and longer inter-genic lengths when compared with genes that are conserved across the various species within the genus (Shi-Kunne et al., 2017). Similarly, LS genes of the fungal wheat pathogen *Z. tritici* were frequently found to be shorter, closer to TEs, and have longer inter-genic lengths, when compared with core genes (Haueisen et al., 2017; Plissonneau et al., 2018; Plissonneau et al., 2016). It has been previously suggested that the localization of effector genes in close proximity to TEs and within gene-poor regions mediate rapid evolution of effector catalogs (Raffaele and Kamoun, 2012; Seidl and Thomma, 2017). Thus, the localization of LS effector genes of *V. dahliae* strains in close proximity to TEs may mediate accelerated evolution of effector catalogs.

In conclusion, our data demonstrate the extensive variability within the effector repertoires of the broad host range pathogen *V. dahliae*. We have demonstrated that LS effectors are highly divergent among *V. dahliae* strains that infect the same host plant, and core effector genes are differentially expressed between hosts. The variability within LS and core effector genes of *V. dahliae* strains may lead to rapid immunity evasion, which may allow pathogen strains to be competitive in the co-evolution with their multiple hosts.

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Supplemental information

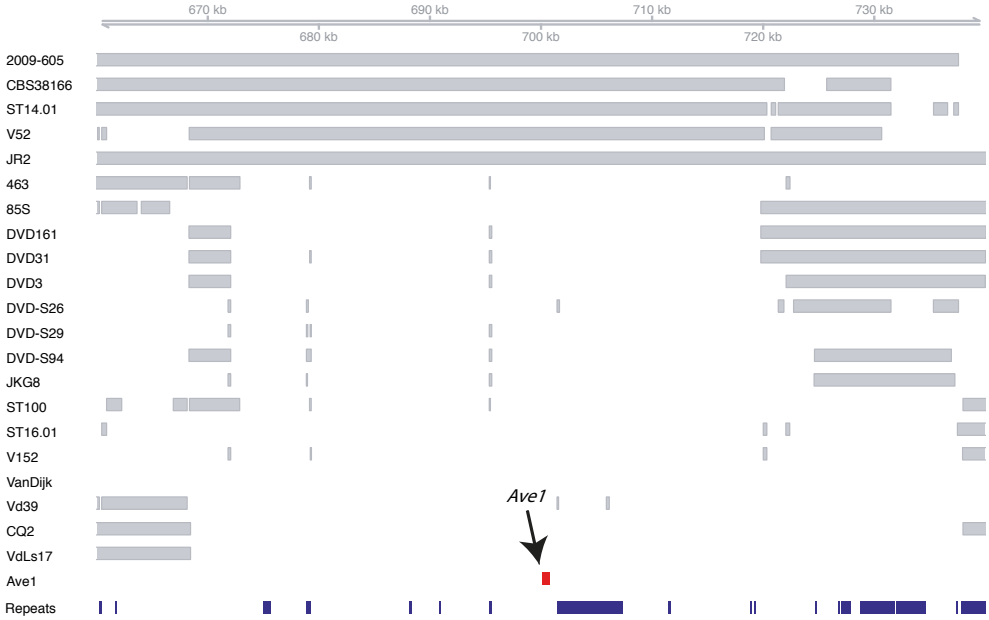

Figure S1. *V. dahliae* effector gene *Ave1* is localized within an LS region. A highly variable, repeat-rich (blue), LS region, which harbors the *Ave1* effector gene (red) of *V. dahliae* is shown. This LS effector gene is only present in a subset of *V. dahliae* strains. Grey bars indicate the genome alignments of *V. dahliae* strains to the reference strain JR2. This effector gene is only present in strains 2009-605, CBS38166, ST14.01, V52, JR2, and absent in the other strains.

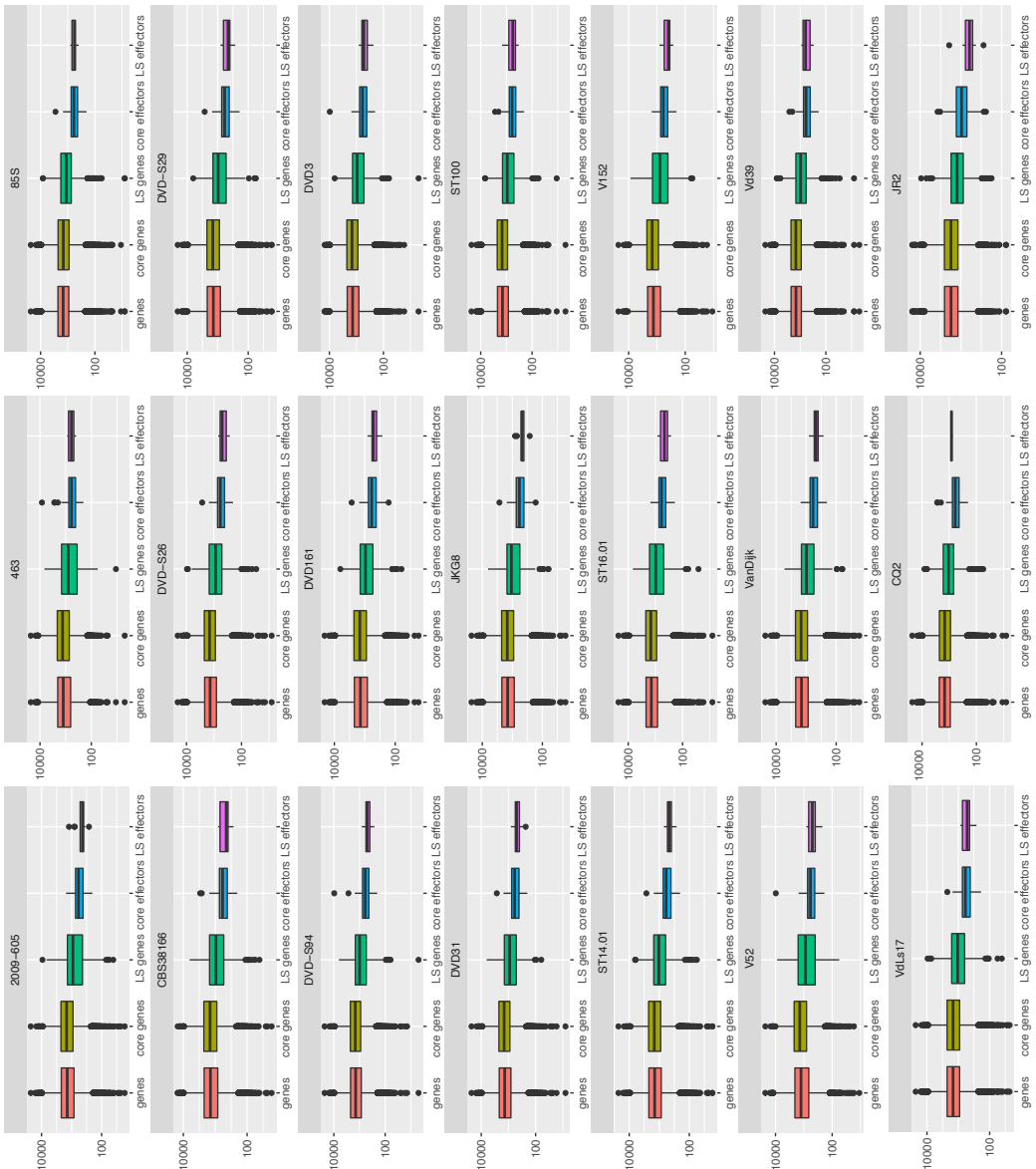

Figure S2. Gene length of genes and effector genes of *V. dahliae* strains. Gene length (bp+log10) is shown for genes, core genes, LS genes, core effectors, and LS effectors for all *V. dahliae* strains.

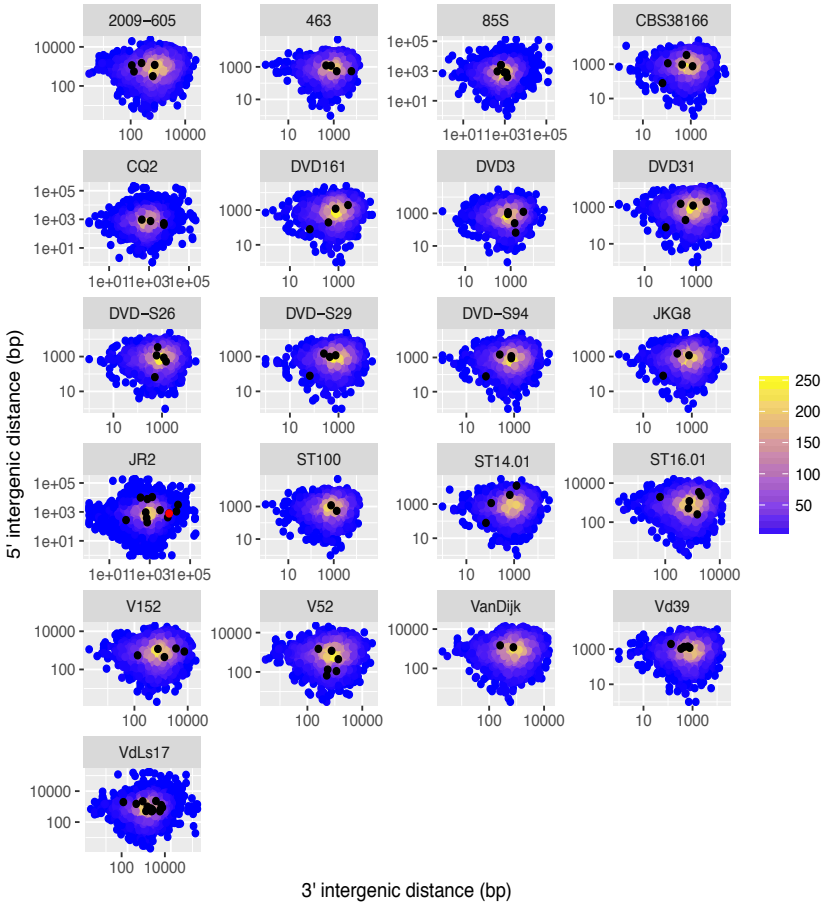

Figure S3. 5' and 3' inter-genic length of genes and effector genes of *V. dahliae* strains. Inter-genic length (bp) is shown for genes (blue) and LS effector genes (black) for all *V. dahliae* strains. *Ave1* effector gene of *V. dahliae* strain JR2 is highlighted in red.

Figure S4. Distance to TEs of genes and effector genes of *V. dahliae* strains. Distance to TEs (log10) is shown for genes, core genes, LS genes, core effectors, and LS effectors for all *V. dahliae* strains.

Table S1. *V. dahliae* strains used in this study.

Strain	Sequencing platform	Reference	Isolated from	Country of isolation
CQ2	PacBio	Depotter et al., 2018	Cotton	China
85S	PacBio	Depotter et al., 2018	Sunflower	France
VdLs17	PacBio	Faino et al., 2015	Lettuce	USA
JR2	PacBio	Faino et al., 2015	Tomato	Canada
CBS381.66	Illumina HiSeq 2000	de Jonge et al., 2012	Tomato	Canada
St14.01	Illumina HiSeq 2000	de Jonge et al., 2012	Pistachio	USA
St100	Illumina HiSeq 2000	de Jonge et al., 2012	Soil	Belgium
DVD-3	Illumina HiSeq 2000	de Jonge et al., 2012	Potato	Canada
DVD-31	Illumina HiSeq 2000	de Jonge et al., 2012	Tomato	Canada
DVD161	Illumina HiSeq 2000	de Jonge et al., 2012	Potato	Canada
DVD-S26	Illumina HiSeq 2000	de Jonge et al., 2012	Soil	Canada
DVD-S29	Illumina HiSeq 2000	de Jonge et al., 2012	Soil	Canada
DVD-S94	Illumina HiSeq 2000	de Jonge et al., 2012	Soil	Canada
JKG8	Illumina HiSeq 2000	Thomma et al. unpublished	Potato	The Netherlands
2009-605	Illumina HiSeq 2000	Thomma et al. unpublished	Bell pepper	Ukraine
463	Illumina HiSeq 2000	Thomma et al. unpublished	Cotton	Mexico
St16.01	Illumina HiSeq 2000	Thomma et al. unpublished	Cotton	Syria
V152	Illumina HiSeq 2000	Thomma et al. unpublished	Oak	Hungry
Vd52	Illumina HiSeq 2000	Thomma et al. unpublished	Pepper	Austria
Vd39	Illumina HiSeq 2000	Thomma et al. unpublished	Sunflower	Germany
van Dijk	Illumina HiSeq 2000	Thomma et al. unpublished	Chrysanthemum	The Netherlands

Table S2. Assembly statistics for the various *Verticillium dahliae* genomes.

Strain	Genome size (Mb)	# scaffolds (>= 0 bp)	# scaffolds (>= 1000 bp)	GC (%)	N50 (Kb)	# N's per 100 kbp	BUSCO (%)
2009-605	34.06	1931	1521	54.76	55.11	123.45	95.4
463	34.03	4188	3562	53.47	17.74	244.06	75
85S	35.93	40	40	53.55	3176.09	0	99.3
CBS38166	34.03	2092	1727	54.24	45.08	316.06	91.6
DVD161	33.47	2155	1819	54.4	41.42	330.79	90.8
DVD31	33.58	2429	2064	54.14	35.75	336.71	89.2
DVD3	34.42	1921	1693	53.62	42.77	378.65	88.9
DVD-S26	34.74	2275	1894	54.2	43.54	367.67	92.2
DVD-S29	33.14	2226	1811	54.56	42.73	306.89	91.6
DVD-S94	34.42	1730	1494	53.92	53.17	287	91.9
JKG8	33.85	1840	1458	54.5	56.35	172.12	95.6
ST100	34.92	2137	1756	53.63	49.89	315.39	93
ST14	34.48	1571	1336	54.03	61.81	221.84	95
ST16	34.22	1821	1454	54.9	57.46	121.71	95.6
v152	33.98	2539	2174	54.28	32.47	146.98	87.6
v52	33.54	3419	2920	54.39	21.80	162.37	78.6
VanDijk	33.17	1769	1389	54.71	61.05	109.26	95.7
Vd39	35.90	1579	1222	53.55	94.44	222.52	98.7
CQ2	35.82	17	17	53.26	3754.19	0	97.5
JR2	36.15	8	8	53.89	4168.63	0	99.4
VdLs17	35.97	8	8	54	5894.01	0	98.9

Table S3. Summary of transposable elements of the various *Verticillium dahliae* strains.

Strain	SINEs		LINEs		LTRs	
	Length occupied (bb)	Percentage of sequence (%)	Length occupied (bb)	Percentage of sequence (%)	Length occupied (bb)	Percentage of sequence (%)
2009-605	0	0	0	0	1403090	4.12
463	0	0	43660	0.13	1601065	4.7
85S	2289.00	0.01	57668.00	0.16	3082210.00	8.58
CBS38166	0.00	0.00	29731.00	0.09	1625629.00	4.78
DVD161	1994.00	0.01	0.00	0.00	1871766.00	5.59
DVD31	15326.00	0.05	14424.00	0.04	1718276.00	5.12
DVD3	1923.00	0.01	17323.00	0.05	2262138.00	6.57
DVD-S26	0.00	0.00	34185.00	0.10	1847224.00	5.32
DVD-S29	22393.00	0.01	7832.00	0.02	1297199.00	3.91
DVD-S94	0.00	0.00	23539.00	0.07	2082451.00	6.05
JKG8	2294.00	0.01	0.00	0.00	1344573.00	3.97
ST100	1720.00	0.01	50597.00	0.14	2693973.00	7.71
ST14	0.00	0.00	30264.00	0.09	1610719.00	4.67
ST16	1582.00	0.00	50167.00	0.15	1009974.00	2.95
v152	0.00	0.00	40614.00	0.12	1660694.00	4.89
v52	3965.00	0.01	28553.00	0.09	1442720.00	4.30
VanDijk	1954.00	0.01	0	0	1337583.00	4.03
Vd39	1582.00	0.00	71392.00	0.20	3201873.00	8.92
CQ2	3965.00	0.01	54386.00	0.15	3156817.00	8.81
JR2	1954.00	0.01	98739.00	0.27	2841008.00	7.86
VdLs17	0.00	0.00	235025.00	0.65	2875975.00	7.99

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Chapter 6

Engineering of a chimeric receptor to mediate improved chitin-triggered immunity in Arabidopsis

Jinling Li[1], Dongli Yu[1], Luigi Faino[1], and Bart P.H.J. Thomma[1*]

[1]Laboratory of Phytopathology, Wageningen University & Research, 6708 PB Wageningen, The Netherlands.

*For correspondence: Bart P.H.J. Thomma, Laboratory of Phytopathology, Wageningen University & Research, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands. Tel. 0031-317-484536, e-mail: bart.thomma@wur.nl

Abstract

Plants employ cell surface-localized immune receptors to detect the presence of potential fungal pathogens and activate appropriate immune responses, for instance by recognizing the conserved fungal cell wall molecule chitin. In Arabidopsis, two lysin motif (LysM) containing receptors, AtCERK1 and AtLYK5, have been implicated in chitin perception. Various fungal pathogens have been shown to secrete LysM effectors to suppress the activation of chitin-triggered host immune responses. For instance, the tomato leaf mould fungus *Cladosporium fulvum* secretes the LysM effector Ecp6 to supress chitin-induced immune responses through direct competition with plant receptors for chitin binding. Chitin binding by Ecp6 is based on intramolecular LysM dimerization that results in increased substrate binding affinity when compared with immune receptors whose LysM domains cannot undergo such intramolecular dimerization. In this study, we attempted to generate chimeric chitin receptors with increased chitin binding affinity by replacing the LysM domains of AtCERK1 and AtLYK5 with the LysM domains of Ecp6. Next, we tested whether the chimeric Ecp6::CERK1 and Ecp6::LYK5 receptors display increased responsiveness to chitin, trigger more robust immune responses, and confer enhanced resistance to fungal infection. Our data suggest that expression of the chimeric receptor Ecp6::LYK5, but not of Ecp6::CERK1, confers responsiveness to fungal chitin. Moreover, we show that Ecp6 treatment fails to suppress chitin-triggered immunity in plants that express the chimeric Ecp6::LYK5 receptor whereas this immune response is suppressed in wild-type plants. This finding suggests that the chimeric Ecp6::LYK5 receptor possesses enhanced chitin-binding characteristics when compared with the native LYK5 receptor. Although the chimeric Ecp6::LYK5 receptor is able to complement enhanced susceptibility of *lyk4/lyk5-2* mutant plants towards the necrotrophic fungal pathogen *Alternaria brassicicola*, no enhanced resistance when compared with wild-type plants was observed.

Introduction

In order to prevent colonization by potentially harmful microbes, plants employ various types of immune receptors to detect pathogen invasion through sensing pathogen-derived or pathogen-induced molecular patterns of various nature, collectively termed invasion patterns (IPs), that betray microbial invasion to activate appropriate immune responses (Cook et al., 2015; Rodriguez-Moreno et al., 2017). A well described IP is chitin, an important structural component of fungal cell walls, which is recognized by cell surface immune receptors (Felix et al., 1993; Sanchez-Vallet et al., 2015; Rovenich et al., 2016). The recognition of chitin fragments by host receptors subsequently prompts the activation of immune responses aimed at halting pathogen invasion (Boller and Felix, 2009; Rovenich et al., 2014).

To date, a number of plant immune receptors for chitin recognition have been characterized from *Arabidopsis thaliana* and from rice (*Oryza sativa*) (Sánchez-Vallet et al., 2015; Shinya et al., 2015). These receptors all contain a varying number of extracellular lysin motifs (LysMs) that are required for chitin-binding (Kombrink et al., 2011; Tanaka et al., 2013). The first chitin receptor that was characterized as chitin elicitor-binding protein (OsCEBiP) was isolated from the plasma membranes of rice cells and is a typical receptor-like protein that contains extracellular LysM domains but lacks an obvious intracellular kinase domain (Kaku et al., 2006). OsCEBiP directly binds chitin and forms a receptor complex with chitin elicitor receptor kinase-1 (OsCERK1) to initiate downstream signalling (Kaku et al., 2006; Hayafune et al., 2014). In contrast to OsCERK1, the *A. thaliana* LysM-containing receptor AtCERK1 has been proposed to directly bind long-chain chitin oligomers through one of the LysM domains, albeit with relatively low binding affinity (Liu et al., 2012). Additionally, the LysM receptor-like kinases AtLYK4 and AtLYK5, both of which carry an inactive kinase domain, were identified as chitin-binding proteins in chitin pull-down assays (Petutschnig et al., 2010). Recently, Cao *et al.* (2014) proposed that AtLYK5 acts as the major chitin receptor in *A. thaliana* due to its higher affinity for chitin binding. AtLYK5 functions in chitin recognition partly overlaps with its paralogue AtLYK4, and *Atlyk4/Atlyk5-2* double mutants showed a loss of chitin responsiveness (Cao et al., 2014).

To overcome chitin-triggered immune responses, fungal plant pathogens employ several strategies, including the secretion of LysM-containing effector proteins (Sánchez-Vallet et al., 2015). For instance, the tomato leaf mould fungus *Cladosporium fulvum* abundantly secretes the LysM effector Ecp6 (extracellular protein 6) during host colonization to compete with plant receptors for chitin binding, and thus prevents the activation of chitin-triggered immune responses (Bolton et al., 2008; de Jonge et al., 2010). Structural analysis of Ecp6 revealed that the concerted action of two of the three LysM domains of Ecp6 results in the formation of a groove with ultra-high chitin-binding affinity that permits to outcompete host immune receptors (Sánchez-Vallet et al., 2013). Interestingly, conserved Ecp6 orthologues widely occur in the fungal kingdom (Bolton et al., 2008; de Jonge and Thomma, 2009), which suggests that the suppression of chitin-triggered immunity is a common strategy used by fungal pathogens (de Jonge et al., 2011). Indeed, various fungal plant pathogens, such as the Septoria tritici blotch fungus *Zymoseptoria tritici*, the Brassicaceae anthracnose fungus *Colletotrichum higginsianum*, the rice

blast fungus *Magnaporthe oryzae* and the vascular wilt fungus *Verticillium dahliae*, have been shown to secrete LysM effectors to perturb the activation of chitin-triggered immunity to promote host colonization (Marshall et al., 2011; Mentlak et al., 2012; Takahara et al., 2016; Kombrink et al., 2017).

Since Ecp6 was shown to outcompete host immune receptors for chitin binding due to increased substrate binding affinity (Sánchez-Vallet et al., 2013), we attempted to generate chimeric chitin receptors with increased affinity for chitin binding by replacing the LysM domains of AtCERK1 and AtLYK5 with the LysM domains of Ecp6. We hypothesized that chimeric receptors with increased affinity for chitin would result in increased responsiveness to fungal chitin, more robust immune responses, and increased resistance to fungal infection.

Materials and methods

Plant materials and chitin treatment

The *A. thaliana* mutant lines used in this study were *cerk1-2* (Miya et al., 2007), *pad-3* (Glazebrook and Ausubel, 1994) and *lyk4/lyk5-2* mutant kindly provided by Prof. Dr. Volker Lipka (Georg-August-University, Göttingen, Germany). *A. thaliana* plants were grown in soil in a growth chamber at 21°C, 65% humidity and a photoperiod of 12 hours. Four-week-old plants were used for all experiments, in which plants were treated by either spraying with water or with 100 µg/ml polymeric chitin (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands).

Construction of chimeric receptors and plant transformation

The primers that were used for the generation of chimeric receptors are listed in Supplemental Table 1. A pGEM-T vector containing the coding sequence of Ecp6 (Sánchez-Vallet et al., 2013) was used as template to amplify the region containing the three LysM domains of Ecp6 (28-220 aa). For construction of the chimeric receptor Ecp6::CERK1, a DNA fragment comprising the *CERK1* promoter sequence and the coding region of *CERK1* was amplified from the *proCERK1:CERK1-GFP* plasmid (Petutschnig et al., 2014). The three LysM domains (35-232 aa) of *CERK1* were then replaced by the LysM domains of Ecp6 via overlap-extension PCR. Similarly, a DNA segment comprising the *LYK5* promoter sequence and the *LYK5* coding sequence was amplified from the *proLYK5:LYK5* mCitrine plasmid (Erwig et al., 2017). The LysM domains of *LYK5* (27-278 aa) were replaced by the LysM domains of Ecp6 (28-220 aa) via overlap-extension PCR as well. All resulting PCR amplicons were cloned into the pDONR207 entry vector using the Gateway® BP Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA). The resulting vectors subsequently recombined into the Gateway-compatible vector pFAST R01 (Shimada et al., 2010) using Gateway® LR Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA). The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) via electroporation and used for Arabidopsis transformation via the floral-dip transformation method (Clough and Bent, 1998). Arabidopsis transformation was confirmed by reverse transcription-PCR (RT-PCR). After two generations of selfing, T2 seeds were used for further experiments.

Oxidative burst assays

Oxidative burst assays were performed as previously described (Rovenich, 2017). Briefly, four-week-old Arabidopsis leaf discs (5 mm diameter) were incubated in 200 µl sterile water overnight in a 96-well plate (one leaf disc per well). The following day, the water was replaced with 100 µl reaction solution containing 100 µM L-012 substrate and 20 µg/ml horseradish peroxidase (HRP). ROS generation was elicited with 100 µg/ml polymeric chitin or 100 nM flg22 peptide (AnaSpec, Fremont, CA, USA), in the presence or absence of 10 µM of the recombinant effector protein Ecp6 that produced in *Pichia pastoris* (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). Luminescence was measured over a 70 min period using an Infinite M200 multimode plate reader (TECAN, Männedorf, Switzerland). Eight leaf discs from three different plants were used for each treatment.

Fungal pathogen inoculation

Inoculation of Arabidopsis plants with the fungus *Alternaria brassicicola* (strain MUCL20297) was performed as previously described (Thomma et al., 1999). Four-week-old Arabidopsis rosette leaves were inoculated with 5 µl droplets of spore suspension (10^6 spores/ml) or mock-inoculated with 5 µl droplets of water. Disease symptoms were examined at 7 days post inoculation and lesion sizes were determined as previously described (Thomma et al., 1999).

Quantitative reverse transcript PCR

To examine the expression of *Ecp6::CERK1*, *Ecp6::LYK5* and the chitin responsive genes *AtWRYK22* and *AtWRKY75* (Egusa et al., 2015; Shinya et al., 2014) in the transgenic plants, leaves of four-week-old Arabidopsis were collected 20 min after plants were treated by spraying 100 µg/ml polymeric chitin or water. Total RNA isolation, cDNA synthesis and quantitative reverse transcript PCR (qRT-PCR) were performed as described earlier (Petutschnig et al., 2014), and the *AtRubisco* gene was used as a reference control (Supplemental Table 1).

Results and discussion

Generation of *Ecp6::CERK1* and *Ecp6::LYK5*-expressing plants

In order to design a host chitin receptor with enhanced chitin-binding affinity, we generated two chimeric receptor constructs, named Ecp6::CERK1 and Ecp6::LYK5, by replacing the LysM domain-containing region of *AtCERK1* and *AtLYK5* with the LysM domain-containing region of Ecp6, respectively (Figure 1). The resulting *Ecp6::CERK1* construct was expressed under the control of the *AtCERK1* promoter in the *cerk1-2* knock-out mutant that completely lost the ability to respond to the chitin (Miya et al., 2007). The *Ecp6::LYK5* construct was expressed under control of *AtLYK5* promoter in the *lyk4/lyk5-2* mutant (Figure S1). Reverse transcription polymerase chain reaction (RT-PCR) confirmed that both the *Ecp6::CERK1* and *Ecp6::LYK5* chimeras are expressed in T2 lines in response to chitin treatment (Figure 2) that were used for further assays to assess the functionality of these chimeric receptors.

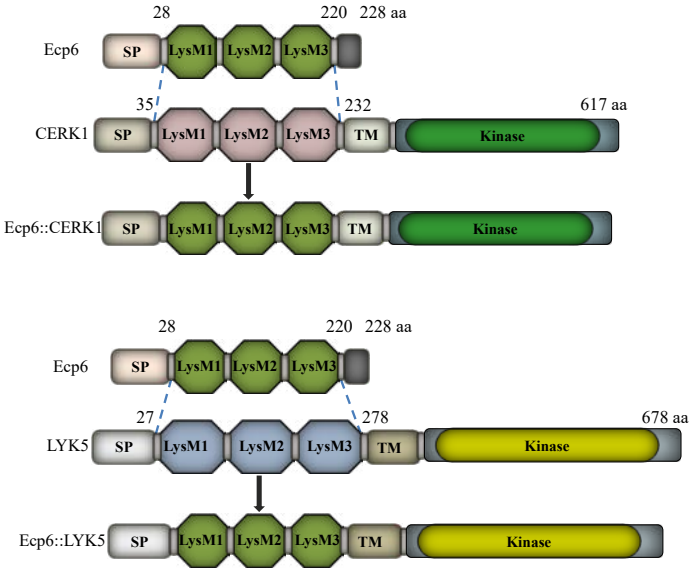

Figure 1. Schematic representation of the chimeric chitin Ecp6::CERK1 and Ecp6::LYK5 receptors. SP: signal peptide, TM: transmembrane region. Amino acids at the fusion points of the protein are indicated.

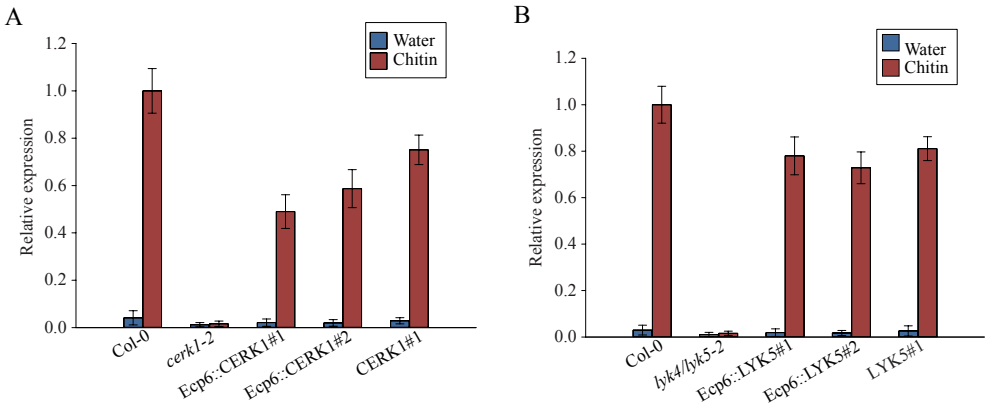

Figure 2. Relative quantification of transgene expression in T2 Arabidopsis lines. Expression of *Ecp6::CERK1* relative to *AtCERK1* **(A)** and expression of *Ecp6::LYK5* relative to *AtLYK5* **(B)**. Transcript levels were normalized to the *AtRubisco* gene and values are expressed as fold changes (± SD) relative to the transcript level of *AtCERK1* or *AtLYK5* in wild type Col-0, which was set at 1. The experiment was performed twice with similar results.

Transgenic expression of *Ecp6::LYK5*, but not of *Ecp6::CERK1*, confers chitin responsiveness

Plant chitin receptors can swiftly prompt the activation of multiple downstream responses upon chitin perception, including the generation of reactive oxygen species (ROS) and the expression of defence-related genes (Shibuya and Minami, 2001). We first examined the functionality of the chimeric receptors by testing their ability to confer chitin-induced ROS production.

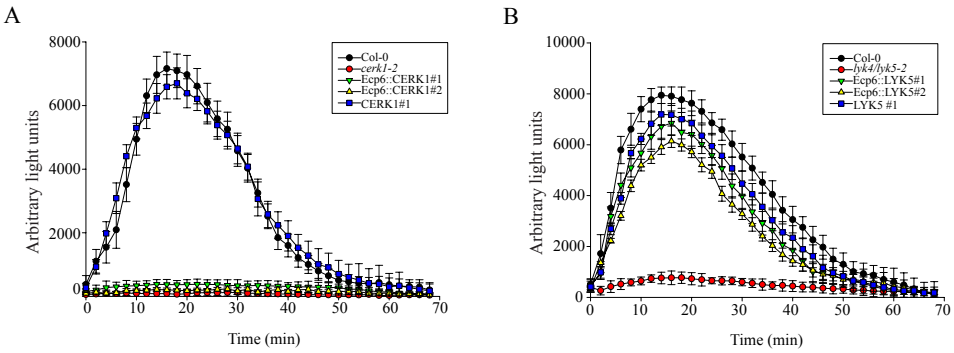

Figure 3. Chitin-induced ROS production in *Ecp6::CERK1* (A) or *Ecp6::LYK5* (B) transgenic plants. Production of ROS was measured by using luminol-dependent chemiluminescence over a period of 70 min following treatment of Arabidopsis leaf discs with 100 µg/ml of polymeric chitin. The relative luminescence was calculated by normalization to water-treated leaf discs. Plotted are means ± SD of 8 leaf discs. The experiment was performed twice with similar results.

To this end, the accumulations of ROS in transgenic lines that express the chimeric receptors were measured upon chitin treatment using a luminol-based chemiluminescence detection assay (de Jonge et al., 2010). Whereas clear a ROS burst occurred with a peak at 15 min following chitin treatment of a wild type Col-0 line and a *AtCERK1* complementation line (CERK1#1), no ROS burst was monitored in two independent *Ecp6::CERK1* transgenic lines or in *cerk1* mutant plants (Figure 3A). Interestingly, whereas chitin-induced ROS production was significantly lower in the *lyk4/lyk5*-2 mutant plants when compared with wild-type plants, treatment of *Ecp6::LYK5*-expression plants with polymeric chitin resulted in high levels of ROS accumulation as similarly observed in wild type plants and a *LYK5* complementation line (LYK5 #1)(Figure 3B). As a control, we monitored ROS production triggered by the well-characterized bacterial elicitor flg22 (Zipfel et al., 2004) in these lines, showing that all genotypes accumulated similar levels of ROS production (Figure S2).

In addition to ROS production, we examined the expression of two well-established chitin-responsive genes, *AtWRKY22* and *AtWRKY75*, in response to chitin treatment (Egusa et al., 2015; Shinya et al., 2014). Similar to the oxidative burst, no clear expression of *AtWRKY22* and *AtWRKY75* was observed in *Ecp6::CERK1* expression lines upon chitin treatment (Figure 4). In contrast, clear expression of *AtWRKY22* and *AtWRKY75* was monitored after chitin treatment of *Ecp6::LYK5* expression lines (Figure 4).

Collectively, our data show that expression of the *Ecp6::LYK5* chimera, but not of the *Ecp6::CERK1* chimera, confers responsiveness to chitin and activates chitin-dependent immune responses. Hence, we focused on further characterization of chimeric Ecp6::LYK5 receptor.

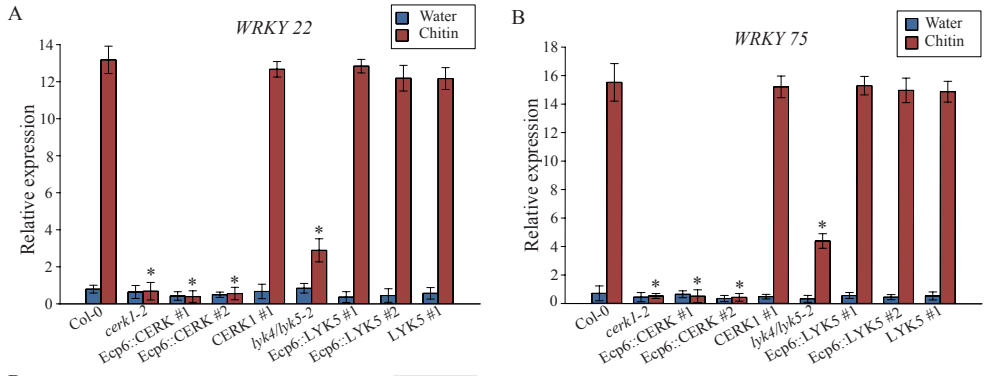

Figure 4. Expression of the chitin-responsive genes *AtWRKY22* and *AtWRKY75* in *Ecp6::CERK1* or *Ecp6::LYK5* transgenic plants upon chitin treatment. The bars represent the transcript levels of *AtWRKY22* **(A)** and *AtWRKY75* **(B)** relative to the constitutively expressed Arabidopsis *Rubisco* gene in various Arabidopsis genotypes. Data represent means ± SD of three biological replicates consisting of pools of three plants from one of a single experiment. The asterisk indicates statistically significant differences from the wild type upon chitin treatment as determined with the Student's *t*-test ($P < 0.05$). The experiment was performed twice with similar results.

Ecp6::LYK5 prompts chitin-induced ROS production in the presence of Ecp6

It has previously been shown that Ecp6 can sequester chitin fragments with ultra-high chitin-binding affinity to effectively prevent the activation of chitin induced immunity, not only in tomato and rice, but also in Arabidopsis plants (de Jonge et al., 2010; Rovenich, 2017). As we have shown that the chimeric receptor Ecp6::LYK5 is able to mediate a chitin response, we further tested if this receptor can compete for chitin binding with Ecp6 to mediate chitin-induced ROS production. Intriguingly, Ecp6 treatment did not compromise ROS production in *Ecp6::LYK5* plants, resulting in the accumulation of similar levels as upon chitin treatment in the absence of Ecp6 (Figure 5). This finding suggests that the Ecp6::LYK5 chimera has gained a significantly higher chitin-binding affinity when compared with the native AtLYK5 receptor, and can no longer be outcompeted by the Ecp6 effector for chitin binding. In contrast, bacterial flg22-induced ROS productions in these plants were not affected in the presence of Ecp6 (Figure S3).

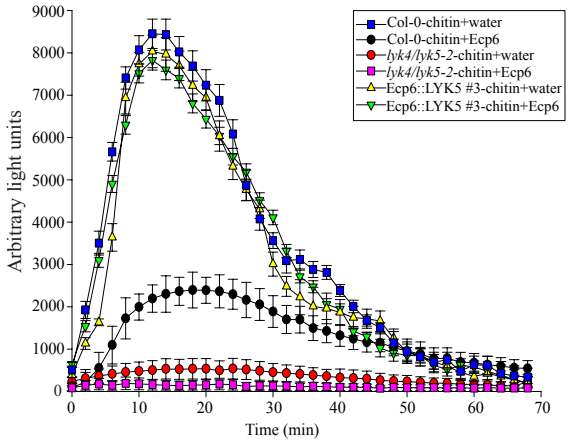

Figure 5. The chimeric receptor Ecp6::LYK5 prompts chitin-induced ROS production in the presence of Ecp6. Production of ROS was measured by using luminol-dependent chemiluminescence over a period of 69 min following treatment of Arabidopsis leaf discs with 100 µg/ml of polymeric chitin, or a mixture of 100 µg/ml of polymeric chitin and 10 µM Ecp6 protein. The relative luminescence was calculated by normalization to water-treated leaf discs. Plotted are means ± SD of 8 leaf discs. The experiment was performed twice with similar results.

Expression of *Ecp6::LYK5* does not confer enhanced resistance to the fungal pathogen *Alternaria brassicicola*

As the expression of *Ecp6::LYK5* enables transgenic Arabidopsis plants to sense and respond to chitin in such manner that it can no longer be abolished by Ecp6 treatment, we further tested whether the enhanced chitin responsiveness results in enhanced disease resistance. To this end, we challenged these transgenic plants with necrotrophic fungal pathogen *A. brassicicola*. The Arabidopsis mutant *pad3-1* that has previously been shown to display enhanced susceptibility towards *A. brassicicola* was used as positive control for successful inoculation (Thomma et al., 1999). As expected, *lyk4/lyk5-2* mutant plants showed increased susceptibility to *A. brassicicola* when compared with Col-0 plants, as inoculated plants showed significantly lager lesions when compared with Col-0 plants at 7 days post inoculation (dpi) (Figure 6). However, lesion sizes on *Ecp6::LYK5* expression plants were similar to those on Col-0 plants or on a LYK5 complementation line (LYK5#1) (Figure 6). Although these data confirm that the chimeric *Ecp6::LYK5* receptor is a functional immune receptor, no enhanced pathogen immunity properties when compared with the native receptor were revealed. However, it needs to be noted that it is not clear to what extent *A. brassicicola* depends on LysM effector activity to subvert chitin-triggered immunity during host invasion. Therefore, more pathogens, preferably alongside with LysM effector gene deletion strains, need to be tested in order to determine to what extent the chimeric *Ecp6::LYK5* receptor can counteract the activity of fungal LysM effectors during pathogen invasion.

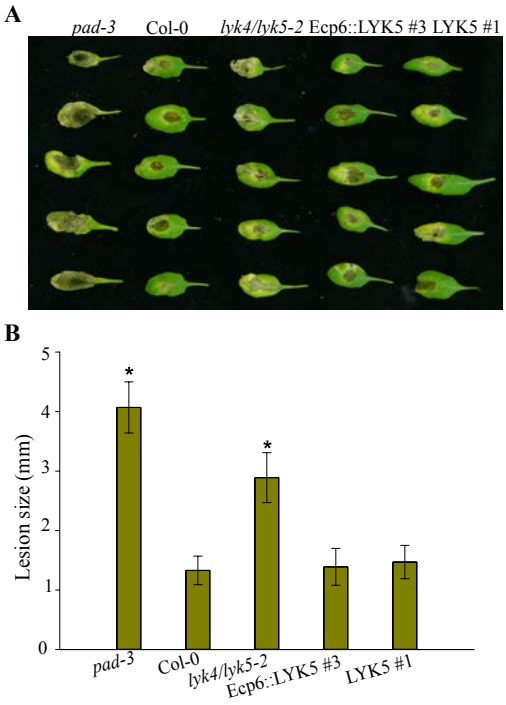

Figure 6. Inoculation assays on *Ecp6::LYK5* transgenic plants with the fungal pathogen *Alternaria brassicicola*. (A) Representative picture showing disease symptoms upon inoculation with *A. brassicicola* at 7 days post inoculation (dpi). The *pad3-1*mutant that displays enhanced susceptibility to *A. brassicicola* was used as positive inoculation control. **(B)** Quantification of the average lesion size at 7 dpi with standard deviations ($n \geq 20$ independent lesions). Asterisks indicate statistically significant differences when compared with Col-0 plants (Student's *t*-test; $P < 0.05$). This experiment was performed two times with similar results.

Conlusion

Our data suggest that the chimeric Ecp6::LYK5 receptor displays enhanced chitin-binding ability, as its chitin-binding activity can no longer be suppressed by treatment with the LysM effector Ecp6. This indicates that the receptor has the potential to mediate enhanced resistance to fungal pathogen infection. Although an initial attempt to demonstrate with the fungal pathogen *A. brassicicola* was unsuccessful, further attempts that included more fungal plant pathogens should be made to verify this hypothesis.

Acknowledgements

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Supplemental information

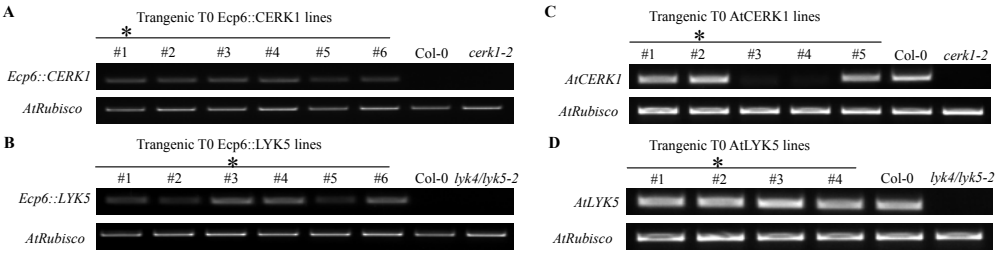

Figure S1. Expression of *Ecp6::CERK1*, *Ecp6::LYK5*, *AtCERK1*, and *AtLYK5* in T0 transgenic *Arabidopsis* plants. Detection of *Ecp6::CERK1* **(A)** or *Ecp6::LYK5* **(B)** transcripts in independent T0 transformation events measured by RT-PCR with primers specific for the LysM domain of Ecp6. The *A. thaliana Rubisco* gene was used as an endogenous control. The transgenic line that was used for selfing is indicated with an asterisk. Detection of *AtCERK1* **(C)** or *AtLYK5* **(D)** transcripts in independent T0 transformation events measured by RT-PCR with primers specific for the kinase domain of *AtCERK1* or *AtLYK5*. The *A. thaliana Rubisco* gene was used as an endogenous control. The transgenic line that used for selfing is indicated with an asterisk.

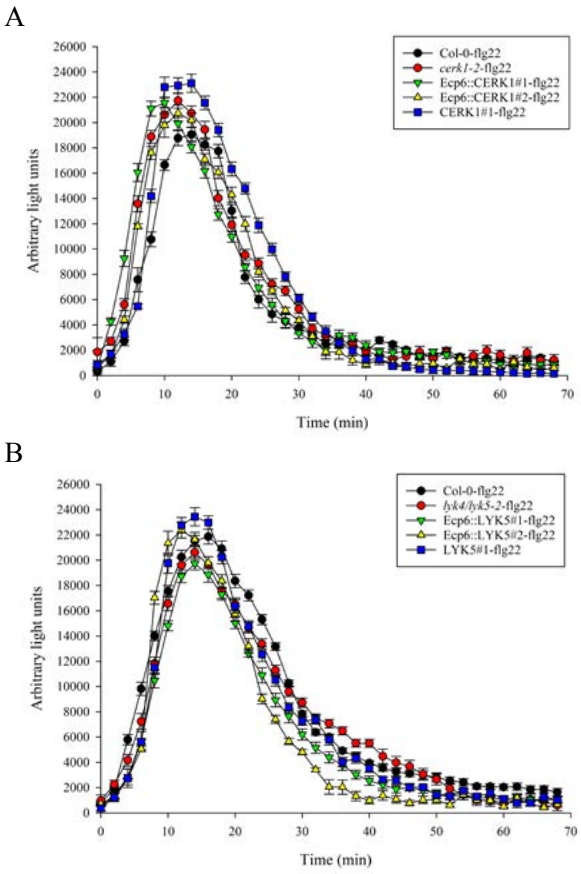

Figure S2. ROS production in *Ecp6::CERK1* (A) or *Ecp6::LYK5* (B) transgenic plants upon bacterial elicitor flg22 treatment. Production of ROS was measured by using luminol-dependent chemiluminescence over a period of 70 min following treatment of Arabidopsis leaf discs with 100 nM of flg22 or water. The relative luminescence was calculated by normalization to water-treated leaf discs. Plotted are means ± SD of 8 leaf discs. The experiment was performed twice with similar results.

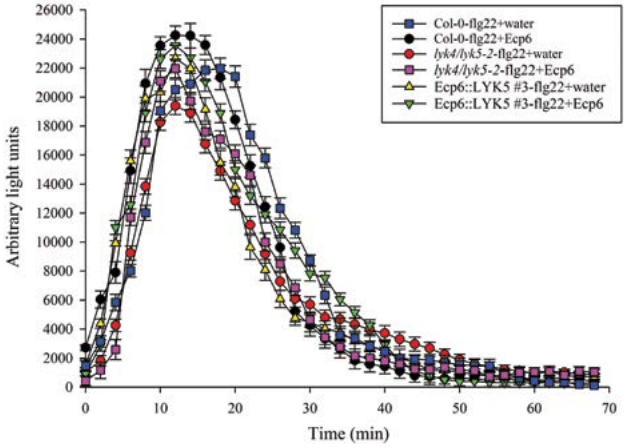

Figure S3. Bacterial flg22-induced ROS production in the presence of Ecp6. Production of ROS was measured by using luminol-dependent chemiluminescence over a period of 70 min following treatment of Arabidopsis leaf discs with 100 nM flg22 in the presence or absence of 10 μM Ecp6 protein. The relative luminescence was calculated by normalization to water-treated leaf discs. Plotted are means ± SD of 8 leaf discs. The experiment was performed twice with similar results.

Table S1. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
Ecp6::CERK1_LysM-F	CTCATTCTTCTTCGCCGTGGAATCTAGCAA-CATCAAATACACTGTC	Amplification of *Ecp6* LysM domains; overhang present in primer for overlap with region of CERK1 is underlined
Ecp6::CERK1_LysM-R	CTATACCAGCAATAACTCCAGCTG-GCAGAATGATCTGCTG	Amplification of *Ecp6* LysM domains; overhang present in primer for overlap with region of CERK1 is underlined
CERK1_pro-F	GGCGCGCCTATATGAAGAAGG	Amplification of *CERK1* promotor region
CERK1_pro-R	GACAGTGTATTTGATGTTGCTAGATTCCAC-GGCGAAGAAG	Amplification of *CERK1* region; overhang present in primer for overlap with region of Ecp6 is underlined
CERK1_ID-F	TTGGTCAGCAGATCATTCTGCCAGCTG-GAGTTATTGCTGGT	Amplification of *CERK1* intracellular domains; overhang present in primer for overlap with region of Ecp6 is underlined
CERK1_ID-R	CTACCGGCCGGACATAAGACTGAC	Amplification of *CERK1* intracellular domains
Ecp6::LYK5_LysM-F	CTTTGCCGTGTCACCGGCGAAAGCTAG-CAACATCAAATACACTGT	Amplification of *Ecp6* LysM domains; overhang present in primer for overlap with region of LYK5 is underlined
Ecp6::LYK5_LysM-R	AGAGAAGCAAACCAGCTCCGGCTTAG-ATGGGTTAACGTTGTTGTT	Amplification of *Ecp6* LysM domains; overhang present in primer for overlap with region of LYK5is underlined
LYK5_pro_F	CCTCTGTTTTTGTTGTGGATTATTATAG	Amplification of *LYK5* promotor region
LYK5_pro_R	ACAGTGTATTTGATGTTGCTAGCTTTCGC-CGGTGACACGGCAAAG	Amplification of *LYK5*, overhang present in primer for overlap with region of Ecp6 is underlined
LYK5_ID-F	AACAACAACGTTAACCCATCTAAGCCG-GAGCTGGTTTGCTTCTCT	Amplification of *LYK5* intracellular domains; overhang present in primer for overlap with region of Ecp6 is underlined
LYK5_ID-R	CTAGTTGCCAAGAGAGCCGGA	Amplification of *LYK5* intracellular domains
CERK1-qPCR-F	AGTGTCTGCAAAAGTAGATG	Reverse Transcription-PCR
CERK1-qPCR-R	CTACCGGCCGGACATAAGACTG	Reverse Transcription-PCR
LYK5-qPCR-F	TCTTTCGGGGAGAGAAGCAG	Reverse Transcription-PCR
LYK5-qPCR-R	AAGGTCATCAGAAGGCTCCC	Reverse Transcription-PCR
WRKY22-qPCR-F	TCCTTCGGAGAGATTCGAGA	Reverse Transcription-PCR
WRKY22-qPCR-R	CTGCTGCTACATGGCACACT	Reverse Transcription-PCR

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
WRKY75-qPCR -F	CCAAAAGGCCGTCAAGAACAACAA	Reverse Transcription-PCR
WRKY75-qPCR-R	TGCTTCTTCACATTGCATCCTCCA	Reverse Transcription-PCR
AtRubisco-F	GCAAGTGTTGGGTTCAAAGCTGGTG	Reverse Transcription-PCR
AtRubisco-R	CCAGGTTGAGGAGTTACTCGGAATGCTG	Reverse Transcription-PCR

Chapter 7

General discussion: How do microbes evolve to become plant pathogens?

Abstract

Microbial plant pathogens cause considerable crop yield losses in agricultural production systems worldwide. In order to establish their parasitic relationships with host plants, microbial pathogens rely on the secretion of effector molecules to support host colonization. To this end, pathogens carry genes to secrete dozens (for bacteria) to hundreds (for fungi and oomycetes) effectors. It is generally demonstrated that significant redundancy occurs among effectors that are present in effector catalogs of a single pathogen. Consequently, deletions of single effector genes most frequently result in modestly attenuated virulence phenotypes. However, in some cases, single effector genes seem to make a major contribution to pathogenicity, even to an extent where pathogenicity relies on a single effector only. Depending on the pathogen, its lifestyle and its host range, the utilization of a single effector that act as a pathogenicity factor on a particular host plant may offer an evolutionary advantage to the microbial pathogen.

Introduction

In nature, plants are teeming with a diverse range of microorganisms, including commensal, symbiotic, and pathogenic ones. Among these, pathogenic microorganisms colonize their plant hosts while adversely affecting them, which typically leads to the expression of disease symptoms (Aung et al., 2018). Commonly referred to as pathogenicity, the ability to cause disease is a widely shared trait among diverse plant-associated microorganisms, including bacteria, fungi, and oomycetes (Plissonneau et al., 2017). Interestingly, phylogenetic studies have shown that pathogenic microbial species do not occur in closely related monophyletic clusters, but are spread as discrete subdivisions within larger phylogenetic groups that also comprise non-pathogenic species (Fitzpatrick et al., 2006). Consequently, pathogens are found in all taxonomic divisions of microorganisms, and it is generally accepted that microbial pathogenicity arose multiple times during evolution (James et al., 2006; Soanes et al., 2007; van der Does and Rep, 2007).

Since plant pathogenic microorganisms cause substantial crop yield losses, they have long been known as major biotic constraints to crop production worldwide. Deciphering mechanisms of microbial pathogenicity is therefore a long-standing objective of plant pathology research (Strange and Scott, 2005; Fisher et al., 2012). Over the years, accumulating evidence has shown that the pathogenicity of a plant pathogen is largely mediated by so-called effector molecules that are secreted during host infection (Jones and Dangl, 2006; de Jonge et al., 2011; Rovenich et al., 2014; Rodriguez-Moreno et al., 2018). These effectors are typically considered to be small cysteine-rich proteins, but may also comprise other types of molecules such as secondary metabolites and small RNAs (Rodriguez-Moreno et al., 2018). Most of the secreted effector molecules are involved in suppression of plant immune responses or in manipulation of other elements of plant physiology to support host colonization (Rovenich et al., 2014; Rodriguez-Moreno et al., 2018).

Because plant pathogens secrete a battery of effector molecules to facilitate host colonization (Büttner et al., 2016; Rovenich et al., 2014), it is not surprising that many effectors appear to be functionally redundant (Win et al., 2012). For instance, the genome of the oomycete pathogen *Phytophthora infestans* encodes over 500 RxLR-motif containing effectors besides other types of effectors (Haas et al., 2009), which are unlikely to all have unique targets (Birch et al., 2008; Stassen and Van den Ackerveken, 2011). Similarly, the maize head smut fungus *Sporisorium reilianum* possesses a pool of functionally redundant effectors to modulate virulence on maize, as individual effector gene deletions only showed a modest impact on virulence (Ghareeb et al., 2018). In the same fashion, the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Pst) carries approximately 30 genes that encode type III secreted effectors (Xin and He, 2013), of which multiple members can be deleted simultaneously without a noticeable effect on virulence (Kvitko et al., 2009). Thus, for various types of pathogens it is generally considered that effector catalogues carry redundant activities and the effectors collectively mediate microbial pathogenicity through the simultaneous manipulation of diverse host processes (de Jonge et al., 2011; Tan et al., 2015; Win et al., 2012).

Pathogen lifestyles are associated with genomic adaption

Plant pathogens have evolved different strategies to interact with their host plants and acquire nutrients. Generally, their pathogenic lifestyles can be assigned to two extremes: biotrophy and necrotrophy (Glazebrook, 2005; Spanu, 2012). While biotrophic pathogens comprise species that can only obtain nutrients from living plant tissue, necrotrophic pathogens kill host cells to secure their nutrient supply (Mengiste, 2012). However, many pathogens reside in the continuum between these two extreme lifestyles and are labelled as hemibiotrophs that start with a biotrophic phase followed, sooner or later, by a transition to a necrotrophic stage (Spanu, 2012).

To date, thousands of genomes of pathogenic microbes have been sequenced and are publically available (Thomma et al., 2016), and so-called pathogenomics has revealed genomic adaptations that are associated with lifestyles (Schmidt et al., 2010; Guttman et al., 2014). For instance, the genomic analysis of several obligate biotrophic pathogens has provided a plausible molecular explanation for their obligate parasitism (Baxter et al., 2010; Spanu et al., 2010; Duplessis et al., 2011; Guttman et al., 2014). A characteristic feature of these genomes is the considerable loss of genes that are involved in primary metabolic pathways (Baxter et al., 2010; Spanu et al., 2010; Guttman et al., 2014). For instance, genes for nitrate and nitrite reductases, as well as for sulfite reductases, are largely missing from the genome of the oomycete pathogen *Hyaloperonospora arabidopsidis* (Baxter et al., 2010). Similar observations were made for powdery mildew fungi (Spanu et al., 2010). Thus, gene losses in primary metabolism may explain why *H. arabidopsidis*, powdery mildew fungi and likely also other biotrophic pathogens became obligate parasites (Spanu et al., 2010, 2012; Guttman et al., 2014). Furthermore, for obligate biotrophs, it is imperative to maintain host cell viability and avoid the activation of host immune responses, especially if these involve apoptosis or other types of host cell death. Thus, another characteristic feature of obligate biotrophic pathogen genomes is that many gene families encoding plant cell wall hydrolases and secondary metabolites are significantly reduced (Spanu et al., 2010; Duplessis et al., 2011; Baxter et al., 2010).

The genome sequences of necrotrophic fungal pathogens, such as the fungal cereal pathogens *Pyrenophora teres* f. *teres* and *Parastagonospora nodorum* (formerly *Stagonospora nodorum*), typically reveal an abundance of genes encoding carbohydrate hydrolases, cytochrome P450 monooxygenases, non-ribosomal peptide synthetases (NRPSs), and polyketide synthases (PKSs) that can be attributed to their necrotrophic lifestyle (Hane et at., 2007; Ellwood et al., 2010; Schmidt et al., 2011). For instance, carbohydrate hydrolases are involved in degradation of plant cell walls (Lombard et al., 2014), while cytochrome P450 monooxygenases, NRPSs and PKSs are typically associated with the biosynthesis of secondary metabolites, some of which are toxic to plant cells (Möbius and Hertweck, 2009; Stergiopoulos et al., 2013). Genomic analysis of the necrotrophic pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea* has shown similar arsenals of genes that can be implicated in necrotrophic processes, such as genes involved in plant cell wall degradation and oxalic acid production that have been involved in tissue maceration (Amselem et al., 2011).

Genome sequencing combined with stage-specific transcriptomic analysis of the hemibiotrophic Arabidopsis pathogen *Colletotrichum higginsianum* and the maize pathogen *Colletotrichum graminicola* revealed a number of pathogenicity-related genes that are transcribed in successive waves and can be linked to the lifestyle transition (O'Connell et al., 2012). Here, the majority of genes encoding effectors and secondary metabolite biosynthesis components are strongly induced during the initial biotrophic phase, whereas lytic enzymes and transporter genes are upregulated in the subsequent necrotrophic phase (O'Connell et al., 2012). It is likely that these fungi need effectors or even particular secondary metabolites for the manipulation of host defence responses during early biotrophic stages, while the pathogen later uses lytic enzymes for destruction of host cells during necrotrophic growth (O'Connell et al., 2012).

The effector arsenal of biotrophic pathogens

As indicated above, for biotrophic pathogens it is vitally important to supress host defences without perturbing host cellular homeostasis (Doehlemann et al, 2014). *Cladosporium fulvum* is a biotrophic fungal pathogen that causes tomato leaf mould disease (Joosten and Wit, 1999). To establish biotrophy and promote colonization of the apoplast, a hostile environment that contains various plant-secreted hydrolytic enzymes including chitinases and glucanases, *C. fulvum* secretes a plethora of effector proteins (de Wit et, 2016; Rovenich et al., 2016). Host-secreted chitinases target fungal cell walls not only to release chitin fragments that activate host immune receptors but also to induce cellular collapse, and thus inhibition of fungal proliferation (Sánchez-Vallet et al., 2015; Rovenich et al., 2016). To prevent this, *C. fulvum* secretes the apoplastic effector Avr4 that binds fungal cell walls through an invertebrate chitin-binding domain to render the cell wall chitin inaccessible to chitinases and consequently protects it against hydrolysis (van den Burg et al., 2006; van Esse et al., 2007). Besides Avr4, *C. fulvum* also secretes the LysM domain-containing effector protein Ecp6 to sequester chitin fragments and prevent the activation of chitin-induced plant immunity during host colonization (Bolton et al., 2008; de Jonge et al., 2010). Besides various types of hydrolytic enzymes, the host apoplast also contains diverse proteases and other types of antifungal compounds that adversely affect fungal invasion (van Esse et al., 2008; Jashni et al, 2015). The *C. fulvum* effector Avr2 was shown to inhibit a set of tomato defense-related proteases to support fungal growth in the apoplast (van Esse et al., 2008). In addition, *C. fulvum* secretes the glycosyl hydrolase CfTom1 to detoxify α-tomatine, an antifungal glycoalkaloid (Ökmen et al., 2013).

Several effectors of the maize smut fungus *Ustilago maydis* have been characterized and implicated in its biotrophic life style (Lanver et al., 2017). For example, Pep1 inhibits the maize peroxidase POX12 to block the peroxidase driven oxidative burst (Doehlemann et al., 2009; Hemetsberger et al., 2012). Like the *C. fulvum* effector Avr2, the apoplastic *U. maydis* effector Pit2 functions as an inhibitor of maize cysteine proteases (Doehlemann et al., 2011, Mueller et al., 2013). In addition, *U. maydis* secretes the chorismate mutase Cmu1 into the host cytoplasm to suppress the synthesis of salicylic acid (SA), a canonical defense hormone that plays a crucial role in the activation of defense responses that are directed against biotrophic pathogens (Thomma et al., 1998; Glazebrook, 2005), by reducing the pool of the salicylic acid precursor chorismate (Djamei et al., 2011).

The effector arsenal of necrotrophic pathogens

Host selective toxins (HSTs) are known to induce necrotic reactions and promote susceptibility on plants that harbour the corresponding toxin target (Wolpert et al., 2002). Therefore, these toxins are frequently called necrotrophic effectors as well (Friesen et al., 2008). For example, victorin is a HST produced by the necrotrophic fungus *Cochliobolus victoriae*, the causal agent of the Victoria blight on oat plants (Meehan and Murphy, 1947; Scheffer et al., 1967; Wolpert et al., 1985, 1986). It has been shown that victorin targets the defense-associated thioredoxin TRX-h5, which is guarded by Arabidopsis resistance protein LOV1, and thus activates a LOV1-mediated cell death response (Lorang et al., 2012). In this manner, *C. victoriae* exploits plant immunity to trigger host cell death and mediate disease establishment (Lorang et al., 2012).

The necrotrophic plant pathogens *P. nodorum* and *Pyrenophora tritici-repentis* produce several necrotrophic effectors that induce disease in wheat cultivars that carry corresponding susceptibility genes (Friesen et al, 2008; McDonald and Solomon, 2018). *P. tritici-repentis* effector PrToxA interacts with the wheat Tsn1 in a gene-for-gene manner to induce necrosis (Ciuffetti et al., 2010; Shi et al., 2016). It was reported that *PrToxA* was horizontally acquired by *P. tritici-repentis* from *P. nodorum* (Friesen et al., 2006; Stukenbrock and McDonald, 2007), and more recently *ToxA* has been discovered in the wheat pathogen *Bipolaris sorokiniana* as well (McDonald et al., 2018). The *P. nodorum* effector SnTox1 was shown to interact with wheat Snn1 to induce hallmarks of apoptosis including an oxidative burst and DNA laddering (Liu et al., 2012). Interestingly, the wheat susceptibility gene *Tsn1* displays disease resistance protein-like features, including S/TPK and NBS-LRR domains (Faris et al., 2010), while Snn1 is a wall-associated kinase receptor (Shi et al., 2016). Collectively, these studies show that, like biotrophic pathogens, also necrotrophic fungal pathogens utilize effectors to modulate host immunity to mediate host interactions (Wolpert et al., 2002; Faris et al., 2010; Liu et al., 2012, 2016; Shi et al., 2016).

The effector arsenal of hemibiotrophic pathogens

Magnaporthe oryzae is a hemibiotrophic fungal pathogen that causes rice blast, which is one of the most devastating diseases of cultivated rice (Wilson and Talbot, 2009). The *M. oryzae* apoplastic effector Slp1, which is a homolog of *C. fulvum* Ecp6, specifically accumulates at the plant-fungus interface during the early stages of rice blast infections (Mentlak et al., 2012). Slp1 competes with the rice chitin receptor CEBiP for binding of chitin oligosaccharides, and thus sequesters chitin oligosaccharides to prevent the activation of chitin-triggered immunity in rice (Mentlak et al., 2012). It was reported that the *M. oryzae* cytoplasmic effector AvrPiz-t suppresses the transcriptional activity of the bZIP-type transcription factor APIP5, which leads to rapid cell necrosis and lesion formation at the necrotrophic stage (Wang et al., 2016). Thus, it is likely that effector AvrPiz-t plays a role in the transition from the biotrophic to the necrotrophic stage of *M. oryzae* (Wang et al., 2016).

The hemibiotrophic oomycete pathogen *P. infestans* is the causal agent of the devastating late blight disease of potato and tomato (Du et al., 2015). During its early biotrophic infection stage, the cytoplasmic effector AVR3a is secreted from haustoria into host cells to stabilize the host E3

ligase CMPG1 to mediate suppression of cell death induced by *P. infestans* elicitor INF1 (Armstrong et al., 2005; Bos et al., 2010). Another *P. infestans* effector protein, SNE1 (suppressor of necrosis 1), is highly expressed during biotrophy to suppress host cell death induced by the *P. infestans* NLP effector PiNPP1.1 (Kelley et al., 2010). Interestingly, at the necrotrophic phase, expression levels of *SNE1* decline, while *PiNPP1* expression increases (Kelley et al., 2010). Thus, *SNE1* and *PiNPP1.1* are coordinately expressed during biotrophy and necrotrophy, respectively, to orchestrate the transition from biotrophy to necrotrophy (Kelley et al., 2010).

These examples suggest that hemibiotrophs carry two types of effectors, namely the ones that are typically produced by biotrophic pathogens to subvert immunity in the early stages of infections when typically no disease symptoms are expressed, and the ones that are typically produced by necrotrophic pathogens for later stages of infection when tissue maceration is induced.

Comparative genomic analyses fail to unravel the molecular basis of pathogenicity in the *Verticillium* genus

Although effectors are typically considered as molecules that are produced by microbial pathogens to support host colonization, it has become evident that also non-pathogenic microbes encode abundant effector catalogs (de Jonge and Thomma 2009; Seidl et al., 2015; Oome and Van den Ackerveken, 2014; Martin et al., 2008; 2010). The accessibility and relatively low cost of modern-day sequencing technologies make that genome sequencing has become accessible to many research laboratories, including those studying non-model organisms (Faino and Thomma, 2014; Thomma et al., 2016). This has also spurred comparative genomic analyses that are aimed at unravelling the molecular basis of microbial pathogenicity.

The *Verticillium* genus comprises soil-borne asexual species that differ significantly in their lifestyles that range from saprophytic to pathogenic (Fradin and Thomma, 2006; Klosterman et al., 2011; Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014). Among them, *Verticillium dahliae*, *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae* and *V. longisporum* are plant pathogens that causes vascular wilt disease (Fradin and Thomma, 2006; Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014). The remaining *Verticillium* spp., namely *V. isaacii*, *V. klebahnii*, *V.nubilum*, *V. tricorpus* and *V. zaregamsianum* are mostly considered saprophytes that thrive on dead organic material and are only occasionally reported as opportunistic pathogens (Inderbitzin et al., 2011; Inderbitzin and Subbarao, 2014). Genome analysis of *V. dahliae* and *V. tricorpus* revealed that the *V. tricorpus* effector repertoire largely resembles that of *V. dahliae* (Seidl et al., 2015). Well-characterized effector families that have been implicated in fungal pathogenicity, such as LysM effectors (de Jonge et al., 2010; Marshall et al., 2011; Mentlak et al., 2012; Kombrink et al., 2017) and NLP effectors (Santhanam et al., 2013), are found in *V. tricorpus* as well (Seidl et al., 2015). Genus-wide genome analyses furthermore showed all *Verticillium* spp. display similar genomic features, including the occurrence of extensive genomic rearrangements and the presence of similarly sized effector catalogs (Shi-Kunne et al., 2018; Shi-Kunne unpublished data). Consequently, no obvious genomic differences can be linked to pathogenicity in the *Verticillium* genus (Seidl et al., 2015; Shi-Kunne et al., 2018; Shi-Kunne unpublished data). This has fuelled the hypothesis that

the ability to cause disease within the *Verticillium* genus is caused by subtle genomic traits that do not easily become apparent from whole-genome comparisons (Seidl et al., 2015; Shi-Kunne unpublished data).

A single to few *V. dahliae* effectors determine pathogenicity on a particular host plant

In this thesis I have shown that two effectors of *V. dahliae* individually are responsible for pathogenicity on different plant hosts. The D effector was shown to act as a pathogenicity determinant of *V. dahliae* strains that belong to the defoliating pathotype to cause disease on cotton and olive, but also for infections on *Nicotiana benthamiana* and *Arabidopsis thaliana* that do not respond to infection with defoliation **(Chapter 2)**. Besides, the Tom1 effector was found to mediate *V. dahliae* pathogenicity on tomato (**Chapter 3**). Our attempt to identify a single such pathogenicity factor for *Verticillium* wilt disease on sunflower failed, perhaps also because the analysis was performed on relatively few strains only. Nevertheless, also this analysis revealed an effector (named as Sun1) with a large impact on disease development (**Chapter 4**). This finding suggests that pathogenicity of *V. dahliae* on sunflower depends on relatively few effectors, such that the deletion of a single one has a marked impact on virulence. Collectively, these findings suggest that pathogenicity of *V. dahliae* on various plant hosts depends on relatively few effectors, and for some host species even on a single one, which challenges the common assumption that effector activities are redundant and that contributions of individual effectors to microbial pathogenicity are dispensable.

Perhaps it is not so surprising that *V. dahliae* as a broad host pathogen utilizes relatively few effectors to cause disease on a single host. On the one hand, it can be hypothesized that broad host-range pathogens possess a collection of "one-fits-all" effectors that interact with targets that are conserved in a wide range of plant species to mediate compatibility on many hosts. On the other hand, broad host-range pathogens may possess multiple sets of more specific effectors that operate on single to few hosts only. This latter option requires that relatively few effectors are required to cause disease on a single host as it is unlikely that a single pathogen would possess extensive effector catalogs for a wide range of host species. In this respect our finding that different strains that are able to infect the same host carry divergent effector catalogs (**Chapter 5**) is interesting, as it suggests that effector catalogs are subject to considerable diversification with a continuous evolution of novel effectors, which argues against the general occurrence of "one-fits-all" effectors that interact with targets that are conserved in a wide range of plant species, as these would be evolutionary more ancient and conserved.

Considering its wide host range, it is conceivable that *V. dahliae* strains display a relatively low degree of co-evolution with individual host species. This is also reflected by its colonization biology, as the fungus thrives in xylem vessels that consists of lignified tracheary elements that have lost their protoplasts, and hence relatively little interaction with the host occurs (Yadeta and Thomma, 2013). Furthermore, the fungus does not develop highly evolved appressoria for penetration, or haustoria for feeding and effector deposition. Finally, little convincing evidence exists that a suite of effector proteins is directed towards the cytoplasm of host cells. Considering

the relatively low degree of co-evolution, it is unlikely that the fungus evolved large numbers of effectors that meticulously manipulate a wide array of host targets of the immune system or other components that mediate the physiology of the different host plants, like for instance powdery mildews and rust fungi do (Spanu et al., 2010; Pliego et al., 2013; Petre et al., 2014; Ramachandran et al., 2017). Therefore it is perhaps not surprising that we found that *V. dahliae* strains only need a single to a few effectors to be able to cause disease on particular host plant species. However, given that strains that are compatible on a particular host show marked differences in their aggressiveness (**Chapters 2-5**), it is evident that other effectors that are likely not crucial for pathogenicity contribute to optimal aggressiveness. The catalog of such effectors differs per strain, as illustrated in **Chapter 5**. For example, whereas the expression of Tom1 is imperative for the ability to cause tomato disease (**Chapter 2**), the additional expression of Ave1 in race 1 strains significantly contributes to aggressiveness on this host plant (de Jonge et al., 2012).

Plant hosts try to intercept pathogen invasion through the evolution of diverse kinds of receptors for invasion patterns (Cook et al., 2015). In this manner, also effectors can become invasion patterns once they become recognized by host immune receptors (Cook et al., 2015). Consequently, the more effectors are secreted during host colonization, the higher the chance that one of the effectors gets recognized by a host immune receptor. Thus, an added advantage for *V. dahliae* of utilizing only few effectors for colonization of a single plant host is that the chance of being recognized by a host immune receptor through effector recognition significantly decreases. Obviously, the flip side of the same coin is that dependency on few effectors makes that recognition of such an effector immediately has large consequences. Once effector recognition occurs, it is important for pathogens to employ strategies to overcome this recognition, often through mutation or loss of the recognized effector (Rovenich et al., 2014). However, loss of an effector that is crucial for pathogenicity, or plays a major role in virulence, has the implication that the pathogen is no longer able to infect the host, or is considerably less aggressive, respectively. Indeed, *V. dahliae* strains that have overcome recognition by the Ve1 immune receptor of tomato have lost the major virulence factor Ave1 and are significantly compromised in their aggressiveness (de Jonge et al., 2012). However, given the fact that *V. dahliae* strains generally possess the ability to infect a wide array of host plants, loss of a crucial pathogenicity factor may impact the ability to cause disease on particular hosts, but the ability to colonize other host plants remains. After all, it is unlikely that multiple host plants simultaneously evolve immune receptors to recognize the same effector. Thus, considering the nature of *V. dahliae* as a rather unsophisticated pathogen that has a low degree of adaptation towards individual plant hosts, the utilization of relatively few effectors for host colonization may have considerable evolutionary advantages, as it perhaps facilitates host jumps and the addition of novel hosts to its host range. In this respect it is interesting to remember that *V. dahliae* is a soil-borne pathogen that does not have the capacity to move over large distances, which foliar pathogens can spread spores over large distances to establish new infections on their host plants. Consequently, *V. dahliae* will have to remain quiescent, in its resting stage, until a root from a new host plant is in the vicinity, or establish compatibility on the hosts that happen to be around.

Concluding remarks

Plant pathogens pose great threats to global food security, and understanding the molecular basis of microbial pathogenesis is crucial for developing effective control strategies. It is generally considered that effector catalogues collectively mediate microbial pathogenicity and that disease establishment requires the concerted action of multiple effectors simultaneously. Pathogens that display a high degree of host specialization, which is typically the consequence of a relatively high degree of co-evolution with their host species, typically evolved large numbers of effectors to meticulously manipulate a wide array of host targets of the immune system. This makes sense considering that such pathogens need to guarantee a successful infection once they come in contact with their host, as the failure to infect may usher an evolutionary dead end. However, *V. dahliae*, which has the ability to infect a wide range of hosts and, consequently, shows a low level of adaptation towards these hosts, seems to rely only on a single to few effectors for host colonization. Perhaps this can be explained when considering that the pathogen is an immobile soil-resident that will have to establish compatibility with plant hosts in its immediate micro-environment, and thus flexibility to interact with a multitude of hosts is of benefit. Given this life style, utilization of a single to a few effectors to mediate compatibility on a single host may confer a considerable evolutionary advantage. In this thesis, the D, Tom1, but also Sun1 effectors were identified as important effectors to cause disease on particular host plants. It can be anticipated that these effectors target essential components of the host immune system or other biological processes that enable host infection. Future research focusing on elucidating the mode of action of these effectors and identification of their targets will not only advance our understanding of microbial pathogenesis and of host immunity, but perhaps also identify novel molecular components that can be targeted for improved disease control.

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Summary

Plants have evolved various types of immune receptors to protect themselves against deleterious microbial invasions. In order to establish symbiotic relationships with host plants, microbes secrete effector molecules to suppress host immune responses and facilitate host colonization. Besides pathogenic microbes, endophytes and mutualists are thought to similarly employ effectors to establish their associations with host plants. The research described in this thesis is focussed on the pathogenic broad host range vascular wilt fungus *Verticillium dahliae* and aims to identify critical effectors that contribute to the establishment of infections on host plants. Such knowledge is essential for designing and developing novel and effective Verticillium wilt disease management strategies. As highly versatile molecules, the role of effectors in niche establishment of symbionts with diverse lifestyles is discussed in **Chapter 1**.

On particular host plants, *V. dahliae* strains are assigned to so-called pathotypes. For example, *V. dahliae* strains that are highly aggressive and cause severe defoliation on cotton, olive, okra and pistachio are referred to as strains of the defoliating (D) pathotype, whereas strains that are moderately virulent and only induce wilting symptoms without defoliation on these hosts are assigned to the non-defoliating (ND) pathotype. In **Chapter 2**, comparative genomics of a collection of D and ND pathotype strains revealed two D pathotype-specific effector genes that are highly expressed during host colonization. Intriguingly, these two effector genes appeared to be identical copies that arose by a segmental duplication. We show that the effector (named D) that is encoded by the duplicated effector gene is a pathogenicity factor on cotton and olive, as targeted deletion of the two gene copies resulted in loss of the ability to cause disease symptoms on these plants. Moreover, application of heterologously produced effector protein to cotton seedling induces defoliation, suggesting that the effector protein itself, rather than extensive fungal proliferation in the xylem vessels, is responsible for the defoliation symptoms. Interestingly, besides on cotton and olive, the D effector is required for pathogenicity of D pathotype strains on *Nicotiana benthamiana* and *Arabidopsis thaliana* as well.

V. dahliae is generally characterized as a broad host range pathogen, although differential pathogenicity between individual strains occurs on nearly all hosts. Currently, the molecular basis underlying differences in pathogenicity between *V. dahliae* strains remains largely unknown. **Chapter 3** focuses on elucidating the molecular basis of *V. dahliae* pathogenicity on tomato. While genome analysis of tomato-pathogenic and non-pathogenic *V. dahliae* strains revealed five effector candidate genes that specifically occur in pathogenic strains, only one of them was expressed during host colonization. Functional analyses showed that this *in planta*-induced effector (named Tom1) gene governs pathogenicity of *V. dahliae* on tomato, as deletion of the effector gene resulted in loss of the ability to colonize tomato plants. Furthermore, transfer of this effector gene into a non-pathogenic *V. dahliae* isolate or into non-pathogenic, saprophytic, sister species *V. tricorpus* and *V. nubilum* resulted in tomato disease. Overall, our findings show that a single effector is required and sufficient to mediate pathogenicity of *V. dahliae* on tomato.

Sunflower cultivation regularly suffers from Verticillium wilt. Intriguingly, most *V. dahliae* isolates that we tested fail to cause sunflower disease. In **Chapter 4**, genome comparisons of a single sunflower-pathogenic strain with several non-pathogenic strains revealed that two candidate effector genes that solely occur in the pathogenic strain and are highly expressed during host colonization. Intriguingly, these two candidate effector genes appeared to be identical copies that arose by a segmental duplication. We further show that this duplicated effector (named Sun1) quantitatively contributes to *V. dahliae* virulence on sunflower. While single deletion mutants exhibited markedly reduced virulence on sunflower when compared with wild-type strain 85S, double deletion mutants showed more significantly compromised virulence than single deletion mutants. Interestingly, the *Sun1* effector gene is dispensable for virulence on the model plants *N. benthamiana* or *A. thaliana*. Thus, we anticipate that the Sun1 effector may specifically mediate fungal virulence on sunflower.

Despite the notion that most *V. dahliae* strains have a broad host range, the pathogenic potential and the severity of symptoms that are induced on a particular host plant may vary considerably between *V. dahliae* strains. In **Chapter 5**, we determined the core and lineage-specific (LS) effector repertoires of a collection of *V. dahliae* strains, as well as the ability of these strains to infect a panel of potential host plants comprising tomato, cotton, *N. benthamiana*, Arabidopsis, and sunflower to assess whether the presence of particular LS effectors correlates with the ability to infect particular plant species. Despite the fact that *V. dahliae* is generally considered as a broad host range pathogen, differential pathogenicity occurs among the collection of 21 *V. dahliae* strains on these five plant species. Surprisingly, we found that *V. dahliae* strains that are able to infect the same host plant harbour highly divergent LS effector repertoires and differential *V. dahliae* core effector gene expression between host plants. This variability may reflect immunity evasion in the co-evolution of individual *V. dahliae* strains with multiple hosts.

Plants employ cell surface-localized immune receptors to detect invasions of fungal pathogens, for instance by recognizing the conserved fungal cell wall molecule chitin. In Arabidopsis, two lysin motif (LysM) containing receptors, AtCERK1 and AtLYK5, have been implicated in chitin perception. The LysM effector protein Ecp6 from tomato leaf mould fungus *Cladosporium fulvum* has been shown to supress chitin-induced immune responses through direct competition with plant receptors for chitin binding. In **Chapter 6**, we aimed to generate chimeric chitin receptors with increased chitin binding affinity by replacing the LysM domains of AtCERK1 and AtLYK5 with those of Ecp6. We show that expression of the chimeric receptor Ecp6::LYK5, but not of Ecp6::CERK1, confers responsiveness to fungal chitin. Moreover, the chimeric Ecp6::LYK5 receptor displays enhanced chitin-binding ability when compared with the native AtLYK5 receptor, and can compete with the Ecp6 effector protein for chitin binding. This indicates that the Ecp6::LYK5 chimeric receptor has the potential to mediate enhanced resistance to fungal pathogen infection.

Finally, in **Chapter 7**, the results described in this thesis are summarized and a perspective on how microbes evolve to become plant pathogens is presented.

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金岭

6th January 2019

Wageningen

About the author

Jinling Li (李金岭) was born on March 16th 1987 in Xinjiang, China. In 2006, he started his Bachelor study in Plant Protection at Northwest A&F University in Yangling, Shaanxi Province, China. After graduation in June 2010, he continued his MSc study on biological control of the pathogenic fungus *Alternaria alternata* on tobacco under the supervision Prof. Derong An. In 2013, he obtained a PhD fellowship from the China Scholarship Council (CSC) to join the laboratory of Phytopathology at Wageningen University, the Netherlands. During his PhD studies he focused on the identification of virulence effectors of the vascular wilt pathogen *Verticillium dahliae* under the supervision of Prof. dr. Bart Thomma and Dr. Luigi Faino. After his PhD, he will work as post-doctoral researcher in the group of Dr. Timothy Friesen at the USDA in Fargo, USA, where he will investigate effector biology of the fungal barley pathogen *Pyrenophora teres* f. *teres*.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Jinling Li**
Date: **13 February 2019**
Group: **Laboratory of Phytopathology**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*
▶ **First presentation of your project**	
Identification and characterization of an ECP6 receptor from tomato	26 Sep 2014
▶ **Writing or rewriting a project proposal**	
Functional analysis of virulence effectors from the vascular wilt fungus *Verticillium dahliae* identified through comparative population genomics comparative population genomics	Oct 2013
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
Plant-Microbe Interaction (PHP-30306)	May-Jun 2014
Subtotal Start-Up Phase	10.5 credits*

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD day 2013, Leiden University	29 Nov 2013
EPS PhD student Day, Get2Together 2015, Soest (NL)	29-30 Jan 2015
EPS PhD student Day, Get2Together 2017, Soest (NL)	09-10 Feb 2017
▶ **EPS theme symposia**	
EPS theme 2 symposium & Willie Commelin Scholten day, University of Amsterdam (NL)	25 Jan 2014
EPS theme 2 symposium & Willie Commelin Scholten day, University of Utrecht (NL)	20 Feb 2015
EPS theme 2 symposium & Willie Commelin Scholten day, Leiden University (NL)	22 Jan 2016
EPS theme 2 symposium & Willie Commelin Scholten day, Wageningen University (NL)	23 Jan 2017
▶ **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren (NL)	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren (NL)	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren (NL)	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren (NL)	10-11 Apr 2017
Host-Microbe Genetics meeting, Wageningen (NL)	27 Oct 2017
▶ **Seminars (series), workshops and symposia**	
Seminar: Prof. Jiayang Li, Understanding the molecular mechanisms underlying rice tillering	15 Nov 2013
Seminar: Prof. Eric Schranz, Whole genome duplications as drivers of evolutions and relations?	21 Nov 2013
Seminar: Prof. Jos Raaijmakers, Back to the Roots: exploring and exploiting the plant microbiome	07 Jan 2014
Seminar: Dr. Jeroen Mesters, From protein solution to single crystal X-ray diffraction: Chitin binding by LysM domains at atomic resolution	31 Mar 2014
Seminar: Prof. Jane Parker, Reprogramming cells for defence in plant innate immunity	09 Apr 2014

Seminar: Dr. Frank van Breusegem, Plant metacaspases	09 Apr 2014
Seminar: Prof. Sophien Kamoun, Genome and effector evolution in the Irish potato famine pathogen lineage	28 May 2014
Farewell symposium: Prof. dr. Pierre de Wit: Fungal plant pathogens and the plant immune system, Wageningen (NL)	05 Jun 2014
Seminar: Prof. Yuanchao Wang, Dissecting the interaction between Phytophthora sojae and soybean: making sense out of signalling and effectors	16 Jul 2014
Seminar: Prof. Hanhui Kuang, Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability	11 Sep 2014
Seminar: Dr. Michael Freitag, Chromatin structure controls centromeres and secondary metabolism in filamentous fungi	11 Oct 2014
Seminar: Dr. Ortrun Mittelsten Scheid, Genetics and epigenetics: a complex relationship	11 Nov 2014
Phytopathology Lab. mini-symposium (Erik Limpens, Tijs Ketelaar, Eric Schranz, Frank Takken & Weixing Shan), Wageningen (NL)	24 Nov 2014
EPS symposium: Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding, Wageningen (NL)	11 Dec 2014
WEES seminar: Prof. Kevin Foster, The evolution of cooperation and competition in microbes	22 Jan 2015
EPS flying seminar: Prof.dr. Yves van de Peer, 'The evolutionary significance of gene and genome duplications	03 Feb 2015
Seminar: Prof. Monica Hofte, Towards understanding rice brown spot, a disease induced by physiological stress	06 Feb 2015
Seminar: Prof. Jiming Jiang, Structure and evolution of centromeres: lessions learned from plants	01 Apr 2015
Phytopathology Lab. symposium: Crop Pathology and Plant-Microbe Interactions, Wageningen (NL)	08 May 2015
Seminar: Joel Salatin, It's the food, my friend!	09 May 2015
Plant sciences seminar: Into the Battle between Plants and Viruses, but what about EVEs?	12 May 2015
EPS flying seminar: Prof. Gero Steinberg, Long-distance endosome trafficking drives fungal effector production during plant infection	05 Jun 2015
EPS flying seminar: Prof. Jane Parker, Plant intracellular immunity: evolutionary and molecular underpinnings	21 Jan 2016
Seminar: Dr. Laura Grennvile Briggs, Molecular Oomycete-Host Interaction: The Good, the Bad and the Ugly	19 Feb 2016
Seminar: Dr. Olivier Hamant, How do plants read their own shape?	16 Mar 2016
Seminar, Prof. Alain Tissier, Insights into the inner working of a metabolic cell factory: the tomato glandular trichome	18 Mar 2016
Seminar: Prof. Douglas Mitchell, Genomics-enabled natural products discovery	31 Mar 2016
Seminar: Dr. Pierre-Marc Delaux, Evolution of symbiotic gene networks in land plants	08 Apr 2016
Seminar: Prof. Caitilyn Allen, How Ralstonia solanacearum succeeds in plant xylem vessels	29 Apr 2016
Seminar: Dr. Katherine Goodrich, The volatile "language" of plants: from attraction to deterrence and back again	23 May 2016
Seminar: Prof. Wenbo Ma, Effectors as molecular probes to understand pathogenesis	20 Jun 2016
Mini-symposium: Dr. Edze Westra and Prof. Jennifer Doudna, Rewriting our genes? CRISPR-CAS systems as tools for genome editing	30 Sep 2016
1st WURomics symposium: Technology-Driven Innovation for Plant Breeding, Wageningen (NL)	15 Dec 2016

	Seminar: Dr. Jan Lochman, Elicitins- What we know and do not know	24 Jan 2017
	Farwell symposium: Prof. Ton Bisseling: The Undergrond Labyrinth: Roots, Friends and Foes, Wageningen (NL)	08 Feb 2017
	EPS flying seminar: Dr. Gerben van Ooijen, Clocks across taxa: Conserved cellular timekeeping mechanisms in plants, algae and other eukaryotes	29 May 2017
	EPS flying seminar: Dr. Martin Cann,The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity	11 Jul 2017
	Seminar: Dr. Sanjay Kapoor, Regulators of reproductive development in rice	29 Aug 2017
	Inauguration Lecture: Prof. Gert Kema, Banana has a future after all	21 Sep 2017
	Seminar: Prof. John Werren, Evolution of new gene functions: lateral gene transfers and expression evolution	06 Oct 2017
	Seminar: Dr. Timothy Friesen, Genome wide association as a tool for identifying fungal effectors important in virulence	10 Apr 2018
	Seminar: Dr. Ronald Snijder, Modern domestication of pelargonium in a commercial environment	09 May 2018
	Seminar: Dr. Bob Schmitz, Epigenomic studies of nature and induced Epialleles in plants	06 Jun 2018
	Seminar: Dr. Mary Wildermuth, Salicylic acid and cell cycle control of plant-microbe interactions	25 Jun 2018
	Seminar: Dr. Yan Wang, A leucine-rich repeat receptor-like protein as PAMP receptor recognising XEG1, a Phytophthora glycoside hydrolase 12	10 Sep 2018
	Seminar: Prof. Antonio Di Pietro, Host adaptation in the fungal cross-kingdom pathogen Fusarium oxysporum	17 Oct 2018
▶	**Seminar plus**	
▶	**International symposia and congresses**	
	The 6th European Plant Science Retreat, University of Amsterdam, the Netherlands	01-04 Jul 2014
	COST SUSTAIN workshop:pathogen-informed strategies for sustainable broad-spectrum crop resistance, Bled, Slovenia	01-03 Mar 2017
	The 5th International Conference on Biotic Plant Interactions, Xiamen, China	17-21 Aug 2017
	SIPIS meeting, Rome, Italy	27 Sep 2018
▶	**Presentations**	
	Poster: Identification and characterization of an Ecp6 receptor from tomato, 6th European Plant Science Retreat	01-04 Jul 2014
	Poster: Identification and functional analysis of the *Verticillium dahliae* "defoliation" effector, COST SUSTAIN workshop	01-03 Mar 2017
	Poster: A single *Verticillium dahliae* effector is responsible for cotton infection of the highly aggressive defoliating pathotype, 5th ICBPI	17-21 Aug 2017
	Talk: Identification and characterization of the *Verticillium dahliae* effector that is responsible for cotton defoliation, EPS Theme 2	23 Jan 2017
	Talk: Engineering of a chimeric receptor to mediate improved chitin-triggered immunity in Arabidopsis, SIPIS meeting	27 Sep 2018
▶	**IAB interview**	
▶	**Excursions**	
	Excursion to Enza Zaden, Enkhuizen	12 Jun 2015
	Excursion to Keygene, Wageningen	12 Sep 2017
	Subtotal Scientific Exposure	20.6 credits*

3) In-Depth Studies	date
▶ **EPS courses or other PhD courses**	
EPS Spring School "Host-Microbe Interactomics", Wageningen (NL)	02-04 Jun 2014
Data analyses and visualizations in R (for biologist)	12-13 Dec 2016
▶ **Journal club**	
Member of literature discussion at Verticillium group in Phytopathology Lab	2012-2016
▶ **Individual research training**	
Subtotal In-Depth Studies	4.5 credits*

4) Personal Development	date
▶ **Skill training courses**	
Scientific Writing	04 Sep -30 Oct 2017
Scientific Integrity	27 Aug 2018
Scientific Artwork - Vector graphics & images	02-03 Oct 2018
▶ **Organisation of PhD students day, course or conference**	
Participated in organizing "the Neighbours Week" Phytopathology Lab	20-21 Apr 2015
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	3.6 credits*

TOTAL NUMBER OF CREDIT POINTS*	**39.2**

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.

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