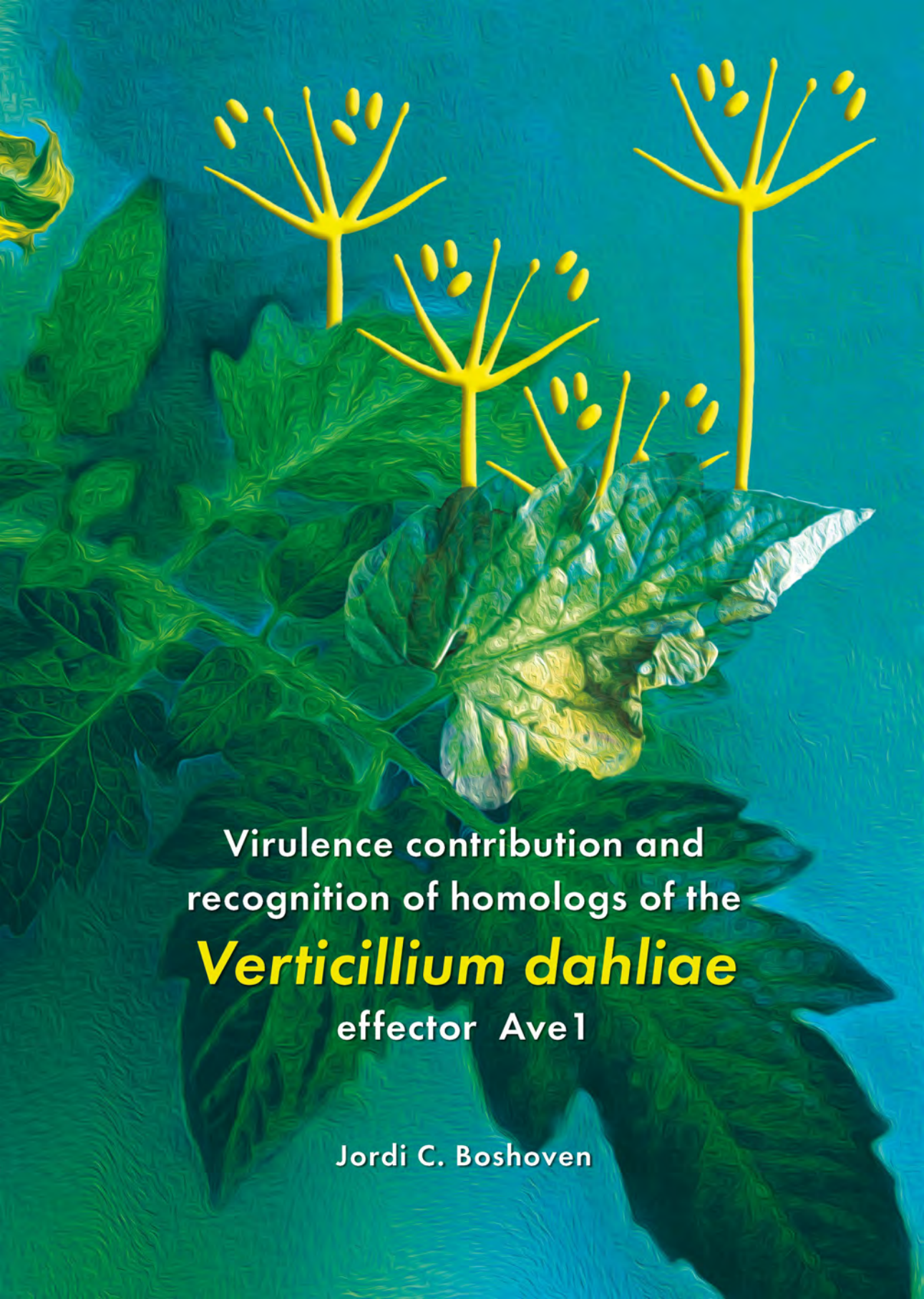

Virulence contribution and recognition of homologs of the *Verticillium dahliae* effector Ave1

Jordi C. Boshoven

Propositions

1. *Verticillium dahliae* effector *Ave1L* has been under selection pressure of an immune receptor.
   (this thesis)

2. Residues at both the N- and C-terminus of the Ave1 effector are required for recognition by tomato immune receptor Ve1.
   (this thesis)

3. Organic agriculture alone cannot feed the future world population.

4. We should be more concerned about the impact of pesticides on the environment, than about growing genetically modified crops that can replace pesticides.

5. Competition between scientists for research grants shows parallels with competition between microorganisms in a microbial community since in both cases occupation of a unique niche increases the chances of success.

6. Open access journals should become the forum of science allowing knowledge to become available to anyone interested.

7. Raising a child during a PhD creates a healthy balance between time spent to work and private life.

8. For a Dutchman it is easier to write a PhD thesis than to learn to speak Greek.

Propositions belonging to the thesis, entitled

Virulence contribution and recognition of homologs of the *Verticillium dahliae* effector Ave1

Jordi C. Boshoven
Wageningen, 10 November 2017

Virulence contribution and recognition of homologs of the *Verticillium dahliae* effector Ave1

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Jordi C. Boshoven

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Contents

To my family

Chapter 1

General introduction

Introduction

Plants provide us with food, feed, fuel, fibre, shelter and drugs and are therefore an important natural resource on which we depend heavily. Since the world population has been, and still is, rapidly growing, the meat and dairy intake has been increasing and also the use of biofuel is rising. Collectively, this still results in an urgent demand for increased crop production. The green revolution that started in the 60s of the 20th century intensified global agricultural practice significantly, often resulting in vast areas of a single crop cultivar known as a monoculture (Jain 2010). Disease outbreaks caused by plant pathogens including fungi, oomycetes, bacteria and viruses can lead to severe yield losses and can even wipe out entire crops, especially when cultivated as monoculture (Oerke 2006; Strange and Scott 2005). To find novel and improved means to combat disease outbreaks, a fundamental understanding of the molecular basis of host-pathogen interactions is essential. Already in the 40s of the 20th century, Harald Flor showed that plants that carry resistance (*R*) genes are resistant to pathogens that carry corresponding avirulence (*Avr*) genes (Flor 1942). Initially, it was thought that R and Avr proteins directly interact (Keen 1990). However, most protein interaction studies failed to confirm this hypothesis, which led to the proposal of the guard hypothesis (Van der Biezen and Jones 1998). This hypothesis states that an R protein guards a plant protein that, when targeted by a pathogen Avr protein, triggers a resistance response. It subsequently became clear that Avr proteins not only initiate resistance responses, but actually act as pathogen virulence factors that manipulate host proteins in absence of host resistance proteins. Therefore, the term effector is more frequently used for Avr proteins nowadays (Boller and Felix 2009). Effectors are considered to be species-, or even race- or strain-specific (Chisholm et al. 2006). Besides sensing effectors or their activities, plants recognize conserved structural components of microorganisms such as bacterial flagellin, fungal chitin and oomycete β-glucans, known as microbe-associated molecular patterns (MAMPs) resulting in MAMP-triggered immunity (MTI) (Dodds and Rathjen 2010). It is generally thought that pathogens evolved effectors to overcome this MTI response. In turn, however, plants evolved receptors to recognize these effectors, resulting in effector-triggered immunity (ETI). These receptors are the earlier discussed R proteins. To avoid recognition by the plant, pathogens have to lose or mutate the recognized effector, or evolve new effectors that can again suppress the immune response. These processes continuously occur in the ongoing arms-race that takes place between plants and pathogens (Chisholm et al. 2006; Jones and Dangl 2006).

About a decade after its introduction, several misconceptions are ubiquitously professed when describing the plant immune system, such as strict separation of MTI versus ETI (Cook et al. 2015; Thomma et al. 2011). Therefore, it was recently proposed that an accurate description of inducible plant immunity should explicitly separate host perception of a given ligand from its physiological or biochemical function. This is captured in the Invasion Model in which host receptors, termed invasion pattern receptors (IPRs), detect ligands that accurately betray pathogen ingress, termed invasion patterns (IPs)(Cook et al. 2015). These IPs can either be an externally encoded or modified-self ligands that indicates invasion. Although any molecule may serve as an IP that will be detected by an IPR, the probability of any given ligand-receptor complex to develop an important role in host immunity increases with increasing ligand molecular constraint to retain function, conservation across organisms, importance in facilitating symbiosis, and accessibility (Cook et al. 2015).

The study system

The genus *Verticillium* includes soil-borne fungal vascular pathogens that are causal agents of Verticillium wilts in a broad range of host plants, including many economically important crops (Agrios 2005), leading to billions of euros of damage annually (Pegg and Brady 2002). We use the species *V. dahliae* as a model system since it has a broad host range and, consequently, can infect model plants such as Arabidopsis, tomato and tobacco. The lifecycle of *V. dahliae* starts with the germination of resting structures, microsclerotia, in the soil after sensing of root exudates. The hyphae that grow from the germinating microsclerotia will progress towards the root and penetrate the root tips (Fitzell et al. 1980; Mol 1995). *V. dahliae* grows through the root cortex into the xylem vessels where it produces conidia that will further spread through the plant (Bishop and Cooper 1983; Fitzell et al. 1980; Garber and Houston 1966). Growth of the fungus in the xylem vessels blocks water transport, which will ultimately lead to wilting and dying of the tissue. Microsclerotia will form on dying tissue, and will eventually end up in the soil after the decomposition of plant material. *V. dahliae* is difficult to control since it has a broad host range and microsclerotia can survive up to 20 years in the soil, which makes crop rotation ineffective. Most fungicides are ineffective as well because they do not reach the fungus which grows protected inside the plant. Although soil fumigation is effective against *V. dahliae* most countries have banned this practice due to its detrimental effects on the environment (Fradin and Thomma 2006).

An effective way to control *V. dahliae* is genetic resistance conferred by tomato immune receptor Ve1 of which homologs are found amongst others in particular tobacco genotypes, mint, hop and cotton (Majer et al. 2014; Vining and Davis 2009; Zhang

et al. 2011; Zhang et al. 2013). Nevertheless, functionality of the *Ve1* homologs was only reported in *Nicotiana glutinosa* and cotton (Zhang et al. 2011; Zhang et al. 2013). Plants carrying *Ve1* are resistant to race 1 strains of *V. dahliae*, but not to race 2 strains (Fradin et al. 2009; Kawchuk et al. 2001). Recently, the effector that is recognized by Ve1 was identified in race 1 strains and named Ave1, for mediating **a**virulence on **Ve1** plants (de Jonge et al. 2012). Besides that *Ave1* deletion strains are no longer recognized by Ve1, they show reduced aggressiveness on tomato plants lacking Ve1, indicating a role in virulence (de Jonge et al. 2012). In addition, *Ave1* deletion was shown to compromise aggressiveness on Arabidopsis as well (de Jonge, van Esse et al. 2012). Remarkably, homologs of Ave1 are mainly found in plants and only in a few microorganisms that all happen to be plant pathogens, including the fungi *Fusarium oxysporum* f. sp. *lycopersici*, *Cercospora beticola*, *Colletotrichum higginsianum* and the bacterium *Xanthomonas axonopodis* (de Jonge et al. 2012). The Ave1 homologs from plant pathogens were coexpressed with Ve1 in *Nicotiana tabacum* to determine whether they can be recognized as well. This experiment showed that homologs from *F. oxysporum* and *C. beticola* are recognized while the homologs from *C. higginsianum* and *X. axonopodis* are not, or only poorly, recognized (de Jonge et al. 2012). Since *F. oxysporum* f. sp. *lycopersici* is a tomato pathogen the fungus was inoculated on transgenic tomato carrying *Ve1*. These *Ve1* tomato plants showed reduced disease symptoms when compared with plants that lack *Ve1*, suggesting that recognition of FoAve1 activates host defense (de Jonge et al. 2012).

The Ave1 homologs that were found in plants are annotated as plant natriuretic peptides or expansin-like proteins that are described to be involved in ion homeostasis, and therefore in many downstream processes including growth, net water uptake, photosynthesis, stomatal opening and gas exchange (Gehring and Irving 2003; Ludidi et al. 2004; Maryani et al. 2001; Pharmawati et al. 2001). Phylogenetic analysis showed that Ave1 homologs found in microbes do not follow phylogeny of the species in which they occur, but rather occur among plant homologs, which is suggestive of horizontal gene transfer. Therefore, our working hypothesis is that *Ave1* homologs found in microbes are acquired from plants through horizontal gene transfer (de Jonge et al. 2012; Gottig et al. 2008). Interestingly, the Ave1 homolog that is found in *X. axonopodis* known as XacPNP is a virulence factor that affects homeostasis and photosynthesis in citrus plants (Garavaglia et al. 2010; Gottig et al. 2008; Nembaware et al. 2004). Since *Ave1* homologs are found in multiple plant pathogens and are likely important for virulence, further studies are needed to gain more understanding of the function of these homologs.

Main objective and research questions

The main objective of this PhD research is to study the *Ave1* homologs derived from *Verticillium* species and other plant pathogens. To this end we addressed the following research questions:

- How are the Ave1 homologs distributed?
- What is the role of Ave1 homologs in virulence in the pathogens that express them?
- What is the function of the Ave1 homologs?
- Do all Ave1 homologs display the same functionality?
- What determines recognition of Ave1 homologs by Ve1?
- How did the Ave1 homologs evolve?

Thesis outline

In **Chapter 2**, a complete overview of the biological function of fungal effectors is provided. Moreover, the most recent views on various manners how fungal effectors contribute to disease establishment is provided. Furthermore we discuss potential roles of fungal effectors outside host-pathogen interactions.

In **Chapter 3**, we studied the *Ave1* homologs of three fungal pathogens: *F. oxysporum* f. sp. *lycopersici*, *C. beticola* and *C. higginsianum* that infect tomato, sugarbeet and Arabidopsis, respectively. We determine whether the *Ave1* homologs are expressed by the respective fungi during host colonization and whether these genes play a role in virulence.

In **Chapter 4**, the recognition of *V. dahliae* Ave1 by the tomato Ve1 immune receptor is studied. Based on comparisons of Ave1 homologs from various species and functional assays with truncated proteins and domain swaps, attempts are made to determine the region of Ave1 that is recognized by Ve1.

In **Chapter 5**, the expanded *Ave1* gene family that is found in the genomes of the apple and pear scab pathogens *Venturia ineaqualis* and *V. pirina* is described. RNAseq of *V. ineaqualis* revealed that some of the paralogs are induced *in planta*. It is studied whether the induced Ave1 homologs are recognized by Ve1 and whether they may perform the same biological function as *V. dahliae* Ave1.

In **Chapter 6**, we describe the occurrence of an *Ave1-like* gene that is found in many *V. dahliae* strains. In our analysis, we identified five different alleles of this gene. We determine whether the various *Ave1-like* gene alleles are expressed during host colonization, and whether Ve1 or its close homolog Ve2 is able to recognize these.

In **Chapter 7**, a random mutagenesis was performed in *V. dahliae* to identify novel virulence and pathogenicity factors. One of the virulence factors that was identified is further characterized.

Chapter 8 is the general discussion and describes how the knowledge of pathogen effectors can be used as a tool to establish durable broad spectrum resistance.

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Chapter 2

Filamentous Pathogen Effector Functions: Of Pathogens, Hosts and Microbiomes

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Abstract

Microorganisms play essential roles in almost every environment on earth. For instance, microbes decompose organic material, or establish symbiotic relationships that range from pathogenic to mutualistic. Symbiotic relationships have been particularly well studied for microbial plant pathogens and have emphasized the role of effectors; secreted molecules that support host colonization. Most effectors characterized thus far play roles in deregulation of host immunity. Arguably, however, pathogens not only deal with immune responses during host colonization, but also encounter other microbes including competitors, (myco)parasites and even potential co-operators. Thus, part of the effector catalog may target microbiome co-inhabitants rather than host physiology.

Introduction

During early microbial colonization stages, plant cell surface-localized pattern recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs), such as fungal chitin, to activate immune responses (Dodds and Rathjen 2010; Thomma et al. 2011). In order to establish themselves, adapted pathogens secrete effector molecules that deregulate immune responses and facilitate host colonization. Simultaneously, hosts evolve effector recognition by novel receptors that reinstall immunity (Dodds and Rathjen 2010; Thomma et al. 2011). Consequently, effectors are subject to various selective forces that drive their evolution, leading to diversified effector repertoires between pathogen lineages. Functional characterization of effectors and determination of their contribution to the microbial lifestyle provides insight in relevant processes for host colonization.

Plant pathogen effectors deregulate host immunity in various subcellular compartments

Many pathogens initially enter the plant apoplast, which contains enzymes that hamper microbial colonization. For example, chitinases target fungal cell walls to release chitin fragments that activate immune receptors, leading to further chitinase accumulation to induce hyphal lysis. In turn, fungal pathogens secrete chitin-binding effectors to protect their cell walls and interfere with immune receptor activation (de Jonge et al. 2010; Kombrink et al. 2011; Marshall et al. 2011; Mentlak et al. 2012). The LysM domain-containing Ecp6 effector of the leaf mold fungus *Cladosporium fulvum* can outcompete host receptors through chitin binding with unprecedented ultrahigh (pM) affinity by intramolecular LysM domain dimerization (Sanchez-Vallet et al. 2013). Additionally, LysM effectors likely interfere with receptor dimerization that is required to activate immune signaling (Hayafune et al. 2014; Liu et al. 2012; Sanchez-Vallet et al. 2013).

Although effectors that directly target chitinases have not yet been identified, some effectors target other apoplastic hydrolytic enzymes, such as proteases. For example, sequence-unrelated effectors of *C. fulvum*, the oomycete *Phytophthora infestans*, and the parasitic nematode *Globodera rostochiensis* inhibit tomato cysteine proteases including Rcr3 (Lozano-Torres et al. 2012; Song et al. 2009; van Esse et al. 2008). The closely related oomycetes *P. infestans* and *P. mirabilis* express an orthologous pair of host protease inhibitor effectors that are subject to positive selection, which was implicated in adaptation to unique protease targets in their respective host plants (Dong et al. 2014). Besides protease inhibitors, *P. infestans* secretes the Avrblb2 effector that interferes with protease secretion (Bozkurt et al.

2011). The smut fungus *Ustilago maydis* inhibits apoplastic proteases via multiple effectors. While Pit2 directly inhibits cysteine proteases (Mueller et al. 2013), Pep1 induces the maize cystatin CC9 that inhibits apoplastic proteases in turn (van der Linde et al. 2012). Pep1 furthermore inhibits the maize peroxidase POX12 to perturb reactive oxygen species balances (Hemetsberger et al. 2012). Thus, the plant apoplast is a dynamic battlefield for plant pathogens.

In addition to apoplastic effectors, many pathogens deliver effectors that act inside host cells, although mechanisms that govern their uptake remain controversial (Petre and Kamoun 2014). The rice blast fungus *Magnaporthe oryzae* was shown to secrete various effectors that enter rice cells, and even move to non-infected neighboring cells, presumably to prepare these for infection (Khang et al. 2010). The AvrPiz-t effector targets proteasome activity through interaction with the RING E3 ubiquitin ligase APIP6, leading to their mutual degradation and suppression of PRR-mediated immunity (Park et al. 2012). Effector diffusion from infected cells into neighboring cells was similarly observed for the *U. maydis* chorismate mutase Cmu1 that targets the shikimate pathway to channel chorismate into the phenylpropanoid pathway, thus adversely affecting salicylic acid (SA) biosynthesis (Djamei et al. 2011). *U. maydis* furthermore secretes the Tin2 effector to stabilize the maize ZmTTK1 kinase that controls anthocyanin biosynthesis, possibly to suppress tissue lignification (Tanaka et al. 2014). Also the oomycete *Hyaloperonospora arabidopsidis* targets SA signaling by secreting a nuclear-localized effector that interacts with the mediator complex that controls interactions between transcriptional regulators and RNA polymerase (Caillaud et al. 2013). Host transcription is furthermore perturbed by effectors that inhibit transcription factor translocation to the nucleus (McLellan et al. 2013). Additionally, nuclear-localized effectors may affect host immunity post-transcriptionally by suppressing the biogenesis of small RNAs in the host (Qiao et al. 2013). Interestingly, *Botrytis cinerea* was recently suggested to deliver even small RNAs into host cells to affect immune responses (Weiberg et al. 2013).

Finally, several effectors target host cell death mechanisms, such as *P. infestans* Avr3a and PexRD2. While Avr3a suppresses INF1-triggered cell death by stabilizing the U-box E3 ligase CMPG1 during biotrophic growth, PexRD2 targets the kinase domain of the cell death regulator MAPKKKε (Bos et al. 2010; King et al. 2014). During later stages of infection, however, *P. infestans* relies on induction of host cell death as it switches to a necrotrophic lifestyle. Necrotrophic pathogens evolved effectors that actually induce cell death. An elegant example is provided by the *Cochliobolus victoriae* effector victorin that binds to thioredoxins including TRXh5, which is required for redox control of the transcriptional immune regulator NPR1. TRXh5 binding activates the NB-LRR-type immune receptor LOV1, facilitating necrotrophic exploitation of host cell death by *C. victoriae* (Lorang et al. 2012).

In conclusion, although information for the vast majority of pathogen effectors, particularly of filamentous pathogens, is still lacking, effector molecules are highly versatile. Clearly, recently uncovered functions revealed that virulence effectors, despite the finding that they converge onto pivotal elements of the plant immune system (Mukhtar et al. 2011), can deregulate any step of immunity in any cellular compartment (Figure 1, Supplemental table 1).

Endophytes and mutualists use effectors to suppress host immunity too

Like pathogens, commensalistic endophytes and mutualists develop intimate host plant associations. During initiation of such symbioses, PRRs continue to perceive MAMPs. Consequently, similar to pathogens, endophytes and mutualists are recipients of immune responses. However, the precise role and fate of host immunity in the establishment of symbiosis has remained enigmatic.

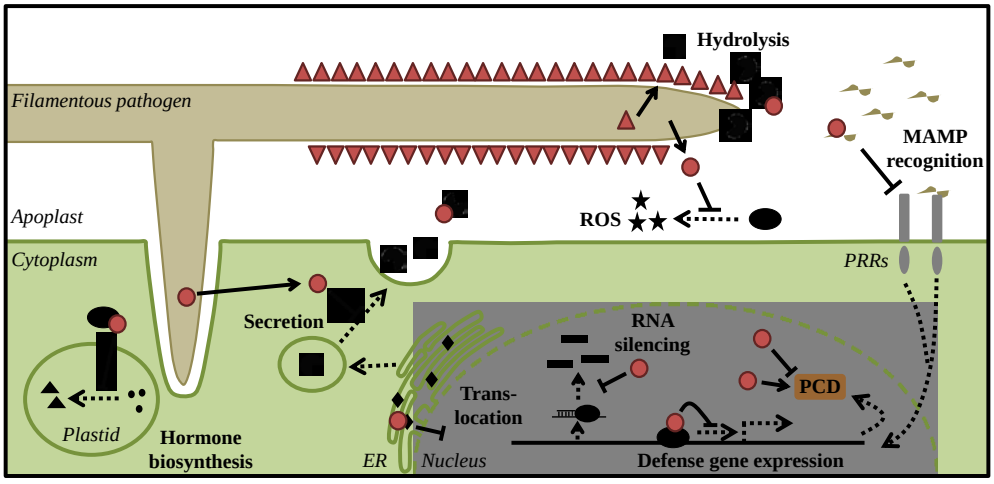

FIGURE 1 | Filamentous pathogen effectors deregulate host immunity in various host subcellular compartments. Pathogens secrete effectors (red symbols) to deregulate plant immunity (see text for details). Whereas one group of effectors (red circles) interacts with host targets that act in immunity (black shapes), another group of effectors (red triangles) acts in self-defense to protect the pathogen from host-derived antimicrobials.

The root endophyte *Piriformospora indica* has a wide host range and induces enhanced growth and stress resistance in colonized hosts. Rather than evading host detection, the fungus actively suppresses immunity (Jacobs et al. 2011). During early biotrophic growth at the onset of symbiosis, about 10% of the transcriptome encodes putative effector proteins (Zuccaro et al. 2011). At later growth stages

the fungus requires host cell death for further colonization, thus resembling hemibiotrophic pathogens such as *Mycosphaerella graminicola* and *M. oryzae*. Like *C. fulvum*, these latter species utilize LysM effectors to suppress immune responses (de Jonge et al. 2010; Marshall et al. 2011; Mentlak et al. 2012). *P. indica* carries an expanded LysM domain-containing effector repertoire that may similarly act in immune suppression (Zuccaro et al. 2011).

Effector-like proteins are also encoded by genomes of other mutualists (Martin et al. 2010; Plett et al. 2011; Plett and Martin 2011). The ectomycorrhiza *Laccaria bicolor* genome encodes hundreds of small secreted proteins, several of which are only expressed in symbiotic tissues. Of these, MiSSP7 was shown to translocate to the nucleus of poplar host cells to stabilize the JAZ6 protein and repress jasmonate signaling (Plett et al. 2014; Plett et al. 2011). Likewise, the ectomycorrhiza *Tuber melanosporum* expresses 125 cysteine-rich small secreted proteins, including a LysM effector, which are highly upregulated during symbiosis (Martin et al. 2010).

It was recently shown that arbuscular endomycorrhizal fungi produce lipochitooligosaccharide mycorrhizal (Myc) factors that stimulate root growth and branching to initiate symbiosis (Maillet et al. 2011). Similar to endophytes and ectomycorrhiza, arbuscular endomycorrhiza secrete effector-like proteins during symbiotic interactions (Kloppholz et al. 2011; Lin et al. 2014; Tisserant et al. 2013). The genome of *Rhizophagus irregularis* encodes a family of CRN-like proteins that are abundantly found in plant pathogenic *Phytophthora* spp. (Lin et al. 2014). *R. irregularis* was furthermore found to encode an effector that interacts with the pathogenesis-related ethylene-responsive transcription factor 19 (ERF19) in the host nucleus to promote mycorrhization, potentially by counteracting MAMP-induced host defense responses that are regulated by ERF19 (Kloppholz et al. 2011).

Collectively, these findings suggest that symbiotic associations that include endophytism, mutualism and parasitism form a continuum in which effectors play essential roles (Supplemental table 1).

Effectors act in self-defense and competition

The ability to establish symbiosis evolved multiple times in microbes, presumably from saprotrophism, and many plant pathogens still display saprotrophic life stages. Saprotrophs generally reside within the soil where they feed on decaying organic matter in the presence of a rich microbiota. In this environment, microbial competition as well as co-operation occurs (Figure 2). Threats are posed by (myco) parasites and competitors that produce antibiotics with specific or broad-spectrum activities. Consequently, microbes require molecules for self-defense and interaction with other microbiome partners.

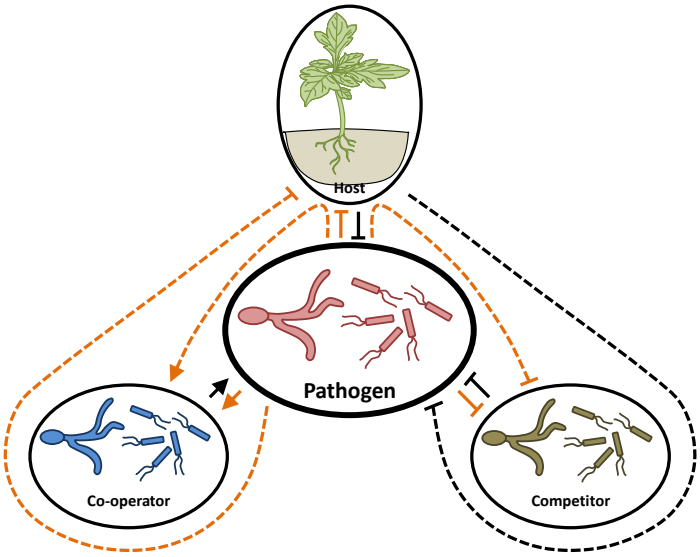

FIGURE 2 | How pathogens influence the local biota by exploiting effector activities. The interaction between microbial pathogens and plant hosts occurs in environments that contain additional microbiome partners that can negatively (competition) or positively (co-operation) impact the pathogen as well as the host. Consequently, the pathogen and host may target each other directly (solid lines) as well as indirectly (dotted lines). Likely, pathogens exploit effector activities (orange lines) to not only directly modulate their hosts, but also to influence the local microbiota that can impact the outcome of the interaction with their hosts.

Similar to infected plants, many mycoparasites secrete hydrolytic enzymes including proteases, chitinases and glucanases to target fungal cell walls. Presumably, chitin-binding effectors that protect hyphal cell walls against plant-derived chitinases similarly protect against mycoparasite-derived chitinases, which may explain abundant LysM effector catalogs of non-pathogenic fungi (Kombrink and Thomma 2013; Kubicek et al. 2011). As LysM domains occur in peptidoglycan-binding proteins of various origins, LysM effector homologs that bind non-chitin substrates likely occur. Indeed, a plant pathogen LysM effector that binds bacterial cell walls was characterized (Kombrink and Thomma, unpublished data), potentially implicating this effector in bacterial competition or protection against bacterial mycoparasites. Genome analyses furthermore revealed that saprotrophic species encode abundant catalogs of small secreted proteins that resemble pathogen effector catalogs (Druzhinina et al. 2012; Kasuga et al. 2009; Kombrink and Thomma 2013; Suh et al. 2012). Although these potential effectors are poorly studied, one such effector, CipC, was implicated in competition with bacteria in *Aspergillus* spp. (Melin et al. 2002; Suh et al. 2012). The genome of the ubiquitous saprophyte and opportunistic mammalian pathogen *A. fumigatus* encodes several effector proteins (Wartenberg

et al. 2011). However, since the vast majority of fungi that cause disease in animals are soil saprophytes that opportunistically infect their hosts, to which they are not highly adapted, it has been speculated that infection does not rely on the activity of effectors (Lowe and Howlett 2012). Rather, their effectors are thought to be required for saprophytic survival (Lowe and Howlett 2012). Nevertheless, effectors that evolved to enable saprophytic survival may be co-opted for opportunistic infection as well.

Likely, competition between plant-associated microbes also occurs within hosts, although perhaps to a lesser extent than in soil due to reduced species diversity. Indeed, the second most abundantly *in planta*-expressed gene of the fungal endophyte *Epichloë festucae* encodes a secreted antifungal protein (Ambrose and Belanger 2012). Thus, effector homologs may play crucial roles in microbial competition in a broad spectrum of environments.

Do pathogens shape local microbiomes?

For various types of multicellular organisms it is increasingly recognized that their microbiome, i.e. the community of microbes that thrives in, on, or immediately near the organism, greatly influences its performance (Ezenwa et al. 2012). For plants, it has been particularly well documented that the rhizosphere microbiota affects plant growth and stress tolerance. In addition, the importance of the phyllosphere microbiota is increasingly recognized (Vorholt 2012). These microbiota comprise members that provide direct as well as indirect pathogen protection through antibiosis and induced immunity, respectively. Whereas soil types have a major impact on root inhabiting bacterial community compositions on Arabidopsis, host genotypes were reported to only have a minor impact (Bulgarelli et al. 2012; Lundberg et al. 2012). In contrast, different Arabidopsis accessions were found to harbor different phyllosphere communities and several host genetic mutations were found to perturb the microbiota composition, demonstrating that host genetic factors shape the associated microbiota (Bodenhausen et al. 2014). It is less clear, however, whether plants evolved to actively recruit phyllosphere communities. Potentially, plants recruit founder species that further shape local microbiomes through inter-microbe interactions (Vorholt 2012). Such interactions may require effectors. Considering that plant factors control the composition of the microbiota, microbiome members may utilize effectors to modulate hosts and control competitors indirectly. Additionally, manipulation of host metabolism could even establish microbial cooperation (Figure 2). Although not immediately addressing inter-microbial interactions, an insect-transmitted phytoplasma was recently shown to utilize an effector to alter floral development of host plants, converting them

into vegetative tissues that attract leafhopper vectors (MacLean et al. 2014). This represents a striking example of the exploitation of effector activity to influence compositions of the local biome. Similarly, the rust fungus *Puccinia monoica* induces floral mimicry in the host *Boechera stricta* to enhance its reproduction and spore dispersal by insects (Cano et al. 2013).

Considering the importance of the microbiome for the ability of plants to withstand pathogen infection, it is conceivable that pathogens evolved to affect host microbiomes, possibly through effector activities (Figure 2).

Different mechanisms drive evolution of effector repertoires

Mechanisms underlying genome plasticity and evolution have been intensely studied, especially for plant pathogens. As genomes are structured and not just a random sequence of genes, effector genes are often found in dynamic genomic compartments, such as gene-sparse regions, subtelomeric regions or conditionally dispensable (pathogenicity) chromosomes (Raffaele and Kamoun 2012). For example, effector localization in gene-sparse regions was recorded for the endophyte *P. indica* (Zuccaro et al. 2011), while in the saprophyte *N. crassa* genes encoding small secreted proteins are found in subtelomeric regions (Kasuga et al. 2009). Genetic plasticity in such compartments is governed by diverse mechanisms including recombination and activity of transposable elements. A direct implication of genomic rearrangement in the evolution of fungal aggressiveness was shown for the vascular wilt fungus *Verticillium dahliae*, leading to the emergence of lineage-specific regions that are enriched for virulence effectors (de Jonge et al. 2013). High genetic variability in effector genes enables rapid evolutionary processes. The importance of dynamic genome compartments for accelerated gene evolution was underlined in the specialization of *P. infestans* after the host jump that separated this species from related species. Uneven evolutionary rates across the genome occur, with *in planta*-induced genes residing in fast-evolving compartments (Raffaele et al. 2010). In turn, effector specialization can lead to diversification and speciation in pathogen lineages (Dong et al. 2014). In this manner, effectors can determine microbial niches. Moreover, composition of effector catalogs can dictate microbial lifestyles. For example, the leaf epiphyte and antagonist of powdery mildews *Pseudozyme flocculosa* lost its ability to parasitize plants like its smut fungi relatives due to loss of virulence effectors (Lefebvre et al. 2013). However, the biocontrol agent has acquired other effectors that are not found in the smut relatives that may have shaped its current lifestyle (Lefebvre et al. 2013). These findings suggest that effector catalogs evolve via different mechanisms and that their composition influences a microbe's lifestyle in a given environment.

Experimental way forward

The interaction between pathogenic (filamentous) microbes and the organisms they encounter in their niches, either while colonizing the host or during free-living stages in the environment, is poorly understood. An extensive characterization of the complex microbial communities in such niches may lead to a better understanding of the interactions that take place beyond the direct interaction between pathogen and host. Detailed transcriptome analyses may lead to the identification of particular triggers of effector gene expression derived from microbial co-inhabitants, and may hint towards functions in inter-microbial interactions (Mathioni et al. 2013; Mela et al. 2011) that can subsequently be tested for in targeted analysis to reveal components that either promote or inhibit other microbes (Kombrink and Thomma 2013).

Conclusions

Although a paradigm in plant pathology dictates that existence of disease requires the interaction of a virulent pathogen with a susceptible host in a favorable environment, plant-microbe interactions are mostly studied as one-on-one relationships. However, in addition to host immune responses, pathogenic microbes continuously encounter other microbes that include competitors and mycoparasites that need to be dealt with simultaneously. Importantly, findings for pathogenic microbes can be extrapolated to other types of symbioses as well. After all, irrespective of the type of symbiosis, the interest of the microbial partner is merely to exploit the host for nutrition and shelter. This may also explain the thin line that is regularly observed between the different types of symbioses (Delaye et al. 2013; Malcolm et al. 2013; Plett and Martin 2011; Zuccaro et al. 2011). In all types of symbioses, the microbial partner needs to suppress host immune responses and ward off microbial antagonists. Using effectors as probes, further critical processes in host colonization will be uncovered, leading to enhanced understanding of the biology of microbes that aim to establish symbioses.

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Supplemental data

SUPPLEMENTAL TABLE 1 | Effectors of filamentous plant-associated microbes for which molecular virulence targets were identified

Effector	Origin	Target	Function	Reference
BEC4	*Blumeria graminis* f.sp. *hordei*	ARF-GAP proteins	Interference with host vesicle trafficking	(Schmidt et al. 2014)
Avr2	*Cladosporium fulvum*	Cysteine proteases	Cysteine protease inhibition	(Shabab et al. 2008; van Esse et al. 2008)
Avr4	*Cladosporium fulvum*	Chitin	Hyphal protection	(van den Burg et al. 2006)
Ecp6	*Cladosporium fulvum*	Chitin	Perturbation chitin-triggered immunity	(de Jonge et al. 2010)
CfTom1	*Cladosporium fulvum*	*α*-tomatine	Detoxification	(Okmen et al. 2013)
Victorin	*Cochliobolus victoria*	TRX-h5	Induction of LOV1-mediated cell death	(Lorang et al. 2012)
SP7	*Rhizophagus irregularis*	ERF19	Deregulation of host gene expression	(Kloppholz et al. 2011)
HaRxL44	*Hyaloperonospora arabidopsidis*	MED19a	Interference with SA-triggered immunity	(Caillaud et al. 2013)
MiSSP7	*Laccaria bicolor*	JAZ6	Deregulation of host gene expression	(Plett et al. 2014)
AvrPiz-t	*Magnaporthe oryzae*	RING E3 ubiquitin ligase APIP6	Suppression of MAMP-triggered immunity	(Park et al. 2012)
Slp1	*Magnaporthe oryzae*	Chitin	Perturbation chitin-triggered immunity	(Mentlak et al. 2012)
MfAvr4	*Mycosphaerella fijiensis*	Chitin	Hyphal protection	(Stergiopoulos et al. 2010)
Mg1LysM	*Mycosphaerella graminicola*	Chitin	Hyphal protection	(Marshall et al. 2011)
Mg3LysM	*Mycosphaerella graminicola*	Chitin	Perturbation chitin-triggered immunity	(Marshall et al. 2011)
Avr3a	*Phytophthora infestans*	CMPG1	E3 ligase stabilization	(Bos et al. 2010)
Avrblb2	*Phytophthora infestans*	C14 protease	Suppression of protease secretion	(Bozkurt et al. 2011)
EPI1	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	(Tian et al. 2004)
EPI10	*Phytophthora infestans*	Serine proteases	Inhibition of serine proteases	(Tian et al. 2005)
EPIC1	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	(Song et al. 2009; Tian et al. 2007)
EPIC2B	*Phytophthora infestans*	Cysteine proteases	Inhibition of cysteine proteases	(Song et al. 2009; Tian et al. 2007)
PexRD2	*Phytophtora infestans*	MAPKKK*ε*	Suppression of host cell death	(King et al. 2014)
Pi03192	*Phytophthora infestans*	NTP1, NTP2	Suppression of transcription factor relocation	(McLellan et al. 2013)
GIP1	*Phytophthora sojae*	*β*-1,3-glucanases	Glucanase inhibition	(Rose et al. 2002)
RTP1p	*Uromyces fabae/U. striatus*	Proteases	Protease inhibition	(Pretsch et al. 2013)
Cmu1	*Ustilago maydis*	Cm2	Interference with SA biosynthesis	(Djamei et al. 2011)
Pep1	*Ustilago maydis*	POX12	Inhibition of peroxidase-mediated ROS production	(Hemetsberger et al. 2012)
Pit2	*Ustilago maydis*	CP2, CP1A/B, XCP2 proteases	Cysteine protease inhibition	(Mueller et al. 2013)
Tin2	*Ustilago maydis*	TmTTK1	Control of anthocyanin biosynthesis	(Tanaka et al. 2014)

Chapter 3

Homologs of *Verticillium dahliae* effector Ave1 contribute to virulence of fungal pathogens of diverse plant hosts

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Abstract

Verticillium dahliae is a soil-borne fungal pathogen with a wide host range including many crops. Tomato immune receptor Ve1 confers resistance to *V. dahliae* race 1 strains that express VdAve1. On plants that do not carry Ve1, Ave1 acts as a virulence factor of *V. dahliae*. Homologs of Ave1 are mostly found in plants and in a handful of fungal plant pathogens including *Fusarium oxysporum* (*FoAve1*), *Cercospora beticola* (*CbAve1*), and *Colletotrichum higginsianum* (*ChAve1*). In the bacterium *Xanthomonas axonopodis* the Ave1 homolog XacPNP was previously described as a virulence factor. The homologs FoAve1, CbAve1 are recognized by Ve1 although not as efficient as VdAve1. In this study, we analysed the role of the individual Ave1 homologs in virulence. We generated targeted gene deletions in *Fusarium oxysporum*, *Cercospora beticola* and *Colletotrichum higginsianum* and showed that both *FoAve1* and *CbAve1* are virulence factors in contrast to *ChAve1* that does not contribute to the virulence of the pathogen. We subsequently tested whether *Ave1* homologs *FoAve1*, *CbAve1*, *ChAve1* and *XacPNP* act in a similar fashion as *VdAve1* by expressing these genes in a *V. dahliae VdAve1* deletion strain. We concluded that VdAve1 on the one hand and FoAve1, CbAve1, ChAve1 and XacPNP on the other hand act in different manners.

Introduction

Plants are surrounded by microbes including oomycetes, fungi, bacteria and viruses. To prevent colonization by harmful microbes, plants evolved pattern recognition receptors (PRRs) that recognize microbe associated molecular patterns (MAMPs) and activate MAMP-triggered immunity (MTI) (Boller and Felix 2009; Dodds and Rathjen 2010). MAMPs generally are structural components such as bacterial elongation factor Tu (EF-Tu), flagellin, lipopolysaccharides (LPS), fungal chitin and oomycete *β*-glucans, which are typically conserved across genera of microbes. Successful pathogens secrete effector proteins that modulate host immunity to overcome PRR-mediated plant defence (de Jonge et al. 2012; Rovenich et al. 2014). Such effectors are often lineage-specific, facilitate colonization of the plant, and therefore contribute to virulence of the pathogen. In turn, plants evolved immune receptors, typically called resistance (R) proteins, which recognize particular effectors or their activities to mount an immune response known as effector-triggered immunity (ETI) (Jones and Dangl 2006). To overcome recognition by the plant, pathogens have to mutate or loose the recognized effector or, alternatively, evolve new effectors to suppress the ETI response. Nevertheless, not all pathogen-secreted molecules follow the strict MAMP-effector dichotomy but rather display traits of either class of molecules, and therefore MTI and ETI responses cannot strictly be separated (Thomma et al. 2011). Typical examples of such pathogen-secreted molecules are Nep1-like proteins, which are virulence factors that are dispersed throughout three kingdoms of life that act as a MAMP at least in Arabidopsis (Böhm et al. 2014; Oome et al. 2014). Although less widely distributed, phenomena have been observed for homologs of the Avr4 effector from the tomato leaf mould fungus *Cladosporium fulvum* (Stergiopoulos et al. 2010), and for homologs of the Ave1 effector from the vascular wilt fungus *Verticillium dahliae* (de Jonge et al. 2012). This has inspired the proposal of the so-called invasion model, which states that plants evolved receptors for all types of molecules, pathogen- as well as host-derived, that can betray microbial invasion to mount the appropriate immune responses (Cook et al. 2015).

V. dahliae is a soil-borne fungal pathogen that causes vascular wilt in over 200 plant species, including important crop species (Fradin and Thomma 2006). Resistance to *V. dahliae* is mediated by the immune receptor Ve1 (Fradin et al. 2009; Kawchuk et al. 2001), of which homologs were found in mint, hop and cotton amongst other plant species (Majer et al. 2014; Vining and Davis 2009; Zhang et al. 2011). Recently, the effector protein that is secreted by *V. dahliae* race 1 strains and that activates Ve1-mediated resistance was identified as Ave1, and all resistance-breaking race 2 strains analysed thus far lack the complete *Ave1* gene (de Jonge et al. 2012; Faino et al.

2016). Importantly, *Ave1* deletion strains show reduced aggressiveness on tomato plants that lack *Ve1*, revealing that *Ave1* acts as a virulence factor on this host species. Additionally, Ave1 was also shown to act as a virulence factor of *V. dahliae* on Arabidopsis plants (de Jonge et al. 2012). However, how Ave1 contributes to *V. dahliae* virulence on these plant hosts remains unknown thus far.

Intriguingly, although pathogen effectors are typically lineage-specific traits, many homologs of Ave1 can be found in public databases (de Jonge et al. 2012). Remarkably, a wealth of *Ave1* homologs was found in plants as well as in a handful of fungal plant pathogens, including *Fusarium oxysporum* (*FoAve1*), *Cercospora beticola* (*CbAve1*) and *Colletotrichum higginsianum* (*ChAve1*), and in the bacterial plant pathogen *Xanthomonas axonopodis* (*XacPNP*) (de Jonge et al. 2012; Gottig et al. 2008). Interestingly, it was shown that Ve1 is not confined to *V. dahliae* Ave1 (VdAve1), as Ve1 is able to also recognize FoAve1 and CbAve1, while ChAve1 is not recognized (de Jonge et al. 2012). Consequently, Ve1 was indeed found to be able to recognize *F. oxysporum* in tomato and mount a defense response (de Jonge et al. 2012). Remarkably, Ve1 was furthermore found to recognize an endogenous Ave1 homolog (SlAve1) that shares a high degree of identity with VdAve1 (de Jonge et al. 2012).

Most plant homologs of Ave1 have been annotated either as plant natriuretic peptides (PNPs) or as expansin-like proteins, and functionally analyzed members were implicated in the regulation of water and ion homeostasis, and consequently in many downstream processes including growth, net water uptake, photosynthesis, stomatal opening and gas exchange (Gehring and Irving 2003; Ludidi et al. 2004; Maryani et al. 2001; Pharmawati et al. 2001). The observation that the microbial-derived Ave1 homologs do not follow the phylogeny of the species in which they occur is generally taken as evidence for horizontal acquisition, and it has thus been speculated that the microbial Ave1 homologs were acquired from plants (de Jonge et al. 2012; Gottig et al. 2008). In this respect it is interesting that the *X. axonopodis* homolog XacPNP affects homeostasis and photosynthesis in citrus plants, and thus promotes bacterial proliferation as a virulence factor in citrus plants (Garavaglia et al. 2010; Gottig et al. 2008; Nembaware et al. 2004). In this study, we investigate whether the Ave1 homologs of several fungal pathogens act as genuine virulence factors.

Results

Characterization of Ve1-mediated tomato defence against *Fusarium oxysporum*

We previously demonstrated that tomato Ve1 can recognize FoAve1, leading to a defence response that affects infection of *Ve1*-carrying tomato by *F. oxysporum* (de Jonge et al. 2012). Because recognition of FoAve1 by *Ve1*-carrying tomato plants does not lead to full immunity against *F. oxysporum* we investigated the occurrence of expression of *FoAve1* in two *F. oxysporum* strains, Bt.01 and Fol4287, in tomato plants lacking *Ve1* with reverse-transcription PCR at 14 dpi. Clear expression of *FoAve1* was monitored at 14 dpi (Figure 1A).

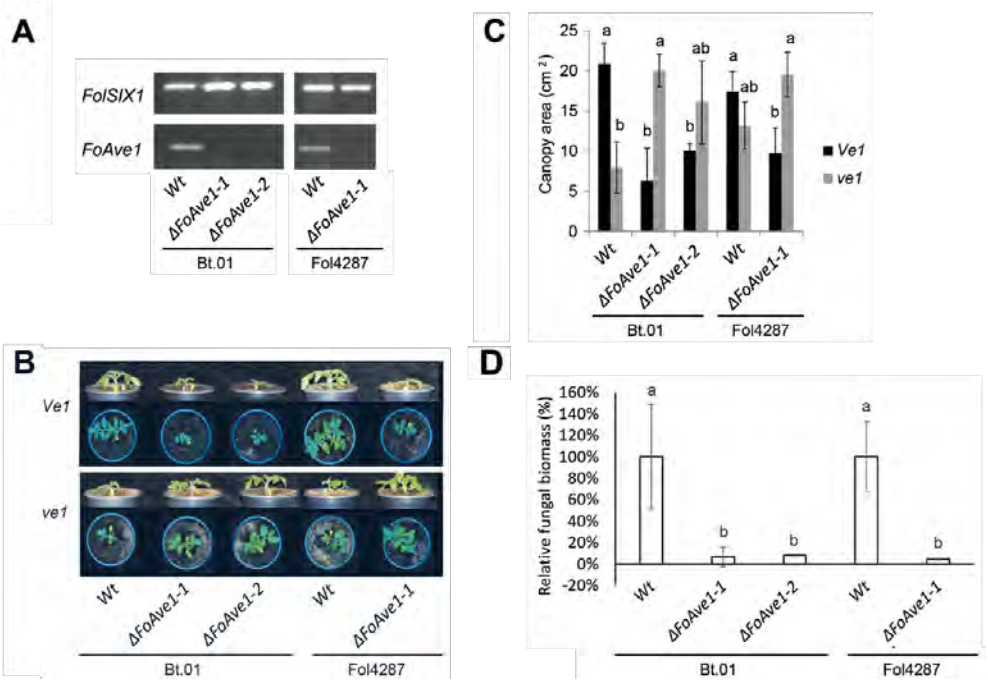

FIGURE 1 | FoAve1 is a virulence factor recognized by tomato Ve1. (A) Expression of *FoAve1* in *F. oxysporum* in wild type (Wt) strains Bt.01 and Fol4287 and the FoAve1 deletion strains (Δ*FoAve1*) inoculated on tomato at 14 dpi. (B) (Upper) *FoAve1* deletion strains (Δ*FoAve1*) of *F. oxysporum* strains Bt.01 and Fol4287 escape recognition by tomato Ve1 compared with the corresponding wild type (Wt) strains evidenced by stunted *Ve1* plants at 14 dpi. (Lower) *FoAve1* deletion strains display reduced virulence compared with the corresponding *F. oxysporum* wild type strains Bt.01 and Fol4287 on tomato lacking *Ve1* (*ve1*) evidenced by reduced stunting at 14 dpi. (C) Canopy area of *Ve1* tomato and tomato lacking *Ve1* after inoculation with *FoAve1* deletion strains and the corresponding *F. oxysporum* wild type strains at 14 dpi. (D) *FoAve1* deletion strains display reduced virulence compared to the corresponding *F. oxysporum* wild type strains Bt.01 and Fol4287 on tomato lacking *Ve1* evidenced by reduced fungal biomass at 14 dpi. 8 plants were pooled per 2 plants (n=4). Different letter labels indicate statistically significant differences ($P<0.05$). Similar results were observed in at least 3 biological replications for all experiments.

To provide further evidence for the role of FoAve1-recognition in Ve1-mediated tomato defence against Fusarium wilt, we generated *FoAve1* deletion mutants in *F. oxysporum* strains Bt.01 and Fol4287 through homologous recombination. Deletion of *FoAve1* was confirmed by PCR (Supplemental Figure 1A) and the deletion strains were subsequently evaluated for their ability to colonize *Ve1* tomato plants. As anticipated, inoculation of *Ve1* tomato plants with *FoAve1* deletion strains resulted in more extensive symptoms of Fusarium wilt disease when compared to tomato plants inoculated with the corresponding wild type strains (Figure 1B (Upper) and 1C). Altogether, these data show that tomato Ve1 is able to recognize FoAve1 and activate a defense response that inhibits colonization by *F. oxysporum*, albeit that the response does not fully arrest the fungus and immunity is not fully established. At later stages after inoculation clear symptoms of wilt disease are observed on *Ve1* plants.

FoAve1 is a virulence factor of *F. oxysporum* during tomato colonization

According to the paradigm that plant immune receptors recognize crucial virulence factors of microbial pathogens, it is expected that *FoAve1* contributes to *F. oxysporum* virulence. To test this hypothesis, *FoAve1* deletion strains were inoculated on tomato plants lacking *Ve1*. Indeed, *FoAve1* deletion strains displayed reduced virulence on tomato plants when compared to the corresponding *F. oxysporum* wild type strain as visualized by the difference in canopy area of the inoculated tomato plants (Figure 1B (Lower) and 1C). In addition, plants inoculated with *FoAve1* deletion strains resulted in less fungal colonization compared to those inoculated with wild type *F. oxysporum* (Figure 1D). These results suggest that *FoAve1* is a virulence factor of *F. oxysporum*, albeit that its contribution to virulence appears to be relatively minor.

ChAve1 is not a virulence factor of *C. higginsianum* during Arabidopsis colonization

Besides the vascular wilt fungi *V. dahliae* and *F. oxysporum*, that belong to the class of Sordariomycetes, an Ave1 homolog is found in the foliar Sordariomycete *C. higginsianum* (*ChAve1*). Using real-time PCR, *ChAve1* expression was monitored in wild type *C. higginsianum* during colonization of Arabidopsis plants from 1 to 4 dpi, by which time the plant tissue was completely macerated. No expression of *ChAve1* was detected at any of these time points. Nevertheless, we investigated whether *ChAve1* contributes to virulence in this foliar pathogen. For this purpose, *ChAve1* deletion strains were generated that, after confirmation with PCR (Supplemental Figure 1B), were evaluated for aggressiveness on *Arabidopis thaliana*. As expected *ChAve1* deletion strains did not show reduced virulence when compared to the

corresponding wild type *C. higginsianum*, as infections resulted in lesions with similar sizes (Figure 2A). Moreover, similar levels of pathogen biomass were recorded in plants inoculated with the wild-type fungus and the deletion strains (Figure 2B). Together these results show that ChAve1 is not a virulence factor of *C. higginsianum* on Arabidopsis, likely due to the lack of *ChAve1* expression.

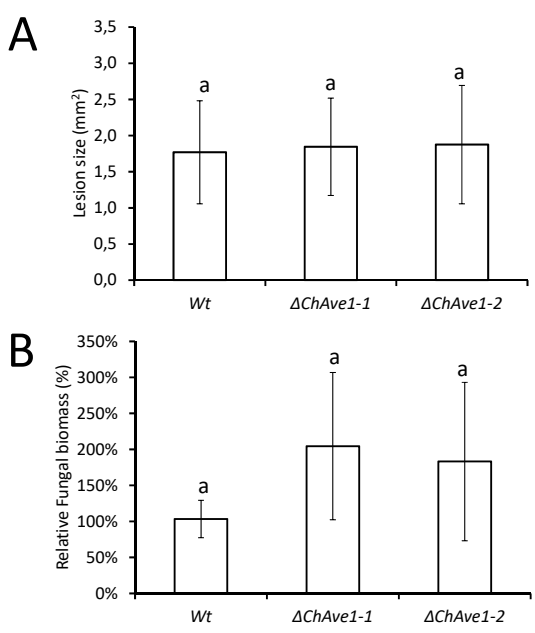

FIGURE 2 | ChAve1 is not a virulence factor of *C. higginsianum*. (A) *ChAve1* deletion strains (Δ*ChAve1*) show a similar lesion size compared to the corresponding *C. higginsianum* wild type strain (Wt) at 3 dpi on 3-week-old *A. thaliana*. Twenty four lesions on three *A. thaliana* plants were measured per strain. No significant difference was observed ($P<0.05$). (B) No difference in fungal biomass was observed at 4 dpi on spray inoculated 3-week-old Arabidopsis plants (n=4). Different letter labels indicate statistically significant differences ($P<0.05$). Similar results were observed in at least 3 biological replications for all experiments in this figure.

CbAve1 is a virulence factor of *C. beticola* during sugarbeet colonization

Besides Sordariomycetes, Ave1 homologs were also identified as we previously identified an Ave1 homolog in the Dothidiomycete *C. beticola* (*CbAve1*). Expression of *CbAve1* was investigated in *C. beticola* during colonization of sugarbeet plants between 3 and 18 dpi using real-time PCR. Clear expression was detected between these time points. To evaluate the contribution of *CbAve1* in *C. beticola* virulence, *CbAve1* deletion strains were generated and confirmed with PCR. For this pathogen, deletion of the *Ave1* homolog resulted in reduced virulence compared to the corresponding wild type strain, as infection resulted in reduced numbers and size of the lesions (Figure 3A; B). As expected, *CbAve1* deletion strains showed reduced colonization on sugarbeet plants compared to the corresponding *C. beticola* wild type strain (Figure 3C). Taken together, these results show that CbAve1 is a virulence factor of *C. beticola*.

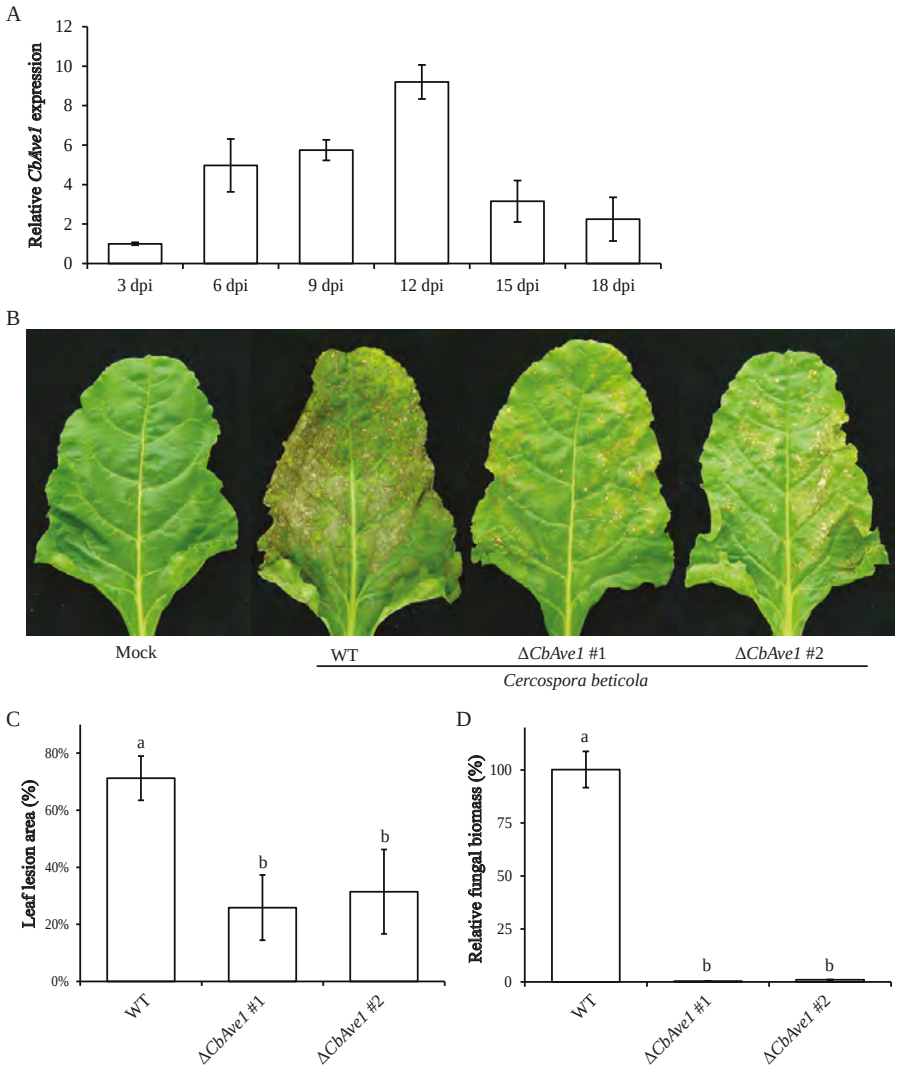

FIGURE 3 | CbAve1 is a virulence factor of *C. beticola*. (A) Expression of *CbAve1* during infection of *C. beticola* on sugar beet. Leaves of 6- to 7-week-old sugar beet plants were inoculated with wild-type *C. beticola* and collected at regular intervals from 3 to 18 days post inoculation (dpi). RT-qPCR was performed to determine the relative expression levels of *CbAve1* using the *C. beticola actin* gene as a reference, and compared with *CbAve1* expression in *C. beticola* in sugar beet plants upon the wild-type *C. beticola* inoculation at 3 dpi, which is set to 1. (B) *CbAve1* deletion strains (Δ*CbAve1* #1 and Δ*CbAve1* #2) show reduced virulence compared to the corresponding *C. beticola* wild type strain (WT) visualized by a reduction in lesion area on sugar beet leaves at 15 dpi. (C) Quantification of leaf lesion caused by *C. beticola* on sugar beet plants at 15 dpi (n>5). Bars represent the average percentage of leaf lesion area of whole leaf area with standard deviations. (D) Fungal biomass determined with qPCR in *Cercospora*-inoculated sugar beet plants at 15 dpi. The fungal biomass in sugar beet plants upon inoculation with the wild-type *C. beticola* is set to 100 % (control). Different letter labels indicate statistically significant differences ($P<0.05$).

Functional diversification among plant and fungal Ave1 homologs

VdAve1 is a virulence factor of *V. dahliae* that shows a high degree of identity with homologs from plants. It has therefore been proposed that *VdAve1* has been acquired by *V. dahliae* from plants through horizontal gene transfer (de Jonge et al. 2012). However, the function of VdAve1 through which it contributes to *V. dahliae* aggressiveness remains presently enigmatic. Similarly, the function of the Ave1 homologs that are found in various pathogens also remains unclear. In order to evaluate whether all homologs share their functionality, we tested whether they can complement the virulence defect that is observed upon *VdAve1* deletion in *V. dahliae*. To this end, we transformed one of the *VdAve1* deletion strains of *V. dahliae* with constructs to drive expression of the plant homologs derived from tomato (*S. lycopersicum*; *SlAve1*) and grape (*V. vinifera; VvAve1*) by the *V. dahliae VdAve1* promotor (de Jonge et al. 2012). To confirm that the plant homologs were expressed in *V. dahliae* upon colonization of tomato we monitored *SlAve1* and *VvAve1* expression with PCR. At 14 dpi we monitored clear expression of *SlAve1* and *VvAve1* in 3 transformants for each construct (Supplemental Figure 2a). Subsequently, we evaluated the ability of these transformants to cause disease on tomato plants lacking *Ve1*. All *VdAve1* deletion strains carrying *SlAve1* and *VvAve1* showed a similar phenotype and fungal colonization as the corresponding *VdAve1* deletion strain (Figure 4; Supplemental Figure 2). These results suggest that two plant Ave1 homologs that share a high identity with VdAve1 are unable to restore virulence in a *VdAve1* deletion strain, suggesting that Ave1 homologs from plants act in a different manner as VdAve1.

Subsequently, we evaluated whether microbial Ave1 homologs can reinstall the compromised virulence of *V. dahliae* that results from *VdAve1* deletion. To this end, we transformed one of the *VdAve1* deletion strains with constructs to drive expression of the Ave1 homologs derived from *F. oxysporum* (*FoAve1*) *C. higginsianum* (*ChAve1*) and *C. beticola (CbAve1*) by the VdAve1 promoter. We also included the Ave1 homolog derived from the plant pathogenic bacterium *Xanthomonas axonopodis* (*XacPNP*) that was previously described as a virulence factor (Gottig et al. 2008). We confirmed in a minimum of 2 transformants that the constructs were expressed (Supplemental Figure 2a). Subsequently, we tested the *V. dahliae* transformants on tomato lacking *Ve1* and compared them with the corresponding wild type *V. dahliae* and the *V. dahliae VdAve1* deletion strain complemented with *VdAve1*. *VdAve1* deletion strains expressing *FoAve1, CbAve1, ChAve1 and XacPNP* showed a similar disease phenotype and fungal colonization as *VdAve1* deletion strains (Figure 4; Supplemental Figure 2). (Figure 4; Supplemental Figure 2). These results show that the *Ave1* homologs *FoAve1*, *CbAve1*, *ChAve1* and *XacPNP* cannot restore virulence in a *VdAve1* deletion strain which may suggest that Ave1 homologs of *V. dahliae* on the one hand, and *F. oxysporum*, *C. beticola*, *C. higginsianum* and *X. axonopodis* on the other hand, contribute to fungal virulence in a different manner.

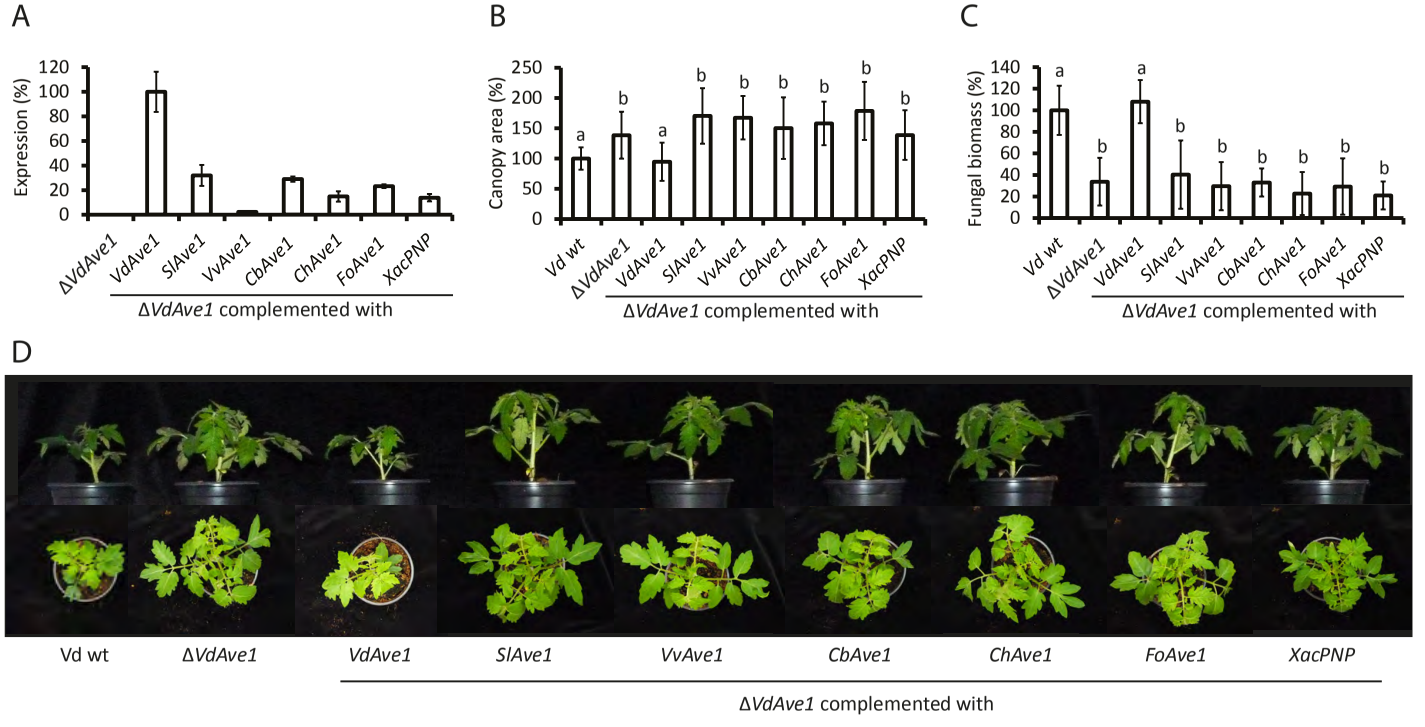

FIGURE 4 | VdAve1 acts in a different manner than Ave1 homologs derived from plant pathogens. *V. dahliae VdAve1* deletion strain (Δ*VdAve1*) complemented with *Ave1* homologs derived from *V. dahliae* (*VdAve1*), tomato (*S. lycopersicum*; *SlAve1*), grape (*V. vinifera; VvAve1*), *F. oxysporum* (*FoAve1*), *C. beticola* (*CbAve1*), *C. higginsianum* (*ChAve1*) and *Xanthomonas axonopodis* (*XacPNP*) inoculated on tomato plants. (A) Gene expression of Ave1 homologs by V. dahliae during colonization of tomato at 14 dpi. (B) Canopy area of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi. (C) Fungal colonization of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi. Different letter labels indicate significant differences ($P<0.05$). (D) Photos taken from the side and the top of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi.

Discussion

The role of FoAve1 in recognition by tomato immune receptor Ve1

We have previously shown that recognition of FoAve1 by Ve1 leads to a defence response (de Jonge et al. 2012) and that Ve1 tomato plants can recognize *F. oxysporum.* To confirm that FoAve1 is involved in recognition we first analysed *FoAve1* expression. A previous study could not detect *FoAve1* expression in *F. oxysporum* after inoculation on tomato (Schmidt et al. 2013). However, in the conditions that we tested clear *FoAve1* expression was monitored in *F. oxysporum* on tomato at 14 dpi. In addition, deletion of *FoAve1* resulted in abolishment of recognition by Ve1. Our data suggest that the incomplete disease resistance cannot be attributed to a lack of *FoAve1* expression, but may be the result of a less efficient detection of this Ave1 homolog by Ve1. Similar differences in recognition efficiencies were previously observed for *Cladosporium fulvum* effector Ecp2 and its homolog MfEcp2 of *Mycosphaerella fijiensis* when coexpressed with tomato immune receptor Cf-Ecp2 (Stergiopoulos et al. 2010).

Some Ave1 homologs act as virulence factors

Previously, we have shown that Ave1 is a virulence factor of *V. dahliae* (de Jonge et al. 2012). In this study, we tested wheter Ave1 homologs in other plant pathogens act as virulence factors as well. We showed that deletion of *Ave1* homologs in *F. oxysporum* and *C. beticola* resulted in reduced aggressiveness on their respective plant hosts. In contrast, the Ave1 homolog *ChAve1* of the foliar pathogen *C. higginsianum* does not contribute to virulence due to the lack of expression *in planta*. Possibly, the lack of expression is due to the availability of effectors with a similar function that render ChAve1 functionality redundant. Alternatively, recognition of ChAve1 by a host immune receptor posed pressure on *C. higginsianum* to avoid recognition, resulting in the lack of *ChAve1* expression. Interestingly, homologs of tomato Ve1 have been identified in many other plant species, of which some within as well as outside the Solanaceae family have been shown to be functional immune receptors (Song et al. 2017a).

Functional diversification among Ave1 homologs

Since Ave1 homologs derived from plants display high similarity to Ave1, it has been suggested that Ave1 was acquired through horizontal gene transfer from plants (de Jonge et al. 2012). As the function of the Ave1 homologs remain unknown we tested whether the Ave1 homologs share the same functionality by complementing a *V. dahiae Ave1* deletion strain with homologs derived from plants and various plant

pathogens. Highly similar Ave1 homologs derived from plants are unable to restore virulence in an Ave1 deletion strain. Therefore we conclude that Ave1 homologs derived from plants act in a different manner as Ave1. Similarly, Ave1 homologs derived from *F. oxysporum, C. beticola*, *C. higginsianum* and *X. axonopodis* cannot restore the virulence penalty caused by the deletion of Ave1. Therefore, we conclude that Ave1 from *V. dahliae* on the one hand, and Ave1 homologs derived from *F. oxysporum*, *C. beticola, C. higginsianum* and *X. axonopodis* on the other hand, contribute to virulence in a different manner. Similarly, functional diversification within effector families has been observed for LysM and NLP effectors (Kombrink and Thomma 2013; Santhanam et al., 2013). Alternatively, it is important to note that *V. dahliae*, *C. beticola, C. higginsianum* and *X. axonopodis* have diverse plant hosts and that the reason for the inability to restore virulence in an *V. dahliae Ave1* deletion strain could be that each homolog targets a host-specific protein. This would for example explain why the homolog of *C. beticola* that likely targets a sugarbeet protein cannot restore virulence in an *V. dahliae Ave1* deletion strain colonizing tomato. Host specific effector adaptation has been suggested for the closely related oomycetes *Phytophtora infestans* and *P. mirabilis* that are pathogens on different hosts. Homologous effectors of both pathogens act more efficiently on their specific host target than on a "foreign" host target (Dong et al. 2014). Nevertheless, the finding that the *Ave1* homolog from *F. oxysporum* f.sp. *lycopersici* cannot complement the *VdAve1* deletion strain for virulence on tomato argues against this hypothesis.

Materials and methods

Deletion and complementation strains

Deletion strains were generated in *F. oxysporum* and *C. higginsianum* by amplifying 1,5 kb sequences (Supplemental table 1) flanking the coding sequence of *FoAve1* and *ChAve1*, respectively, and cloned as previously described (Frandsen et al. 2008) into vector pRF-HU2 containing a nourseothricin cassette for selection. For the complementation of *V. dahliae Ave1* deletion strains *FoAve1, CbAve1, ChAve1, SlPNP*, *VvPNP and XacPNP* were obtained by gene synthesis (Eurofins Genomics, Ebersberg, Germany). The synthesized genes were then cloned into vector pFBT005 under the *VdAve1* promoter, containing a nourseothricin cassette for selection. *F. oxysporum* (Fol4287 and Bt.01) and *C. higginsianum* (IMI349063A) conidiospores were transformed on a Hybond-N^+ filter by *A. tumefaciens* carrying the pRF-HU2 plasmid containing the constructs and a *V. dahliae Ave1* deletion strain (JR2) was transformed by *A. tumefaciens* carrying the pFBT005 plasmid containing the constructs as previously described (Santhanam 2012). Transformants

were then selected on potato dextrose agar (PDA) (Thermo Fisher Scientific Inc, Breda, The Netherlands) containing hygromycin B (Duchefa Biochemie BV, Haarlem, The Netherlands) for selection of *F. oxysporum*, *C. beticola* and *C. higginsianum* transformants or nourseothricin sulphate (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) for selection of *V. dahliae* transformants. After five to seven days at room temperature, individual transformants were transferred from the filter to fresh PDA plates with the appropriate selection and incubated for ten days (Santhanam 2012). To verify the transformants, fungal spores were collected and genomic DNA was extracted followed by a PCR to test the presence of the hygromycin or nourseothricin cassette and the presence of the construct in the genome (Supplemental table 1).

CbAve1 deletion mutants of *C. beticola* were generated using the split-marker approach described by Catlett *et al.* (2003). Genomic DNA of the wild type *C. beticola* strain 09-40 and pDAN vector (Friesen *et al.*,2006) served as PCR templates to generate split-marker PCR constructs used for transformation. Primers are listed in Supplemental table 1. PEG-mediated transformation of the wild type *C. beticola* strain 09-40 was performed as previously described (Bolton *et al.*,2016). Site-directed gene replacement was confirmed by the absence of PCR product using split-marker CbAve1 1F forward primer of the 5' flank of the target gene and MDB-760 reverse primer designed on the coding sequence of *CbAve1*.

Pathogen inoculations

Plants were grown in soil in the greenhouse at 21°C/19°C during 16-h/8-h day/night periods, respectively, with 70% relative humidity and 100 W m^{-2} supplemental light when the intensity dropped below 150 W m^{-2}.

For *V. dahliae* and *F. oxysporum* inoculations, 10-day-old tomato (*Solanum lycopersicum*; cv. MoneyMaker or MoneyMaker *35S:Ve1*) (Fradin et al. 2009) were uprooted, rinsed in water and dipped for 5 minutes in a suspension of 10^6 conidiospores per mL of water harvested from 1- to 2-week-old *V. dahliae* or *F. oxysporum* cultures on PDA as previously described (Fradin et al. 2009). Control plants received the same treatment, but the roots were dipped in water without conidiospores. After replanting in fresh soil, plants were incubated at standard greenhouse conditions. Disease development was monitored up to 21 days post inoculation (dpi).

For *C. higginsianum* inoculations 3-week-old Arabidopsis (*A. thaliana* ecotype Co-0 or Col-0 *35S:Ve1*) (Fradin et al. 2011) were inoculated on the leaves with either 2 µl drops or sprayed with a suspension of 10^6 conidia per mL of water harvested from 1- to 2-week-old *C. higginsianum* cultures on Mathurs' agar as previously

described (O'Connell et al. 2004; Sherriff et al. 1994). Control plants received the same treatment, but the leaves were sprayed with water without conidiospores. After sealing the plants inside a transparent closed box lined with wet tissue paper to provide high humidity, they were incubated at 25°C, under a 16-h/ 8-h light/dark regime (O'Connell et al. 2004). Disease development was monitored up to 4 dpi.

For *C. beticola* inoculations 6- to 7-week-old sugarbeet plants (*Beta vulgaris*) were inoculated by evenly spaying spore suspension of 10^5 conidia per mL of water on the lower side of the leaves harvested from *C. beticola* cultures growing on V8 solid medium as previously described (Bolton et al. 2012). Control plants received the same treatment, but the leaves were sprayed with water. Plants were incubated at standard greenhouse conditions and disease development was monitored up to 18 dpi.

Fungal biomass and gene expression

Stem sections of tomato, cut from the base of the stem up to the cotyledons, were collected at 7 and 14 dpi from plant inoculated with *F. oxysporum* or *V. dahliae*. Arabidopsis leaves were collected at 1-4 dpi after inoculation with *C. higginsianum*. Sugarbeet leaves were collected at regular intervals between 3 and 18 dpi from plants inoculated with *C. beticola*. Collected plant tissue was flash frozen in liquid nitrogen and ground to powder, of which an aliquot of ~100 mg was used for RNA extraction with the Quick-RNA™ Miniprep kit (Zymo Research Europe GmbH, Freiburg, Germany), and cDNA was synthesized using M-MLV Reverse Transcriptase (Promega Benelux BV, Leiden, The Netherlands). Fungal biomass was determined by quantifying the expression of *VdGAPDH*, *FoTUB*, *ChTUB* and *CbAct* relative to the expression of *SlRUB* (tomato), *AtRub* (Arabidopsis), *BvAct* (sugarbeet), respectively. Expression of *VdAve1*, *FoAve1*, *CbAve1*, *ChAve1*, *SlPNP* and *VvPNP* was determined by quantifying the expression relative to the expression of *VdGAPDH*, *FoTUB*, *ChTUB* and *CbAct*, respectively (Supplemental table 1). Real-time PCR was carried out on an ABI7300 PCR machine (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, The Netherlands) in combination with the qPCR SensiMix kit (BioLine, GC Biotech BV, Alphen aan den Rijn, The Netherlands). The following Real-time PCR conditions were used: an initial 95°C denaturation step for 10 minutes followed by denaturation for 15 seconds at 95°C, annealing for 60 seconds at 60°C, and extension at 72°C for 40 cycles.

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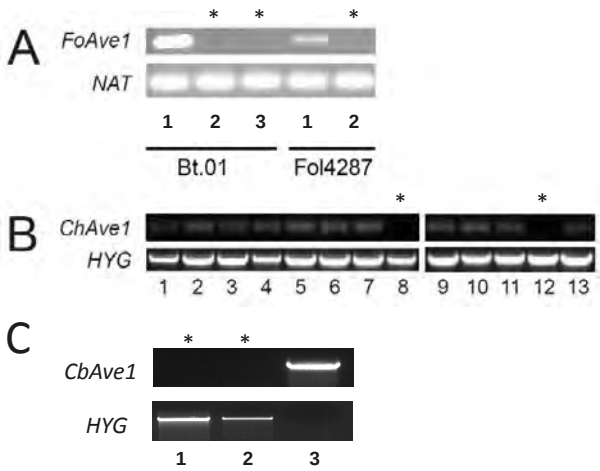
Supplemental data

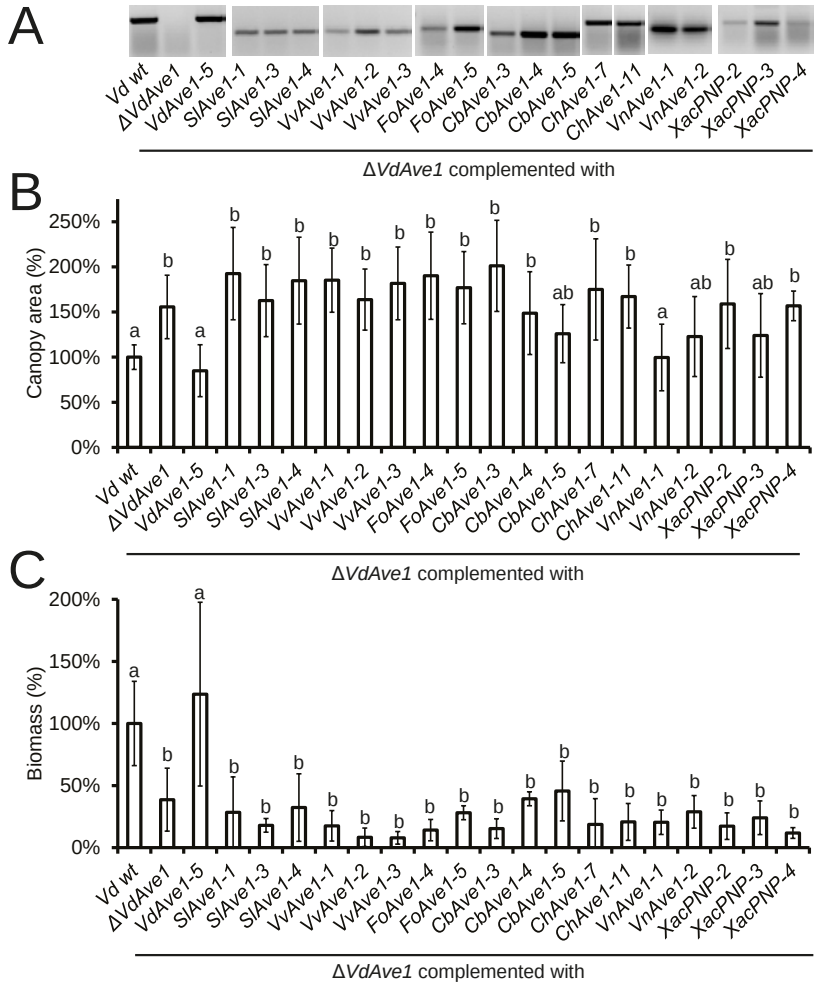
SUPPLEMENTAL TABLE 1 | Primers used in this study

Primer	Sequence (5'- 3')	Notes
SlRub_QPCR_F	GAACAGTTTCTCACTGTTGAC	*S. lycopersicum Rubisco*
SlRub_QPCR_R	CGTGAGAACCATAAGTCACC	*S. lycopersicum Rubisco*
FolSIX1_F	GTCTCACGAGCCAAGTCTACC	*F. oxysporum Six1*
FolSIX1_R	GAACCGCAGCCTCTTGAGCAT	*F. oxysporum Six1*
FolTub_F	CTCTGGCAACAAGTATGTTCCC	*F. oxysporum Tubulin*
FolTub_R	TTGTCGGGACGGAAGAGCTGA	*F. oxysporum Tubulin*
FolAve1_QPCR_F3	ATATCGGAACTGCAAATATTCTCAAC	*F. oxysporum Ave1*
FolAve1_QPCR_R3	CTTATACATTTCATCGTATACAGTCTGC	*F. oxysporum Ave1*
AtRub_QPCR_F	GCAAGTGTTGGGTTCAAAGCTGGTG	*A. thaliana Rubisco*
AtRub_QPCR_R	CCAGGTTGAGGAGTTACTCGGAATGCTG	*A. thaliana Rubisco*
ChELF1a_F	CTGGTACAAGGGTTGGGAGA	*C. higginsianum Elongation factor*
ChELF1a_R	ACCGCCGATCTTGTAGACAT	*C. higginsianum Elongation factor*
ChAve1_QPCR_F5	CAAGATGCTATGGCAACAATATGAAC	*C. higginsianum Ave1*
ChAve1_QPCR_R5	GTCTTGAGGAAAATCTATCGTATTTCTG	*C. higginsianum Ave1*
BvAct_QPCR_F	GATTTGGCACCACACCTTCT	*B. vulgaris actin*
BvAct _QPCR_R	TCTTTTCCCTGTTTGCCTTG	*B. vulgaris actin*
CbAct_QPCR_F	ACATGGCTGGTCGTGATTTG	*C. beticola actin*
CbAct_QPCR_F	TGTCCGTCAGGAAGCTCGTA	*C. beticola actin*
CbAve1_QPCR_F	ATTCCCTTCAGGCAACCTCT	*C. beticola Ave1*
CbAve1_QPCR_R	CGGACAAGCTTCGCAATAAT	*C. beticola Ave1*
VdGapdh_F	CGAGTCCACTGGTGTCTTCA	*V. dahliae GAPDH*
VdGapdh_R	CCCTCAACGATGGTGAACTT	*V. dahliae GAPDH*
VdAve1-Fw5	ATCCTACTATAACCCACCCTACCTTC	*V. dahliae Ave1*
VdAve1-Rv5	CATCATATGAGTCCTGAGATAAGATCA	*V. dahliae Ave1*
XacAve1_QPCR_F	GCAATCGGTTTGCTCTTTTC	*X. axonopodis XacPNP*
XacAve1_QPCR_R	AGCACCGTTATCCCACAGAC	*X. axonopodis XacPNP*
VvAve1-QPCR-F2	CGTTATAGGTTAAGGTGCCTGAGT	*V. vinifera Ave1*
VvAve1-QPCR-R2	TTCATGCTAGGAGAGTGTGAAATG	*V. vinifera Ave1*
SlAve1_QPCR_F	CGTCGGGGAATCTATTTGTG	*S. lycopersicum Ave1*
SlAve1_QPCR_R	AAAGCATCCGTTGACAAAGC	*S. lycopersicum Ave1*
FolAve1_LB_F3	GGTCTTAAUAACCTAACCTGTATCTAGACCAGAGTGTCT	Left border *FolAve1*
FolAve1_LB_R3	GGCATTAAUCAGCAGGTGTTTAGGTATTGTTAAGATAAG	Left border *FolAve1*
FolAve1_RB_F	GGACTTAAUGTTCTTAGAGCTTAAGGTCAATGTAGC	Right border *FolAve1*
FolAve1_RB_R	GGGTTTAAUCTGCAGCTTTCACGGGGCTA	Right border *FolAve1*
ChAve1_LB_F	GGTCTTAAUGGCAGGAGGTCTAGGTGAGA	Left border *ChAve1*
ChAve1_LB_R	GGCATTAAUCGACTTGAGTTTTGCTGCAC	Left border *ChAve1*
ChAve1_RB_F	GGACTTAAUAAAGATTTTCGCAGTGCTTCA	Right border *ChAve1*
ChAve1_RB_R	GGGTTTAAUAGGCTGCAGAATGAGTTTCG	Right border *ChAve1*
pRF-HU2_Hyg_Fw	CTATTCCTTTGCCCTCGGACGAGTGC	Hygromycin primers
pRF-HU2_Hyg_Rv	CGATGTAGGAGGGCGTGGATATGTCC	Hygromycin primers
NAT_Fw	CGGGCCGGATTGGTCAAGATTTGC	Nourseothricin primers
NAT_Rv	CGATTCGTCGTCCGATTCGTCG	Nourseothricin primers

Primer	Sequence (5'- 3')	Notes
MDB-277	GACGTTGTAAAACGACGGCCAGTG	Split-marker HYG-F
MDB-258	GGATGCCTCCGCTCGAAGTA	Split-marker HY-R
MDB-259	CGTTGCAAGACCTGCCTGAA	Split-marker YG-F
MDB-278	CACAGGAAACAGCTATGACCATGA	Split-marker HYG-R
MDB-1145	GGCAGGTAGATGACGACCAT	HYG R2
CbAve1 1F	CAAGATTGGGCCTTCGTATG	*CbAve1* split-marker left
CbAve1 2R	CACTGGCCGTCGTTTTACAACGTCTCTTCAATGGATCC GGACTG	*CbAve1* split-marker left border
CbAve1 3F	TCATGGTCATAGCTGTTTCCTGTGTGAGTGGGGTTTTT GGTTTC	*CbAve1* split-marker right border
CbAve1 4R	AAGCATACCTCTTCGGCAAA	*CbAve1* split-marker right
MDB-760	CAACGTCTGCCACAAGCTGCT	*CbAve1* reverse



SUPPLEMENTAL FIGURE 1 | Verification of deletion strains. (A) Identification of *F. oxysporum FoAve1* deletion strains by amplification of *FoAve1* and selection marker nourseothricin (*NAT*) from DNA obtained from transformants. DNA bands were observed at the expected size. Asterisks indicate positive transformants, while transformants that lack an asterisk are ectopic transformants. (B) Identification of *C. higginsianum ChAve1* deletion strains by amplification of *ChAve1* and selection marker hygromycin (*HYG*) from DNA obtained from *C. higginsianum* transformants. DNA bands were observed at the expected size. Asterisks indicate positive transformants, while transformants that lack an asterisk are ectopic transformants. (C) Verification of *CbAve1* deletion strains (1 and 2) based on absence of *CbAve1* using gene-specific primers and presence of the hygromycin resistance cassette. Amplification on *C. beticola* Wt gDNA (3) as a control showing the presence of *CbAve1* and absence of the hygromycin resistance cassette at the expected size. Asterisks indicate positive transformants.



SUPPLEMENTAL FIGURE 2 | VdAve1 acts in a different manner than Ave1 homologs derived from plant pathogens. *V. dahliae VdAve1* deletion strain (Δ*VdAve1*) complemented with *Ave1* homologs derived from *V. dahliae* (*VdAve1*), tomato (*S. lycopersicum*; *SlAve1*), grape (*V. vinifera; VvAve1*), *F. oxysporum* (*FoAve1*), *C. beticola (CbAve1*), *C. higginsianum* (*ChAve1*) and *Xanthomonas axonopodis* (*XacPNP*) inoculated on tomato plants. (A) Expression of *VdAve1*, *SlAve1*, *VvAve1*, *FoAve1*, *CbAve1*, *ChAve1* and *XacPNP* in a minimum of 2 transformants. (B) Canopy area of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs in a minimum of 2 transformants compared to *V. dahliae* wild type (Vd wt) at 14 dpi. (C) Fungal colonization of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi. Different letter labels indicate significant differences (P<0.05). (D) Photos taken from the side and the top of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi.

Chapter 4

A nine amino acid peptide of *Verticillium dahliae* effector Ave1 is required but not sufficient for recognition by tomato immune receptor Ve1

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Abstract

Effectors are molecules secreted by plant pathogens to facilitate infection, often through deregulation of plant immune responses. During infection, race 1 strains of the soil borne vascular wilt fungus *Verticillium dahliae* secrete the effector protein Ave1 that is recognized by tomato immune receptor Ve1. Homologs of *V. dahliae* Ave1 (VdAve1), have been identified from the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* (XacPNP), and in the fungal pathogens *Fusarium oxysporum* (FoAve1), *Colletotrichum higginsianum* (ChAve1) and *Cercospora beticola* (CbAve1). In this study, we identified a novel *Ave1* homolog from *V. nubilum* (VnAve1), which is mainly known as saprophyte and opportunistic pathogen. The Ave1 homologs are differentially recognized by Ve1, with the most efficient recognition of VdAve1 and VnAve1, and little to no recognition of ChAve1 and XacPNP. Interestingly, C-terminal GFP-tagging of VdAve1 resulted in loss of recognition by Ve1, suggesting that exposure of the C-terminus of VdAve1 is essential for recognition by Ve1. Truncations of VdAve1 and domain swaps between VdAve1 and XacPNP narrowed down the sequence that is required for Ve1 recognition to the C-terminal nine amino acids. Site-directed mutagenesis was performed to analyse the contribution of individual amino acid residues to recognition by Ve1, revealing the importance of five amino acids from Val127 to Phe131. Nevertheless, fusions of the VdAve1 C-terminal nine amino acids to GFP or the *Cladosporium fulvum* effector Avr9 showed that this sequence is not sufficient to activate Ve1-mediated immunity. Finally, with a complementation experiment in *V. dahliae* we confirmed that the C-terminal nine amino acids of Ave1 are required but not sufficient to activate Ve1-mediated immunity.

Introduction

In nature, plants are threatened by diverse groups of potential microbial pathogens. However, only few of these potential pathogens actually cause disease, as plants have developed innate immunity to protect themselves against microbial attack (Dodds and Rathjen 2010; Thomma et al. 2011). In its simplest form, plant immunity against pathogen attack is governed by immune receptors that sense pathogen-derived ligands to activate defense. Originally, the interaction between plant immune receptors and pathogen ligands was described in the "gene-for-gene" model, stating that the products of plant resistance (*R*) genes induce race-specific resistance upon recognition of the products of corresponding pathogen avirulence (*Avr*) genes (Flor 1971). Decades later, an updated view of plant innate immunity has been introduced as the "zigzag" model, by incorporating pathogen-secreted effector molecules that suppress host immune responses, but that may subsequently be recognized by newly evolved immune receptors, in turn (Jones and Dangl 2006). In this model, the first line of defense is governed by plasma membrane-localized pattern recognition receptors (PRRs) that detect conserved microbe-associated molecular patterns (MAMPs) to activate MAMP-triggered immunity (MTI). In subsequent layers of defense effectors are recognized by corresponding resistance proteins (R proteins), resulting in effector-triggered immunity (ETI). Although initially portrayed as separate layers of defense, numerous studies on various plant-microbe interactions have revealed that the delineation between MTI and ETI is not strict, but rather a continuum (Thomma et al. 2011). Moreover, the conceptual conflict that MAMPs are defined from the perspective of the host whereas effectors are defined from the perspective of the invader creates a conceptual conflict and has recently inspired the formulation of the Invasion Model, in which host receptors (termed invasion pattern receptors; IPRs) detect either an externally encoded or modified-self ligand that betrays invasion (termed invasion patterns; IPs) (Cook et al. 2015). In this model, any molecule can serve as an IP that is detected by an IPR, but the probability of a particular ligand-receptor complex to evolve within the framework of host immunity increases with increasing ligand probability to retain function, conservation across organisms, importance in establishment of symbiosis, and accessibility (Cook et al. 2015).

Verticillium dahliae is a xylem invading fungal pathogen that causes Verticillium wilt diseases in a wide range of plant species worldwide (Fradin and Thomma 2006). *V. dahliae* persists in the soil and enters plants through their roots. Once inside the root, the fungus grows intercellularly and invades the xylem vessels, where it sporulates to spread through the vascular system. Typical symptoms of *V. dahliae* infection include stunting, wilting, chlorosis, and necrosis (Fradin and Thomma 2006). In tomato, a single dominant locus that confers *Verticillium* resistance has

been identified that controls isolates that are assigned to race 1 (Schaible et al. 1951). The locus comprises two genes, *Ve1* and *Ve2*, that both encode extracellular leucine rich repeat (eLRR) receptor-like proteins (RLPs), and of which only *Ve1* acts as a functional *Verticillium* resistance gene in tomato (Fradin et al. 2011; Fradin et al. 2009; Kawchuk et al. 2001; Wang et al. 2010). Functional homologs of Ve1 were reported from *Nicotina glutinosa* and from cotton (Chen et al. 2016; Yang et al. 2014; Zhang et al. 2012; Zhang et al. 2011; Zhang et al. 2013).

Through comparative population genomics, the *V. dahliae* effector protein that is recognized by the Ve1 immune receptor of tomato was identified as Ave1 (for **A**virulence on **Ve1** tomato 1) (de Jonge et al. 2012; Fradin et al. 2009). Although the intrinsic function of *V. dahliae* Ave1 (VdAve1) remains elusive, it is clear that Ave1 contributes to fungal virulence on susceptible plant genotypes (de Jonge et al. 2012). Interestingly, Ave1 homologs were identified from a number of fungal pathogens, including the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), the sugar beet pathogen *Cercospora beticola* (CbAve1) and the Brassicaceae pathogen *Colletotrichum higginsianum* (ChAve1). Strikingly, however, most Ave1 homologs were found in plants, with the most closely related homologs derived from tomato (*Solanum lycopersicum*; SlPNP) and grape (*Vitis vinifera*; VvAve1). Finally, a more distantly related homolog was identified in the plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (XacPNP) (de Jonge et al. 2012; Nembaware et al. 2004). Co-expression of *SlPNP*, *FoAve1*, and *CbAve1* with tomato *Ve1* in tobacco triggers a hypersensitive response (HR), whereas co-expression of *ChAve1* with tomato *Ve1* did not lead to an HR (de Jonge et al. 2012). Consequently, Ve1 was found to mediate resistance towards *F. oxysporum* in tomato, demonstrating involvement of this tomato immune receptor in resistance against multiple fungal pathogens (de Jonge et al. 2012).

It has previously been demonstrated that eLRR-containing cell-surface immune receptors recognize peptide sequences as epitopes of their pathogen ligands. For example, flg22 is the 22 amino acid peptide derived from bacterial flagellin that is perceived by the RLK-type immune receptor FLS2 (Zipfel et al. 2004), while the Arabidopsis EFR RLK-type immune receptor was shown to recognize elf18, an 18 amino acid peptide derived from bacterial EF-Tu (Zipfel et al. 2006). Similarly, a surface-exposed pentapeptide TKLGE of the 22 kDa ethylene-inducing xylanase (EIX) from the biocontrol fungus *Trichoderma viride* determines recognition by the tomato RLP-type receptor LeEIX2 (Ron and Avni 2004; Rotblat et al. 2002). Furthermore, the tyrosine-sulfated 21 amino acid sequence RaxX21-sY present in *Xanthomonas oryzae* pv. *oryzae* RaxX is sufficient to activate XA21-mediated immunity (Pruitt et al. 2015). Finally, a conserved 20-24 amino acid sequence derived from Nep1-like proteins (NLPs) is sufficient to activate RLP23-mediated immunity in

Arabidopsis (Albert et al. 2015; Böhm et al. 2014; Oome et al. 2014). In the present study, we aimed to identify a minimal motif in VdAve1 that determines recognition by tomato immune receptor Ve1. Our approach was based on alignment of differentially recognized Ave1 homologs, followed by a combination of deletions, domain swaps among Ave1 homologs and mutagenesis.

Results

Conservation among Ave1 homologs

Previously, we reported the cloning of *Ave1* from *V. dahliae* and described the absence of allelic variation among 85 *Ave1* alleles of *Verticillium* strains (de Jonge et al. 2012). The *Ave1* alleles were derived from *V. dahliae* and *V. albo-atrum* (presently *V.* alfalfae; (Inderbitzin et al. 2011)), whereas *Ave1* alleles were not identified in any of the race 2 strains analysed, nor in *V. dahliae* and *V. albo-atrum* strains that are not pathogenic on tomato, nor in *V. longisporum* or *V. tricorpus* (de Jonge et al. 2012). To further explore *Ave1* diversity, we assessed its presence in a collection of 97 *Verticillium* strains isolated from various host plants and different geographical locations, resulting in the identification of 17 novel *Ave1* alleles (Supplemental Table 1). No allelic variation was found among the newly identified *Ave1* alleles amplified from *V. dahliae* as well as from *V. alfalfae* and *V. nonalfalfae*, two novel species that have recently been separated from *V. albo-atrum* (Inderbitzin et al. 2011). However, an *Ave1* homolog was identified in four isolates of *V. nubilum* (*VnAve1*), a species that is mainly known as a saprophytic and opportunistic pathogen (Isaac 1953). While the predicted VnAve1 protein sequence displays 13 amino acid polymorphisms when compared with VdAve1 (Figure 1A), the four *VnAve1* alleles were found to be identical to each other.

Alignment of the amino acid sequences of VdAve1 with homologs from plants (SlAve1 and VvAve1), and plant pathogens (VnAve1, FoAve1, CbAve1, ChAve1 and XacPNP) reveals blocks of highly conserved amino acids that are alternated with more variable regions (Figure 1A). Based on prediction by SignalP 4.0 (Petersen et al. 2011), all Ave1 homologs contain N-terminal signal peptides that direct secretion of the molecules into the extracellular space (Figure 1A; D-cutoff score>0.6). Moreover, the four cysteine residues that are present in VdAve1 are conserved among all homologous proteins (Figure 1A), and *in silico* analysis using DISULFIND (Ceroni et al. 2006) suggests the formation of disulphide bridges between Cys35 and Cys63, as well as between Cys71 and Cys79. From the alignment it is apparent that XacPNP is the most divergent, while all other homologs are relatively comparable (Figure 1A).

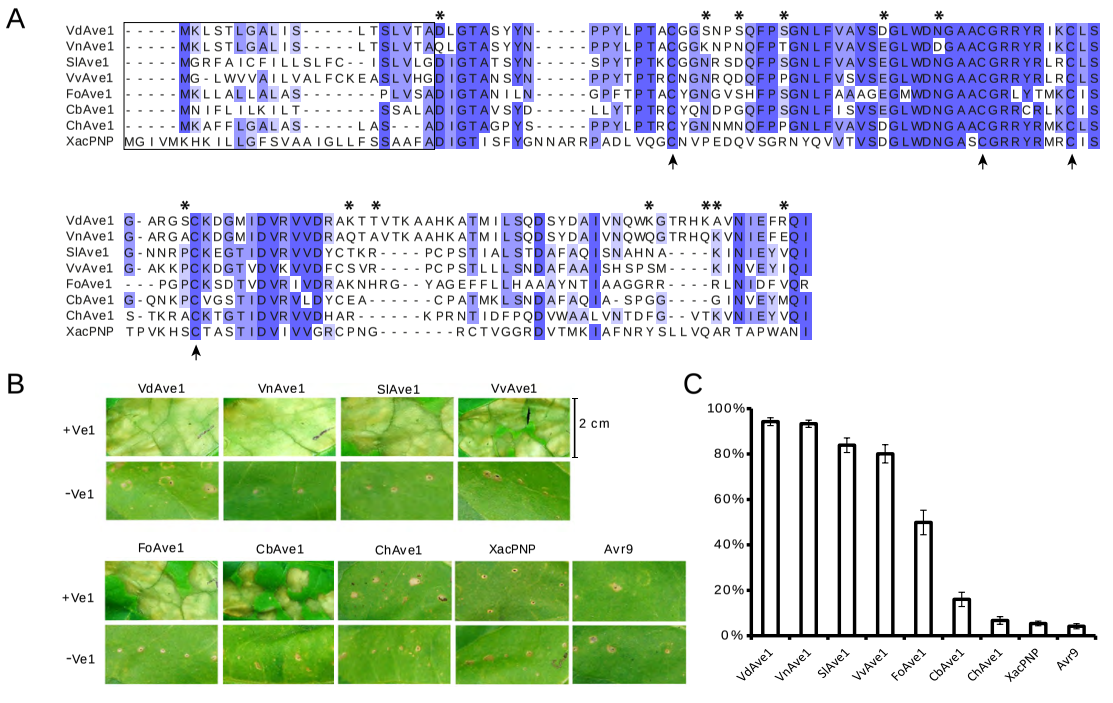

FIGURE 1 | Comparison of necrosis induced by *Ave1* homologs through co-expression with *Ve1* in *Nicotiana tabacum*. (A) Amino acid sequence alignment of Ave1 homologs from *Verticillium dahliae* (VdAve1), *V. nubilum* (VnAve1), *Solanum lycopersicum* (SlAve1), *Vitis vinifera* (VvAve1), *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), *Cercospora beticola* (CbAve1), *Colletotrichum higginsianum* (ChAve1), and *Xanthomonas axonopodis* pv. citri (XacPNP). Purple shading indicates identical amino acids while the color intensity represents the frequency. Asterisks indicate the 13 amino acid polymophisms between VdAve1 and VnAve1. The positions of four conserved cysteine residues are indicated with arrows in the bottom. Predicted signal peptides are framed. (B) Coexpression of tomato *Ve1* and *Ave1* homologs VdAve1, VnAve1, SlAve1, VvAve1, FoAve1, CbAve1, ChAve1, and XacPNP in *N. tabacum*. Expression of the Avr9 effector from the tomato leaf mold fungus *Cladosporium fulvum* in combination with Ve1 is shown as negative control. Pictures were taken at five days post infiltration. (C) Quantification of cell death resulting from recognition of Ave1 homologs by Ve1. The average percentage of necrotic leaf area of infiltration zones at five days post infiltration (n=24). Data are presented as mean with standard errors.

Comparison of necrosis-inducing activity of Ave1 homologs

It was previously demonstrated that Ve1 recognizes not only VdAve1, but also SlPNP, FoAve1 and CbAve1 (de Jonge et al. 2012). We now also tested the HR-inducing capacity of VnAve1, VvAve1 and XacPNP that were isolated from *V. nubilum, V. vinifera* and *X. axonopodis*, respectively. Co-expression of the sequence-unrelated effector *Avr9* from the tomato leaf mould fungus *Cladosporium fulvum* (van Kan et al. 1991) in combination with *Ve1* served as a negative control. Whereas expression of *VnAve1* or *VvAve1* together with *Ve1* in *Nicotiana tabacum* resulted in strong HR, co-expression of *XacPNP* or *Avr9* with Ve1 triggered little to no necrosis in addition to the small wounds that were caused by the infiltration procedure (Figure 1B). To compare the HR induced by the various Ave1 homologs, they were co-expressed with Ve1 in *N. tabacum* and HR development was measured at five days post infiltration by quantification of the leaf area that developed necrosis (Zhang et al. 2013). Importantly, none of the Ave1 homologs induced necrosis in absence of Ve1 (Figure 1B). Whereas agroinfiltration of either *VdAve1* or *VnAve1* with *Ve1* resulted in complete necrosis of the infiltrated leaf area, agroinfiltration of *FoAve1* with *Ve1* resulted in large necrotic spots in the infiltrated leaf area, although no complete collapse of the infiltrated area was observed. Upon agroinfiltration of *CbAve1* with *Ve1*, spreading of smaller and larger necrotic spots was observed in all infiltrated areas, but the infiltrated leaf area did not turn completely necrotic. For *ChAve1*, *XacPNP* and *Avr9*, no spreading necrosis was observed beyond the wounded infiltration sites (Figure 1B). Upon agroinfiltration of the tomato and grape homologs, *SlAve*1and *VvAve1*, most of the infiltrated leaf area developed necrosis, occasionally affecting the complete infiltrated leaf sector. To confirm that reduced levels of HR are not due to instability of the proteins in the expression assay, GFP-tagged Ave1 homologs were detected by immunoblotting. Similar to GFP-tagged VdAve1 protein or GFP-tagged VnAve1 that induce the strongest HR, all other GFP-tagged Ave1 homologs accumulated to clearly detectable protein levels (Supplemental Figure 1).

The C-terminus of VdAve1 is required for recognition by Ve1

In order to permit functional analyses, a construct encoding C-terminal protein GFP fusion of VdAve1 was generated. However, C-terminal fusion of a GFP tag to Ave1 resulted in loss of recognition by Ve1. Considering that the GFP tag is relatively large, we engineered C-terminal fusions to VdAve1 with smaller protein tags. Nevertheless, all C-terminal tags abolished, or significantly reduced, HR development (Figure 2A; C). Importantly, the C-terminal GFP fusions could be detected by immunoblotting (Supplemental Figure 2), suggesting that accessibility of the VdAve1 C-terminus is important for recognition by Ve1.

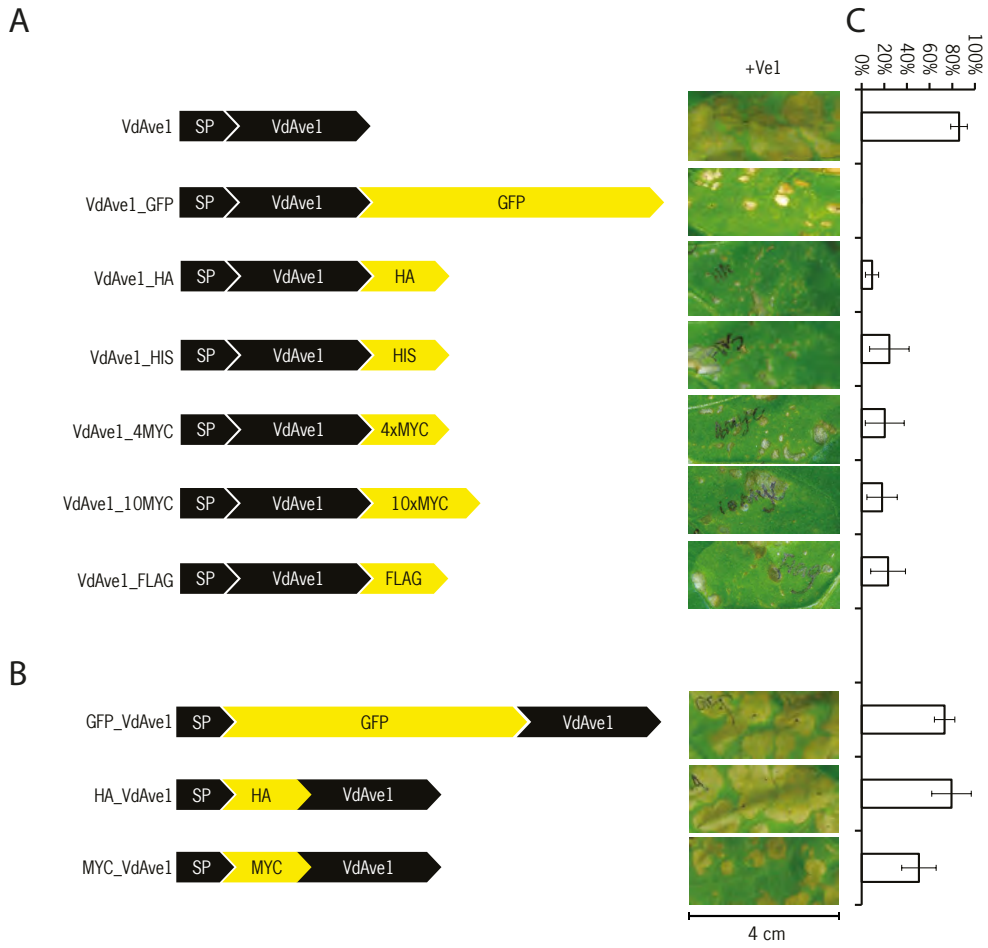

FIGURE 2 | C-terminal fusion of a GFP tag to VdAve1 results in loss of recognition by Ve1. (A) The signal peptide of VdAve1 (SP) directs secretion of untagged VdAve1 (VdAve1), C-terminally tagged VdAve1 (VdAve1_GFP, VdAve1_HA, VdAve1_HIS, VdAve1_4MYC, VdAve1_10MYC, VdAve1_FLAG) into the extracellular space. The constructs were co-expressed with Ve1 in *N. tabacum* upon agroinfiltration and the occurrence of the hypersensitive response was monitored. (B) The signal peptide of VdAve1 (SP) directs secretion of N-terminally tagged VdAve1 (GFP_VdAve1, HA_VdAve1 and MYC_VdAve1) into the extracellular space. The constructs were co-expressed with Ve1 in *N. tabacum* upon agroinfiltration and the occurrence of the hypersensitive response was monitored. All pictures were taken at five days post infiltration. (C) Quantification of cell death resulting from recognition of tagged VdAve1 proteins by Ve1. The graph shows the average percentage of necrotic leaf area of infiltration zones at five days post infiltration (n>5). Data are presented as mean with standard deviations.

To further investigate the role of the C-terminus in recognition of VdAve1 by Ve1, a number of C-terminal truncations was generated. Deletion of 42 amino acids of the C-terminus, from Lys93 to Ile134 (VdAve1_M1), resulted in loss of recognition by

Ve1 (Figure 3). Subsequent analysis of step-wise smaller truncations revealed that already a C-terminal deletion of nine amino acids (VdAve1_M4) resulted in loss of Ve1 recognition (Figure 3), despite the expression of stable protein (Supplemental Figure 2).

We subsequently performed complementation experiments in *V. dahliae* to confirm the importance of the C-terminus for immune activation. To this end we expressed the *VdAve1_M4* construct (Figure 3) in a *V. dahliae VdAve1* deletion strain and inoculated *Ve1* tomato with this transgenic fungus. Plants that were inoculated with *V. dahliae* expressing this *VdAve1* construct showed a similar disease phenotype as plants inoculated with a *VdAve1* deletion strain, whereas plants inoculated with wild type *V. dahliae* and the *VdAve1* complementation strain resembled mock-inoculated plants (Figure 4A; B). Collectively, our results show that the C-terminal nine amino acids of VdAve1 are required to establish Ve1-mediated immunity.

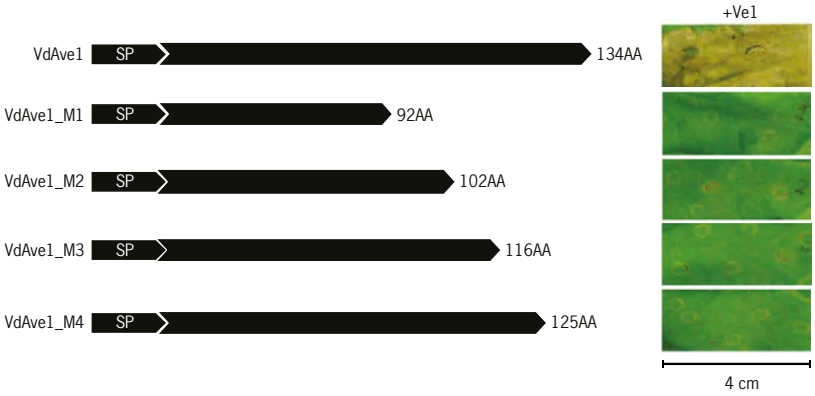

FIGURE 3 | Deletion of the C-terminal nine amino acids from VdAve1 results in loss of recognition by Ve1. Occurrence of the hypersensitive response in *N. tabacum* upon co-expression of VdAve1 truncations with Ve1. Constructs encoding full length VdAve1 with its native signal peptide (SP) and four truncations that lack the C-terminal 42 (VdAve1_M1), 32 (VdAve1_M2), 18 (VdAve1_M3) and 9 (VdAve1_M4) amino acids of VdAve1 were tested. Pictures were taken at five days post infiltration.

Ave1 recognition cannot be assigned to a single amino acid residue

Since the C-terminal nine amino acids appear to be essential for VdAve1 recognition, and the bacterial homolog XacPNP that is significantly divergent in this region (Figure 1A) is not recognized by Ve1 (Figure 1B and C), an expression construct for a chimeric Ave1 protein was engineered in which the C-terminal nine amino acids of VdAve1 were replaced by those of XacPNP (Vd_Xac9AA; Figure 5A; D). As expected, co-expression of the Vd_Xac9AA chimera with Ve1 in *N. tabacum* resulted in absence of recognition, as only small necrotic spots were observed at

most (Figure 5A). Conversely, a chimeric Xac_Vd9AA protein was constructed, in which the last nine amino acids of XacPNP were replaced by those of VdAve1. Co-expression of Xac_Vd9AA with Ve1 in *N. tabacum* resulted in a relatively strong HR, although full necrosis was not observed in the infiltrated leaf area (Figure 5A; D). With immunodetection the stability of the chimeric Vd_Xac9AA and Xac_Vd9AA proteins was confirmed (Supplemental Figure 3).

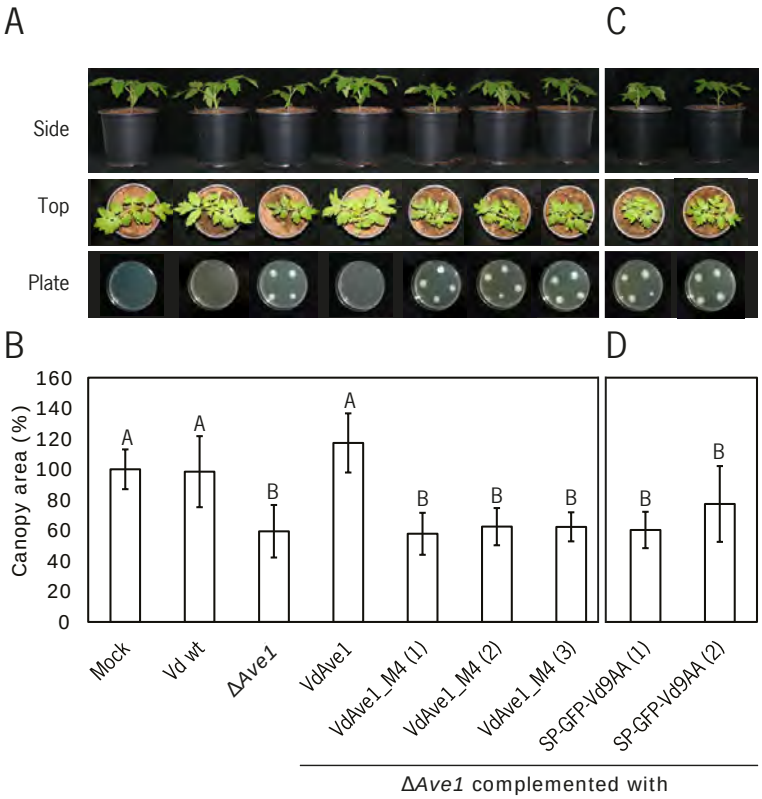

FIGURE 4 | Complementation experiments in *V. dahliae* confirm that the C-terminal nine amino acids are required to activate Ve1 immunity in tomato. (A) *V. dahliae Ave1* deletion strains expressing a construct encoding VdAve1 lacking the C-terminal 9 amino acids (VdAve1_M4) are unable to activate Ve1 immunity in tomato when compared with *V. dahliae* wild-type (Vd wt) and genetic complementation (VdAve1). (B) Quantification of the tomato canopy area (n=8). Data are presented as mean with standard deviations. Different letters indicate significant differences (P<0.05; One-Way ANOVA). (C) *V. dahliae Ave1* deletion strains expressing a construct that encodes the C-terminal 9 amino acids fused to GFP (SP-GFP-9AA) are unable to activate Ve1 immunity in tomato compared to *V. dahliae* wild-type (Vd wt) and genetic complementation (VdAve1). (D) Quantification of the tomato canopy area (n=8). Data are presented as mean with standard deviations. Different letters indicate significant differences (P<0.05; One-Way ANOVA).

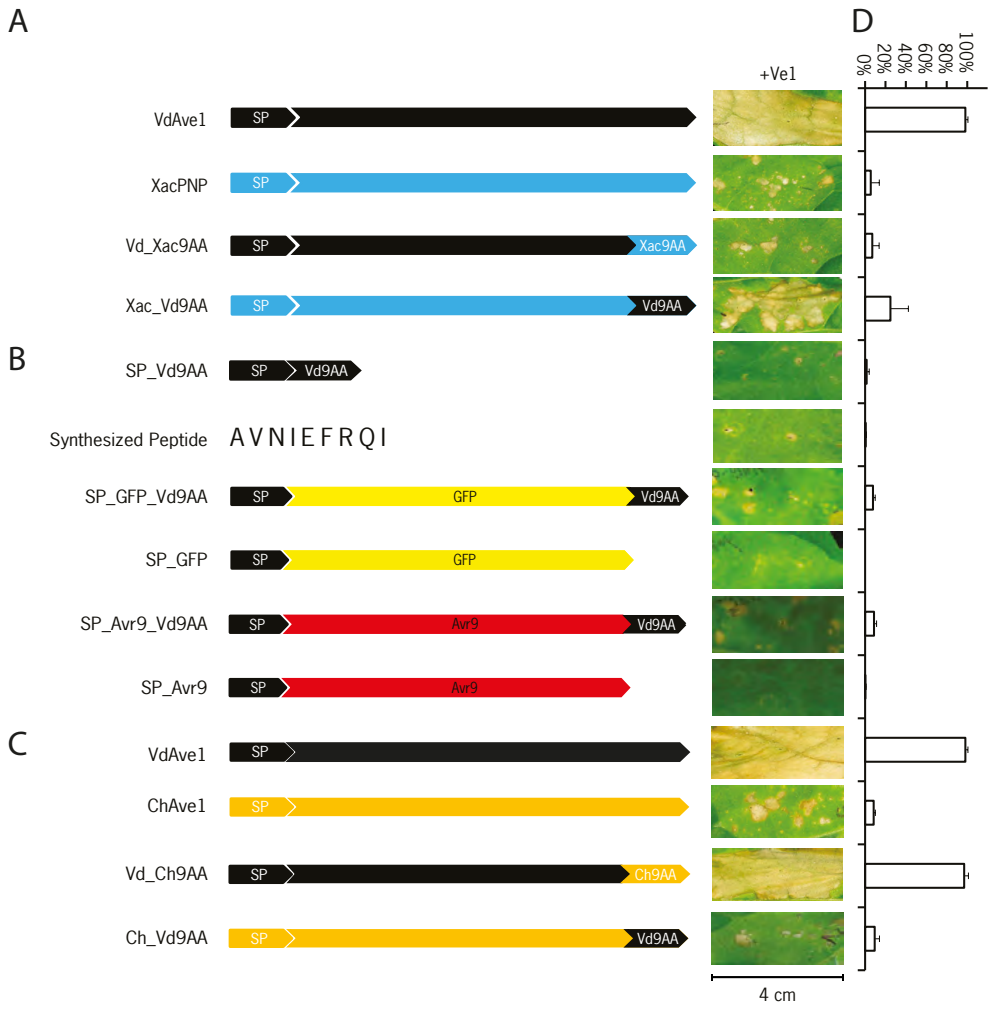

FIGURE 5 | Domain swaps reveal the importance of the C-terminal nine amino acids of Ave1 homologs for recognition by Ve1. (A) Occurrence of the hypersensitive response in *N. tabacum* upon co-expression of *Ave1* chimeras with *Ve1*. Constructs encoding full length VdAve1 and XacPNP with their native signal peptides (SP) and two chimeras in which their C-terminal nine amino acids were exchanged were tested. In chimera Vd_Xac9AA the C-terminal nine amino acids of VdAve1 (Vd9AA) were replaced with those of XacPNP (Xac9AA), while in chimera Xac_Vd9AA Xac9AA was replaced by Vd9AA. (B) Construct SP_9AA encodes the C-terminal nine amino acids of VdAve1 (Vd9AA) fused to the VdAve1 signal peptide (SP). SP_GFP_Vd9AA encodes GFP that is C-terminally tagged with Vd9AA, and N-terminally fused to the VdAve1 signal peptide (SP) to establish extracellular targeting, while SP_GFP lacks the C-terminal Vd9AA fusion. Furthermore, a chemically synthetized peptide encompassing Vd9AA (AVNIEFRQI) was used. (C) Constructs encoding full length VdAve1 and ChAve1 with their native signal peptides (SP) and two chimeras in which their C-terminal 9 amino acids were exchanged were tested. In chimera Vd_Ch9AA the C-terminal nine amino acids of VdAve1 (Vd9AA) were replaced with those of ChAve1 (Ch9AA), while in chimera Ch_Vd9AA Ch9AA was replaced byVd9AA. All pictures were taken at five days post infiltration. (D) Quantification of cell death resulting from recognition of chimera proteins by Ve1. The average percentage of necrotic leaf area of infiltration zones at five days post infiltration (n>10). Data are presented as mean with standard deviation.

To further unravel how the C-terminal nine amino acids contribute to recognition of VdAve1, we performed site-directed mutagenesis. Since the first of the nine residues (Ala126) is shared with XacPNP, we conclude that this one likely does not contribute to VdAve1 recognition. Thus, eight alanine scanning constructs were engineered in which the remaining residues were substituted by alanine (V127A, N128A, I129A, E130A, F131A, R132A, Q133A, and I134A, respectively; Figure 6A; B). The mutant constructs were tested in *N. tabacum* upon co-expression with *Ve1*, and HR development was measured at five days post infiltration by quantification of the leaf area that developed necrosis (Figure 6C). Similar to VdAve1, mutants R132A and Q133A activated strong Ve1-mediated HR, as >90% of the infiltrated leaf area developed necrosis (Figure 6C). In contrast, mutants N128A, I129A, E130A and F131A triggered clearly reduced necrosis, with <70% of the infiltrated leaf area developing necrosis (Figure 6C). Alanine substitution of Val127 and Ile134 (V127A and I134A, respectively) resulted in an intermediate reduction of necrosis-inducing activity, with 70-80% of the infiltrated leaf area developing necrosis (Figure 6C).

To further investigate the contribution of individual amino acids to recognition by Ve1, we engineered five additional mutants in which individual residues of the VdAve1 C-terminal nine amino acids were substituted by corresponding residues from XacPNP (V127R, N128T, E130P, F131W, and Q133N; Figure 6A; B). Whereas expression of F131W and Q133N induced similar necrosis as wild type VdAve1, mutants N128T and E130P exhibited significantly compromised HR upon co-expression with *Ve1*, with <60% of the infiltrated leaf area developing necrosis (Figure 6C). Mutant V127R did not induce HR at all in the presence of Ve1 (Figure 6C). Importantly, all site-directed mutants that showed compromised HR-inducing capacity accumulated to similar levels as VdAve1 or XacPNP (Supplemental Figure 4). These observations suggest that the region from Val127 to Phe131 is critical for recognition by Ve1. Collectively, we conclude that the C-terminal nine amino acids of VdAve1 are required for recognition by Ve1, and that recognition cannot be assigned to a single amino acid residue.

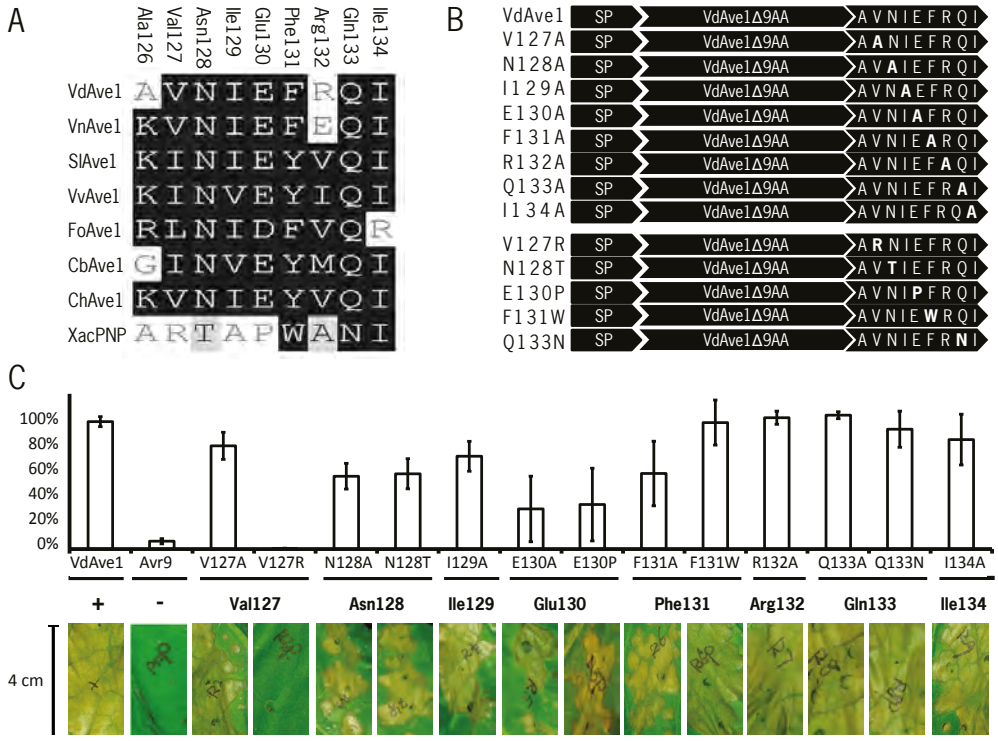

FIGURE 6 | Contribution of individual amino acid residues to recognition of the C-terminal nine amino acids of VdAve1. (A) Amino acid sequence alignment of the C-terminal nine amino acids of various Ave1 homologs. Identical and highly similar residues are indicated with black shading whereas weakly similar residues are indicated with grey shading. (B) Schematic representation of mutant proteins V127A, N128A, I129A, E130A, F131A, R132A, Q133A, I134A, V127R, N128T, E130P, F131W, and Q133N, respectively. Bold characters indicate the mutated residues. (C) Co-expression of tomato *Ve1* and *VdAve1* mutants in *N. tabacum*. The quantification of cell death resulting from recognition of VdAve1 mutants by Ve1 is shown as the average percentage of necrotic leaf area of infiltration zones at five days post infiltration (n>5). Data are presented as mean with standard errors.

The C-terminal nine amino acids of VdAve1 are not sufficient for recognition by Ve1

To determine whether the C-terminal nine amino acids are sufficient to trigger Ve1-mediated recognition, we generated a construct encoding the C-terminal nine amino acids of VdAve1 fused to the VdAve1 signal peptide (SP_Vd9AA) (Figure 5B; D). This construct was co-expressed with *Ve1* in *N. tabacum*, but no necrosis was observed in the infiltrated leaf (Figure 5B). Furthermore, also infiltration of a chemically synthetized peptide encompassing the C-terminal nine amino acids of VdAve1 was not able to trigger an HR in *Ve1*-expressing tobacco up to a

concentration of 1 mg/mL (Figure 5B; D). We speculated that the nine amino acid peptide may not be stable upon secretion in the apoplast, irrespective whether it is *in planta* expressed or injected upon chemical synthesis. In an attempt to overcome such complication, we generated constructs in which the coding sequence of GFP or Avr9 was N-terminally fused to the VdAve1 signal peptide and C-terminally fused to the C-terminal nine amino acids of VdAve1 (SP_GFP_Vd9AA and SP-Avr9_Vd9AA; Figure 5B; D). As negative controls, we generated constructs in which the coding sequence of GFP or Avr9 was N-terminally fused to the VdAve1 signal peptide without the C-terminal nine amino acids of VdAve1 (SP_GFP and SP_Avr9; Figure 5B; D). All constructs were co-expressed with *Ve1* in *N. tabacum* and HR development was monitored at five days post infiltration. However, only slight necrosis was found in the infiltrated sector despite the observation that the chimeric GFP proteins could be detected upon immunoblotting (Supplemental Figure 2).

Subsequently, a complementation assay was performed in *V. dahliae* to confirm that the C-terminal nine amino acids are not sufficient to activate immunity. To this end we expressed the SP_GFP_Vd9AA construct in the *Ave1* deletion strain of *V. dahliae* (Figure 4C; D) and inoculated *Ve1* tomato. Plants inoculated with strains expressing SP_GFP_Vd9AA showed similar symptoms as plants inoculated with the *VdAve1* deletion strain, whereas plants inoculated with wild type *V. dahliae* and the *VdAve1* complementation strain displayed similar symptoms as mock-inoculated plants (Figure 4A; D). Collectively, our results show that the C-terminal nine amino acids of VdAve1 are required but not sufficient to establish Ve1-mediated immunity.

Evidence for contribution to recognition in addition to the C-terminal nine amino acids

ChAve1 is closely related to VdAve1 as six of the nine C-terminal amino acids are identical. Nevertheless, only weak HR is detected upon co-expression of *ChAve1* and *Ve1* in *N. tabacum* (Figure 1B; C). To further investigate recognition of the C-terminal nine amino acids of ChAve1, an expression construct encoding a chimeric Ave1 protein was engineered in which the C-terminal nine amino acids of VdAve1 were replaced by those of ChAve1 (Vd_Ch9AA). Intriguingly, expression of Vd_Ch9AA resulted in complete necrosis of the infiltrated leaf area upon co-expression with *Ve1* in *N. tabacum*, whereas the reciprocal swap in which the C-terminal nine amino acids of ChAve1 were replaced by those of VdAve1 (Ch_Vd9AA) was not recognized by Ve1 (Figure 5C; D). With immunodetection the stability of the chimeric Vd_Ch9AA and Ch_Vd9AA proteins was confirmed (Supplemental Figure 5). These data provide further support for the finding that the C-terminal nine amino acids of VdAve1 are not sufficient to establish Ve1-mediated recognition, and confirm that additional requirements for the occurrence of the HR reside outside the stretch of the C-terminal nine amino acids.

A structural model of Ave1 suggests a contribution of both the N- and C-terminus of Ave1 to Ve1 recognition

In an attempt to get a better insight in Ave1 recognition by Ve1 we generated a structural model of Ave1. Structural comparison with the protein databank (RCSB PDB) (Rose et al. 2013) revealed the maize protein EXPB1 (PDB ID: 2HCZ) (Yennawar et al. 2006) as a potential structural analogue (TM-Score 0.84) of VdAve1. The Ave1 structural model shows that the C-terminal nine amino acids (shown in orange in Figure 7) are exposed at the surface of the Ave1 protein. The model also predicts that the N-terminus (shown in blue in Figure 7) congregates with the C-terminus, which led us to hypothesize that Ve1 potentially recognizes a patch of the Ave1 protein that includes both the N- and C-terminal. To test this hypothesis, we fused various tags to the N-terminal of Ave1, between the signal peptide and the mature peptide. Surprisingly, while N-terminal tagging of Ave1 with GFP and HA did not affect recognition, N-terminal tagging with MYC compromised recognition (Figure 2B; C). Immuno blotting confirmed that the GFP fusions were stably expressed (Supplemental Figure 2).

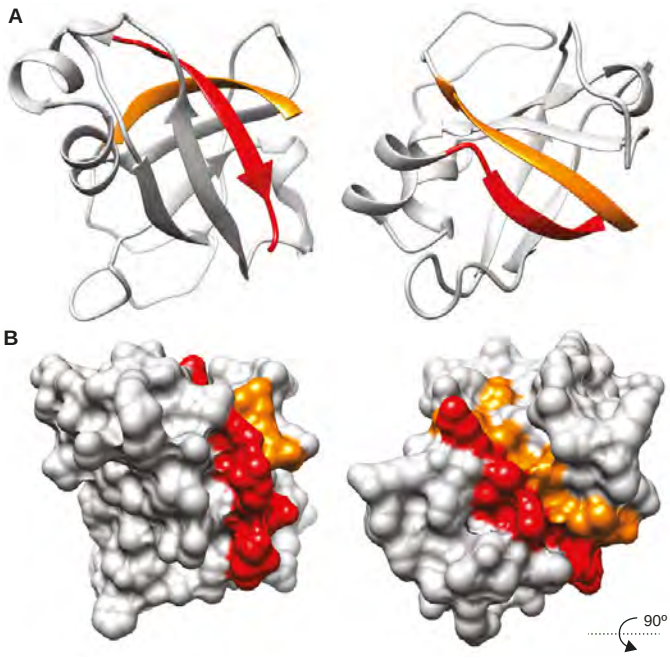

FIGURE 7 | Model for the 3D structure of the *Verticillium dahliae* effector VdAve1. The *V. dahliae* VdAve1 structure was predicted using I-TASSER (Zhang 2008). The inferred VdAve1 structure is of high quality indicated by a confidence score (C-Score) of 1.22, and displayed as a ribbon (A), and surface (B) model from the side (left) and the top (right). The C-terminal nine amino acid sequence of VdAve1 is indicated in orange, while the N-terminal eight amino acid sequence is indicated in blue.

Discussion

The C-terminal nine amino acids of Ave1 are required for Ve1-mediated recognition

We have previously shown that the VdAve1 effector of *V. dahliae* is recognized by the tomato immune receptor Ve1, and we identified several homologs in other species that are differentially recognized by Ve1 (Figure 1B; C) (de Jonge et al. 2012). In this study, we demonstrate that the C-terminal nine amino acids of VdAve1 are required for the recognition by Ve1, as truncations of VdAve1 lose recognition. The sequence of the C-terminal nine amino acids of the Ave1 homologs that are recognized (VdAve1, VnAve1, SlPNP, VvAve1, FoAve1, CbAve1 and also ChAve1) only share Asn128 and Gln133 that are completely conserved (Figure 6A). However, the other amino acids in this region are characterized by a high degree of conservation. A similar sequence variation in peptide epitopes, allowing substitutions by amino acids with similar chemical properties has previously been observed, for instance in flagellin, CLV3 and AtPep1 (Kondo et al. 2008; Naito et al. 2008; Pearce et al. 2008; Song et al. 2012).

To further analyse the contribution of individual amino acids in the C-terminal nine amino acids to recognition by Ve1, we performed site-directed mutagenesis on VdAve1. The sequence alignment and mutagenesis analysis suggest that the five neighbouring residues Val127, Asn128, Ile129, Glu130 and Phe131 are the major determinants of recognition of the C-terminal nine amino acids. Whereas alanine substitution of Val127 (V127A) only slightly reduced the HR-inducing capacity of VdAve1, VdAve1 cannot be recognized when Val127 is substituted by an arginine (V127R) that occurs in XacPNP on that position. Conversely, the HR-inducing capacity of VdAve1 is significantly impaired when Phe131 was replaced by the non-similar amino acids alanine (F131A), while substitution by tryptophan (F131W), an amino acid with similar chemical properties that occurs in XacPNP on that position, still triggers strong HR.

The C-terminal nine amino acids are not sufficient to trigger Ve1-mediated recognition

To test whether the C-terminal nine amino acids are sufficient to trigger Ve1-mediated HR a synthesized peptide and a construct in which the C-terminal nine amino acids of Ave1 were fused to GFP were co-expressed with Ve1. The C-terminal nine amino acids are not sufficient to trigger Ve1-mediated recognition as neither construct encoding the C-terminal nine amino acids peptide alone nor a GFP fusion construct can activate Ve1-mediated HR. With a *V. dahliae* complementation assay

we confirmed that the C-terminal nine amino acids of Ave1 are not sufficient to stop the fungus from colonizing *Ve1* tomato. Altogether, these results show that the C-terminal nine amino acid sequence is required, but not sufficient, to trigger Ve1-mediated immunity. Thus, recognition may involve additional residues besides the nine amino acid sequence to initiate Ve1-mediated immunity. To test if recognition involves residues immediately adjacent to the nine amino acids, GFP fusion constructs should be generated with longer C-terminal sequences.

We noticed that expression of ChAve1 with Ve1 does not trigger the activation of HR although an Ave1 chimera containing the C-terminal nine amino acids of ChAve1 have the potential to be recognized. Conversely, a ChAve1 chimera containing the C-terminal nine amino acids of VdAve1 does not trigger the activation of an HR. This also supports the hypothesis that a stretch outside the nine amino acid sequence is required to activate Ve1 immunity. However, obviously it needs to be realized that Ve1 evolved in tomato to recognize attempted host colonization by *V. dahliae*, while *C. higginsianum* is not a tomato pathogen and thus Ve1 did not have the chance to co-evolve with ChAve1.

A structural model of Ave1

Plasma membrane-localized PRRs are often activated upon recognition of a short peptide sequence on the surface of their PAMP ligands, such as flg22 or flgII-28 derived from flagellin, elf18 or EFa50 derived from EF-Tu, and pentapeptide TKLGE derived from EIX (Cai et al. 2011; Furukawa et al. 2014; Rotblat et al. 2002; Zipfel et al. 2006; Zipfel et al. 2004). Similarly, a 20-24 peptide in NLP effectors conserved in organisms from three kingdoms of life was shown to be sufficient to activate the PRR receptor RLP23 in Arabidopsis (Albert et al. 2015; Böhm et al. 2014; Oome et al. 2014). However, for the flax-rust effectors AvrM and AvrL567 it has been suggested that multiple contact points are required for recognition by the corresponding flax R-proteins (Ve et al. 2013; Wang et al. 2007). Similarly, multiple residues at separate locations on the surface of the ATR1 effector of *Hyaloperonospora arabidopsidis* are required for recognition by the R-protein RPP1 in Arabidopsis (Chou et al. 2011; Goritschnig et al. 2016). Fusion of various tags to the C-terminus of Ave1 resulted in loss of recognition and fusion of a MYC tag to the N-terminus of Ave1 resulted in reduced Ve1-mediated HR. We generated a structural model of Ave1 to get a better insight in the recognition by Ve1. This model shows that the N- and C-terminal congregate and are exposed at the protein surface. We therefore hypothesize that residues at both the N- and C-terminus of Ave1 are required for recognition by Ve1. This can be tested by generating N-terminal deletions of VdAve1 for co-expression with Ve1.

Involvement of C-terminal nine amino acids in Ave1 virulence

Ave1 has been characterized as an ortholog of plant natriuretic peptides (PNPs) (de Jonge et al. 2012). Natriuretic peptides were originally identified in animals as atrial natriuretic peptides (ANPs); polypeptide hormones that are secreted by heart muscle cells that are involved in the homeostatic control of salt and water balance and blood pressure (Potter et al. 2009). It has been demonstrated that *X. axonopodis* pv. *citri* utilizes a PNP homolog, XacPNP, to modulate the regulation of host plant homeostasis to establish infection (Gottig et al. 2008). The high homology between tomato SlAve1and *V. dahliae* VdAve1, likely due to acquisition of VdAve1 by *V. dahliae* from plants through horizontal gene transfer (HGT), in combination with the fact that VdAve1 acts as a virulence factor of *Verticillium* in susceptible tomato plants, suggests that *V. dahliae* utilizes VdAve1 to modulate host plant responses to generate conditions that favour colonization.

Absence of allelic variation in Ave1 alleles from *V. dahliae* and *V. albo-atrum* suggests that identical alleles are required for maximum virulence (de Jonge et al. 2012). However, the importance of the C-terminal amino acids for the function of VdAve1 related to virulence function is unknown. Mutational analysis within Pep-13, a surface-exposed fragment of a calcium-dependent cell wall transglutaminase (TGase) from the oomycete plant pathogen *Phytophthora sojae* that activates plant immune responses, identified the same amino acids indispensable for both TGase activity and immune activation (Brunner et al. 2002). Similarly, amino acid residues that are required for immunity-inducing activity of the flagellin-derived peptide flg22 were found to be important for the intrinsic flagellar function, as mutant flagella were unstable and impaired in flagellar motility (Naito et al. 2008). However, in contrast, the surface exposed pentapeptide TKLGE epitope of the *Trichoderma viride* ethylene-inducing xylanase (EIX) that is essential for recognition by the eLRR receptor LeEIX2 is not involved in xylanase enzymatic activity (Ron and Avni 2004; Rotblat et al. 2002). Further research, involving the complementation of VdAve1 deletion strains in *V. dahliae* with truncated versions of VdAve1, will reveal the particular involvement of the C-terminal nine amino acids in VdAve1 function.

Processing of the VdAve1 protein into smaller peptides might occur during infection. This is the case for the biologically active peptide in animal atrial natriuretic peptides (ANPs) which is a C-terminally derived 28 amino acid peptide that is cleaved from the 126 amino acid ANP precursor (Potter et al. 2009). The importance of precursor maturation has been demonstrated for the recognition of the CLV3 peptide by the eLRR-containing cell-surface receptor CLV2. Receptor CLV2 directly recognizes a 12 amino acid CLE motif of the Arabidopsis CLV3 peptide, that is also conserved in the nematode CLE-like GrCLE peptide, to modulate shoot apical meristem maintenance

during development (Guo et al. 2011; Ni et al. 2011; Wang et al. 2010). Both types of CLE peptides are cleaved from their larger precursor proteins. Studies have suggested that maturation of the CLE peptide is essential for its function (Fiers et al. 2006; Fiers et al. 2005; Guo et al. 2011; Ni and Clark 2006). In addition, elongation of chemically synthesized CLE peptides was found to decrease its activity (Kondo et al. 2008; Kondo et al. 2006).

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) and transgenic tomato (*Solanum lycopersicum* cv. MoneyMaker p*35S:Ve1*) (Fradin et al. 2011) plants were grown in the greenhouse at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity and 100 W/m^{-2} supplemental light when the light intensity dropped below 150 W/m^{-2}. After agroinfiltration, tobacco plants were grown in the climate room at 22°C/19°C during 16-h/8-h day/night periods, respectively, with 70% relative humidity.

Generation of expression constructs for *Ave1* homologs

To generate constructs for constitutive expression of the Ave1 homologs, the coding sequences of the homologs were cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction, and subsequently transferred into the Gateway-compatible destination vector pSol2092 (Zhang et al. 2013) to generate expression constructs driven by the constitutive CaMV35S promoter. *VdAve1*, *VnAve1* and *FoAve1* were amplified from cDNA using the primers attB-Vd-F and attB-Vd-R, attB-Vn-F and attB-Vn-R, attB-Fo-F and attB-Fo-R, respectively (Supplemental Table 2). *ChAve1*, *CbAve1*, *VvAve1*, *Sl*Ave1and *XacPNP* were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany).

Generation of expression constructs for GFP-VdAve1 fusion

To generate *VdAve1* fused at the C-terminus to the green fluorescent protein (GFP), the *VdAve1* coding sequence without stop codon was PCR amplified using primers containing Gateway attB sites (attB-Vd-F and attB-Vd-R-SC). The PCR fragment was cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction to generate entry vector pDONR207::VdAve1-SC. Subsequently, pDONR207::VdAve1-SC was transferred into the Gateway-compatible destination vector pSol2095 (Zhang et al. 2013) to generate an expression construct driven by the constitutive CaMV35S promoter and C-terminally tagged to GFP. Similarly,

pDONR207::VdAve1-SC was transferred into the Gateway-compatible destination vector pGWB14, pGWB8, pGWB17, pGWB20 and pGBW11 (Nakagawa et al. 2007) to generate VdAve1_HA, VdAve1_HIS, VdAve1_4MYC, VdAve1_10MYC, VdAve1_FLAG, respectively. To generate VdAve1 fused at the N-terminus to a GFP tag, the GFP coding sequence lacking the stop codon was fused at the N-terminus to the sequence encoding the signal peptide of VdAve1 to ensure extracellular targeting, and at the C-terminus to the *VdAve1* coding sequence (without signal peptide). This construct was obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany), and subsequently cloned into Gateway destination vector pSol2092. Fusion constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Generation of deletion and domain swap constructs for VdAve1

Deletion constructs VdAve1_M1, VdAve1_M2, VdAve1_M3 and VdAve1_M4 were amplified from cDNA using the forward primers attB-Vd-F in combination with reverse primers attB-VdM1-R, attB-VdM2-R, attB-VdM3-R, and attB-VdM4-R, respectively. Vd_Ch9AA, Ch_Vd9AA, and SP_GFP-Vd9AA were amplified from cDNA using the primers attB-Vd-F and attB-VdCh9AA-R, attB-Ch-F and attB-ChVd9AA-R, attB-Vd-F and attB-GFP-Vd9AA-R respectively (Supplemental Table 2). Vd_Xac9AA, Xac_Vd9AA and SP_Vd9AA were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany). The AVNIEFRQI peptides were synthesized by GenScript company (Piscataway, USA; Purity>75%). The deletion and domain swap constructs were cloned into pDONR207 and subsequently transferred into pSol2092 to generate expression constructs.

Generation of site-directed mutagenesis constructs for VdAve1

Site-directed mutagenesis constructs V127A, V127R, N128A, N128T, I129A, E130A, E130P, F131A, F131W, R132A, Q133A, Q133N, I134A were amplified from pDONR207::VdAve_M4 plasmid using the forward primers attB-Vd-F in combination with corresponding reverse primers (Supplemental Table 2). The PCR products were cloned into pDONR207 and subsequently transferred into pSol2092 to generate expression constructs. Subsequently, pDONR207 carrying mutant constructs were transferred into pSol2092 for expression *in planta*.

***Agrobacterium tumefaciens*-mediated transient expression**

Overnight cultures of *A. tumefaciens* strain GV3101 containing expression constructs were harvested at OD600 of 0.8 to 1 by centrifugation and resuspended to a final OD of 2 in infiltration medium as described previously (Zhang et al. 2013).

A. tumefaciens cultures containing constructs to express VdAve1 and Ve1 proteins were mixed in a 1:1 ratio and infiltrated into leafs of five- to six-week-old tobacco plants. At five days post infiltration (dpi), necrosis was examined and quantified by measuring the area of necrosis as percentage of the total infiltrated area.

Generation of *Verticillium dahliae* strains carrying VdAve1 constructs

pDONR207::*VdAve1*_M4 and pDONR207::SP_GFP-Vd9AA were used to clone the constructs into vector pFBT005 under the *VdAve1* promoter, containing a nourseothricin cassette for selection. *V. dahliae* was transformed by *A. tumefaciens* carrying pFBT005 as previously described (Santhanam 2012) and selected on PDA plates containing nourseothricin sulphate (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands). After five to seven days at room temperature, individual transformants were transferred to fresh PDA plates and incubated for ten days. Genomic DNA was extracted from the transformants and PCR was performed to test presence of the inserted nourseothricin cassette and of the inserted swap constructs.

Verticillium inoculation

For *V. dahliae* inoculations 10-day-old tomato plants were uprooted, rinsed in water and then dipped for 5 minutes in a suspension of 10^6 conidia per mL of water harvested from 1- to 2-week-old *V. dahliae* cultures on PDA as previously described (Fradin et al. 2009). Control plants got the same treatment, but the roots were dipped in water without conidiospores. After replanting in fresh soil, plants were incubated at standard greenhouse conditions. Disease development was monitored up to 21 dpi. The canopy area of 8 plants was measured with ImageJ software and a One-Way ANOVA was performed with IBM SPSS statistics software.

Generation of the structural model of VdAve1

The *V. dahliae* VdAve1 structure was predicted using I-TASSER v4.3 (Zhang 2008) and rendered using UCSF Chimera v1.10.1 (Pettersen et al. 2004). Structural predictions with C-Scores > -1.5 are generally considered to have a correct fold (C-Scores are typically in the range of [-5,2]) (Roy et al. 2010). The structural analog in the protein data bank (RCSB PDB) (Rose et al. 2013)was identified using the TM-align program which is part of the I-TASSER package. Analogous structures with TM-Scores > 0.5 are considered to have a similar fold (TM-Scores in the range [0,1]) (Roy et al. 2010).

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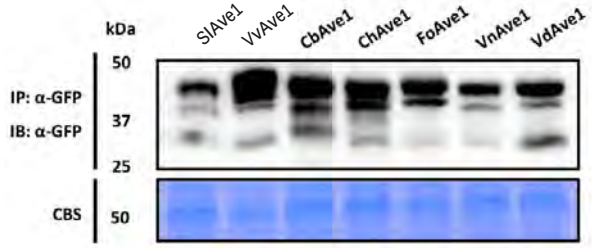
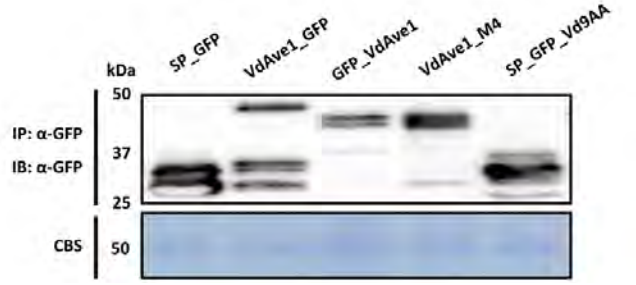
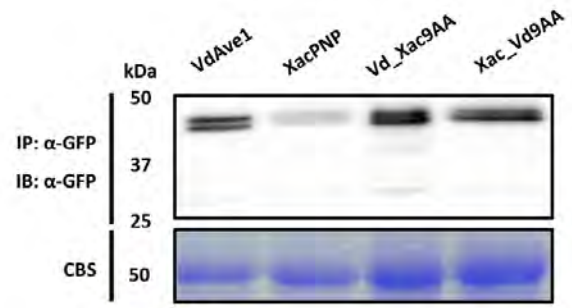
Supplemental data



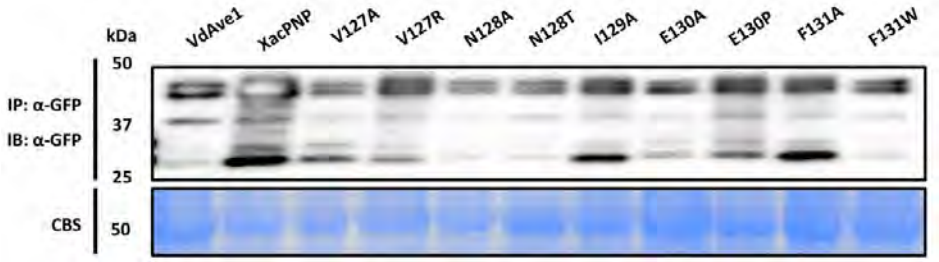
SUPPLEMENTAL FIGURE 1 | Immunoblotting of GFP-tagged Ave1 homologs. GFP-tagged Ave1 homologs were detected by western blotting using GFP antibody (α-GFP). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading.



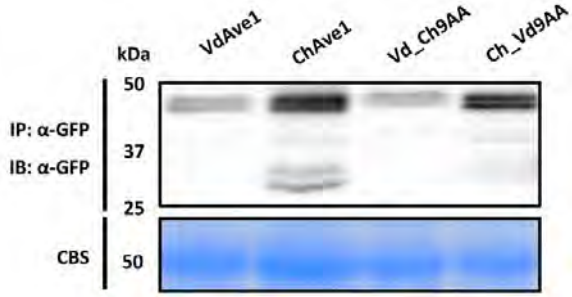
SUPPLEMENTAL FIGURE 2 |Stability of truncated Ave1 proteins. SP_GFP, VdAve1_GFP, GFP_VdAve1, GFP-tagged VdAve1_M4 and SP_GFP_Vd9AA proteins were detected using GFP antibody (α-GFP). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading.



SUPPLEMENTAL FIGURE 3 | Stability of chimeric Ave proteins Vd_Xac9AA and Xac_Vd9AA. GFP-tagged VdAve1, XacPNP, chimeric constructs Vd_Xac9AA and Xac_Vd9AA were detected by immunoblotting using GFP antibody (*α*-GFP). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading.



SUPPLEMENTAL FIGURE 4 | Stability of site-directed mutagenesis constructs that showed compromised HR-inducing capacity. GFP-tagged VdAve1, XacPNP, site-directed mutagenesis constructs V127A, V127R, N128A, N128T, I129A, E130A, F131A, F131W, R132A, Q133A, I134A were detected by immunoblotting using GFP antibody (*α*-GFP). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading.



SUPPLEMENTAL FIGURE 5 | Stability of chimeric Ave proteins Vd_Ch9AA and Ch_Vd9AA. GFP-tagged VdAve1, ChAve1, chimeric constructs Vd_Ch9AA and Ch_Vd9AA were detected by immunoblotting using GFP antibody. Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading.

SUPPLEMENTAL TABLE 1 | *Verticillium* strains analysed for presence of *Ave1* homologs

Species	Isolate	Original host	Origin	*Ave1*
V. nonalfalfae	P10	hop	Germany	-
	P114/1	hop	Germany	-
	P34/1	hop	Germany	-
	P15	hop	Germany	-
	P55	hop	Germany	-
	P83	hop	Germany	-
	6/99	hop	Germany	-
	14/93	hop	Germany	-
	15/98	hop	Germany	-
	P84/2	hop	Germany	-
	16/00	hop	Germany	-
	T2	hop	Slovenia	-
	TABOR6	hop	Slovenia	-
	Ledina09-V.aa	hop	Slovenia	+
	BIZ	hop	Slovenia	-
	VranBis09	hop	Slovenia	-
	Sent4	hop	Slovenia	-
	MO3	hop	Slovenia	-
	OCer	hop	Slovenia	-
	zup	hop	Slovenia	-
	Rec91	hop	Slovenia	-
	KRES98	hop	Slovenia	-
	Gajsek	hop	Slovenia	-
	1985a	hop	U.K.	-
	11041	hop	U.K.	-
	11055	hop	U.K.	-
	11047	hop	U.K.	-
	11097	hop	U.K.	-
	11100	hop	U.K.	-
	1974	hop	U.K.	-
	298099	hop	U.K.	-
	298100	hop	U.K.	-
	298101	hop	U.K.	-
	298102	hop	U.K.	-
	11052	hop	U.K.	-
	1953	hop	U.K.	-
	298092	hop	U.K.	-
	298095	hop	U.K.	-
	Sol	hop	Poland	+
	CBS393.91	hop	Belgium	-
	kum	cucumber	Slovenia	-
	Surf	surfinias	Slovenia	-
	11077	*Galinsoga ciliata*	U.K.	-
	11081	chrysanthemum	U.K.	+
	CBS102.464	cynara	Italija	-
	CBS241.82	catalpa	Italija	+
	CBS454.51	potato	U.K.	-
	CBS682.88	potato	Netherlands	-
	11066	potato	U.K.	-
	T179	tomato	U.K.	+
	CBS321.91	tomato	Netherlands	-

Species	Isolate	Original host	Origin	*Ave1*
	AR01/067	tomato	U.K.	-
	AR0/140	tomato	U.K.	-
	AR01/JS1	tomato	U.K.	-
	PD83/53a	tomato	Netherlands	-
	PD2000/4186a	tomato	Netherlands	+
V. alfalfae	Luc	alfalfa	U.K.	+
	41	alfalfa	Canada	-
	CBS392.91	alfalfa	Netherlands	-
	107	alfalfa	USA	-
	PD693	potato	Iran	-
	314193	potato	Australia	-
	340646	potato	Spain	+
	11	alfalfa	Slovenia	-
V. dahliae	JKG2	catalpa	Netherlands	-
	CIG3-Vd	hop	Slovenia	-
	JKG1	potato	Netherlands	-
	JKG8	potato	Netherlands	-
	A56	potato	Slovenia	+
	DJK	chrysanthemum	Netherlands	-
	MH	chrysanthemum	Netherlands	-
	Mint	mint	USA	-
	GAJ09	hop	Slovenia	-
	PDRENU	hop	Slovenia	-
	CasD	hop	Slovenia	-
	KresD	hop	Slovenia	-
	MoD	hop	Slovenia	-
	Oset	hop	Slovenia	-
	12099	hop	U.K.	-
	12042	hop	U.K.	-
	PD335	cabbage	unknown	-
	PD584	mint	unknown	-
	V-176l	cotton	Netherlands	+
	V-138l	cotton	Germany	-
	PAPmb	pepper	Slovenia	-
	PAP	pepper	Slovenia	+
	Pap99	pepper	Slovenia	+
	Pap2008	pepper	Slovenia	+
V. nubilum	CBS456.51	potato	UK	+
	CBS457.51	soil	UK	+
	PD621	mushroom compost	UK	+
	PD702	potato	UK	+
V. tricorpus	CBS227.84	potato	Netherlands	-
	JKG20	linden	Netherlands	-
V. longisporium	CBS110218	*Brassica napus*	Sweden	-
	PD330	cabbage	unknown	-

SUPPLEMENTAL TABLE 2 | Primers used in this study.

Primer name	Sequence (5′-3′)
attB-Vd-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGCTTTCTACGCTT
attB-Vd-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATATCTGTCTAAATTC
attB-Vn-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGCTTTCTACGCTT
attB-Vn-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATATCTGTTCAAACTC
attB-Fo-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAACTACTCGCACTA
attB-Fo-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCATCTTTGTACAAAATCGATATTT
attB-Vd-R-SC	GGGGACCACTTTGTACAAGAAAGCTGGGTCTATCTGTCTAAATTC
attB-VdM1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTATGCCTCGTTCCCTT
attB-VdM2-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAACAATGGCATCATATGAGT
attB-VdM3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTGTGTGCTGCTTTGGTAA
attB-VdM4-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTAAGCTCTGTCAACCACCCGCA
attB-VdCh9AA-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTCAAATTTGTACGTACTCAATGTTTACTTTCT-TATGCCTCGTTCCCTT
attB-Ch-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAGGCCTTCTTTCTAGGAG
attB-ChVd9AA-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATATCTGTCTAAATTCGATGTTGACC-GCAGTTACGCCAAAGTCCG
attB-GFP-Vd9AA-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTATATCTGTCTAAATTCGATGTTGACCGC TTTGTATAGTTCATCCATGC
V127A R	TTATATCTGTCTAAATTCGATGTTCGCCGCCTTATGCCTCGTTCCCTTC
N128A R	TTATATCTGTCTAAATTCGATCGCGACCGCCTTATGCCTCGTTCCCTTC
I129A R	TTATATCTGTCTAAATTCCGCGTTGACCGCCTTATGCCTCGTTCCCTTC
E130A R	TTATATCTGTCTAAACGCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
F131A R	TTATATCTGTCTCGCTTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
R132A R	TTATATCTGCGCAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
Q133A R	TTATATCGCTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
I134A R	TTACGCCTGTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
V127R R	TTATATCTGTCTAAATTCGATGTTTCTCGCCTTATGCCTCGTTCCCTTC
N128T R	TTATATCTGTCTAAATTCGATTGTGACCGCCTTATGCCTCGTTCCCTTC
E130P R	TTATATCTGTCTAAAGGGGATGTTGACCGCCTTATGCCTCGTTCCCTTC
F131W R	TTATATCTGTCTCCATTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
Q133N R	TTATATATTTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCTTC
Vd_Xac_V2 R	TTAAATATTTGCCCAGGGCGCTGTGACTGCCTTATGCCTCGTTCCCTTC
Vd_Xac_V5 R	TTAAATATTTGCCCATTCCGCTGTTCTTGCCTTATGCCTCGTTCCCTTC
Vd_Xac_V2V5 R	TTAAATATTTGCCCATTCCGCTGTGACTGCCTTATGCCTCGTTCCCTTC
Vd_Xac_V2V6 R	TTAAATATTTGCAAATTCCGCGTTGACTGCCTTATGCCTCGTTCCCTTC

Chapter 5

Characterization of an expanded family of Ave1 homologs in the apple and pear scab fungi *Venturia inaequalis* and *V. pirina*

Abstract

Venturia inaequalis and *V. pirina* are fungal pathogens that cause scab on apple and pear, respectively. Although several gene-for-gene interactions have been identified for *V. inaequalis* and apple, no avirulence gene has been cloned thus far. Recently, a family of *Ave1* homologs was identified in *V. pirinia*. *Ave1* was first identified in race 1 strains of *Verticillium dahliae* that has a wide host range including many crops, and was shown to be recognized by the tomato Ve1 immune receptor. In absence of *Ve1*, *Ave1* contributes to virulence of *V. dahliae*. Homologs of *Ave1* have been identified in a wide range of plants and in a handful of phylogenetically unrelated plant pathogens, including the fungi *Fusarium oxysporum*, *Cercospora beticola* and *Colletotrichum higginsianum* and the bacterium *Xanthomonas axonopodis*. In this study we found that also *V. inaequalis* contains a family of *Ave1* homologs. We show that the tomato Ve1 immune receptor is unable to recognize a selection of Ave1 homologs from *V. inaequalis* and *V. pirina*. Transcriptome analysis revealed that *V. inaequalis* *ViAve1-1*, *ViAve1-5* and *ViAve1-9* are expressed *in planta*. We show that *in planta* expressed *ViAve1-1*, *ViAve1-5* and *ViAve1-9* are unable to complement a *V. dahliae VdAve1* deletion strain, suggesting that these homologs have different functions than *V. dahliae VdAve1*.

Introduction

Plants evolved an immune system that detects conserved microbe-associated molecular patterns (MAMPS) to activate defense responses against invading pathogens (Cook et al. 2015). Successful plant pathogens evolved appropriate effectors; secreted molecules of which many can deregulate immunity in the respective host (Rovenich et al. 2014) and that are typically species, strain or even isolate specific. In turn, host plants typically evolve immune receptors that can recognize such effectors and mount an immune response to prevent further invasion. However, recognition of effectors and subsequent immune responses pose a huge selection pressure on pathogens to either evolve new effectors or alter or loose recognized effectors to prevent the activation of immune responses. This continuous co-evolution between host and pathogen is elegantly visualized in the zig-zag model (Jones and Dangl 2006). However, this model is focused from the point-of-view of the plant, while the point-of-view of the pathogen is largely underrepresented. Additionally, effectors and MAMPs are depicted as two distinct and opposing features of pathogen molecules, while this distinction is often not clear cut (Thomma et al. 2011). The recently proposed invasion model states that any signal that betrays an invading organism can potentially serve as a cue to activate an appropriate host response, either to a beneficial or detrimental organism (Cook et al. 2015).

Verticillium dahliae is a fungal plant pathogen with a broad host range that includes many crops (Fradin and Thomma 2006). *V. dahliae* race 1 strains express the effector Ave1 that activates immunity in tomato plants carrying the Ve1 receptor (de Jonge et al. 2012). In addition, it was shown that Ave1 contributes to the virulence of *V. dahliae*. Homologs of Ave1 are mainly found in plants and only in a handful of plant pathogens, including the fungi *Fusarium oxysporum*, *Cercospora beticola* and *Colletotrichum higginsianum* and the bacterium *Xanthomonas axonopodis*. Only one homolog of *Ave1* is found in the genome of each of these organisms, and these homologs share between 52-95% sequence similarity. In contrast, each *V. dahliae* strain analyzed thus far contains an additional *Ave1-like* (*Ave1L*) allele. Five alleles have been identified, denoted as *Ave1L1* to *Ave1L5* that share between 64 and 68% sequence similarity with *V. dahliae VdAve1* (Chapter 6, this thesis). Co-expression of Ave1 homologs derived from *Fusarium oxysporum*, *Cercospora beticola* and *Colletotrichum higginsianum* and *Xanthomonas axonopodis* with tomato immune receptor Ve1 revealed differential recognition of the various homologs (de Jonge et al. 2012).

Venturia inaequalis and *V. pirina* cause devastating scab diseases on apple and pear, respectively (Bowen et al. 2011; Shabi et al. 1973). Although a number of gene-for-gene interactions have been identified for *V. inaequalis* and its host apple (Bus et al. 2011; Gessler et al. 2006), no avirulence genes have been cloned thus far (Bus et al. 2011). AvrLm6 is an effector protein of the fungal pathogen *Leptosphaeria maculans* that triggers an immune response in Brassica plants expressing the immune receptor Rlm6 (Fudal et al. 2007). Homologs of *AvrLm6* have been identified in other plant pathogens, such as *Colletotrichum* and *Fusarium* species (Grandaubert et al. 2014). Interestingly, an expanded family of *AvrLm6* effector homologs was identified in the genomes of *V. inaequalis* and *V. pirina* (Shiller et al. 2015). However, the *V. inaequalis* AvrLm6 homolog with the highest sequence similarity to *L. maculans* AvrLm6 was unable to trigger an immune response in Brassica plants expressing the immune receptor Rlm6 (Cooke et al. 2014). Recently it was found that the *V. pirina* genomes also contains an expanded *Ave1* effector gene family, which is in contrast to the plant pathogens *Fusarium oxysporum*, *Cercospora beticola* and *Colletotrichum higginsianum* and *Xanthomonas axonopodis* that carry only one homolog of *Ave1* (Cooke et al. 2014; de Jonge et al. 2012). In this study we test whether any of the homologs in the expanded *Ave1* family derived from *Venturia* spp. is recognized by the tomato Ve1 immune receptor.

Results

Ave1 homologs derived from *Venturia inaequalis* and *V. pirina* are not recognized by tomato Ve1

Previously, 14 loci with homology to *V. dahliae VdAve1* were identified in the genome of *V. pirina*, including 12 genes (*VpAve1-1* to *VpAve1-12*) and 2 pseudogenes (*VpAve1-13* and *VpAve1-14*) (Cooke et al. 2014) (Joanna Bowen personal communication). We similarly analysed the genome of *V. inaequalis* for the presence of *Ave1* homologs. In this analysis, 17 loci were identified comprising 9 genes (*ViAve1-1* to *ViAve1-5* and *ViAve1-8* to *ViAve1-10*) and 8 pseudogenes (*ViAve1-6*, *ViAve1-7* and *ViAve1-11* to *ViAve1-16*) (Kim Plummer personal communication). The *Ave1* homologs derived from *V. inaequalis* and *V. pirina* display between 51-63% sequence similarity to *V. dahliae Ave1* (*VdAve1*). Alignment of the gene products of *V. inaequalis* and *V. pirina* Ave1 homologs with *V. dahliae* VdAve1 shows a high degree of sequence conservation distributed over the whole protein sequence (Figure 1). We hypothesized that since Ave1 is recognized by tomato immune receptor Ve1, *Venturia* Ave1 homologs are potentially recognized by Ve1 as well. To test this hypothesis, we co-expressed *Ve1* with *Ave1* homologs from *V. inaequalis* (*ViAve1-1*, *ViAve1-2*, *ViAve1-4*, *ViAve1-5*, *ViAve1-8*, *ViAve1-9*, *ViAve1-10*), and *Ave1*

homologs from *V. pirina* (*VpAve1-1*, *VpAve1-2*, *VpAve1-3*, *VpAve1-4*, *VpAve1-5*, *VpAve1-6*, *VpAve1-7*, *VpAve1-11*). Unfortunately, we were unable to clone *Ave1* homologs *ViAve1-3*, *VpAve1-8*, *VpAve1-9*, *VpAve1-10* and *VpAve1-12*. Whereas at 5 days post inoculation (dpi) a clear hypersensitive response (HR) was observed upon co-expression of the control combination of *Ve1* and *V. dahliae Ave1*, no HR was observed in any of the co-inoculations of Ve1 with the selected *V. inaequalis* and *V. pirina Ave1* homologs, except for some small necrotic spots in the case of *ViAve1-4* and *ViAve1-9* (Figure 2A and B). These results suggest that the tomato immune receptor Ve1 is unable to recognize Ave1 homologs derived from the apple and pear scab fungi *V. inaequalis* and *V. pirina*, respectively.

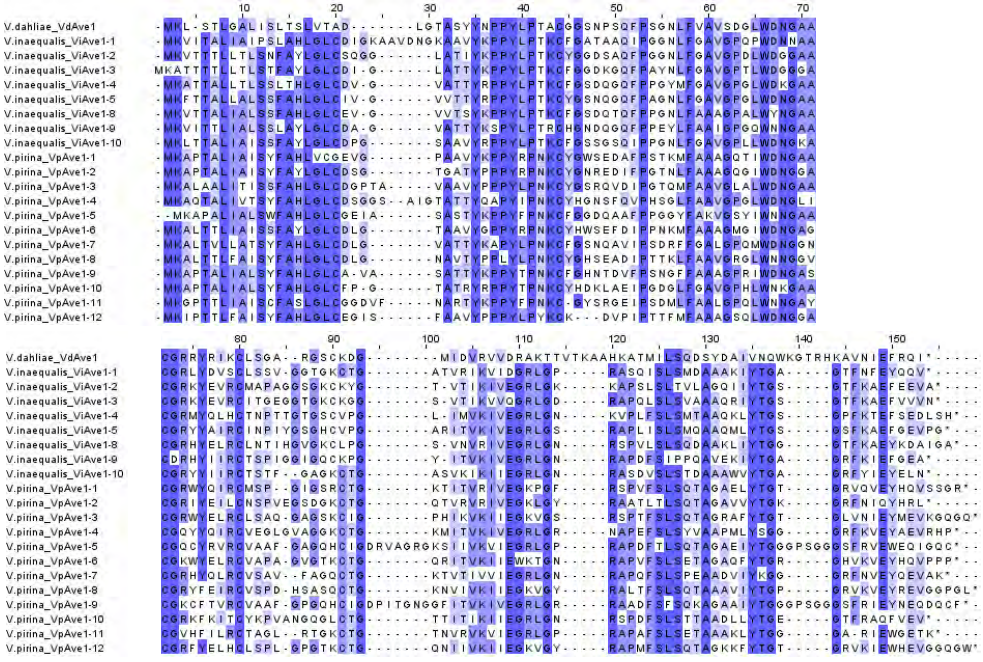


FIGURE 1 | Alignment of Ave1 homologs indicates regions with high conservation Alignment of Ave1 homologs derived from *Venturia inaequalis* and *V. pirina* with *V. dahliae* VdAve1. The intensity of the blue color indicates the degree of conservation.

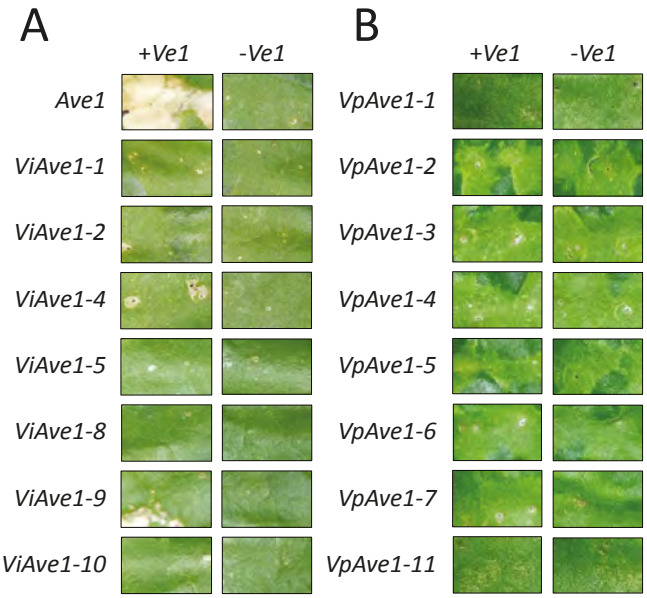


FIGURE 2 | Ave1 homologs derived from *Venturia inaequalis* and *V. pirina* are not recognized by tomato Ve1 (A) Co-expression of selected *Ave1* homologs from *Venturia inequalis* with tomato *Ve1*. (B) Co-expression of selected *Ave1* homologs from *Venturia pirina* with tomato *Ve1*.

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Ave1 homologs derived from *Venturia inaequalis* cannot complement loss of *Verticillium dahliae* Ave1

A method to mimic leaf or fruit surface penetration and *in planta* expression is to grow *V. inaequalis* on cellophane discs. Therefore, transcriptome analysis was performed on *V. inaequalis* grown on cellophane discs (Kucheryava et al. 2008). This analysis revealed that the *Ave1* homologs *ViAve1-1, ViAve1-5* and *ViAve1-9* are induced *in planta* (Kim Plummer personal communication). To test whether the *Ave1* homologs from *V. inaequalis* share the same functionality as *V. dahliae Ave1* to promote fungal virulence, we transformed a *V. dahliae Ave1* deletion strain with constructs to express *ViAve1-1*, *ViAve1-5* and *ViAve1-9* driven by the *V. dahliae Ave1* promotor. After confirmation that the strains contain the expression construct, the transformants were inoculated onto tomato plants that lack the Ve1 immune receptor and screened for virulence. Tomato plants that were inoculated with wild type *V. dahliae* and the *V. dahliae Ave1* deletion strain that was complemented with *VdAve1* as a control showed clear symptoms of Verticillium wilt. In contrast, the *Ave1* deletion strain and the transformants carrying expression constructs for *ViAve1-1*, *ViAve1-5* and *ViAve1-9* only induced reduced Verticillium wilt symptoms when compared with the symptoms induced by wild type *V. dahliae* (Figure 3A; B). To confirm that the transgenes were expressed, expression of *Ave1*, *ViAve1-1*, *ViAve1-5* and *ViAve1-9* by *V. dahliae*

in planta was analyzed. All genes were expressed at a similar level as *Ave1* by *V. dahliae* wild type (Figure 3D). Thus, *ViAve1-1*, *ViAve1-5* and *ViAve1-9* are unable to complement a *V. dahliae VdAve1* deletion strain, suggesting that these homologs display (a) different function(s) than *V. dahliae VdAve1.*

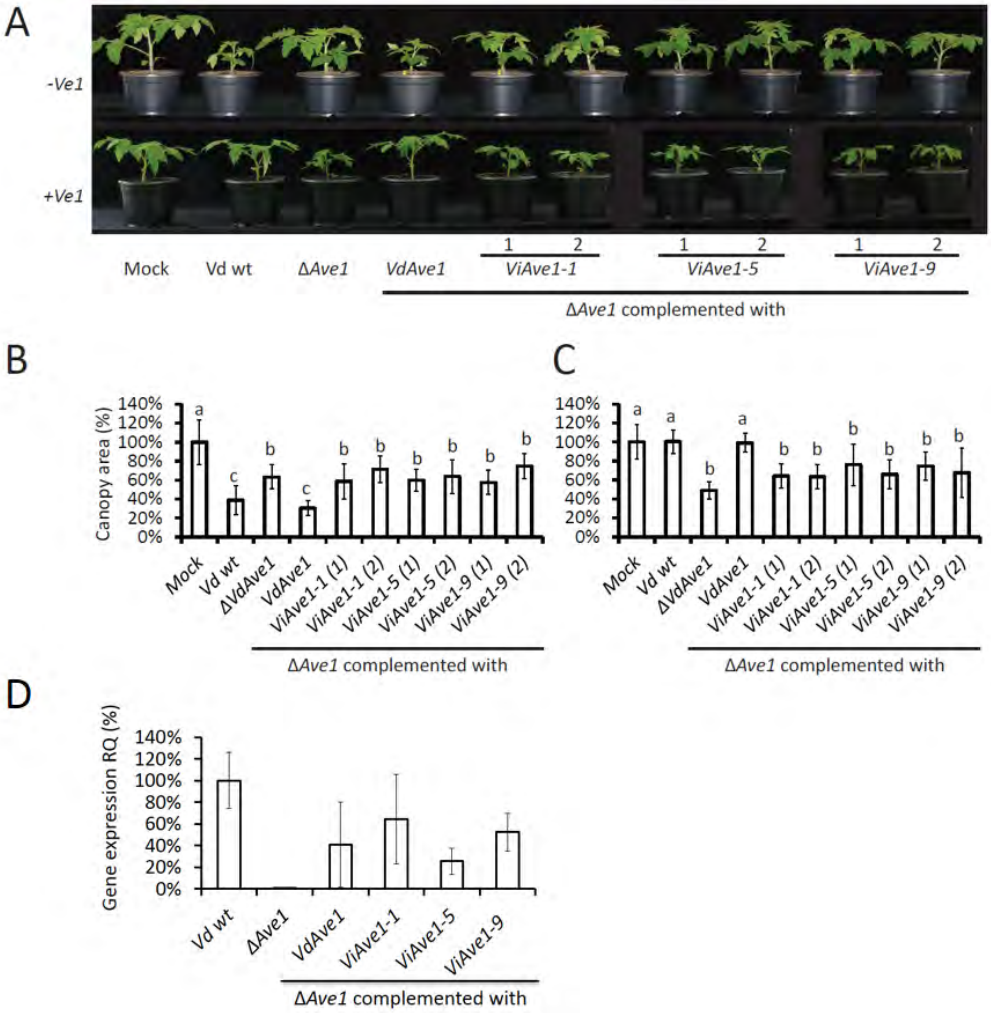

FIGURE 3 | *Ave1* homologs derived from *Venturia inaequalis* cannot complement loss of *Verticillium dahliae Ave1* (A) Complementation of *V. dahliae* Δ*Ave1* with *ViAve1-1*, *ViAve1-5* and *ViAve1-9* on tomato plants lacking *Ve1* (top) and *Ve1* tomato (bottom) (B) Canopy area of tomato plants lacking *Ve1* inoculated with *V. dahliae* Δ*Ave1* expressing *ViAve1-1*, *ViAve1-5* and *ViAve1-9* at 14 dpi (n=8). (C) Canopy area of *Ve1* tomato plants inoculated with *V. dahliae* Δ*Ave1* expressing *ViAve1-1*, *ViAve1-5* and *ViAve1-9* at 14 dpi (n=8). Data are presented as the mean ± standard deviation. Different letters indicate significant differences (P<0.05; One-Way ANOVA). (D) Gene expression of transgenes in a *V. dahliae VdAve1* deletion strain when compared with *VdAve1* expression in wild type *V. dahliae*.

Ave1 homologs derived from *Venturia inaequalis* are unable to initiate an immune response in *Ve1* tomato plants

From the co-expression analysis on *N. benthamiana* we concluded that the *Venturia* Ave1 homologs are not recognized by tomato Ve1 due to lack of a visible HR. Nevertheless, we have previously shown that Ve1 is able to establish immunity without signs of an HR under particular conditions (Zhang et al. 2013a). We therefore tested whether the *V. dahliae* transformants expressing *ViAve1-1*, *ViAve1-5* and *ViAve1-9* are contained when inoculated on tomato plants carrying *Ve1* (Figure 3A; C). Tomato plants that were inoculated with wild type *V. dahliae* and the *V. dahliae Ave1* deletion strain that was complemented with *VdAve1* as a control showed no symptoms of Verticillium wilt, indicating that they are successfully contained upon recognition by Ve1. In contrast, the tomato plants inoculated with the *Ave1* deletion strain and the transformants carrying expression constructs for *ViAve1-1*, *ViAve1-5* and *ViAve1-9* showed clear symptoms of Verticillium wilt. Together with the co-expression data this confirms that ViAve1-1, ViAve1-5 and ViAve1-9 are unable to induce a Ve1-mediated immune response.

Discussion

We have previously shown that the *Verticillium dahliae* effector *VdAve1* is recognized by the tomato immune receptor Ve1, and that homologs of Ave1 identified in particular other plant pathogens that share between 52 and 95% similarity are differentially recognized by Ve1 (de Jonge et al. 2012). Only one homolog was found in each of those plant pathogens, except for *V. dahliae* where subsequent analysis has shown that most isolates contain an additional *Ave1*-like (*Ave1L*) allele that shares between 64 and 68% sequence similarity with *Ave1* (Chapter 6 of this thesis). In the genomes of the apple scab fungus *V. inaequalis* and the pear scab fungus *V. pirinia* largely expanded families of *Ave1* homologs were identified (Cooke et al. 2014). These homologs share between 51 and 63% sequence similarity with Ave1. Alignment of VdAve1 and the *Venturia* Ave1 homologs shows that there is no specific region of the protein that is highly conserved. In this study we showed that Ave1 homologs derived from *V. inaequalis* and *V. pirina* are not recognized by Ve1. A reason for the lack of recognition might be the fact that *V. inaequalis* and *V. pirina* never co-evolved with Ve1 since tomato is not a host for these pathogens. Homologs of Ve1 have been found in many plant species, including functional homologs in various plants including tobacco, potato, eggplant and hop (Song et al. 2017a). Possibly, Ve1 homologs occur in apple and pear as well, that may potentially recognize one or more of the Ave1 homologs derived from *V. inaequalis* and *V. pirina*, respectively. Possible recognition by an immune receptor can be tested by

performing a transient expression assay with *ViAve1-1*, *ViAve1-5* and *ViAve1-9* on apple or pear (Li et al. 2004; Vleeshouwers and Oliver 2014).

We also showed that *Ave1* homologs *ViAve1-1*, *ViAve1-5* and *ViAve1-9* derived from *V. inaequalis* that are highly expressed *in planta* cannot complement the virulence function of a *V. dahliae Ave1* deletion strain on tomato. Possibly, to exert its function, Ave1 targets a host specific protein. If ViAve1-1, ViAve1-5 and ViAve1-9 have a similar function as Ave1 and target a host-specific protein, this protein may be absent from the *Verticillium*-tomato interaction and therefore Ave1 homologs cannot exert their function in other host plants. Host specificity of effectors has been described for the closely related oomycetes *Phytophtora infestans* and *P. mirabilis* that are pathogens on different hosts (Dong et al. 2014). Effector homologs derived from both pathogens are more efficient on their specific host target than on the foreign target. To test whether one or more of the Ave1 homologs are contributing to the virulence of *V. inaequalis*, individual deletion strains of *ViAve1-1*, *ViAve1-5* and *ViAve1-9* should be generated or gene silencing of multiple genes should be performed (Fitzgerald et al. 2004; Fitzgerald et al. 2003) and analyzed for full virulence on their host plant apple. However, unfortunately, targeted gene deletion is not straightforward in *Venturia* spp.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) and tomato (*Solanum lycopersicum* cv. MoneyMaker) plants were grown in the greenhouse at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity and 100 W/m^{-2} supplemental light when the light intensity dropped below 150 W/m^{-2}.

Generation of expression constructs

The coding sequences (CDSs) of *ViAve1-1*, *ViAve1-2*, *ViAve1-4*, *ViAve1-5*, *ViAve1-8*, *ViAve1-9*, *ViAve1-10* and *VpAve1-1*, *VpAve1-2*, *VpAve1-3*, *VpAve1-4*, *VpAve1-5*, *VpAve1-6*, *VpAve1-7*, *VpAve1-11*, were amplified from genomic DNA of *Venturia inaequalis* and *V. pirina*, respectively. The CDS was then cloned into pENTR-D (Invitrogen, Carlsbad, California) and subsequently transferred to the Gateway-compatible destination vector pSol2092 (Zhang et al. 2013b) to generate expression constructs driven by the constitutive CaMV35S promoter for *Agrobacterium tumefaciens*-mediated transient expression. The CDSs of *ViAve1-1*, *ViAve1-5* and *ViAve1-9* were cloned into destination vector pFBT005 to generate expression constructs driven by the *VdAve1* promoter for expression in *V. dahliae*.

Agrobacterium tumefaciens-mediated transient expression

A. tumefaciens GV3101 strains containing the expression constructs in pSol2092 were grown for two nights in liquid Yeast Extract Broth (YEB) and then resuspended to an OD_{600} of 2 in infiltration medium as previously described (Zhang et al. 2013b). *A. tumefaciens* strains that carry the expression constructs for the *V. dahliae* and *Venturia Ave1* homologs and tomato *Ve1* were mixed in a 1:1 ratio and infiltrated in the lower side of 5- to 6 weeks-old tobacco leafs. At 5 dpi the leafs were inspected for the presence or absence of a hypersensitive response.

Complementation of *Verticillium dahliae Ave1* deletion strains

The *A. tumefaciens* Agl1 strain containing the expression construct for *ViAve1-1*, *ViAve1-5* or *ViAve1-9* in pFBT005, respectively, driven by the *VdAve1* promoter, was used to transform a *V. dahliae* strain JR2 *Ave1* deletion strain (de Jonge et al. 2012) as previously described (Santhanam 2012). Genomic DNA was extracted from transformants to verify the presence of *ViAve1-1*, *ViAve1-5* or *ViAve1-9* and the presence of the nourseothricin cassette.

V. dahliae assay on tomato

For *V. dahliae* inoculations 10-day-old tomato seedlings were uprooted, rinsed in water and then dipped for 5 minutes in a suspension of 10^6 conidiospores per mL of water harvested from 1- to 2-week-old *V. dahliae* cultures on PDA as previously described (Fradin et al. 2009). Control plants received the same treatment, but the roots were dipped in water without conidiospores. After replanting in fresh soil, plants were incubated at standard greenhouse conditions. Disease development was monitored up to 21 dpi. The canopy area of 8 plants was measured with ImageJ software and a One-Way ANOVA was performed with IBM SPSS statistics software.

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Chapter 6

An *Ave1*-like effector displays allelic variation within the *Verticillium dahliae* population

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Abstract

Verticillium dahliae is a soil-borne fungal pathogen that causes vascular wilt disease on a broad range of host plants. The tomato immune receptor Ve1 is able to recognize race 1 strains of *V. dahliae* that secrete the Ave1 effector. We identified a gene in *V. dahliae* strain JR2 with high similarity to the *Ave1* effector gene that we called *Ave1L* (for *Ave1*-like). Subsequently, we identified in total five alleles (*Ave1L1* to *Ave1L5*) in the *V. dahliae* population. While some of the *V. dahliae* strains do not have *Ave1L*, other strains have one of the five *Ave1L* alleles in presence or absence of *Ave1*. The *Ave1L* alleles contain multiple single nucleotide polymorphisms that result in a stop codon in some of the alleles. In addition, one of the alleles contains a retrotransposon. These observations suggest that Ave1L experienced significant selection pressure, potentially to evade recognition by an immune receptor. Thus, we attempted to predict the common progenitor sequence of the five *Ave1L* alleles. However, we show that neither of the *Ave1L* alleles, nor the progenitor, are recognized by the tomato immune receptor Ve2 that shows high homology to Ve1. We furthermore show that *Ave1L2* is expressed during host colonization but does not seem to contribute to virulence on tomato. Since *V. dahliae* has a wide host range, *Ave1L* potentially contributes to virulence on other host plants than tomato where it may have been recognized by an immune receptor that drove its divergence.

Introduction

Plants can sense invading microbial pathogens through the recognition of conserved microbe-associated molecular patterns (MAMPs) such as bacterial flagellin, fungal chitin and oomycete β-glucans (Dodds and Rathjen 2010; Thomma et al. 2011). Recognition of MAMPs leads to the activation of immune responses to stop pathogen ingress. To gain further access to the host, pathogens produce effectors; secreted molecules that support host colonization. Although effectors may contribute to host colonization in many ways, most effectors that have been functionally characterized up to now specifically target host immune responses (Rovenich et al. 2014). In response to pathogen exploitation of effectors, plants evolved immune receptors that can monitor (the activity of) specific effectors to re-initiate immune responses, typically referred to as effector-triggered immunity (ETI) (Dodds and Rathjen 2010; Thomma et al. 2011). To overcome effector recognition, pathogens in turn may lose the recognized effector, evolve mutants that are no longer recognized or, alternatively, evolve novel effectors that can suppress the immune response. As a result of all this, plant immunity evolved to a robust system to detect invasion in which immunogenic molecules form a continuum and can be host- or microbe-derived and can have various intrinsic functions. Ultimately host receptors, termed invasion pattern receptors (IPRs), detect externally encoded as well as modified-self ligands, termed invasion patterns, that betray microbial invasion (Cook et al. 2015; Thomma et al. 2011).

The *Cladosporium fulvum*-tomato interaction is a well-established fungal model system that has been used to study effector-receptor interactions (de Wit 2016; Thomma et al. 2005). The first fungal avirulence gene was cloned from *C. fulvum* and to date a dozen additional effector genes have been cloned most of which act as avirulence genes (Bolton et al. 2008; Joosten et al. 1994; Lauge et al. 2000; Luderer et al. 2002; Mesarich et al. 2014; Stergiopoulos et al. 2007; Van den Ackerveken et al. 1993; van Kan et al. 1991; Westerink et al. 2004). As a consequence, variation within a fungal effector was also first reported in *C. fulvum* for which isoforms of the Avr4 effector were identified that are no longer recognized by the corresponding tomato immune receptor Cf-4 as a result of a frameshift mutation or a single point mutation in the *Avr4* gene that induces a single amino acid change (Joosten et al. 1997; Joosten et al. 1994). Other alterations were observed in *C. fulvum* effector Avr2 where, besides a frameshift mutation, the introduction of a premature stop codon or a retrotransposon in the gene results in a truncated protein that is no longer recognized by the corresponding tomato immune receptor Cf-2 (Luderer et al. 2002). In the case of *C. fulvum* effectors Avr4E and Avr9 some alleles contain point mutations while in other strains the complete gene is

deleted to avoid recognition by the corresponding tomato immune receptor Cf-4E and Cf-9, respectively (van Kan et al. 1991; Westerink et al. 2004). Importantly, while effector deletion or mutation can overcome effector recognition in plants carrying the corresponding immune receptor, such modification may compromise virulence on plants that lack this immune receptor. Although not yet observed in *C. fulvum*, pathogens may also evolve novel effectors to suppress recognition of a particular effector. This has been demonstrated for Avr1 of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* that can suppress immune responses that are activated by the tomato immune receptors I-2 and I-3 that recognize the Avr2 and Avr3 effectors, respectively (Houterman et al. 2008). Collectively, these examples illustrate that pathogens have evolved many ways to circumvent recognition by immune receptors of their host plants.

Verticillium dahliae is a soil-borne vascular pathogen and causal agent of vascular wilt disease in a broad range of plant species (Fradin and Thomma 2006). In contrast to the situation for *C. fulvum* for which many complex races have been described on tomato, the situation for *V. dahliae* is simple on that same host due to the existence of only a single dominant resistance source that divides the population in only two races. On tomato, race 1 strains of *V. dahliae* are contained by the Ve1 immune receptor whereas race 2 strains are not recognized (Fradin et al. 2009; Kawchuk et al. 2001). Recently, the race 1-specific effector that is recognized by Ve1 was identified as Ave1 (for Avirulence on *Ve1* tomato) (de Jonge et al. 2012). Interestingly, it was found that all race 2 strains that were analysed lack the complete *Ave1* gene. Moreover, it was shown that Ave1 contributes to the virulence of *V. dahliae* on plants that lack *Ve1*. Intriguingly, the *Ave1* CDS is completely identical in all 85 race 1 strains that have been analyzed to date, suggesting that sequence conservation is important for virulence (de Jonge et al. 2012). Intriguingly, many homologs of Ave1 are found in plants as well as in a few plant pathogens (de Jonge et al. 2012). Most of the plant homologs are annotated as plant natriuretic peptides (PNPs) or expansin-like proteins that are thought to be involved in water and ion homeostasis and therefore in many fundamental plant processes including growth, net water uptake, photosynthesis, stomatal opening and gas exchange (Gehring and Irving 2003; Ludidi et al. 2004; Maryani et al. 2001; Pharmawati et al. 2001). Interestingly, the *Ave1* homolog of the bacterial pathogen *X. axonopodis*, known as *XacPNP*, acts as a virulence factor by affecting homeostasis and photosynthesis in citrus to stimulate bacterial proliferation (Garavaglia et al. 2010; Gottig et al. 2008). The Ave1 homologs that occur in plant pathogens do not follow the phylogeny of the species in which they occur, which is interpreted as a sign of horizontal gene transfer, leading to believe that the microbial *Ave1* homologs have been acquired from plants (de Jonge et al. 2012; Gottig et al. 2008)

In this study, a BLAST search using *Ave1* against the recently finished gapless genome of *V. dahliae* strain JR2 (Faino et al. 2015) revealed an *Ave1-like* gene. Subsequent BLAST searches identified allelic variation in a population of *V. dahliae* strains.

Results

Identification of an *Ave1-like* gene in *Verticillium dahliae*

Using the genome of *V. dahliae* strain JR2 (Faino et al. 2015), a BLAST analysis using *Ave1* as a query identified a gene with 67% similarity that we will further refer to as *Ave1L* (for *Ave1-like*). Intriguingly, while *Ave1* encodes a protein of 134 amino acids (de Jonge et al. 2012), the CDS of *Ave1L* likely encodes a protein of only 24 amino acids due to a predicted stop codon. To determine whether *Ave1L* can be found in other *V. dahliae* strains as well, we performed BLAST analyses using *Ave1L* as a query on twenty recently sequenced *V. dahliae* genomes (Table 1) (de Jonge et al. 2012; Faino et al. 2015; Klosterman et al. 2011). While no *Ave1L* sequence was found in four genomes, the genomes of 16 *V. dahliae* strains were each found to carry a single *Ave1L* copy that displays 93-100% similarity to the initially identified *Ave1L*, while displaying 65-68% similarity to *Ave1*. Collectively, five different *Ave1L* alleles were identified, designated *Ave1L1* to *Ave1L5*. Following the phylogeny of the *V. dahliae* strains, *Ave1L1* was found in five strains that include JR2 and VdLs17 (de Jonge et al. 2012; Faino et al. 2015; Klosterman et al. 2011), *Ave1L2* in three strains, *Ave1L3* in five strains, *Ave1L4* in one strain and *Ave1L5* in two strains (Table 1, Figure 1B). Similar to the initially identified *Ave1L1*, also *Ave1L3* and *Ave1L4* are predicted to encode proteins of 24 amino acids. In contrast, *Ave1L2* and *Ave1L5* are predicted to encode proteins of 134 amino acids similar to *Ave1* (Figure 1A). Interestingly, the presence of *Ave1L* alleles did not correlate with presence of *Ave1*, as *Ave1L* alleles were found in race 1 as well as in race 2 strains (Table 1, Figure 1B).

Like Ave1, Ave1L shows similarity to plant natriuretic peptides (PNPs) or expansin-like proteins (de Jonge et al. 2012). To analyse whether Ave1L carries a conserved PNP domain as has been characterized in *Arabidopsis thaliana* PNP (AtPNP-A) (Ludidi et al. 2004; Morse et al. 2004) the amino acid sequences of Ave1, Ave1L and various PNPs were aligned to AtPNP-A and similarity was determined (Fig. 1C). Intriguingly, there does not seem to be a particularly conserved region among the proteins, and also the region that aligns with the PNP domain of AtPNP-A is not particularly conserved (Fig. 1C). The bacterial pathogen *X. axonopodis* secretes a PNP-like effector known as *XacPNP* that modulates host responses to facilitate bacterial proliferation on citrus. XacPNP comprises a 12 amino acid peptide that

displays a high degree of similarity to the PNP domain of AtPNP-A, and that exerts the biological activity of XacPNP (Gottig et al. 2008). However, Ave1L does not carry this 12 amino acid peptide, in contrast to Ave1 and the other PNPs included in the alignment (Figure 1C) (Gottig et al. 2008). This finding suggests that Ave1L does not function as a natriuretic peptide.

TABLE 1 | *Ave1L* alleles identified in sequenced *V. dahliae* genomes

Strain	Origin	Isolated from	Race**	*Ave1*	*Ave1L* allele	Number and position of premature stop codon(s)
JR2$	Canada	*Solanum lycopersicum*	1	Yes		
VdLs17Δ	USA	*Lactuca sativa*	2	No		
St16.01*	Syria	*Gossypium spp*	2	No	*Ave1L1*	1 (25W)
Vd57*	Germany	*Fragaria x ananassa*	2	No		
V#*	Netherlands	*Chrysanthemum spp.*	2	No		
CBS38166$	Canada	*Solanum lycopersicum*	1	Yes		
St14.01$	USA	*Pistacia vera*	1	Yes	*Ave1L2*	No
DVD-S26$	Canada	Soil	2	No		
DVD-3$	Canada	*Solanum tuberosum*	2	No		
DVD-31$	Canada	*Solanum lycopersicum*	2	No		
DVD-S94$	Canada	Soil	2	No	*Ave1L3*	4 (25W, 57Q, 120Q, 128Q)
DVD-161$	Canada	*Solanum tuberosum*	2	No		
JKG8#*	Netherlands	*Solanum tuberosum*	2	No		
DVD-S29$	Canada	Soil	2	No	*Ave1L4*	1 (25W)
St.100µ	Belgium	Soil	2	No	*Ave1L5*	No
463*	Mexico	*Gossypium spp.*	2	No		
2009-605*	Ukraine	*Capsicum annuum*	1	Yes		
Vd152*	Hungary	*Quercus spp*	2	No	No	x
Vd52*	Austria	*Capsicum annuum*	1	Yes		
Vd39*	Germany	*Helianthus annuus*	2	No		

* newly sequenced *V. dahliae* genomes

** The race nomination in *V. dahliae* is restricted to strains that are pathogenic on *Ve1* tomato in which race 1 strains are contained and race 2 strains are not.

$ de Jonge et al., 2012

Δ Klosterman et al., 2011

Ispahani et al., 2008

µ de Jonge et al., 2013

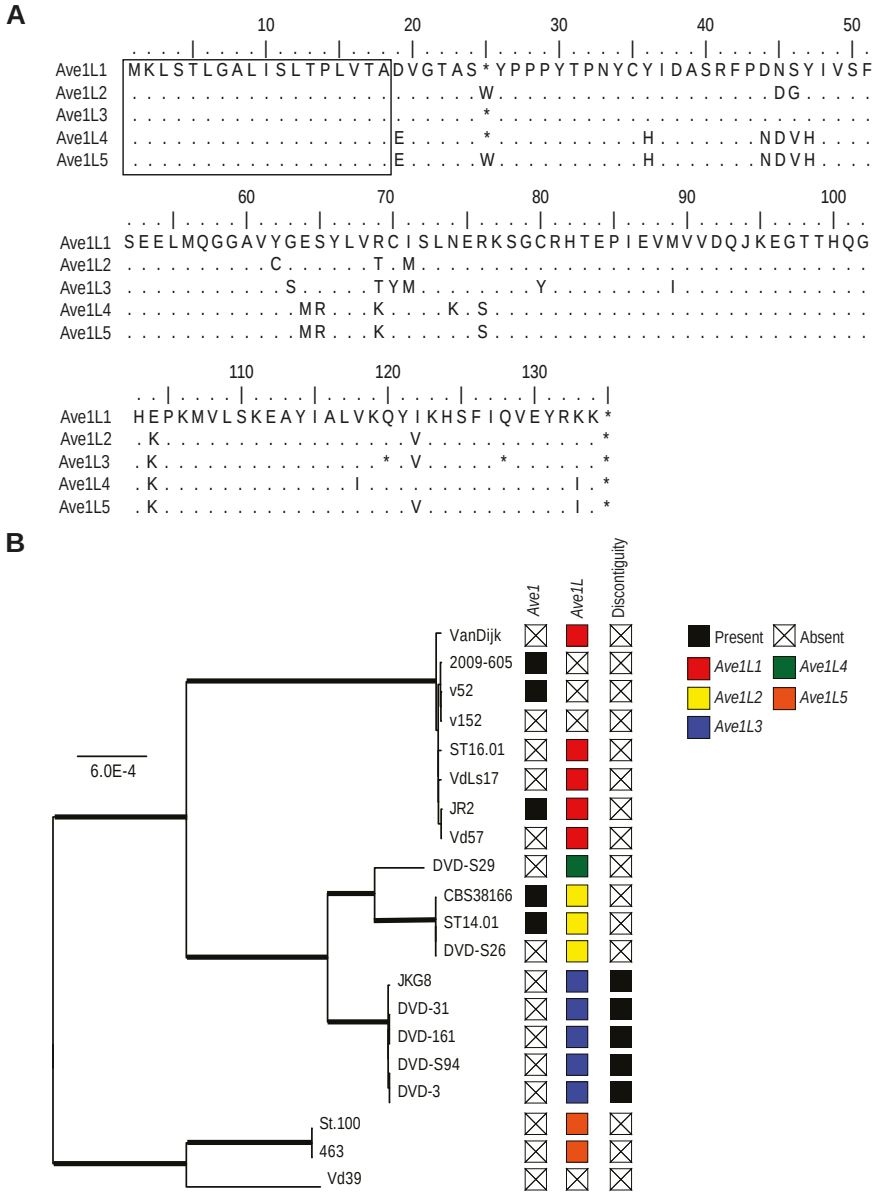

FIGURE 1 | The identification of five *Ave1L* alleles. (A) Alignment of predicted Ave1L proteins Ave1L1 to Ave1L5. The black box indicates the predicted signal peptide, while asterisks (*) indicate predicted stop codons and the hashtag (#) indicates discontiguity. (B) Phylogenetic tree based on the genomes of *V. dahliae* strains and the presence of *Ave1* and *Ave1L* alleles in the respective genomes. (C) continues at next page.

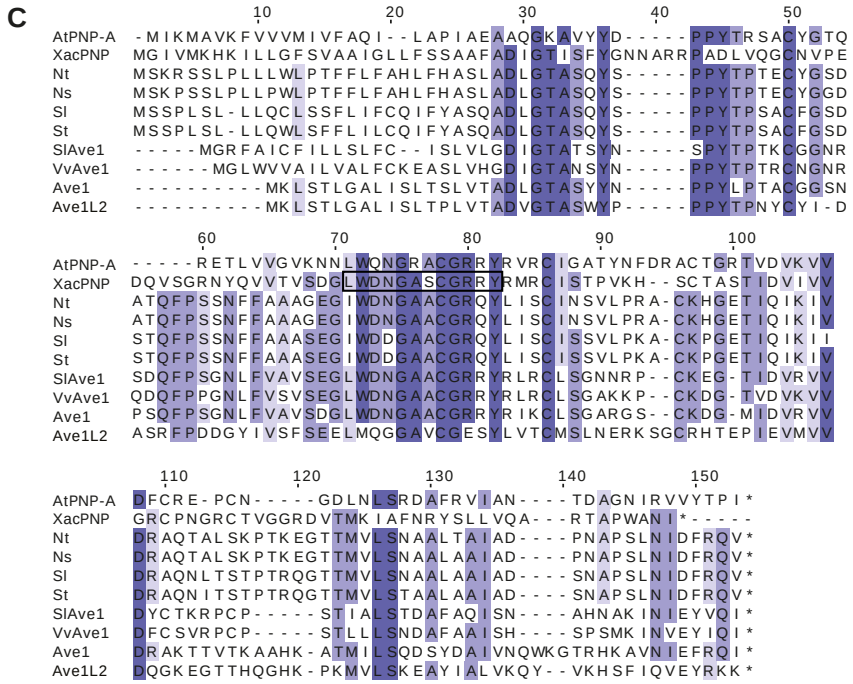

FIGURE 1 | The identification of five *Ave1L* alleles. (C) Alignment of AtPNP-A, XacPNP, PNPs (*Nicotiana tomentosiformis* Nt; *N. sylvestris* Ns; *Solanum lycopersicum* Sl; *S. tuberosum* St; *Vitis vinifera* Vv), Ave1 and Ave1L. The purple colour indicates amino acid conservation. The black box indicates the active PNP domain of XacPNP (Gottig et al. 2008).

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Ave1L alleles occur in a highly dynamic, lineage-specific, genomic region

It was previously shown that extensive chromosomal rearrangements occur among strains of *V. dahliae* (de Jonge et al. 2013). The occurrence of these rearrangements coincides with the occurrence of lineage-specific (LS) regions that are only present in sub-sets of *V. dahliae* strains and that are significantly enriched for *in planta*-expressed genes that also include effectors (de Jonge et al. 2013). Consequently, it has been suggested that chromosomal rearrangements facilitate adaptive evolution in *V. dahliae*, giving rise to lineage-specific segmental duplications that may evolve new traits (de Jonge et al. 2013; Faino et al. 2015; Seidl and Thomma 2014). Since *Ave1L* does not occur in all *V. dahliae* strains, the gene likely occurs in an LS region. To confirm this, we aligned reads from the sixteen *Ave1L*-carrying *V. dahliae* strains to the gapless genome of *V. dahliae* strain JR2 (Faino et al. 2015). We indeed found that the five *Ave1L* alleles are positioned in the same genomic environment in a repeat-rich LS region (Figure 2A).

Ave1L3 is disrupted by a transposable element

We noticed that each *Ave1L3* allele was consistently located on two contigs, which suggests discontiguity of this particular allele (Figure 2B). To confirm this *in silico* observation, we designed PCR primers on conserved sequences that are present in all *Ave1L* alleles, spanning the predicted site where the discontiguity occurs, as well as PCR primers that amplify a conserved region up- and downstream of this site. We used these primers on genomic DNA of a panel of *V. dahliae* strains that represent the five different *Ave1L* alleles. The primers spanning the site of the observed discontiguity in *Ave1L3* amplified a 200 bp PCR product from strains VdLs17 (*Ave1L1*), Dvd-S26 (*Ave1L2*), Dvd-S29 (*Ave1L4*) and St.100 (*Ave1L5*), while no PCR product was amplified from the *Ave1L3*-carrying strains DVD-3, DVD-31, DVD-S94, DVD-161. However, primers that were targeted to a region upstream and downstream of the predicted discontiguity amplified a product from all strains, including those carrying *Ave1L3* (Figure 2C). These results confirm that *Ave1L3* is discontiguous in the genome of all strains that carry this allele.

Discontiguity of *Ave1L3* may be caused by the insertion of a transposable element or by a gross chromosomal rearrangement that occurred within the allele. To test whether *Ave1L3* is disrupted by an insertion we repeated the PCR with primers spanning the site of the discontiguity using a significantly enhanced elongation time to allow for amplification of considerably larger PCR fragments. We used an *Ave1L1*-carrying *V. dahliae* strain as a control for absence of discontiguity. A fragment of around 7 kb was amplified from *V. dahliae* strain DVD-3 that carries *Ave1L3* (Figure 2D). To determine the sequence of the PCR fragment we used an Oxford Nanopore MinION sequencer. Subsequently, we obtained a consensus sequence from the reads and performed a BLAST analysis. This analysis revealed that *Ave1L3* is interrupted by a long terminal repeat retrotransposon that is classified as VdLTRE3 (Faino et al. 2016).

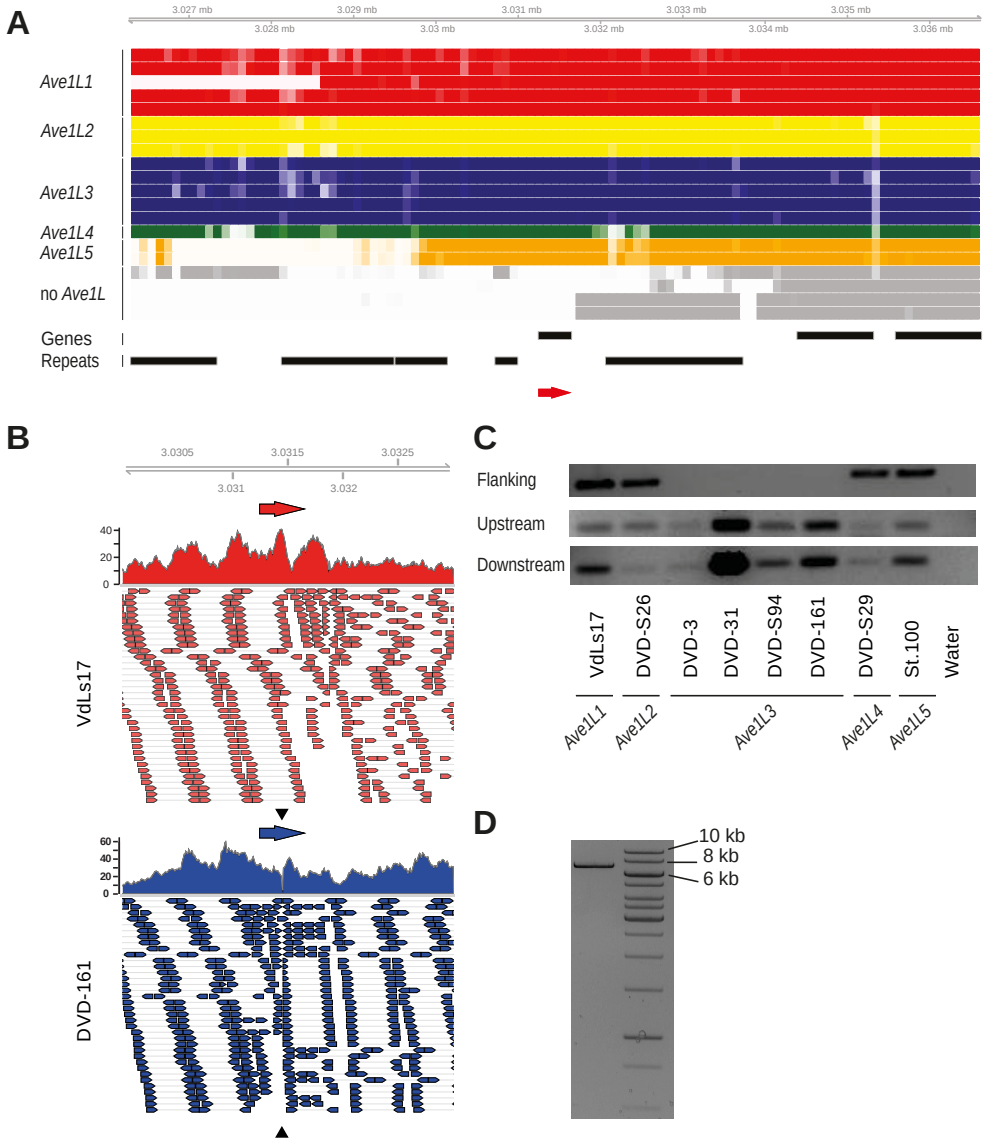

FIGURE 2 | The genomic context of the *Ave1L* alleles. (A) Each coloured bar represents the genomic context of *Ave1L* in different *V. dahliae* strains. The red arrow indicates the position of the *Ave1L* gene while the black bars indicate other genes and repeats in the region. (B) Genomic region flanking *Ave1L* and the read coverage in *V. dahliae* strain VdLs17 and DVD-161. The horizontal red and blue arrows indicate the position of *Ave1L*. The black arrows indicate discontiguity of *Ave1L3* in DVD-161. (C) Amplification products on gel using primers spanning, are upstream, and downstream of (*) the predicted side. The absence of an amplification product on gel when primers were used spanning the predicted side where discontiguity occurs confirms discontiguity in *V. dahliae* strains that carry *Ave1L3*. (D) The presence of an amplification product (left lane) confirms that *Ave1L3* contains an insert of approximately 7 kb deduced from the 10 kb ladder that was run alongside (right lane).

Ave1L2 is expressed during host colonization

To assay whether any of the *Ave1L* alleles is expressed during host colonization, we inoculated Arabidopsis plants with *V. dahliae* strains VdLs17 (*Ave1L1*), DVD-S26 (*Ave1L2*), DVD-S94 (*Ave1L3*), DVD-S29 (*Ave1L4*) and St.100 (*Ave1L5*) and expression of the *Ave1L* alleles was monitored with real-time PCR at 14 and 21 days post inoculation, showing that only *Ave1L2* is expressed *in planta* (Figure 3). To further confirm this observation, we monitored the expression of *Ave1L2* in another strain as well. To this end, we used the race 1 strain St14.01 that carries *Ave1L2* in addition to *Ave1*, in contrast to the race 2 strain DVD-S26 that carries *Ave1L2* in absence of *Ave1*. Interestingly, also in this strain *Ave1L2* was found to be expressed (Figure 3).

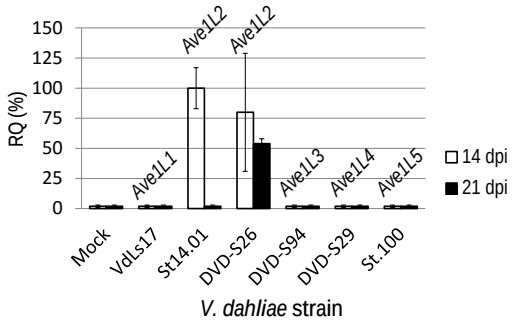

FIGURE 3 | *Ave1L2* is expressed by *V. dahliae* on Arabidopsis. *Ave1L* expression in *V. dahliae* on Arabidopsis at 14 and 21 dpi after mock treatment or inoculation with *V. dahliae* strain VdLs17, St14.01 DVD-S26, DVD-S94, DVD-S29 or St.100.

Ave1L2 is not a virulence factor on tomato plants

Since *Ave1L2* is expressed *in planta* we tested whether *Ave1L2* is a virulence factor of *V. dahliae*. To this end, we deleted *Ave1L2* from the genomes of *V. dahliae* strains St14.01 and CBS38166 (Supplemental Figure 1). We then inoculated three transformants of St14.01 and four transformants of CBS38166 on tomato plants and tested their aggressiveness in comparison to the corresponding wild type *V. dahliae* strain. However, tomato plants inoculated with the *Ave1L2* deletion strains showed a similar level of stunting as the corresponding wild type strains (Figure 4A;B). Moreover, a similar amount of fungal biomass was detected in tomato upon inoculation with the *Ave1L2* deletion strains as well as with the corresponding wild type strains (Figure 4C). Therefore, we conclude that *Ave1L2* does not contribute to the virulence of *V. dahliae* on tomato.

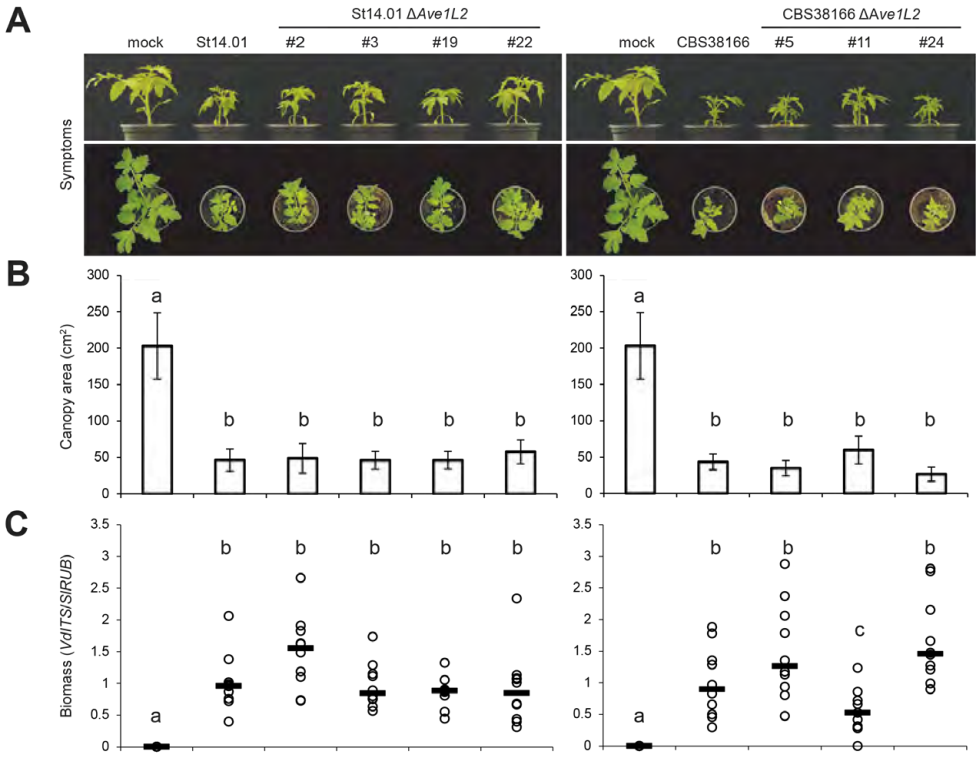

FIGURE 4 | Ave1L2 does not markedly contribute to virulence on tomato. (A) Tomato plants inoculated with *Ave1L2* deletion strains (#) and the corresponding wild strain (St14.01 or CBS38166). (B) The average canopy area of inoculated tomato plants at 14 dpi (n=10). Error bars represent standard errors. (C) Data points indicate the accumulated fungal biomass in tomato plants at 14 dpi. The horizontal line indicates the average fungal biomass.

Ave1L proteins are not recognized by Ve2

Ve1 is the only plant immune receptor known to confer resistance to *V. dahliae* (Fradin et al. 2009; Kawchuk et al. 2001). In tomato, the *Ve1* gene is located at the *Ve* locus, together with *Ve2*, a gene that similarly encodes a receptor-like protein and that has high (~85%) sequence similarity to Ve1 (Fradin et al. 2009). *Ve2* is expressed similarly as *Ve1*, but the encoded protein does not recognize Ave1 and thus far its function remains unknown (de Jonge et al. 2012; Fradin et al. 2009). The occurrence of *Ave1L* alleles that encode proteins that display considerable sequence divergence, or that are severely truncated and thus likely not functional due to premature stop codons in the coding sequences, suggests that the progenitor of these alleles has experienced significant selection pressure, potentially to avoid recognition by a plant immune system. Although such selection pressure may have occurred in any plant species, considering the broad host range

of *V. dahliae*, we tested the possibility that Ve2 was able to recognize ancestral forms of Ave1L. To this end, a library of potentially ancestral Ave1L alleles was constructed for co-expression with *Ve2* in *N. tabacum.* The predicted non-functional *Ave1L* alleles *Ave1L1*, *Ave1L3* and *Ave1L4* were chosen to reverse the disruptive mutations using site-directed mutagenesis to yield the derivatives *Ave1L1**, *Ave1L3** and *Ave1L4* by replacing the nucleotides that are responsible for generating the stop codons by nucleotides that are present at the same positions in *Ave1L2* and *Ave1L5*, respectively (Figure 5A and B). As *Ave1L3* contains multiple mutations and a retrotransposon, we synthesized *Ave1L3** in which the stop codons were replaced and the retrotransposon was removed (Figure 5A and B). While each of the artificial derivatives (*Ave1L1**, *Ave1L3** and *Ave1L4**) is derived from only one of the current Ave1L alleles in which only stop codons and the retrotransposon were replaced, we aimed to determine their most likely common ancestor based on the observed substitutions in the current Ave1L alleles. Therefore, a consensus sequence was generated from the alignment of *Ave1L* alleles, leading to replacement of the various point mutations by the most frequently occurring nucleotide at the same position in the various *Ave1L* alleles, that we synthesized as *Ave1L*** (Figure 5A and B). To test the hypothesis that Ve2 potentially recognized any of these potentially ancestral forms of Ave1L, we co-expressed the artificially generated alleles with *Ve2* and screened for the occurrence of a hypersensitive response. However, at five days post infiltration, no necrosis was observed in leaf tissue where *Ve2* was co-expressed with either *Ave1L**, Ave1L1**, *Ave1L2*, *Ave1L3**, *Ave1L4*, Ave1L5,* as well as with the negative control *Ave1* (Figure 5C). As expected, in leaf tissue where *Ve1* was co-expressed with *Ave1* as a control, clear necrosis was observed (Figure 5C). Moreover, none of the Ave1Ls is recognized by Ve1 (Figure 5C). Therefore, we conclude that it is unlikely that Ve2 recognized an *Ave1L* progenitor allele, and was thus responsible for its diversification.

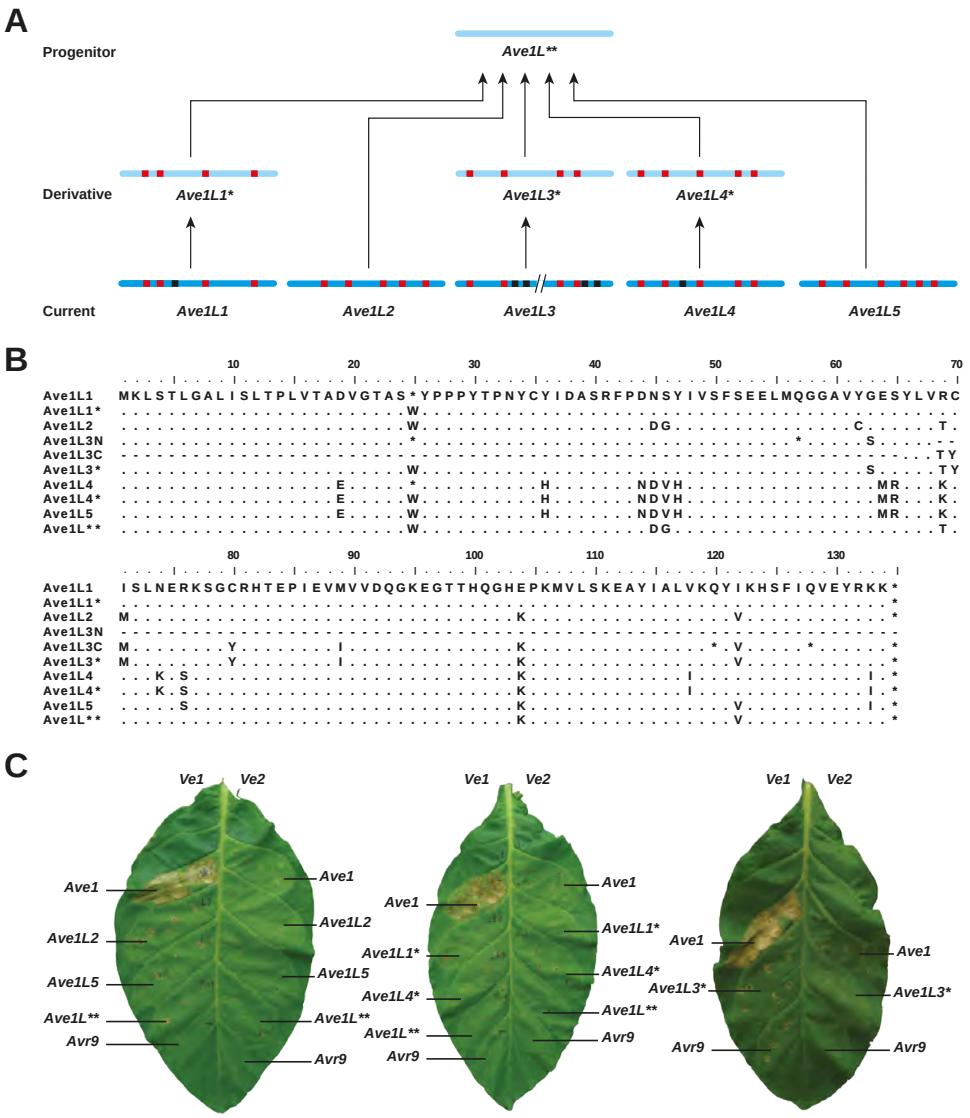

FIGURE 5 | Generation and recognition of the predicted derived alleles and the progenitor allele. (A) Schematic overview of the relationship between the currently identified *Ave1L* alleles (*Ave1L1-Ave1L5*), the predicted derived alleles *Ave1L1**, *Ave1L3**, *Ave1L4** and the predicted progenitor allele (*Ave1L***). The blue bars indicate the alleles, the line with arrows indicates the relationship between the current alleles, the artificial derivatives and the progenitor. Within the blue bars the red blocks indicate point mutations, the black blocks indicate premature stop codons and the double slash (//) indicates discontiguity. (B) Alignment of Ave1L proteins (Ave1L1-Ave1L5), derivatives (Ave1L1*, Ave1L3* and Ave1L4*), the progenitor (Ave1L**), the N-terminal sequence of Ave1L3 (Ave1L3N) and the C-terminal sequence of Ave1L3 (Ave1L3C). Dots (.) indicate similar amino acids, lines (-) indicate the absence of an amino acid, and asterisks (*) indicates a stop codon at the respective position. (C) Co-expression of *Ave1L* alleles with *Ve1* and *Ve2*. The left side of the leaf is co-infiltrated with *Ve1* while the right side of the leaf is co-infiltrated with *Ve2*. Ave1 serves as positive control for the HR, whereas the sequence unrelated *C. fulvum* effector Avr9 serves as a negative control for the HR.

Discussion

In this study we identified a gene in *V. dahliae* with high similarity to the previously characterized *V. dahliae* effector *Ave1* (de Jonge et al. 2012) that we named *Ave1L*. BLAST analyses of this gene in twenty sequenced *V. dahliae* strains revealed four additional alleles. These alleles follow the phylogenetic distribution of *V. dahliae* strains as previously described (de Jonge et al. 2013). Some strains do not have an *Ave1L* allele while other strains have a single *Ave1L* allele. Intriguingly, presence or absence of *Ave1L* does not correspond to presence or absence of *Ave1*. Particular strains contain *Ave1* but no *Ave1L* allele. Moreover, several of the alleles contain point mutations, including mutations that cause stop codons, suggesting they experienced selection pressure to inactivate the gene product. It is tempting to speculate that this pressure arose to avoid recognition by an immune receptor. However, unequivocal evidence for this hypothesis, including knowledge of the nature of the immune receptor or the host plant that carried it, remains absent today.

Since we found that *Ave1L2* is expressed during host colonization we hypothesized that Ave1L2 is a virulence factor. However, *Ave1L2* deletion strains of *V. dahliae* did not display attenuated virulence on tomato. The *Ave1L2* deletion strains were generated in two *V. dahliae* race 1 strains that also contain *Ave1*. Since Ave1 makes a major contribution to virulence it cannot be excluded that redundancy in virulence functions obscures the identification of virulence defects upon *Ave1L2* deletion. Unknowingly, the virulence function of *Ave1* was unravelled in the JR2 strain that does not carry a functional *Ave1L* homolog, as the *Ave1L1* homolog that is found in this strain is inactivated by an early stop codon. It can thus be explained that a potential redundancy in virulence function between Ave1 and Ave1L remained unnoticed thus far. Therefore, deletion strains should be generated in *V. dahliae* race 2 strain DVD-S26 to test the contribution to virulence in absence of *Ave1*.

Some virulence factors have previously been shown to be host-specific, which is particularly relevant for broad host-range pathogens such as *V. dahliae*. For example, the *V. dahliae* effector NLP2 acts as a virulence factor on Arabidopsis but not on *Nicotiana benthamiana* (Santhanam et al. 2013). Similarly, the EPIC effectors of *Phythophthora infestans* and *P. mirabilis* are only functional on their respective plant hosts (Dong et al. 2014). Arguably, Ave1L2 may act as a virulence factor on other host plants of *V. dahliae*. Potent virulence factors pose a strong selection pressure on host plants to evolve recognition (Cook et al. 2015; Thomma et al. 2011). Therefore, Ave1L2 is likely to act as a virulence factor on the host where recognition occurs.

Ave1 is recognized by the tomato immune receptor Ve1 that is encoded by a gene at a locus that also encodes the highly identical orphan receptor Ve2. Considering the high degree of identity between the Ave1 homologs, we tested the possibility that Ve2 recognized the progenitor of the current Ave1L alleles or any of the current alleles. However, the current Ave1L alleles nor the predicted progenitor or any of the intermediate alleles appears to be recognized by Ve2. We therefore conclude that Ve2 does not recognize Ave1L. The fact that Ve2 does not recognize Ave1L is not surprising since we do not know whether Ave1L was recognized in tomato in the first place. Moreover, if recognition occurred in tomato this may have been mediated by any of the hundreds of potential immune receptors that are encoded in the genome. However, it is not unlikely that recognition occurred in another host than tomato since *V. dahliae* has a very wide host range. The observation that *Ave1L2* does not contribute to virulence of *V. dahliae* on tomato supports the latter hypothesis.

Ave1L and *Ave1* are both located in highly dynamic lineage-specific regions of the genome, which could have contributed to the duplication of one of the two genes resulting in the other one. It has been suggested that *Ave1* was acquired from plants through horizontal gene transfer (de Jonge et al. 2012) and then was lost multiple times to result in the current population of race 2 strains (Faino et al. 2016). Ave1 shows higher sequence similarity to PNPs than Ave1L and the active PNP site appears to be degenerated and no longer functional in Ave1L (Figure 1C), whereas the PNP site in Ave1 is conserved. The sequence of *Ave1* is conserved in the population and either present or absent, whereas the *Ave1L* alleles display variation that includes various mutations in addition to presence-absence polymorphisms. Thus, for *Ave1* it has been suggested that sequence conservation is essential for its function (de Jonge et al. 2012).In addition, *Ave1L* alleles are present in both *V. dahliae* race 1 and race 2 strains. Collectively, these observations suggest that selection pressure on *Ave1* is more recent than selection pressure on *Ave1L* and argues that not *Ave1* but *Ave1L* was acquired from plants first.

Materials and methods

Verticillium dahliae genomics

Genome sequence data in this study was partly obtained from previous studies (de Jonge et al. 2013; de Jonge et al. 2012; Faino et al. 2015), and additionally contains nine newly sequenced *V. dahliae* genomes (Table 1). Library preparation (500 bp insert size) and genomic sequencing (100 bp paired-end reads) were conducted at the Beijing Genome Institute (BGI, Hong Kong, China). The genome of each individual *V. dahliae* strain was assembled using the A5 pipeline (Tritt et al. 2012). Discontiguity in a single *Ave1L* allele was analyzed by mapping the genomic

paired-end sequences to the *V. dahliae* strain JR2 reference genome using BWA (Faino et al. 2015; Li and Durbin 2009).

The genetic diversity and the population structure of the sequenced *V. dahliae* strains was assessed using the reference sequence alignment based phylogeny builder (REALPHY) v. 1.11 (Bertels et al. 2014). Briefly, short paired-end genomic reads were mapped against the reference genome (*V. dahliae* strain JR2 (Faino et al. 2015)) using Bowtie2 (Langmead and Salzberg 2012). Polymorphic as well as non-polymorphic sites were extracted (per base quality [20], coverage [10] and polymorphism frequency [0.95]) and sites that were present in all species were retained. With this pseudo-molecule, a maximum likelihood phylogenetic tree was inferred using PhyML (Guindon et al. 2009) with the generalized time reversible (GTR) nucleotide substitution model, and the robustness of the phylogeny was assessed by 500 bootstrap replicates.

Ave1L sequence analysis

To identify *Ave1L* sequences in the twenty *V. dahliae* genome assemblies, we used the CDS of *Ave1* as a BLASTn query (Altschul et al. 1990). Sequence alignments of the five unique *Ave1L* alleles were performed online by using Clustal Omega (EMBL-EBI, Hinxton, UK), and locally using the software package MEGA6 (Tamura et al. 2013).

Phylogeny analyses of Ave1(-like) homologs in plant pathogenic microbes and in plants was conducted using MrBayes (Huelsenbeck and Ronquist 2001). Ave1(-like) homologs in plant pathogenic microbes were previously identified, and plant homologs were identified by sequence similarity search (BLASTp) against the predicted proteomes deposited in Phytozome v9.1 (Goodstein et al. 2012), supplemented by the proteome from *Amborella trichopoda*, and spurious sequence hits were subsequently manually filtered. Protein sequences were subsequently aligned using MAFFT (Katoh et al. 2002), and the alignment was curated using trimAl by removing alignment positions that contained >5 % gaps (Capella-Gutiérrez et al. 2009). Phylogenetic analysis was performed using MrBayes (Huelsenbeck and Ronquist 2001) with the Whelan and Goldman amino acid substitution matrix, and the gamma-shaped rate categories with a proportion of invariable sites were estimated using four rate categories. Markov Chain Monte Carlo (Marjoram et al. 2003) was run with two independent runs of four chains (T = 0.1) and a sample frequency of 500 for 40,000,000 generations. At this point, the average standard deviation of split frequency, a measure of convergence, was well below 0.02. The first 25% of samples of each run were discarded as burn-in, and the remaining samples were used to infer tree and branch topology.

Ave1L gene expression and gene deletions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 and tomato (*Solanum lycopersicum*) cultivar MoneyMaker were inoculated with *V. dahliae* strains VdLs17 (containing *Ave1L1*), DVD-S26 (*Ave1L2*), St14.01 (*Ave1L2*), DVD-S94 (*Ave1L3*), DVD-S29 (*Ave1L4*) and St.100 (*Ave1L5*) as previously described (Fradin et al. 2011; Fradin et al. 2009). The stems of tomato plants were cut from the base to the first cotyledons and flash frozen in liquid nitrogen. The base of Arabidopsis plants of which the leaves were removed were harvested and flash frozen in liquid nitrogen. Plant material was ground to a powder and RNA was extracted using Quick-RNA™ Miniprep (Zymo Research Europe GmbH, Freiburg, Germany) and cDNA was synthesized using M-MLV Reverse Transcriptase (Promega Benelux BV, Leiden, The Netherlands). Real-time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, The Netherlands) in combination with the qPCR SensiMix kit (BioLine, GC Biotech BV, Alphen aan den Rijn, The Netherlands). Primers that were used for gene expression and fungal biomass are described in supplemental table 2. Real-time PCR conditions were as follows: an initial 95°C denaturation step for 10 minutes followed by denaturation for 15 seconds at 95°C, annealing for 60 seconds at 60°C, and extension at 72°C for 40 cycles. Gene expression and fungal biomass was analyzed using the software package 7300 System Software (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, The Netherlands). To generate *Ave1L2* deletion strains, primers were designed to amplify approximately 1500 bp up- and downstream of the *Ave1L2* CDS (Supplemental table 2). Both amplicons were used to generate a USER friendly cloning construct to replace *Ave1L2* by a hygromycin cassette through homologous recombination (Frandsen et al. 2008).

Production of derived *Ave1L* alleles

The potential progenitor of the *Ave1L* alleles was obtained by generating a consensus sequence from an alignment of the CDS of *Ave1L* alleles and was synthesized as *Ave1L***. The predicted derivatives of *Ave1L1* and *Ave1L4* were obtained by replacing the predicted premature stop codons. To this end primers were designed (Supplemental table 2) to replace 1 nucleotide in the stop codon with a nucleotide at the same position in *Ave1L2* and *Ave1L5* that do not contain a predicted premature stop codon. Overlap PCR was performed to introduce the mutation in *Ave1L1* and *Ave1L4.* The *Ave1L1* and *Ave1L4* sequences without a predicted premature stop codon were designated as *Ave1L1** and *Ave1L4**, respectively (Figure 4A). The derivative of *Ave1L3* was obtained by synthesizing the gene without stop codons and without the retrotransposon and was designated as *Ave1L3** (Eurofins Genomics, Ebersberg, Germany) (Figure 4A).

Functional analysis of *Ave1L* alleles

V. dahliae strains were grown on potato dextrose agar (PDA) (Thermo Fisher Scientific Inc, Breda, The Netherlands) for 1-2 weeks and conidia were harvested for DNA extraction with high-salt CTAB buffer (100 mM TrisHCl, 20 mM EDTA, 2 M NaCl, 3% CTAB). PCR primers were designed to amplify *Ave1*, *Ave1L* or to detect discontiguity (Supplemental table 2). For constitutive expression the CDS of *Ave1L* alleles was amplified from genomic DNA and cloned in the adjusted, Gateway compatible, pBIN vector variant pSol2092 (Zhang et al. 2013) and transformed into *Agrobacterium tumefaciens* strain GV3101. *Nicotiana tabacum* cv. Petite Havana SR1 was infiltrated in a 1:1 ratio with *A. tumefaciens* that express either *Ve1* or *Ve2* and an *Ave1*(*-like*) construct, as previously described (Zhang et al. 2013). After infiltration, plants were transferred to a climate chamber and incubated at 22°C and 19°C during 16-h day and 8-h night periods, respectively, with 70% relative humidity. At 5 dpi leaves were inspected for necrosis development.

Oxford Nanopore MinION sequencing

Library preparation of the PCR fragment was performed according to the protocol of Oxford Nanopore (https://wiki.nanoporetech.com/display/BP/Nanopore+Sequencing+Kit+protocol+SQK-MAP005) skipping the DNA fragmentation step. The library was loaded on the Nanopore flowcell and the sequencing was stopped after ~4 hr. The run generated about 870 high quality long reads with an average length of 3,869 bp and the longest read of about 14 kb. Using Nanocorrect software (https://github.com/jts/nanocorrect), we used all reads to correct the longest 50 reads. Out of the 50 longest reads, only 28 were corrected to generate a consensus. The average protected read length was of 5,666.8 bp with a median of 6,483 bp. All the reads were used in BLAST analysis to confirm the presence of *Ave1L3* fragments at both ends. Out of the 28 corrected fragments, eight showed high similarity (~99%) to *Ave1L3* at both ends.

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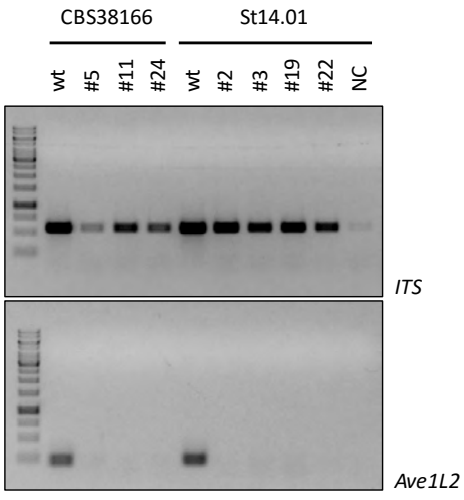
Supplemental data

SUPPLEMENTAL TABLE 2 | Primers used in this study

Primer	Sequence (5′-3′)	Notes
Ave1L-Break-Fw	TCGATGCGAGCAGATTCC	Primers spanning discontiguity
Ave1L-Break-Rv	GAGTAGTACCTTCCTTGCCTTGA	Primers spanning discontiguity
Ave1L-Up-Fw	CTACGCTTGGAGCCCTCAT	Primers upstream of discontiguity
Ave1L-Up-Rv	AGGAGTGTAGGGTGGAGGAT	Primers upstream of discontiguity
Ave1L-Down-Fw	GGCATACTGAACCGATTGAAG	Primers downstream of discontiguity
Ave1L-Down-Rv	CTCCTTAGAAAGAACCATTTTCG	Primers downstream of discontiguity
VdLs17del.stop_Fw	CGCATCCT**G**GTATCCTCCACC	Replace stop codon in *Ave1L1* and *Ave1L4*
VdLs17del.stop_Rv	TGGAGGATAC**C**AGGATGCGGT	Replace stop codon in *Ave1L1* and *Ave1L4*
Topo_Ave1L_Fw	CACCATGAAGCTTTCTACGCTTGGAG	Combined with primer VdLs17del.stop_Rv
Topo_St14.01_Rv	TTATTTTTTTTCTATACTCAACTTGAATGAAC	Combined with primer VdLs17del.stop_Fw
Topo_St.100_Rv	TTATTTTATTCTATACTCAACTTGAATGAAC	Combined with primer VdLs17del.stop_Fw
Ave1L2-LB-F3	GGTCTTAAUTTTTATCTCTCCCTTCCTCTTATCT	1500bp fragment upstream of *Ave1L2*
Ave1L2-LB-R3	GGCATTAAUTTTTTAAGCCTTTCTAGCTTATTCTT	1500bp fragment upstream of *Ave1L2*
Ave1L2-RB-F3	GGACTTAAUGCTATCTTCACGAGAGCAGAGT	1500bp fragment downstream of *Ave1L2*
Ave1L2-RB-R3	GGGTTTAAUTTGCGCGTTTATATATTCTTATCTT	1500bp fragment downstream of *Ave1L2*
QPCR-Ave1L-Fw3	ATCCTCCACCCTACACTCCT	*Ave1L* expression
QPCR-Ave1L-Rv3	CCTTGATGAGTAGTACCTTCCTTG	*Ave1L* expression
Vd-ITS-Fw	AAAGTTTTAATGGTTCGCTAAGA	*V. dahliae* biomass
Vd-ITS-Rv	CTTGGTCATTTAGAGGAAGTAA	*V. dahliae* biomass
Sl-RUB-Fw	GAACAGTTTCTCACTGTTGAC	Tomato rubisco gene
Sl-RUB-Rv	CGTGAGAACCATAAGTCACC	Tomato rubisco gene



SUPPLEMENTAL FIGURE 1 | Verification of *Ave1L2* deletion strains by amplification of *VdITS* and *Ave1L2* from *V. dahliae* strains CBS38166 and St14.01.

Chapter 7

Rhamnose synthase activity is required for pathogenicity of the vascular wilt fungus *Verticillium dahliae*

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Abstract

The initial interaction of a pathogenic fungus with its host is complex and involves numerous metabolic pathways and regulatory proteins. Here we present a random genetic screen that enabled us to identify 58 novel candidate genes that are involved in the pathogenic potential of the fungal pathogen *Verticillium dahliae* that causes vascular wilt diseases in over 200 dicotyledonous plant species, including economically important crops. One of the candidate genes that was identified concerns a putative biosynthetic gene involved in nucleotide-sugar precursor formation as it encodes a putative nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER). This enzyme has homology to bacterial enzymes involved in biosysnthesis of the nucleotide sugar dTDP-rhamnose, a precursor of L-rhamnose that was shown to be required for virulence in several human pathogenic bacteria. Rhamnose is known as a minor cell wall glycan in fungi and has therefore not been suspected as a crucial molecule in fungal-host interactions. Nevertheless, our study shows that deletion of the VdNRS/ER gene from the *V. dahliae* genome results in complete loss of pathogenicity on tomato and *N. benthamiana* plants, whereas vegetative growth and sporulation are not affected. We demonstrate that VdNRS/ER is a functional enzyme in the biosynthesis of UDP-rhamnose, and further analysis revealed that *VdNRS/ER* deletion strains are impaired in colonization of tomato roots. Collectively, our results demonstrate that rhamnose, although only being a minor cell wall component, is essential for pathogenicity of *V. dahliae*.

Introduction

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

Verticillium wilt diseases are difficult to control due to the longevity of the microsclerotia and inability of fungicides to eliminate the fungus once it has entered the xylem tissues of the host plant (Fradin and Thomma 2006; Wilhelm 1955). Moreover, crop rotation is ineffective due to the broad host range of *V. dahliae*. The only effective control measure, soil fumigation, is expensive and has harmful environmental effects (Rowe et al. 1987). As a consequence, genetic resistance is presently preferred to control *Verticillium* wilt diseases. Importantly, *Verticillium* resistance has been described in several plant species, including crops (Bolek et al. 2005; Hayes et al. 2011; Schaible et al. 1951; Simko et al. 2004). From tomato (*Solanum lycopersicum*) the *Ve1 Verticillium* resistance gene has been cloned that mediates resistance to race 1 strains of *V. dahliae* that express the secreted Ave1 effector (de Jonge et al. 2012; Fradin et al. 2009). Putative *Ve1* orthologs have been identified within and outside the Solanaceae family, suggesting that *Ve1*-mediated *Verticillium* resistance may be widespread in plants (Thomma et al. 2011). Interestingly, Ave1 contributes to *V. dahliae* virulence on susceptible tomato plants, and homologs occur in a handful of taxonomically unrelated plant pathogenic fungi (de Jonge et al. 2012).

Various other virulence genes have been identified in *V. dahliae* including members of the expanded LysM effector and NEP-like (NLP) protein family (de Jonge et al. 2013; Klimes et al. 2015; Klosterman et al. 2011; Santhanam et al. 2013). Genome analysis furthermore revealed the relative abundance of particular families of cell-wall degrading enzymes (CWDE) when compared with other fungi (Klosterman et al. 2011). The importance of CWDE during pathogenicity was demonstrated by the disruption of the CWDE regulator VdSNF1 which reduced *V. dahliae* virulence (Tzima

et al. 2011). In addition, genes involved in nitrogen and amino acid metabolism where found to be important for establishment in the nutrient-poor vascular system (Singh et al. 2010; Timpner et al. 2013). Finally, virulence genes were described that are also involved in stress tolerance (Klimes et al. 2015).

The identification and subsequent characterization of novel genes involved in disease establishment is of fundamental importance to understand the interaction between *V. dahliae* and its hosts, and to potentially design novel strategies for disease control. An effective way to identify novel pathogenicity genes in plant pathogenic fungi is the use of random mutagenesis coupled with a quick manner to screen the mutants for phenotypes. *Agrobacterium tumefaciens* is a Gram-negative soil bacterium that can infect hosts as diverse as fungi, oomycetes and plants, and can be used to randomly insert a transfer–DNA (T-DNA) into the host genome. Over recent years, *A. tumefaciens*-mediated transformation (ATMT) was used to identify genes involved in disease development in various plant pathogens (Giesbert et al. 2012; Hüser et al. 2009; Jeon et al. 2007; Korn et al. 2015; Maruthachalam et al. 2011; Michielse et al. 2009; Munch et al. 2011; Ramos et al. 2013; Schumacher et al. 2014; Xu and Chen 2013). In this study, we report on the identification of pathogenicity and virulence genes in *V. dahliae* through ATMT. A collection of 900 transformants of *V. dahliae* was generated and tested for reduced disease development on susceptible tomato plants. We subsequently studied one of the candidates in detail, a gene that putatively encodes a biosynthetic gene involved in nucleotide-sugar precoursor formation, to reveal its role in pathogenicity.

Results

Identification of random *V. dahliae* transformants affected in aggressiveness

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

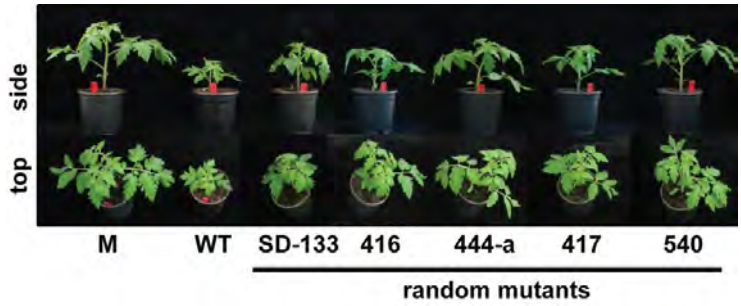

FIGURE 1 | Typical assay to identify transformants with reduced pathogenicity or virulence. Ten-day-old tomato seedlings were mock-inoculated (M) or inoculated with conidiospores of wild-type *V. dahliae* (WT) or random T-DNA insertion mutants. At 21 days post inoculation the plants were scored for disease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

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

Inverse PCR (iPCR) was carried out on the 80 selected transformants to determine the T-DNA insertion sites. The genomic borders flanking the T-DNA were amplified, sequenced, and the resulting sequences were queried against the *V. dahliae* genome (Faino et al. 2015). In this manner, sequences flanking the T-DNA were determined for 65 mutants, while T-DNA flanking regions repeatedly could not be amplified from any of the borders of the remaining 15 mutants. Of the 65 flanking sequences, 12 sequences corresponded to the backbone of the vector that was used for ATMT while 10 sequences could not unambiguously be assigned to a single genomic location. The remaining 43 sequences resulted in single genomic hits, allowing determination of T-DNA integration sites. In total, 14 insertions were found within predicted open reading frames (ORFs; Table 1) of which two transformants carried an insertion in the same ORF, while the remaining 29 insertions occurred in non-coding regions. For these 29 insertions the predicted genes flanking the T-DNA were identified. In one case we found that for two transformants the T-DNA was integrated in the same non-coding region, albeit at a slightly different location, resulting in the identification of the same flanking genes. In another case the same candidate gene was identified twice in two transformants due to an insertion upstream and downstream of the gene. One flanking gene was only identified at 55,077 bp from the insertion site, for which we disqualified it as a candidate gene. In total, this resulted in the identification of 55 candidate genes from the 29 mutants with insertions in non-coding regions.

In order to try to determine which of the two genes flanking a particular insertion site in the non-coding region is likely causal to the reduced disease development, *in planta* expression of the flanking genes was assessed making use of previously generated RNAseq data of *V. dahliae*-inoculated *Nicotiana benthamiana* plants (de Jonge et al. 2012; Faino et al. 2012). For 10 insertions, this revealed that only one of the two flanking genes is expressed during infection, disqualifying the 10 candidate genes that are not expressed. Thus, 45 candidate genes were considered from mutants with an insertion in the non-coding region and 13 candidate genes were identified from mutants with an insertion in the ORF (Table 1). Subsequently, the 58 candidate genes were queried against the pathogen-host interaction (PHI) database that contains experimentally verified pathogenicity and virulence factors of plant and animal pathogens (Winnenburg et al. 2006; Winnenburg et al. 2008), which resulted in the identification of homologs ($E<10^{-6}$) for only 19 of the 58 candidate genes (Table 2).

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

	T-DNA insertion within the CDS				
Mutant	**Old locus[b]**	**New locus[c]**	**Annotation**	**Expr.[d]**	**CDS[e]**
78	Not available	VDAG_JR2_Chr1g08240	glycoside hydrolase	+	-
73	Not available	VDAG_JR2_Chr1g08240	glycoside hydrolase	+	-
407	VDAG_00500	VDAG_JR2_Chr1g19180	carboxypeptidase d	+	-
659	VDAG_02200	VDAG_JR2_Chr2g03300	alpha-beta hydrolase fold family	-	-
SD-133	VDAG_04418	VDAG_JR2_Chr3g02140	ran gtp-ase activating protein 1	+	-
444-B	VDAG_08830	VDAG_JR2_Chr3g07650	acetate kinase	+	-
211	VDAG_06985	VDAG_JR2_Chr4g06020	leucine rich-repeat protein	+	-
375	VDAG_07314	VDAG_JR2_Chr4g10770	copper amine oxidase 1	+	-
416	VDAG_10292	VDAG_JR2_Chr5g06230	conserved hypothetical protein	-	-
174	VDAG_05684	VDAG_JR2_Chr5g09640	hypothetical protein	+	-
657-2	VDAG_04769	VDAG_JR2_Chr6g09500	phospho-2-dehydro-3-deoxyheptonate aldolase	+	-
367	VDAG_06253	VDAG_JR2_Chr7g00300	cyclic nucleotide-binding protein	+	-
618	VDAG_08151	VDAG_JR2_Chr8g01560	hypothetical protein	+	-
48	VDAG_08150	VDAG_JR2_Chr8g01570	caib baif family enzyme	-	-

T-DNA insertion in intergenic region						
Upstream direction						
Mutant	Dist.[a]	Old locus[b]	New locus[c]	Annotation	Expr.[d]	CDS[e]
459-1	2485	VDAG_00838	VDAG_JR2_Chr1g01770	predicted protein	+	-
143	374	VDAG_00843	VDAG_JR2_Chr1g01820	not annotated	+	-
SD-102	270	VDAG_00599	VDAG_JR2_Chr1g20260	histidinol-phosphate aminotransferase	+	-
44	545	VDAG_08030	VDAG_JR2_Chr1g22770	deuterolysin metalloprotease	-	-
11	100	VDAG_08003	VDAG_JR2_Chr1g23050	f-box protein	-	-
SD-107	165	VDAG_07796	VDAG_JR2_Chr1g25310	hydrolase	+	-
182	470	VDAG_09439	VDAG_JR2_Chr2g01480	smc n-terminal containing protein	+	-
525	895	VDAG_02343	VDAG_JR2_Chr2g01760	cell wall glucanase	+	100
SD-7	215	VDAG_02014	VDAG_JR2_Chr2g05250	myb-like dna-binding protein	+	-
540	622	VDAG_01960	VDAG_JR2_Chr2g05800	kh protein	+	-
402	1788	VDAG_01891	VDAG_JR2_Chr2g06520	domain-containing protein	+	400
417	88	VDAG_01651	VDAG_JR2_Chr2g09280	f-box protein	+	-
622	700	Not available	VDAG_JR2_Chr3g02050	unknown	+	-
SD-89	335	VDAG_07703	VDAG_JR2_Chr4g00600	transcriptional regulatory protein pro-1	-	-
SD-78	1214	VDAG_07011	VDAG_JR2_Chr4g05750	subunit of golgi mannosyltransferase complex	+	-
414	4	Not available	VDAG_JR2_Chr4g08330	Myomesin	-	-
584	51	VDAG_09007	VDAG_JR2_Chr4g08540	family protein	+	-
534	737	VDAG_09007	VDAG_JR2_Chr4g08540	family protein	+	-
199	64	VDAG_05141	VDAG_JR2_Chr5g01900	low-affinity potassium transport protein	-	-
412	1647	VDAG_09301	VDAG_JR2_Chr5g03950	potassium sodium efflux p-type fungal type	+	-
550	78	VDAG_04591	VDAG_JR2_Chr6g07500	calmodulin	+	-
SD-94	716	VDAG_04810	VDAG_JR2_Chr6g09950	nadph dehydrogenase	+	-
75	1060	VDAG_09054	VDAG_JR2_Chr6g10940	ubiquitin fusion degradation protein	+	-
374	1023	VDAG_06275	VDAG_JR2_Chr7g00090	maintenance of telomere capping 1	+	
389	56	VDAG_06010	VDAG_JR2_Chr7g02960	nad dependent epimerase dehydratase	+	-
438	977	VDAG_10534	VDAG_JR2_Chr7g06650	ctp synthase	+	-
59	47	VDAG_10192	VDAG_JR2_Chr7g10460	benzoate 4-monooxygenase cytochrome P450	-	-
646	246	VDAG_10191	VDAG_JR2_Chr7g10470	methyltransferase	+	-
575	466	VDAG_03431	VDAG_JR2_Chr8g09910	vegetative cell wall protein gp1-like	-	-

T-DNA insertion in intergenic region					
Downstream direction					
Dist.[a]	Old locus[b]	New locus[c]	Annotation	Expr.[d]	CDS[e]
1784	VDAG_00839	VDAG_JR2_Chr1g01780	transcription initiation factor tfiid subunit 12	+	-
100	VDAG_00844	VDAG_JR2_Chr1g01830	exosome complex exonuclease	+	-
746	VDAG_00600	VDAG_JR2_Chr1g20270	acetyl esterase	+	-
669	VDAG_08031	VDAG_JR2_Chr1g22760	saff protein	+	-
3330	VDAG_08988	VDAG_JR2_Chr1g23040	umta methyltransferase	+	-
1586	VDAG_07797	VDAG_JR2_Chr1g25300	briggsae cbr-smu-2 protein	+	-
257	VDAG_09440	VDAG_JR2_Chr2g01470	sgt1-like protein	+	-
71	VDAG_02344	VDAG_JR2_Chr2g01750	phosphoribosylglycinamide formyltransferase	+	-
1004	VDAG_02015	VDAG_JR2_Chr2g05240	zinc nfx1-type containing protein	-	-
336	VDAG_01961	VDAG_JR2_Chr2g05790	duf974 protein	+	-
1318	VDAG_01892	VDAG_JR2_Chr2g06510	hypothetical protein	+	100
731	VDAG_01652	VDAG_JR2_Chr2g09270	cytochrome p450 protein	-	-
101	VDAG_04427	VDAG_JR2_Chr3g02040	agc akt protein kinase	+	-
423	VDAG_07704	VDAG_JR2_Chr4g00580	pathway-specific nitrogen regulator	+	-
1914	VDAG_07012	VDAG_JR2_Chr4g05740	fungal specific transcription factor	+	-
1466	Not available	VDAG_JR2_Chr4g08340	umta methyltransferase	-	-
1221	VDAG_09008	VDAG_JR2_Chr4g08550	zinc-binding dehydrogenase	+	-
535	VDAG_09008	VDAG_JR2_Chr4g08550	zinc-binding dehydrogenase	+	-
703	VDAG_05142	VDAG_JR2_Chr5g01910	sodium ion /h ion antiporter	+	-
1459	VDAG_09302	VDAG_JR2_Chr5g03940	glycoside hydrolase family 43 protein	+	100
667	VDAG_04592	VDAG_JR2_Chr6g07510	ap-1 complex subunit gamma-1	+	-
1014	VDAG_04811	VDAG_JR2_Chr6g09960	hypothetical protein	-	100
196	VDAG_09055	VDAG_JR2_Chr6g10930	ubiquitin fusion degradation protein	+	-
279	VDAG_06276	VDAG_JR2_Chr7g00080	major facilitator superfamily transporter	+	-
2363	VDAG_06011	VDAG_JR2_Chr7g02950	squalene synthetase	+	-
55077	VDAG_06595	VDAG_JR2_Chr7g06640	sad1 unc protein	+	-
1565	VDAG_10193	VDAG_JR2_Chr7g10450	cellulose-binding family II	+	-
422	VDAG_10192	VDAG_JR2_Chr7g10460	benzoate 4-monooxygenase cytochrome p450	-	-
1040	VDAG_03432	VDAG_JR2_Chr8g09920	duf221 protein	+	-

[a] Distance from the T-DNA to the coding sequence (CDS).

[b] Locus corresponds to the *V. dahliae* strain VdLs.17 assembly deposited on http://genome.jgi.doe.gov/programs/fungi/ (Klosterman et al. 2011).

[c] Locus corresponds to the *V. dahliae* strain JR2 assembly deposited on http://fungi.ensembl.org/ (Faino et al. 2015).

[d] Evidence for *in planta* expression.

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

TABLE 2 | Homologs identified in the PHI database among the 58 potential *V. dahliae* pathogenicity genes.

Old locus[a]	New locus[b]	Annotation	E-value	Accession[c]
VDAG_00839	VDAG_JR2_Chr1g01780	transcription initiation factor tfiid subunit 12	7E-19	PHI:2168
VDAG_00844	VDAG_JR2_Chr1g01830	exosome complex exonuclease	5E-12	PHI:3472
VDAG_01461	VDAG_JR2_Chr1g08260	ring finger protein	2E-09	PHI:1393
VDAG_00500	VDAG_JR2_Chr1g19180	carboxypeptidase d	4E-55	PHI:901
VDAG_09440	VDAG_JR2_Chr2g01470	sgt-1-like protein	1E-156	PHI:1615
VDAG_09439	VDAG_JR2_Chr2g01480	smc n-terminal containing protein	2E-10	PHI:359
VDAG_02343	VDAG_JR2_Chr2g01760	cell wall glucanase	9E-30	PHI:816
VDAG_02014	VDAG_JR2_Chr2g05250	myb-like dna-binding protein	1E-105	PHI:1540
VDAG_01960	VDAG_JR2_Chr2g05800	kh protein	3E-22	PHI:1662
VDAG_04427	VDAG_JR2_Chr3g02040	agc akt protein kinase	0E+00	PHI:3131
VDAG_07704	VDAG_JR2_Chr4g00580	pathway-specific nitrogen regulator	0E+00	PHI:1960
VDAG_07012	VDAG_JR2_Chr4g05740	fungal specific transcription factor	0E+00	PHI:1954
VDAG_09008	VDAG_JR2_Chr4g08550	zinc-binding dehydrogenase	7E-13	PHI:2895
VDAG_07314	VDAG_JR2_Chr4g10770	copper amine oxidase 1	1E-106	PHI:2171
VDAG_09301	VDAG_JR2_Chr5g03950	potassium sodium efflux p-type fungal type	0E+00	PHI:2098
VDAG_04591	VDAG_JR2_Chr6g07500	calmodulin	4E-20	PHI:2640
VDAG_06253	VDAG_JR2_Chr7g00300	cyclic nucleotide-binding protein	2E-10	PHI:372
VDAG_10193	VDAG_JR2_Chr7g10450	cellulose-binding family II	6E-13	PHI:2388
VDAG_10191	VDAG_JR2_Chr7g10470	methyltransferase	9E-40	PHI:2315

[a] Locus corresponds to the *V. dahliae* strain VdLs.17 assembly deposited on http://genome.jgi.doe.gov/programs/fungi/ (Klosterman et al. 2011).

[b] Locus corresponds to the *V. dahliae* strain JR2 assembly deposited on http://fungi.ensembl.org/ (Faino et al. 2015).

[c] Pathogen-host interaction database accession.

Analysis of a T-DNA insertion site links *VdNRS/ER* to virulence

In random mutant 389, the T-DNA was integrated 56 bp upstream of the coding region of a nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER) homolog VDAG_06010 (Table 1; (Klosterman et al. 2011)), which corresponds to VDAG_JR2_Chr7g02960 in the *V. dahliae* JR2 assembly deposited at http://fungi.ensembl.org/ (Faino et al. 2015). The first CDS downstream of the T-DNA insertion site is found only at 2.4 kb, encoding a squalene synthetase (VDAG_06011 or VDAG_JR2_Chr7g02950), an enzyme that has been implicated in sterol and triterpene biosynthesis. However, considering the distance between the T-DNA insertion and the CDS of this gene it is unlikely that it is affected and causal to the reduced pathogenicity phenotype of the RM-389 mutant. To assess whether any genes have been overlooked by the automated gene prediction in the region between the

T-DNA insertion and the squalene synthetase CDS, RNAseq reads from *V. dahliae*-inoculated *N. benthamiana* (de Jonge et al. 2012; Faino et al. 2012) were queried. However, no reads were found to map to this region suggesting that no *in planta* transcribed genes reside in this region. Finally, with real-time PCR on genomic DNA, using the single copy *Ave1* gene as a reference (de Jonge et al. 2012), it was determined that only a single T-DNA insertion was present in the genome of the RM-389 mutant.

V. dahliae NRS/ER (VdNRS/ER) shows weak (17%) amino-acid sequence similarity to the *Salmonella enterica* dTDP-rhamnose biosynthesis component rmlD, a bacterial protein involved in the last step in the conversion of dTDP-glucose to dTDP-rhamnose. In bacteria the nucleotide sugar dTDP-rhamnose is the precursor that facilitates the incorporation of L-rhamnose into glycan, which has been implicated in virulence of pathogenic bacteria such as *Salmonella enterica*, *Vibrio cholerae* and *Streptococcus mutans* (Giraud et al. 2000). Thus, considering all of the above, functional analysis of the *VdNRS/ER* gene was pursued.

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

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

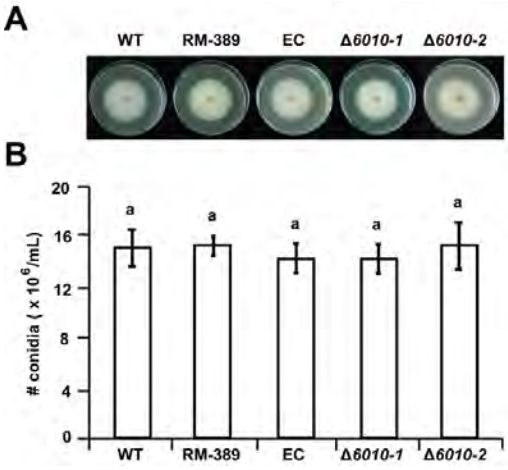

FIGURE 2 | Targeted deletion of *VdNRS/ER* does not impair growth and conidiogenesis. (A) Radial growth and colony morphology of wild type *V. dahliae* (WT), random transformant RM-389, an ectopic transformant (EC) and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) after 7 days of incubation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

Subsequently, the role of *VdNRS/ER* in *V. dahliae* pathogenicity was addressed. To this end, the expression of *VdNRS/ER* was assessed in wild-type *V. dahliae* during infection of tomato plants in a time course harvested at regular intervals after inoculation, showing that *VdNRS/ER* is induced during *in planta* growth with a peak in expression at around 12 days post inoculation (Figure 3). Subsequently, the *VdNRS/ER* deletion strains were inoculated on tomato plants alongside the random mutant (RM-389) and an ectopic transformant to investigate the role of *VdNRS/ER* in fungal virulence. While inoculation of the plants with the wild-type *V. dahliae* strain and the ectopic transformant resulted in the development of symptoms of *Verticillium* wilt disease that include stunting of the plants and wilting, inoculation with RM-398 or the *VdNRS/ER* deletion strains did not result in disease development (Figure 4A). The absence of disease symptoms was confirmed by measuring the surface area of the foliage of the plants that were inoculated with the various fungal strains. Plants that were inoculated with *VdNRS/ER* deletion strains developed similar foliage (canopy) areas as mock-inoculated plants and plants inoculated with RM-389 (Figure 5A). In contrast, less foliage developed for plants that were inoculated with the wild-type *V. dahliae* strain and the ectopic transformant (Figure 5A). Thus, we conclude that *VdNRS/ER* is crucial for disease development by *V. dahliae*. To assess whether *VdNRS/ER* deletion strains retained the capability to colonize tomato plants, fungal outgrowth assays were performed by plating of stem sections harvested from the hypocotyls of the inoculated plants. Interestingly, while the ectopic transformants and the wild-type strain had extensively colonized the stems of the tomato plants, no outgrowth was observed for the *VdNRS/ER* deletion strains and RM-389, demonstrating that these genotypes were not able to colonize the xylem vessels of the inoculated tomato plants (Figure 4A). The absence of tomato colonization by *VdNRS/ER* deletion strains was confirmed with real-time PCR quantification of fungal biomass (Figure 5B). Importantly, pathogenicity was restored upon complementation of the *VdNRS/ER* deletion strains with a genomic construct containing the wild-type *VdNRS/ER* locus (Supplemental Figure 2). Thus, it can be concluded that *VdNRS/ER* is required for pathogenicity on tomato.

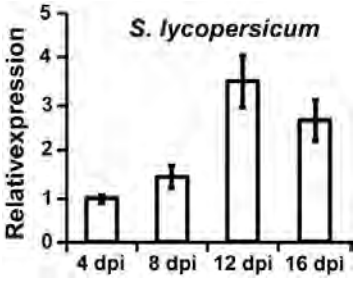

FIGURE 3 | Expression of *VdNRS/ER* during infection of *Verticillium dahliae* on tomato. Ten-day-old tomato (*S. lycopersicum*) cultivar MoneyMaker plants were root-inoculated with *V. dahliae* and plants were harvested at regular intervals from 4 to 16 days post inoculation (dpi). After RNA isolation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

To investigate whether the observed role of *VdNRS/ER* in pathogenicity is confined to tomato only, or also extends to other host species, we tested the pathogenicity of the *VdNRS/ER* deletion strains on the Solanaceous model plant *Nicotiana benthamiana*. Similar to tomato, targeted deletion of *VdNRS/ER* resulted in compromised pathogenicity, as also these plants remained devoid of *Verticillium* wilt symptoms (Figure 4B). Again, loss of pathogenicity was confirmed by measurement of the canopy surface area (Figure 5C), and loss of pathogenicity was confirmed by real-time PCR quantification of fungal biomass (Figure 5D).

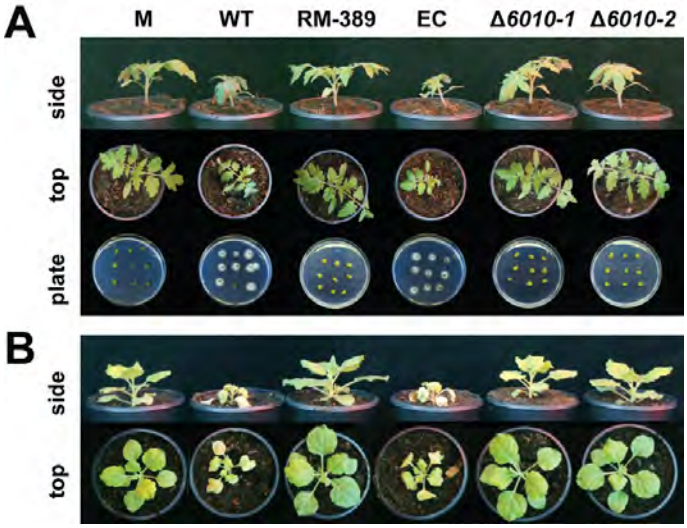

FIGURE 4 | *VdNRS/ER* is required for pathogenicity of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. (A) Top and side view of tomato cultivar MoneyMaker plants that were mock-inoculated (M), or inoculated with wild type *V. dahliae* (WT), random transformant RM-389, an ectopic transformant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

Absence of VdNRS/ER variation in a collection of *V. dahliae* strains

Allelic variation can be a sign of selection pressure and adaptation. To analyze potential VdNRS/ER diversity, we mined the genomes of 39 sequenced *V. dahliae* strains for the *VdNRS/ER* gene sequence (de Jonge et al. 2012) (Faino and Thomma, unpublished data). Intruigingly, only two single nucleotide polymorphisms (SNPs) were identified in two strains, each at a different position and both resulting in a synonymous substitution that does not affect the sequence of the encoded protein. We thus conclude that these substitutions do not affect the function of VdNRS/ER.

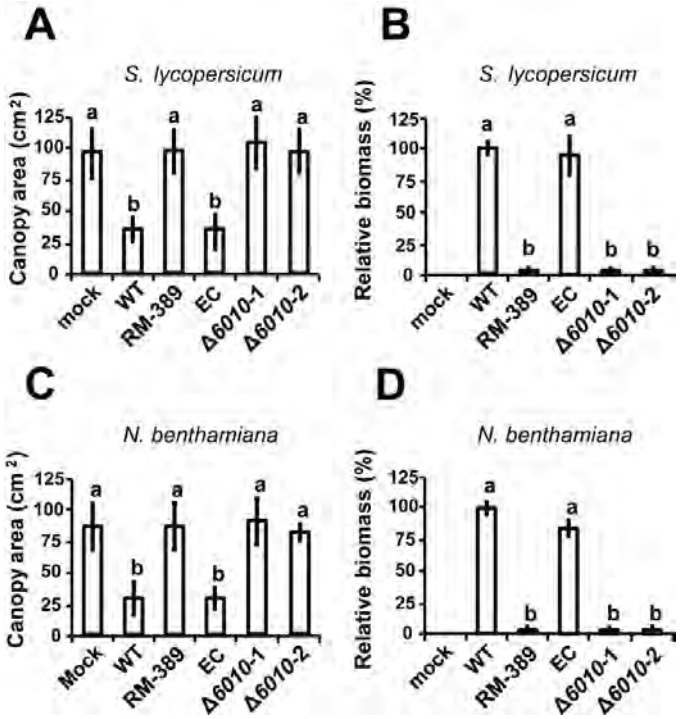

FIGURE 5 | *VdNRS/ER* is required for pathogenicity of *Verticillium dahliae* on tomato and *Nicotiana benthamiana*. (A) Average canopy area of 6 tomato plants, and (B) real-time PCR quantification of fungal biomass at 14 days after mock-inoculation (mock), or inoculation with wild type *Verticillium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

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

To examine the role of *VdNRS/ER* in cell wall integrity and osmotic stress resistance, the random mutant (RM-389), the *VdNRS/ER* deletion strains and the ectopic transformant were grown on minimal agar medium supplemented withmannitol, sodium chloride, sorbitol or congo red. After seven days of growth the colony diameter was measured, showing that mutant and wild type strains were equally sensitive to the cell wall stress reagents (Figure 6). These findings suggest that *VdNRS/ER* is not involved in cell wall integrity or osmotic stress resistance during mycelium development.

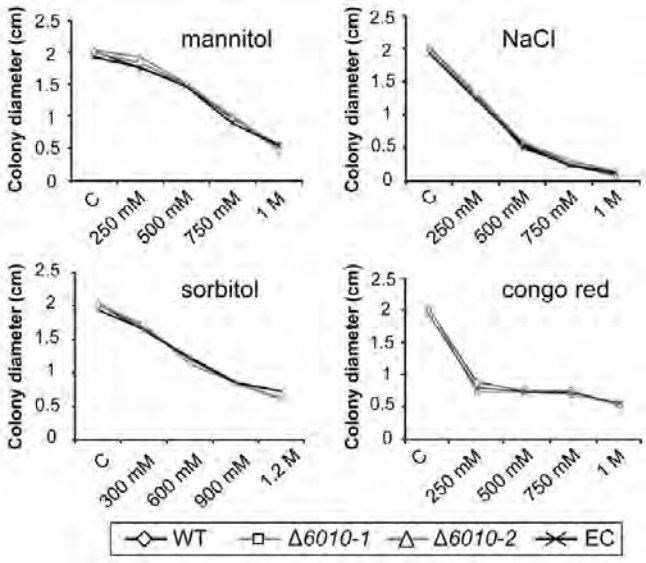

FIGURE 6 | Targeted deletion of *VdNRS/ER* does not affect cell wall integrity and osmotic stress resistance. Stress sensitivity assays were performed by placing a 5 µL droplet (10^6 conidia/mL) of wild type *V. dahliae* (WT), an ectopic transformant (EC) and two *VdNRS/ER* deletion strains (Δ*6010-1* and Δ*6010-2*) on Czapek-dox medium (C), or Czapek-dox supplemented with congo red (250 mM, 500 mM, 750 mM and 1 M), NaCl (250 mM, 500 mM, 750 mM and 1 M), mannitol (250 mM, 500 mM, 750 mM and 1 M), or sorbitol (300 mM, 600 mM, 900 mM, 1.2 M). The colony diameter was measured after 7 days of incubation at 22°C.

VdNRS/ER is required for tomato root colonization

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

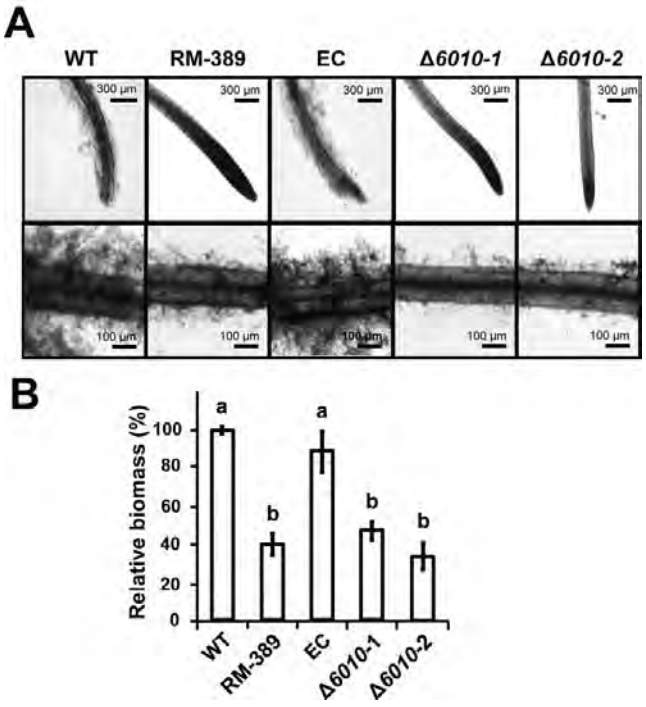

FIGURE 7 | *VdNRS/ER* is required for colonization of tomato roots. Roots of ten-day-old tomato cultivar MoneyMaker seedlings were immersed in one-fifth PDB containing 10^6 conidia/mL of wild type *V. dahliae* (WT), random transformant (RM-389), an ectopic transformant (EC) and two *VdNRS/ER* deletion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

VdNRS/ER is a functional enzyme that converts UDP-KDG to UDP-rhamnose

To examine whether *VdNRS/ER* is involved in the biosynthesis of UDP-rhamnose and determine whether this activated sugar is the precursor for the synthesis of rhamnose-containing glycan, we examined the polysaccharide composition of *V. dahliae*. Polysaccharides were extracted from conidiospores of wild type *V. dahliae*, the *VdNRS/ER* deletion strain and the complementation strain, and analysed by GC-MS. In the samples derived from wild type *V. dahliae* and the complementation strain a peak was detected that corresponds to rhamnose, which was absent in the *VdNRS/ER* deletion strain (Figure 8). This finding confirms that *VdNRS/ER* is involved in rhamnose biosynthesis.

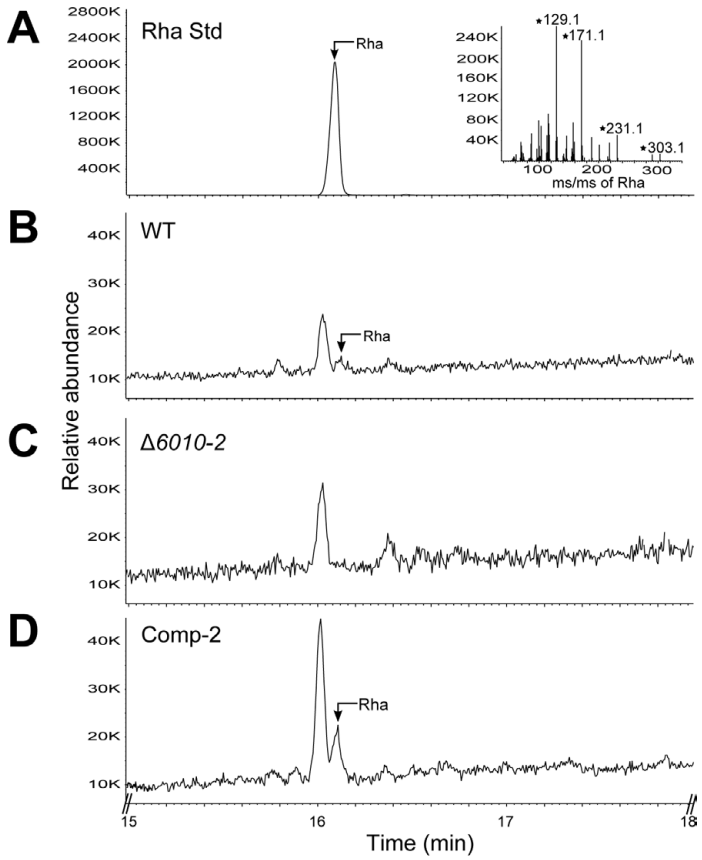

FIGURE 8 | *VdNRS/ER* deletion strains are depleted in UDP-rhamnose. GC-MS analysis of alditol acetate derivatives of the glycosyl residues from conidiospore polysaccharides (A) Total ion count chromatogram of alditol-acetate derived standard rhamnose (Rha), and mass spectra (insert) showing fragmentation pattern fingerprints with m/z 129, 171, 231, 303. (B) Polysaccharides from wild type *V. dahliae* (WT) conidiospores display a peak eluting at 16.10 minutes that has the same retention time as rhamnose standard. (C) *VdNRS/ER* deletion strain (Δ*6010-1*) lacks a peak eluting at 16.10 minutes. (D) *VdNRS/ER* deletion strain complemented with native *VdNRS/ER* gene (Comp-2), displays a peak eluting at 16.10 minutes that has the same retention time as the rhamnose standard.

If rhamnose-containing glycans play a role during early stages of host colonization, such as attachment of conidiospores to the root, we anticipated that such glycans are produced during conidiospore germination. Therefore, we determined the sugar composition of germinating conidiospores. Indeed, the sample derived from wild type *V. dahliae* comprised a clear rhamnose peak (Figure 9A;B) that was absent in samples from the *VdNRS/ER* deletion strain (Figure 9C). Taken together, *VdNRS/ER* plays a role in the formation of rhamnose-containing glycan in germinating conidiospores during early stages of host colonization.

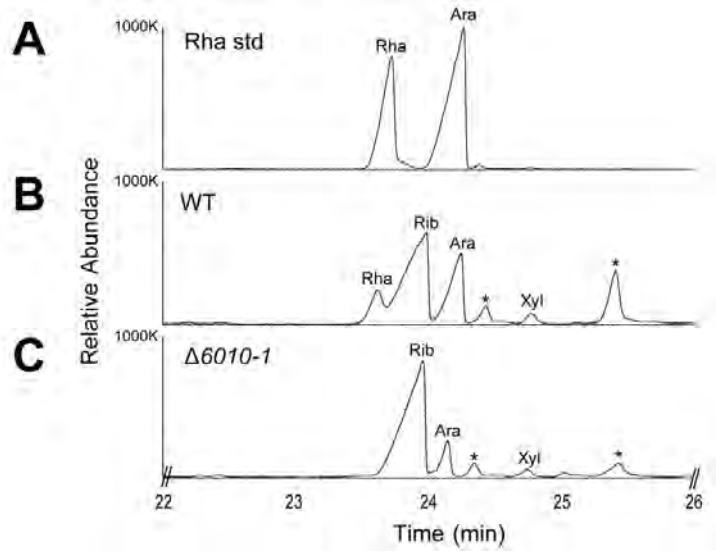

FIGURE 9 | UDP-rhamnose is produced in germinating conidiospores. GC-MS analysis of alditol acetate derivatives of the glycosyl residues from germinating conidiospore polysaccharides (A) Total ion count chromatogram of alditol-acetate derived standard rhamnose (Rha) (B) Polysaccharides of conidiospores from wild type *V. dahliae* (WT) display a peak with the same retention time as the rhamnose standard. (C) *VdNRS/ER* deletion strain (Δ*6010-1*) does not display a rhamnose peak. Other peaks in the chromatogram correspond to ribose (rib), arabinose (ara) and xylose (xyl). Asterisks indicate unidentified compounds that are not sugars.

In contrast to bacteria that produce dTDP-rhamnose in a three-step pathway, plants follow a two-step pathway to synthesize UDP-rhamnose (Oka et al. 2007; Watt et al. 2004). Genes involved in UDP-rhamnose were recently also identified in other fungi, including the rice pathogen *Magnaporthe oryzae* and the broad host range pathogen *Botrytis cinerea* (Martinez et al. 2012). In *B. cinerea*, UDP-glucose is converted by a 4,6-dehydratase (BfDh) into an intermediate UDP-KDG (UDP-4-keto-6-deoxy-glucose) that is subsequently converted by a bifunctional 3,5-epimerase/4-reductase (BfER, i.e. NRS/ER) into UDP-rhamnose (Martinez et al. 2012). Thus, we examined whether VdNRS/ER, like BfER, is a functional enzyme that can convert UDP-KDG to UDP-rhamnose. To this end, the dual enzyme activities of recombinant VdNRS/ER and BfDh was analyzed by LC-MS using UDP-glucose as a substrate (Martinez et al. 2012). The first enzyme in the pathway is capable to convert UDP-Glc to UDP-KDG (Figure 10B, and D) as expected, and with the addition of the NRS/ER activity (VdNRS/ER) UDP-KDG was converted to UDP-rhamnose (Figure 10C). The authenticity of UDP-rhamnose was determined by its elution and by its mass and ms/ms ion fragmentation (see insert in Fig 10C). The enzyme activity of recombinant VdNRS/ER unambiguously shows that VdNRS/ER is a bifunctional enzyme with a 3,5-epimerase and 4-reductase

activities (Figure 10E). Thus, we conclude that deletion of *VdNRS/ER* contributed to the loss of UDP-rhamnose formation and the lack of rhamnose-containing glycans during spore germination and early stages of infection.

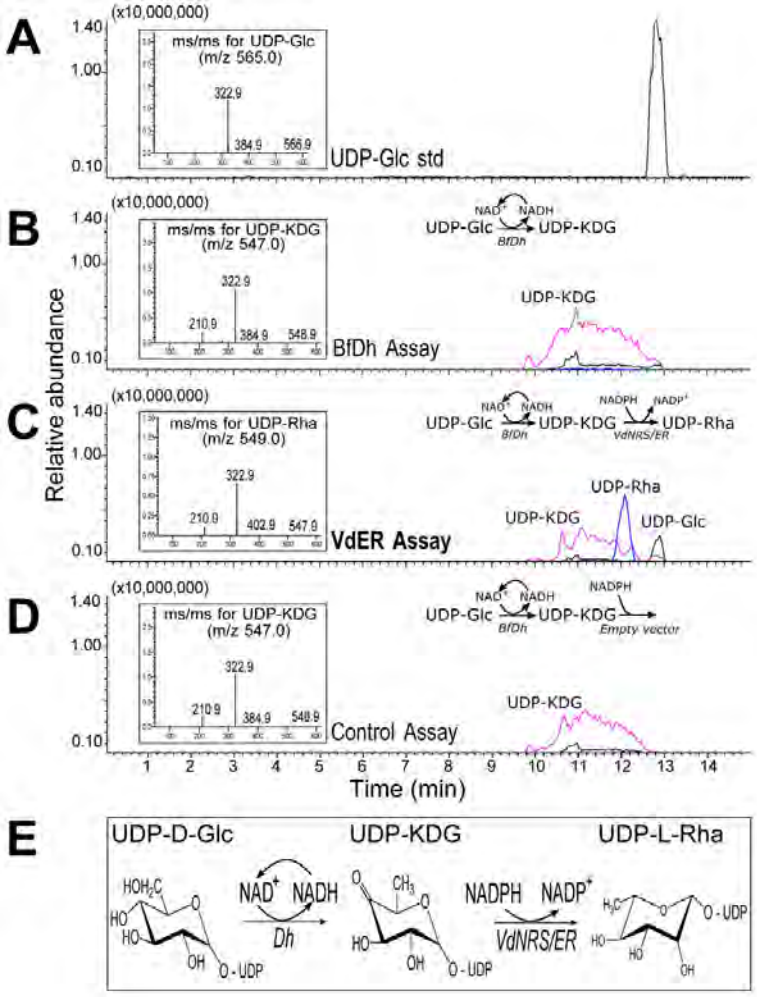

FIGURE 10 | VdNRS/ER converts UDP-KDG to UDP-rhamnose. (A) UDP-glucose (UDP-Glc) standard elutes from Hilic column at 12.8 minutes and is detected by MS with m/z 565 $[M-H]^-$. The ms/ms of the parent ion (insert) gave m/z 323 and 385 diagnostic ion fragments $[UMP-H]^-$ and $[UDP-H2O-H]^-$, respectively. (B) Hilic separation of products from the enzymatic reaction with BfDh displays a peak with the same rentention time as UDP-4-keto-6-deoxyglucose (UDP-KDG) with diagnostic $[M-H]^-$ m/z 547 and ms/ms ion fragment with m/z 323. (C) Hilic separation of of products from the dual enzymatic reaction with BfDh and VdNRS/ER displays a peak with the same rentention time as UDP-rhamnose (UDP-Rha) with diagnostic $[M-H]^-$ m/z 549, and ms/ms of 403, 323, 210. (D) Dual enzymatic reaction with BfDh and an empty vector control displays a peak with the same rentention time as UDP-4-keto-6-deoxyglucose (UDP-KDG) with diagnostic $[M-H]^-$ m/z 547 and ms/ms ion fragment with m/z 323. (E) UDP-rhamnose metabolic pathway in fungi showing the enzymes involved in the sequential conversion of UDP-Glc to UDP-Rha.

Discussion

In our study on a random mutant library that was generated in the vascular wilt fungus *V. dahliae*, we show for the first time that rhamnose containing macromolecules are required for fungal pathogenicity through their contribution to colonization of tomato roots. In random mutant 389, the T-DNA was integrated 56 bp upstream of the coding region of a putative nucleotide-rhamnose synthase/epimerase-reductase (NRS/ER). Our biochemical analyses of cell wall polysaccharides (Figures 8 and 9) and enzyme activity (Figure 10C) confirmed that VdNRS/ER is involved in UDP-rhamnose biosynthesis and shows that this activated sugar is the precursor for rhamnose-containing glycan.

VdNRS/ER shows similarity to the bacterial rmlD substrate binding domain, which is involved in biosynthesis of dTDP-rhamnose. In Gram-negative bacteria such as *S. enterica*, *V. cholerae* or *Escherichia coli*, L-rhamnose is an important residue in the O-antigen of lipopolysaccharides, which are essential for resistance to serum killing and intestinal colonization (Chiang and Mekalanos 1999). In mycobacteria such as *Mycobacterium tuberculosis*, L-rhamnose maintains the structural integrity of the cell wall through connecting the inner peptidoglycan layer to the arabinogalactan polysaccharides (Giraud et al. 2000). Moreover, disruption of dTDP-rhamnose biosynthesis in the plant growth-promoting rhizobacteria *Azospirillum brasilense* and *Azorhizobium caulinodans* resulted in reduced root attachment and colonization (Gao et al. 2001; Jofré et al. 2004). Work on *Colletotrichum graminicola*, causal agent of stalk rot of cereals and anthracnose of maize revealed the secretion of mucilaginous matrix by the fungus to promote survival of fungal spores, the major component of which appeared to be high molecular weight glycoproteins containing rhamnose and mannose oligomers (Ramadoss et al. 1985). Subsequently, this matrix was proposed to allow for the rapid adhesion of even ungerminated conidia to the hydrophobic plant surface to ensure germination and appressorium formation and establishment of an infection court (Nicholson and Kunoh 1995). Interestingly, further rhamnose-containing extracellular matrix is released upon germ tube elongation to surround and extend between hyphae (Sugui et al. 1999). Finally, a recent study identified two genes encoding UG4,6-Dh and U4k6dG-ER enzymes from *Magnaporthe oryzae* and *Botrytis cinerea* involved in UDP-rhamnose formation, although their contribution to pathogenicity was not investigated (Martinez et al. 2012). Based on our study, however, strong evidence is presented that fungal UG4,6-Dh and U4k6dG-ER enzymes are involved in the formation of UDP-rhamnose that is incorporated into rhamnose-containing polysaccharides that are crucial for fungal pathogenicity. Here, we have shown that targeted deletion of *VdNRS/ER* contributes to loss of *V. dahliae* pathogenicity on various host plants.

The role of rhamnose-containing macromolecules in cell wall integrity and oxidative stress tolerance in fungi is largely unknown. *VdNRS/ER* deletion strains were able to grow and sporulate without any visible defects, suggesting that rhamnose-containing glycans such as glycoproteins, polysaccharides or exopolysacharides, are not essential for vegetative growth or sporulation. In addition, the growth rate of *VdNRS/ER* knock-out mutants was not affected in the presence of inducers of cell wall stress and osmotic stress, suggesting that the rhamnose containing glycans are not involved in functional cell wall structure and osmotic stress tolerance.

Based on the findings reported in this study, we propose a model to explain the contribution of UDP-rhamnose to the virulence of *V. dahliae* during infection on host plants. In the cytoplasm UDP-glucose is converted into UDP-rhamnose in two steps using VdUG4,6-Dh and VdNRS/ER. The rhamnose of UDP-rhamnose is then transferred to glycan(s). The nature of these glycans remains unknown, but they consist of NaOH-solubilized cell wall polysaccharides and potentially glycoproteins. Presently, we cannot exclude the possibility that the rhamnose-containing polysaccharide is not an extracellular polysaccharide (EPS) as shown to be the case in *Botrytis cinerea*. We propose that the rhamnose-containing glycan, produced during spore germination, plays a role in the attachment of spores to the root surface which results in successful colonization of host plants. As *VdNRS/ER* deletion strains are unable to synthesize UDP-rhamnose and unable to cause disease, inhibitors of UDP-rhamnose synthesis may result in impaired pathogenicity and could present a novel strategy for disease control.

Materials And Methods

Agrobacterium tumefaciens mediated transformation (ATMT)

The binary vector (pBHt2) harboring the hygromycin B resistance gene (*hph*) under control of the *Aspergillus nidulans trpC* promoter was used for ATMT (Mullins et al. 2001). This vector was introduced to *Agrobacterium tumefaciens* strain SK1044 to transform conidia of race 1 *V. dahliae* strain JR2 as described previously (Faino et al. 2015; Santhanam 2012). Briefly, *A. tumefaciens* was grown at 28°C for 2 days in minimal medium supplemented with kanamycin (25 µg/mL). The *A. tumefaciens* cells were diluted to an OD_{600} of 0.15 in induction medium (IM), supplemented with 200 µM acetosyringone (AS). The cells were grown for an additional six hours before mixing them with an equal volume of *V. dahliae* conidiospore suspension (10^6 conidia/mL). 200 µl of this mixture was plated onto a Hybond-N^+ filter placed on induction medium supplemented with 200 µM acetosyringone. The plates were incubated in the dark at room temperature for 48 h after which the filter was

transferred onto a selection plate (PDA supplemented with 50 µg/mL of hygromycin B and 200 µM of cefotaxime). After 10 to 14 days, individual transformants were transferred to 24-well culture plates containing one mL of selection medium and incubated for 7 to 10 days. Spores from these cultures were stored in 30% glycerol at -80°C until further analysis.

Plant inoculations

Pathogenicity assays were performed as described previously (Santhanam and Thomma 2013). Briefly, individual transformants were sub-cultured in six-welled culture plates for 7 to 10 days. Subsequently, 10 glass beads (~3 mm) and 3 mL of tap water were added to each of the wells and the plates were sealed. The conidiospores were released by shaking the plates for 15 min at 200 rpm on a reciprocal shaker after which the sealing was removed. The roots of 10-day-old tomato (*Solanum lycopersicum* cv. MoneyMaker) seedlings were rinsed in water and dipped into the conidiospore suspension for 5 minutes. Seedlings were replanted in soil and scored for symptom development (wilting and stunting) up to 14 days. Seedlings that exhibited reduced *Verticillium* wilt symptoms when compared to inoculation with wild type *V. dahliae* inoculated plants were identified and the corresponding mutants were retained for rescreening.

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

Identification of T-DNA insertion sites

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

and sequenced. The obtained sequences were used as query to blast against the *V. dahliae* genome (http://fungi.ensembl.org/Verticillium_dahliaejr2/Info/Index) (Faino et al. 2015).

Targeted mutagenesis and complementation

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

To generate a *VdNRS/ER* complementation construct, a 2404 bp *EcoR*I/*Pac*I fragment containing the VDAG_06010 (VDAG_JR2_Chr7g02960) coding sequence with 1000 bp upstream and 450 bp downstream sequence was amplified from *V. dahliae* strain JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al. 2008). Complementation transformants were selected on PDA supplemented with 200 µg/mL of cefotaxime and 100 µg/mL phleomycin (InvivoGen, San Diego, USA).

Growth, conidiogenesis and stress assays

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

Root colonization assay

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

Expression of Recombinant VdNRS/ER

The following expression constructs were expressed in *E. coli* strain Bl21(DE3): pET-6hSUMO-VDAG_06010, contains a 6-histidine-SUMO tag fused to the N-terminal of *V. dahliae VdNRS/ER* (VDAG_06010); pET-6hSUMO was used as a empty vector control (pET-SUMO); pET28-BfDH4.1 (Martinez et al. 2012) was used to express and purify the 6His tagged *Botrytis fuckeliana* UDP-Glc 4,6-dehydratase (BfDH). The *E. coli* strains carrying the different constructs were cultured for 16 h at 37°C in LB medium supplemented with 50 µg/mL kanamycin and 35 µg/mL chloramphenicol. The following day, 5 mL cultures were transferred into 245 mL of fresh LB liquid medium supplemented with the same antibiotics and cell growth was resumed at 37°C at 220 rpm until the cell density reached an OD_{600} of ~0.6. Gene expression was then induced by the addition of isopropyl β-D-thiogalactoside (0.5 mM), and cultures were grown for additional 20 h at 18°C at 220 rpm. Cells were collected by centrifugation (6,000 × *g* for 10 min at 4°C) and suspended in 10 mL lysis buffer [50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.5 mM PMSF, with or without 50 mM NaCl]. Cell were lysed in an ice bath with a S-4000 sonicator (Misonix Inc, Farmingdale, NY, USA) equipped with 1/8-inch microtip probe using 12 sonication cycles each (10-s pulse; 20-s off). The lysed cells were centrifuged (6,000 × *g* for 10 min at 4°C); the supernatant was supplemented with 1 mM DTT and centrifuged again (20,000 × *g* for 30 min at 4°C). The resulting supernatant was kept at −20°C. Protein purification was carried out as previously described (Martinez et al. 2012).

Enzyme Assays

The dual activity of recombinant enzymes BfDH and *VdNRS/ER* was analyzed by LC-MS, in a total volume of 50 µl, containing 50 mM Tris-HCl pH 6.5, 0.25 mM UDP-Glc, 0.5 mM NAD^+, 0.5 mM NADPH, 5 µl of purified *B. fuckeliana* BfDH with or without the addition of 2 µl of *VdNRS/ER*. The reaction was incubated at 37°C for 1 h and terminated by heating for 5 min at 95°C. After the addition of chloroform and centrifugation (10,000 x g 4 min) an aliquot (20 µl) of the upper layer phase was

removed and mixed with 38 µl acetonitrile and 2 µl 0.5 M ammonium-acetate pH 4.35. A portion (25 µl) of this mixture was analyzed using HILIC chromatography and mass spectrometry analyses. This was done using a LC-MS/MS IT-TOF MS system (Shimadzu Scientific Instruments, Columbia, MD, USA) operating in the negative ion mode with a Nexera UFPLC LC-30AD pump, autosampler (Sil30), and column heater (set to 37°C). Nucleotide sugars were separated on an Accucore 150-amid HILIC column (150 × 4.6 mm, 2.6 µm particle size, ThermoScientific) using 40 mM ammonium acetate, pH 4.3 (solvent A), and acetonitrile (solvent B) with the gradient conditions as in (Li et al. 2015).

Isolation of polysaccharide and sugar analyses by GC-MS

Conidiospores were harvested from 7-day-old *V. dahliae* cultures grown on potato dextrose agar (PDA) (Thermo Fisher Scientific Inc, Breda, The Netherlands). Germinating spores were prepared by inoculating 30 µl of conidiospores into 12.5 mL of Gamborg's Glc (2%) in a medium sized plate (9 cm). The plates were incubated for 66 hours at room temperature under regular light to allow the spores to germinate. Polyssaccharides were either directly extracted from conidospore samples or from germinating conidiospores (< 100 µg) *s*tarting by incubation for 3h at 80°C while stirring with 5 mL 1 M NaOH (5 mL). After centrifugation (10,000 × *g*, 4°C, 10 min) the alkaline extract was mixed with an equal volume of 96% ethanol. Following centrifugation (10,000 × *g*, 4°C, 15 min), the pellet was resuspended in 10 mL water, and dialyzed (3,000 molecular weight cutoff) against two 10-liter changes of deionized water. The dialyzed solution was centrifuged (10,000 × *g*, 4°C, 15 min) and the water-soluble supernatant (aWS) was collected and freeze-dried. The glycosyl residue compositions of these extracts were then determined.

An aliquot of each extract (1 mg) was supplemented with 10 µg inositol and hydrolyzed with 1 mL of 2 M trifluoroacetic acid *at* 120°C. The released monosaccharides were reduced to their alditols (York et al. 1986), and acetylated. The resulting alditol acetate derivatives were analyzed by GC-MS system consisting of GC (Agilent 7890a) (Agilent Technologies, Santa Clara, CA, USA) equipped with an EI-MS detector (5975c). A 1 µl sample or std was injected via Agilent 7693 autosampler injector port (Agilent Technologies, Santa Clara, CA, USA) (set at 250°C; 3 mL/min helium) in a split (1:50) mode into a non-polar Equity-1 capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness). The chromatography gas carrier was helium (1 mL/min) and the column oven temperature program used to separate sugar derivatives was as follows: initial injection temperature was 60°C for 1 min, followed by an increase of 27.5°C min^{-1} to 170°C, then at 4°C min^{-1} to 235°C, and a hold at 235°C for 2 min. The column was then kept at 260°C for 12 min, cooled to 60°C and kept

at 60°C for 1 min prior to the next sample injection. The temperature of the transfer line between the column ends to the MS was 250°C. Detection was achieved using EI-MS operating with an electron impact ionization of energy 70 V with the temperature of MS ion source set at 230°C and that of the quad at 150°C. MS data was collected in full scan monitoring mode at *m/z* range 50-550 from 5-50 min. The spectra were analyzed using Software MSD ChemStation D.02.00.275 (Agilent Technologies, Santa Clara, CA, USA). Alditol-acetate derivatives of monosaccharide standards (50 µg each of rhamnose, fucose, xylose, mannose, glucose, and 30 µg each ribose, arabinose, galactose) supplemented with 10 µg inositol (internal std.) were prepared under the same conditions as the samples. For data analysis, sugar residue peaks were identified based on retention times of standard monosaccharides and their characteristic mass spectrum.

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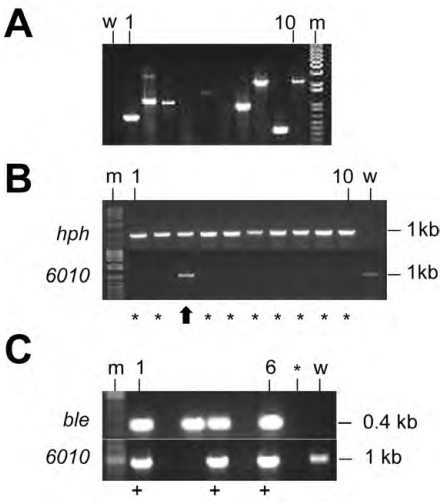
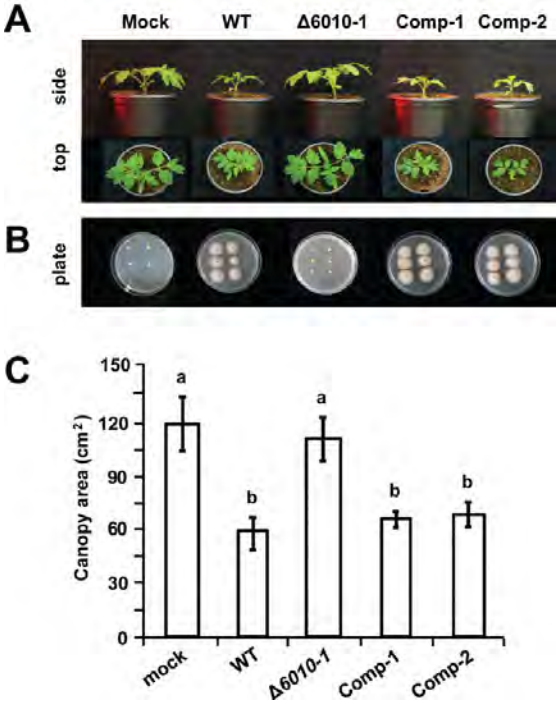
Supplemental data

SUPPLEMENTAL TABLE 1 | Primers used in this study

Primer	Sequence (5′- 3′)	Remarks
MLBF	GGATTTTGGTTTTAGGAATTAGA	*Msp*I left border, forward
MLBR	AATTCGGCGTTAATTCAGTACA	*Msp*I left border, reverse
MLSeq	TCAGTACATTAAAAACGTCCGCAA	*Msp*I left border, sequencing
MRBF	CAACTGTTGGGAAGGGCGATC	*Msp*I right border, forward
MRBR	CAGCCTGAATGGCGAATGCTA	*Msp*I right border, reverse
MRSeq	GAATGCTAGAGCAGCTTGAGCT	*Msp*I right border, sequencing
NLBF	AGTGTATTGACCGATTCCTTGC	*Nco*I left border, forward
NLBR	AGGGTTCCTATAGGGTTTCGCTCATG	*Nco*I left border, reverse
NLSeq	GAATTAATTCGGCGTTAATTCAGT	*Nco*I left border, sequencing
NRBF	CGTTATGTTTATCGGCACTTTG	*Nco*I right border, forward
NRBR	GGCACTGGCCGTCGTTTTACAAC	*Nco*I right border, reverse
NRSeq	CCCTTCCCAACAGTTGCGCA	*Nco*I right border, sequencing
KO-6010-LF	GGTCTTAAUACTGCGCTGATGACCTCAC	Left border, forward
KO-6010-LR	GGCATTAAUCAGTGGATGCTGTTGTCGAT	Left border, reverse
KO-6010-RF	GGACTTAAUAATGCGAAATGCGTAAGGAG	Right border, forward
KO-6010-RR	GGGTTTAAUGCGGTAGGCCTTCTTGATCT	Right border, reverse
6010-F	GCGGCCGCATGTCAGTTACGAACGGTGC	Full length, forward
6010-R	CCATGGTCAATTAACACCAGCAGCCTT	Full length, reverse
6010 -comp_F	GAATTCTGCGCTGATGACCTCACC	Complementation, forward
6010 -comp_R	TTAATTAACCGCCTTGGATCACCAC	Complementation, reverse
qPCR-6010-F	ACACGATCCTCCACG ATCTC	Real-time PCR, forward
qPCR-6010-R	GCGAGAAGTTCTTCCAGGTG	Real-time PCR, reverse
VdELF1-a-F	CCATTGATATCGCACTGTGG	Real-time PCR, forward
VdELF1-a-F	TGGAGATACCAGCCTCGAAC	Real-time PCR, reverse
qPCR-Hyg-F	ATAGGTCAGGCTCTCGCTGA	Real-time PCR, forward
qPCR-Hyg-R	GATGTAGGAGGGCGTGGATA	Real-time PCR, reverse
6010-BamH1-F	GGATCCTCAGTTACGAACGGTGCCA	pET-SUMO cloning, forward
6010-Hind3-R	AAGCTTCTATTATCAATTAACACCAGCAGCCTT	pET-SUMO cloning, reverse



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



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

Chapter 8

General discussion: From effector discovery to breeding for durable pathogen resistance in crops

Abstract

One of the major challenges in agriculture is to prevent yield loss caused by plant pathogens. Arguably, the most sustainable way to control disease is the deployment of genetic resistance. Pathogen resistance in plants is often conferred by immune receptors that, directly or indirectly, recognize pathogens or their activity. Effectors are pathogen-secreted molecules that support host colonization and therefore pose a strong selection pressure on plant hosts to recognize them. Effector identification and characterization has shed light on our understanding of plant-pathogen interactions, and on the various molecular mechanisms of pathogens and hosts that are involved in shaping those interactions. Here, I discuss the current understanding of pathogen effectors and how they can be exploited in breeding programs to establish durable pathogen resistance in crops.

Introduction

Crop production is continuously threatened by plant pathogens, and genetic resistance is the most important source to prevent disease development. Already in the 40's of the 20th century, Harold Flor proposed the "gene-for-gene" theory, stating that for any given plant that carries a resistance (*R*) gene, a corresponding avirulence (*Avr*) gene is present in the pathogen to which it confers resistance (Flor 1942). This led to the assumption that R proteins directly interact with the corresponding Avr proteins (Keen 1990). However, most studies on R-Avr pairs failed to demonstrate such direct interaction, which led to the formulation of "the guard hypothesis" (Van der Biezen and Jones 1998). This hypothesis states that plant R proteins monitor, or "guard", the status of a plant protein that may be targeted by pathogen effectors. Once this indeed happens and the "guardee" is modulated by a pathogen effector, the guard becomes activated and triggers an immune response. This hypothesis was formulated because meanwhile it had become evident that Avr proteins often have functions to contribute to the virulence of the pathogen, for instance by targeting host proteins that are involved in defence responses, and were therefore referred to as effectors (Boller and Felix 2009). Currently, pathogen effectors are best described as secreted molecules that support host colonization (Rovenich et al. 2014). Besides pathogen effectors, plant immune receptors may also recognize conserved microbial signatures such as fungal chitin and bacterial flagellin, also known as microbe-associated molecular patterns (MAMPs). Whereas recognition of effectors is referred to as effector-triggered immunity (ETI), recognition of MAMPs is referred to as MAMP-triggered immunity (MTI) in the so-called "zigzag model" (Chisholm et al. 2006; Jones and Dangl 2006). It is generally accepted that successful plant pathogens use effectors to overcome MTI. In turn, to overcome ETI upon recognition of an effector by a host receptor, the pathogen needs to mutate or purge this effector, or evolve a novel effector to suppress the immune response and successfully colonize the plant.

Effectors and MAMPs cannot be strictly separated

The discovery and characterization of various effectors and MAMPs from plant pathogens, including fungi, oomycetes and bacteria, have resulted in molecules that cannot be strictly separated as was proposed in "the zigzag model" (Thomma et al. 2011). In this model, effectors are described as species-, race- or even isolate-specific and contribute to pathogen virulence, whereas MAMPs are described as molecular patterns that are conserved in a broad range of species and contribute to microbial fitness. Arguably, these two groups represent the ends of a spectrum of molecules that should be seen as a continuum. Indeed, these definitions do not apply to pathogen molecules such as the NEP1-like proteins (NLPs) that are

found in a multitude of species belonging even to multiple kingdoms, and act as effectors that stimulate virulence on the one hand, and as MAMP on the other hand (Oome et al. 2014; Pemberton and Salmond 2004). Similarly, lysin-motif containing (LysM) effectors are conserved and widespread in the fungal kingdom (de Jonge and Thomma 2009; Kombrink and Thomma 2013). Also *Verticillium dahliae* effector Ave1 has homologs in other plant pathogens, including the fungi *Fusarium oxysporum*, *Colletotrichum higginsianum*, *Cercospora beticola* and the bacterium *Xanthomonas axonopodis* (de Jonge et al. 2012). In these organisms, the Ave1 homologs act as virulence factor as well (Ficarra et al. 2017; Gottig et al. 2008), with the exception of *C. higginsianum* (Chapter 3, this thesis). In addition, multiple Ave1 homologs were identified in the fungi *Venturia inaequalis* and *V. pirina* that are pathogens of apple and pear, respectively (Chapter 5, this thesis)(Cooke et al. 2014; Deng et al. 2017). However, whether these homologs act as virulence factors too remains to be demonstrated. Tomato immune receptor Ve1 differentially recognizes Ave1 effector homologs from various plant pathogens (Chapter, 4 this thesis)(de Jonge et al. 2012; Song et al. 2017). Finally, the effector proteins Pep1 and Pee1 of the maize smut fungus *Ustilago maydis*, which are essential effectors at the biotrophic stage, are conserved in other *Ustilago* species (Hemetsberger et al. 2012; Redkar et al. 2015). These examples illustrate that pathogen effectors are not always lineage-specific but can be conserved even across multiple kingdoms of life.

Bacterial flagellin is a MAMP that is broadly conserved between species. However, within species polymorphisms in flagellin have been identified in which some versions are recognized and others are not (Cai et al. 2011; Clarke et al. 2013; Sun et al. 2006). This shows that MAMPs are not always conserved and can also mutate to avoid recognition. Therefore, MAMPs and effectors and, consequently, MTI and ETI cannot be strictly separated, but rather form a continuum (Thomma et al. 2011).

The zigzag model adequately describes the interaction of biotrophic pathogens with their hosts, as they are generally affected by immune responses that involve host cell death. However, necrotrophic pathogens that actively kill host cells to access nutrients actually benefit from cell death activated by immune receptors (Glazebrook 2005). This has been shown for the fungal pathogen *Cochliobolus victoriae* effector victorin that binds to TRXh5 which activates immune receptor LOV1, resulting in cell death that benefits the pathogen instead of stopping the pathogen (Lorang et al. 2012). Thus, although recognition of effectors of biotrophic and necrotrophic pathogens similarly leads to cell death, the outcome for the interaction is significantly different. Thus, the interaction between necrotrophic pathogens and their hosts is generally not well described by the zigzag model.

Similarly, the zigzag model does not account for the interaction between plants and mutualistic microbes that use effectors as well (Plett and Martin 2015). Cell death does not benefit the 'desired' outcome of the interaction between plants and mutualists, and therefore the zigzag model does not accurately describe these interactions.

Examples such as those above have been the inspiration to propose an alternative model to describe plant-microbe interactions, in which a continuum between MAMPs and effectors is appreciated on the one hand, and similarly between MTI and ETI on the other hand. This alternative model is named the "the invasion model" (Cook et al. 2015), which states that any signal that reveals invasion is a so-called invasion pattern (IP) that can potentially be detected by the plant through invasion pattern receptors (IPRs), leading to an appropriate response (IP-triggered response; IPTR) that either supports or obstructs microbial colonization of the host.

Population genomics to identify genomic regions that are associated with pathogenicity

The ease and low cost of present-day genome sequencing (Faino and Thomma 2014) has made it possible to sequence pathogen populations instead of a single genome per species. This has led to new insights in genome and effector evolution. Certain regions of fungal pathogen genomes are enriched in effector genes. For example, in the genome of the asexual pathogen *V. dahliae* chromosomal rearrangements are associated with highly dynamic lineage-specific regions that are enriched for *in planta*-induced effector genes and active transposons (de Jonge et al. 2013; Faino et al. 2016). Also in the fungal Brassica pathogen *Leptosphaeria maculans* and the potato famine oomycete *Phythophtora infestans* it was shown that gene-poor and repeat-rich regions of the genome are highly dynamic and enriched for *in planta* expressed effector genes, in contrast to gene-rich and repeat-poor regions of the genome (Dong et al. 2015). These observations illustrate the "the two-speed genome" model, in which effectors reside in fast evolving regions of the genome (Croll and McDonald 2012; Stukenbrock et al. 2010).

Various pathogens carry conditionally dispensable chromosomes (CDCs) that are enriched in effector genes. In the genome of the tomato pathogen *Alternaria alternate* a CDC contains all the genes responsible for AAL-toxin production (Akagi et al. 2009). More strikingly, in the genome of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* a CDC contains all but one SIX (for secreted in xylem) effector genes (Ma et al. 2010; Schmidt et al. 2013; Van Dam et al. 2016), including *FoAve1* (Chapter 3, this thesis)(de Jonge et al. 2012). When this chromosome, which is also referred to as a pathogenicity chromosome, is transferred to a non-pathogenic isolate of *F. oxysporum*, this isolate gains the capacity to infect tomato (Ma et al. 2010).

Effector discovery based on motifs and similarity

Many effectors have been identified based on their characteristics as small secreted cysteine-rich proteins. Depending on the (type of) organism, additional motifs have been associated with effectors, such as the RXLR or CRN (Crinkler) motif in *Phytophthora* species (Haas et al. 2009), MIMP (miniature impala) motif presence in the promotor of *F. oxysporum* effectors (Schmidt et al. 2013), lysin motif (LysM) in fungal effectors (de Jonge and Thomma 2009), the type-III secretion signal in effectors of gram-negative bacteria and the TAL (transcription activator-like) effector DNA binding motif in *Xanthomonas* species (Boch and Bonas 2010). However, for most pathogens effector motifs have not yet been identified and effectors often do not display sequence homology to other effectors, which makes it challenging to identify novel effectors. Comparison of the 3D-structure of two sequence-unrelated effector proteins of the fungal rice pathogen *Magnaporthe oryzae* revealed structural similarity (de Guillen et al. 2015). Subsequent structure-informed pattern searches identified novel effectors in *M. oryzae* and *M. grisea* that were named MAX (***M****agnaporthe* **A**vrs and To**x**B like) effectors (de Guillen et al. 2015). A similar approach to identify novel effectors can likely be applied to other pathogens as well (Franceschetti et al. 2017).

Identification of effectors through random mutagenesis

Since the majority of effector genes that have been identified thus far are unique and do not have homologs in other pathogens, an unbiased approach should be applied to identify them. Indeed, using random mutagenesis through *Agrobacterium tumefaciens*-mediated transformation (ATMT) followed by a screen for reduced virulence on the host plant has proven to be successful in identifying novel effectors and pathogenicity factors in fungal pathogens including for example *F. oxysporum* (Michielse et al. 2009), *Colletotrichum graminicola* (Munch et al. 2011) and *Botrytis cinerea* (Giesbert et al. 2012). In a random mutagenesis screen on *V. dahliae* using ATMT 900 mutants were generated that, after thorough virulence testing and sequence analysis, led to the identification of 58 candidate pathogenicity and effector genes. One of the candidate genes, *VdNRS/ER*, was functionally analyzed and led to the finding that rhamnose synthase activity is required for pathogenicity of *V. dahliae* (Chapter 7, this thesis) (Santhanam et al. 2017).

Population genomics as a tool for effector identification

Population genomics has contributed to the discovery of effectors that are recognized by host receptors. For example, by sequencing the genomes of 11 race 1 and race 2 strains of *V. dahliae* and subsequent RNA sequencing, the race

1-specific Ave1 effector that is recognized by tomato immune receptor Ve1 was identified (de Jonge et al. 2012). A similar approach was used to identify the Avr5 effector of the tomato leaf mould fungus *Cladosporium fulvum* that is recognized by the tomato immune receptor Cf-5 (Mesarich et al. 2014). Finally, the *F. oxysporum* f. sp. *melonis* effector AvrFom2 that is recognized by melon immune receptor Fom-2 was identified using a comparable approach (Schmidt et al. 2016). Potentially, this powerful strategy may be applied to identify potent virulence factors that are differentially present in other pathogen populations.

Effectors define host specificity

Population genomics has revealed that effectors may be responsible for host specificity of a pathogen. Within the species complex of *F. oxysporum*, the *formae speciales* indicates on which host plant an isolate is pathogenic. *Formae speciales* are generally polyphyletic and therefore hard to identify based on conserved genes. However, effectors identified in *F. oxysporum* isolates from various host plants group together based on the same *formae speciales*, providing an identification tool based on effector presence or absence (Lievens et al. 2009; Van Dam et al. 2016). However, it should be taken into account that effector presence alone is not always proof that an isolate is pathogenic as non-pathogenic Fusarium isolates where found that carry effector genes too (Jelinski et al. 2017). Also, comparative genomics between strains of the bacterium *Ralstonia solanacearum* that are pathogenic on different host plants revealed effector repertoires associated with a specific host (Ailloud et al. 2015). Finally, *P. infestans* and its close relative *P. mirabilis* that is pathogenic on *Mirabilis jalapa* share a homologous effector that is only effective on their corresponding plant host (Dong et al. 2014).

Gene and effector expansions and contractions are linked to the host range of a pathogen. In *V. dahliae*, NLP effector expansion has been associated with functional diversification (Santhanam et al. 2013; Zhou et al. 2012). Only a subset of the NLP effectors in *V. dahliae* is cytotoxic (typical for NLPs) while others are non-cytotoxic. Besides a role in virulence, NLP1 was also shown to be involved in asexual reproduction and vegetative growth. NLP1 and NLP2 are virulence factors on tomato and Arabidopsis, but not on cotton, and only NLP1 is required for virulence on *Nicotiana benthamiana* (Santhanam et al. 2013; Zhou et al. 2012). This suggests that NLP diversification might have contributed to host range expansion. A comparison of species in the genus *Colletotrichum* that have a broad host range with species that have a limited host range revealed that species with broad host ranges rely on secreted enzymes and NLPs, whereas narrow host range species have lost proteases and are more reliant on lineage-specific effectors (Baroncelli et al. 2016). The examples above illustrate that effector repertoires define the host

(range) of a pathogen. Moreover, as effectors are important virulence factors of pathogens that are potentially recognized by related or unrelated plant species of the host, they can be exploited as tools to identify novel resistance sources.

Effectors as tools for pathogen resistance breeding

Traditional plant breeding is a tedious and time-consuming practice in which marker-assisted selection has significantly increased the speed and efficiency of the selection process. However, pathogen assays are still an essential aspect for the development and verification of markers. Pathogen assays have limitations since they require extensive knowledge of the pathogens life cycle and can often only be performed with a single species or race of a given pathogen as resistance is usually species- or even race- specific. Once effectors have been identified for a given pathogen, these effectors can be used to screen germplasm for novel recognition specificities, and thus new resistance sources (Vleeshouwers and Oliver 2014). This can be done by transient expression of effector genes in germplasm by *Agrobacterium*-mediated expression or potato virus X (PVX) (Chapman et al. 1992; Janssen and Gardner 1990). In this manner, many effectors can be tested in parallel, circumventing the limitations of disease assays, while simultaneously providing the possibility of screening germplasm that is a non-host to the pathogen or the possibility of screening effectors of pathogens with a complex lifecycle.

Since many effectors are race- or even strain- specific it is not very efficient to use these in a germplasm screen for resistance. Recognition specificity towards effectors that are conserved in a population of pathogens, also referred to as core effectors, is far more valuable. In a population genomics study on 65 isolates of the cassava bacterial blight pathogen *Xanthomonas axonopodis* pv. *manihotis* 9 effectors were identified that are conserved in the population (Bart et al. 2012). In a similar approach, 13 conserved effectors were identified in 67 isolates that belong to three *Xanthomonas* species that are pathogenic on tomato and pepper (Potnis et al. 2011; Schwartz et al. 2015). Population genomics on strains of *V. dahliae* also revealed effectors that are conserved in the population (de Jonge et al. 2013). Identification and exploitation of *R* genes that recognize these conserved effectors might potentially lead to durable pathogen resistance.

Knowledge of the effectors that are present in a given population can also aid in developing recognition specificity towards (a) certain effector(s). A single amino acid substitution can already broaden the specificity of an *R* gene. This was shown for potato NB-LRR immune receptor R3a by performing a gain-of-recognition screen after random mutagenesis in which the recognition specificity was extended to isoforms of the recognized *P. infestans* effector Avr3A that are predominant in *Phytophthora* populations world-wide (Segretin et al. 2014). I2 is a NB-LRR immune receptor of

tomato that confers resistance to *F. oxysporum* and partial resistance to *P. infestans* and shows high similarity to R3a (Giannakopoulou et al. 2015). Mutations that extended recognition in potato R3a were also mutated in tomato I2 which resulted in a broader and enhanced specificity to both *P. infestans* effector isoforms of Avr3a and *F. oxysporum* effector isoforms of Avr2 (Giannakopoulou et al. 2015). These examples illustrate that with relatively small adjustments the recognition specificity of an immune receptor can be extended. Potentially, this strategy can be exploited to increase the recognition specificity of Ve1 towards pathogens that secrete homologs of Ave1 that are poorly recognized.

Like *I2*, other *R* genes or combinations of genes have been described to provide resistance to two or more pathogens. Tomato immune receptor Cf-2 guards Rcr3 that is targeted by effectors of the fungus *C. fulvum* and the nematode *Globodera rostochiensis* resulting in a defence response (Lozano-Torres et al. 2012). Thus, naturally occurring *R* genes can be exploited that confer resistance to multiple pathogens.

In a map-based cloning approach combined with natural variation analysis in Arabidopsis a dual *R* gene system consisting of the nucleotide binding, leucine-rich repeat (NLR) domain receptors *RPS4-Ws* and *RRS1-Ws* was identified that provides resistance to the fungus *C. higginsianum*, the bacteria *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tomato* (Deslandes et al. 2002; Narusaka et al. 2009). Other dual *R* gene systems have been characterized as well, such as the rice NLRs RGA4 and RGA5 that interact to provide resistance to *M. oryzae* (Césari et al. 2014a). In these NLR pairs integrated domains (NLR-IDs) have been identified that serve as a bait for effectors to activate immunity as has been described in the integrated decoy hypothesis (Césari et al. 2014b). NLR-ID integration in plant immune receptors is broadly distributed in the plant kingdom (Kroj et al. 2016; Sarris et al. 2016) and analyses of grass genomes suggests the presence of NLR 'acceptor' genes that likely acquire these domains from elsewhere in the genome (Bailey et al. 2017). Potentially, modification or transfer of NLR-IDs to other NLRs can contribute to synthesizing novel *R* genes (Kim et al. 2016; Rodriguez-Moreno et al. 2017).

Often *R* genes are identified or characterized in wild species or model organisms and cannot easily be introduced into crops by crossing. However, interfamily transfer of receptor-like proteins (RLPs) and receptor kinases (RKs) has been quite successful. For example, the *Arabidopsis thaliana* receptor kinase EFR that recognizes bacterial elongation factor Tu (EF-Tu) was transferred to tomato (*Solanum lycopersicum*) providing broad bacterial resistance (Lacombe et al. 2010). Similarly, the *A. thaliana* receptor kinase *LecRK-I.9* which provides resistance to *P. infestans* was functionally transferred to potato (*S. tuberosum*) (Bouwmeester et al. 2014). Interfamily transfer of NB-LRRs seems to be less efficient compared to transfer of RLPs and RKs. Perhaps, for NLRs it is more efficient to transfer genes from wild relatives into crops (Bent 2016).

Susceptibility genes as a source of resistance

Resistance is often overcome within a couple of years after the introduction of an *R* gene (Rouxel et al. 2003; Sprague et al. 2006a; Sprague et al. 2006b). It has been proposed that mutation of host components that are required for a pathogen to successfully colonize its host, also known as susceptibility (*S*) genes (Eckardt 2002), is a more durable strategy to provide pathogen resistance (McDonald and Linde 2002). An example of an *S* gene is the *Mlo* gene that provides resistance to powdery mildew in many crops including monocots and dicots (Kusch and Panstruga 2017). In barley, a recessive mutant of this gene was introduced 70 years ago and is still providing resistance to all powdery mildews in the field (Jørgensen 1992). Effector targets can be mutated with the consequence that the corresponding effector becomes useless. However, *S* genes have other functions in the plant as well. Therefore it should be taken into account that mutating an *S* gene can have detrimental effects on the plant (van Schie and Takken 2014).

Genome editing in crops

Genome editing in crops has made considerable steps forward over the last few years. With synthetic nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and Cas9 of the clustered regularly interspaced short palindromic repeats (CRISPR) system it is possible to knockout genes, delete or insert sequences without selection markers (Puchta 2017; Schiml and Puchta 2016). Arguably, CRISPR/Cas9 is the most efficient and flexible system to work with (Puchta 2017). Concerns about off-target mutations seem to be a problem of the past and efficiencies of 4-9% have been reached. The strength of the CRISPR/Cas9 system is that it is possible to make precise changes in a sequence without the introduction of foreign DNA and marker genes. This was elegantly shown by editing the genomes of maize and bread wheat (Liang et al. 2017; Svitashev et al. 2016). In tomato, CRISPR/Cas9 was used to inactivate the *S* gene orthologue *DMR6* (for downy mildew resistance 6) that was characterized in *A. thaliana* (Van Damme et al. 2008), resulting in resistance to *Pseudomonas syringae*, *Phytophtora capsici* and *Xanthomonas* species (de Toledo Thomazella et al. 2016). Similarly, a deletion generated in an *Mlo* orthologue by CRISPR/Cas9 in tomato resulted in the variety Tomelo which is resistant to the powdery mildew fungal pathogen *Oidium neolycopersici* (Nekrasov et al. 2017). For this variety it was shown with whole-genome sequencing that it does not carry any foreign DNA, does not have off-target mutations and carries a single deletion that is indistinguishable from naturally occurring mutations. Whether genome editing using CRISPR/Cas9 will skip regulation as a genetically modified organism (GMO) in the EU should become clear in the near future (Waltz 2016; Wolter and Puchta 2017).

Management strategies to prevent resistance breakdown

Unfortunately, current agricultural practice is still based on planting vast areas of monocultures, often carrying only a single *R* gene against a given pathogen, season-after-season. This puts a huge selection pressure on pathogen populations to overcome resistance. Resistance breakdown can occur within a few years after the introduction of an *R* gene (Rouxel et al. 2003; Sprague et al. 2006a; Sprague et al. 2006b).

R gene stacking, the introduction of multiple *R* genes in a single variety, significantly increases the hurdle for a pathogen to overcome resistance (McDonald and Linde 2002; Michelmore et al. 2013). *R* gene stacking can be achieved by gene transfer or marker-assisted breeding (Dangl et al. 2013). Using a cisgenic approach, broad spectrum *Rpi* (for resistance against *P. infestans*) genes were stacked and introduced in the susceptible potato cultivar Désirée to provide durable resistance to *P. infestans* and were subsequently monitored in field trials in the Netherlands (Haverkort et al. 2016; Tan et al. 2010; Zhu et al. 2013; Zhu et al. 2012). Several combinations of *Rpi* genes provided resistance to *P. infestans* and spray applications were significantly reduced (Haverkort et al. 2016). With marker-assisted breeding multiple *R* genes were stacked in soybean to provide durable resistance to *Soybean mosaic virus* (SMV)(Maroof et al. 2008) and in wheat to provide resistance to powdery mildew (Liu et al. 2000). Criteria for stacking genes should be taken into account to prevent rapid resistance breakdown. These include: the stacked *R* genes should target the same pathogen species, they should be introduced as a tandem construct to prevent segregation, have similar levels of expression in the same tissue and should target species that are susceptible to at least two of the stacked *R* genes (Gressel et al. 2017). Obviously, extensive knowledge on *R* genes and their corresponding effectors is essential to make educated decisions on which genes to stack. *R* gene catalogues are available for multiple crop-pathogen interactions including, for example, potato and *P. infestans,* and lettuce and *Bremia lactucae* (Parra et al. 2016; Vleeshouwers et al. 2011). Monitoring pathogen populations in the field is essential to know which effectors are present in the pathogen population and to know at an early time point whether resistance breakdown occurs (Howlett et al. 2015; Michelmore et al. 2013). Subsequently, *R* genes should be selected that provide resistance to conserved core effectors present in a pathogen population to increase the durability of resistance (Dangl et al. 2013). In addition, stacks of different *R* genes should be used either within a variety or between different varieties to provide distinct selection pressure and therefore increased durability (Chin and Wolfe 1984; Michelmore et al. 2013). Finally, different *R* genes or *R* gene stacks should be used in time and space to break the continuous selection pressure that is put on pathogens. The criteria described above will ultimately lead to durable broad spectrum resistance in crops (Figure 1).

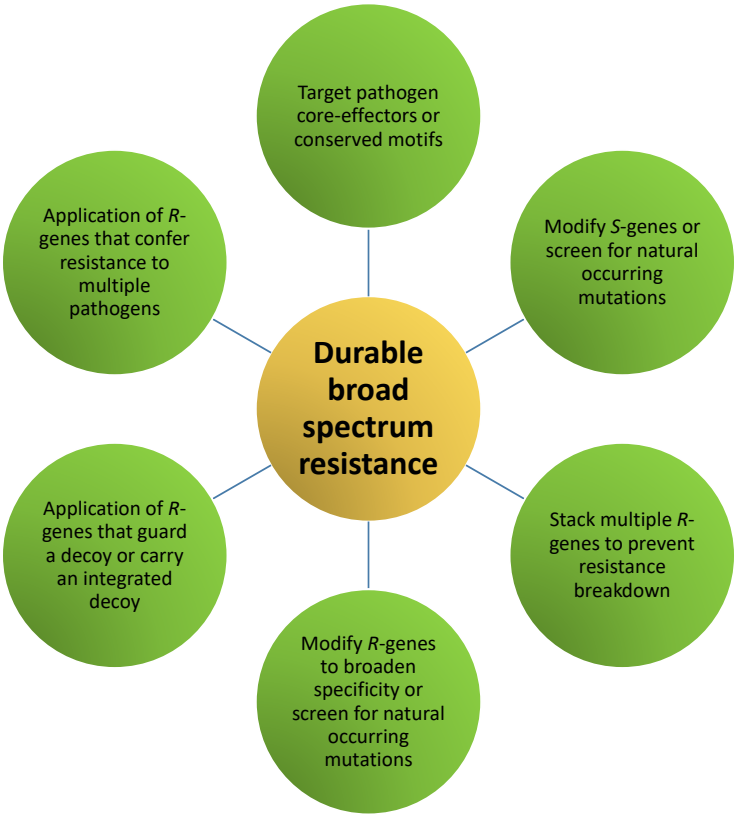


FIGURE 1 | Summarizing figure to visualize the many strategies that potentially lead to durable broad spectrum resistance in crops.

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Summary

Disease resistance in crops is an important aspect of securing global food security. Resistant plants carry immune receptors that sense pathogen invasion often through the recognition of important pathogen virulence factors, known as effectors. Thus, identification and characterization of effectors is important for the fundamental understanding of virulence mechanisms and to aid in resistance breeding. In **Chapter 1**, the motivation for selecting the plant pathogen *Verticillium dahliae* as a study object is explained. The main objectives and research questions are described and an outline of the thesis is provided. **Chapter 2** reviews the various roles of effectors of filamentous plant pathogens and explains that effectors can basically deregulate any step of plant immunity in any cellular compartment. Besides in plant pathogens, endophytes and mutualistic organisms carry effectors that suppress host immunity as well. In addition, effectors have been identified in saprophytes and other non-pathogenic plant-associated microbes and are proposed to act in microbial competition and cooperation. We propose a role for effectors in shaping the plant microbiome and argue that it is important for a microbe to rapidly diversify its effector repertoire to establish a microbial niche.

The genus *Verticillium* contains soil-borne fungal pathogens causing Verticillium wilts in a wide range of host plants. The most effective means of controlling these pathogens is genetic disease resistance. The tomato immune receptor Ve1 provides resistance to race 1 *V. dahliae* strains that carry *Ave1* (*VdAve1*). Previously, it was shown that VdAve1 contributes to the virulence of *V. dahliae* and that VdAve1 is the effector that is recognized by Ve1. Homologs of VdAve1 were mostly found in plants and in some plant pathogens, including the fungi *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), *Colletotrichum higginsianum* (ChAve1), *Cercospora beticola* (CbAve1) and the bacterium *Xanthomonas axonopodis* (XacPNP). In **Chapter 3** we analysed the role of the various Ave1 homologs in fungal virulence. Targeted *Ave1* deletions were generated in *F. oxysporum*, *C. beticola* and *C. higginsianum* and we showed that *FoAve1* and *CbAve1* are virulence factors. This is in contrast to *ChAve1*, which does not contribute to fungal virulence on *Arabidopsis thaliana* plants, likely because this gene is not expressed during infection. In a complementation experiment we found that none of the fungal Ave1 homologs can restore virulence in a *V. dahliae Ave1* deletion strain. Therefore we conclude that VdAve1 on the one hand and FoAve1, CbAve1, ChAve1 and XacPNP on the other hand, act in different manners. These homologs are differentially recognized by Ve1, with the most efficient recognition of VdAve1, and little to no recognition of ChAve1 and XacPNP. In **Chapter 4** we show that C-terminal GFP-tagging of VdAve1 resulted in loss of recognition by Ve1, suggesting that exposure of the C-terminus of VdAve1 is essential for recognition by Ve1. Truncations of VdAve1,

and domain swaps between VdAve1 and XacPNP, narrowed down the sequence that is required for Ve1 recognition to the C-terminal nine amino acids. Site-directed mutagenesis was performed to analyse the contribution of individual amino acid residues to recognition by Ve1, revealing the importance of five amino acids from Val127 to Phe131. Nevertheless, fusions of the VdAve1 C-terminal nine amino acids to GFP or to the *Cladosporium fulvum* effector Avr9 showed that this sequence is not sufficient to activate Ve1-mediated immunity. Finally, with a complementation experiment in *V. dahliae* we confirmed that the C-terminal nine amino acids of Ave1 are required, but not sufficient, to activate Ve1-mediated immunity. **Chapter 5** describes the identification of an expanded family of Ave1 homologs in the apple and pear scab pathogens *Venturia inaequalis* and *V. pirina*, respectively. We show that tomato immune receptor Ve1 is unable to recognize Ave1 homologs derived from *V. inaequalis* and *V. pirina*. Transcriptome analysis revealed that *V. inaequalis ViAve1-1*, *ViAve1-5* and *ViAve1-9* are expressed *in planta* and that these homologs are unable to complement a *V. dahliae VdAve1* deletion strain, suggesting that these homologs have different functions than *V. dahliae VdAve1*.

In **Chapter 6**, a gene with high similarity to *VdAve1* was identified in *V. dahliae* strain JR2 as an *Ave1-like* (*Ave1L*). Subsequently, we identified a total of five alleles (*Ave1L1* to *Ave1L5*) in the *V. dahliae* population. While some of the *V. dahliae* strains do not have *Ave1L*, other strains have one of the five *Ave1L* alleles in presence or absence of *Ave1* itself. Some of the *Ave1L* alleles contain multiple single nucleotide polymorphisms, some of which result in a premature stop codon. In addition, one of the alleles contains a retrotransposon insertion. These observations suggest that *Ave1L* experienced significant selection pressure, potentially to evade recognition by an immune receptor. Thus, we attempted to predict the common progenitor sequence of the five *Ave1L* alleles. However, we show that neither of the *Ave1L* alleles, nor the predicted progenitor, are recognized by tomato immune receptor Ve2 that shows high homology to Ve1. We furthermore show that *Ave1L2* is expressed during host colonization, but does not seem to contribute to virulence on tomato. Since *V. dahliae* has a wide host range, *Ave1L* potentially contributes to virulence on other host plants than tomato where it may have been recognized by an immune receptor that drove its divergence.

In **Chapter 7** random mutagenesis was performed on the *V. dahliae* genome, followed by a pathogenicity screen to identify genes that are important for host invasion. This resulted in the identification of 58 novel candidate genes. We focused on one of these candidates and showed that deletion of the VdNRS/ER gene from the *V. dahliae* genome results in complete loss of pathogenicity on tomato and *N. benthamiana* plants, whereas vegetative growth and sporulation are not affected.

We demonstrate that VdNRS/ER is a functional enzyme in the biosynthesis of UDP-rhamnose, and further analysis revealed that *VdNRS/ER* deletion strains are impaired in colonization of tomato roots. Collectively, our results demonstrate that rhamnose, although only being a minor cell wall component, is essential for pathogenicity of *V. dahliae*.

Chapter 8 is the general discussion and describes how the knowledge of pathogen effectors can be used as a tool to establish durable broad spectrum resistance.

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About the author

Jordi C. Boshoven was born in Alphen aan den Rijn, The Netherlands, on April 1st 1986. At the age of 5 he moved to Maasdijk where he went to the PC Basisschool Het Kompas. His secondary education was performed at the Interconfessionele Scholengroep Westland (ISW) in Naaldwijk. During his holidays Jordi was working in the company of his uncle that is growing freesias in the greenhouse. In 2002, he went to the ROC Mondriaan in Delft where he was educated at Mbo level with a specialization in Botany to work in a laboratory. His first internship was performed at Rijk Zwaan in De Lier under the supervision of Theresia van Luijk where he studied seed pathology. His second internship was performed at Groen Agro Control in Delfgauw under the supervision of Dr. Adriaan Vermunt and Laurens Hanemaaijer where he studied the molecular detection of plant pathogens in diagnostic samples.

In 2006, he continued his studies at Hbo level at the Hogeschool van Arnhem en Nijmegen (HAN) in Nijmegen where he studied Plant Biotechnology. During his education in Nijmegen he performed a minor, an internship and a thesis at Wageningen University. His internship was performed at the Laboratory of Phytopathology under the supervision of Prof. Dr. Bart Thomma where he studied plant recognition of the human bacterial pathogen *Staphylococcus aureus*. His thesis was performed in the Laboratory of Nematology under the supervision of Dr. Jose Lozano-Torres where he studied the recognition of the nematode effector Gr-VAP-1 by the tomato immune receptor Cf-2 that resulted in a publication in the scientific journal Proceedings of the National Academy of Sciences of the United States of America.

After his studies in Nijmegen he moved to Wageningen in 2009 to do a MSc in Plant Biotechnology with the specialization Breeding and Pathology at Wageningen University. He performed his MSc internship in the group of Prof. Dr. Brian Staskawicz at the University of California in Berkley, USA under the supervision of Dr. Sandra Goritschnig where he studied the recognition of the oomycete *Hyaloperonospora arabidopsidis* effector ATR1 by the Arabidopsis immune receptor RPP1. His MSc thesis was performed at the Plant-Microbe Interactions group at Utrecht University under the supervision of Dr. Dieuwertje van der Does where he studied the jasmonic acid and salicylic acid plant defense signaling pathways in Arabidopsis.

After obtaining his MSc degree he started in 2012 as a PhD candidate in the Laboratory of Phytopathology under the supervision of Prof. Dr. Bart Thomma. He studied the virulence and recognition of *Verticillium dahliae* effector Ave1 homologs that resulted in the publication of this PhD thesis. During his PhD he organized several activities as a member of the PhD council of the graduate school of Experimental Plant Sciences (EPS). Since 2016 Jordi is working as a researcher phytopathology at Bejo Zaden in Warmenhuizen and he lives with his wife and two children in Koedijk.

List of publications

Lozano-Torres, J. L., Wilbers, R. H. P., Gawronski, P., **Boshoven, J. C.**, Finkers-Tomczak, A., Cordewener, J. H. G., America, A. H. P., Overmars, H. A., Van 't Klooster, J. W., Baranowski, L., Sobczak, M., Ilyas, M., van der Hoorn, R. A. L., Schots, A., de Wit, P. J. G. M., Bakker, J., Goverse, A., and Smant, G. 2012. Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode. Proc Natl Acad Sci U S A 109:10119-10124.

Rovenich, H., **Boshoven, J. C.**, and Thomma, B. P. H. J. 2014. Filamentous pathogen effector functions: of pathogens, hosts and microbiomes. Curr Opin Plant Biol 20:96-103.

Santhanam, P., **Boshoven, J. C.**, Salas, O., Bowler, K., Islam, M. T., Saber, M. K., van den Berg, G., Bar-Peled, M., and Thomma, B. P. 2017. Rhamnose synthase activity is required for pathogenicity of the vascular wilt fungus *Verticillium dahliae*. Mol Plant Pathol 18:347-362.

Song, Y., Zhang, Z., **Boshoven, J. C.**, Rovenich, H., Seidl, M., Jakse, J., Maruthachalam, K., Liu, C.-M., Subbarao, K., and Javornik, B. 2017b. Tomato immune receptor Ve1 recognizes surface-exposed co-localized N-and C-termini of *Verticillium dahliae* effector Ave1. BioRxiv:103473.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to:	**Jordi C. Boshoven**
Date:	**10 November 2017**
Group:	**Laboratory of Phytopathology**
University:	**Wageningen University & Research**

1) Start-up phase	*date*
▶ **First presentation of your project**	
Title: The role of Verticillium dahliae Ave1	16 Jan 2012
▶ **Writing or rewriting a project proposal**	
Title: The role of Verticillium dahliae Ave1 in fungal virulence and its recognition by the tomato Ve1 immune receptor	25 jan 2012
▶ **Writing a review or book chapter**	
Title: Filamentous Pathogen Effector Functions: Of Pathogens, Hosts and the Microbiome, Current Opinion in Plant Biology 20, 2014, 96-103; DOI: 10.1016/j.pbi.2014.05.001	31 Jan 2014
▶ **MSc courses**	
Laboratory use of isotopes	
Subtotal Start-up Phase	*8.5 credits**

2) Scientific Exposure	*date*
▶ **EPS PhD student days**	
EPS PhD student day, Amsterdam, NL	30 Nov 2012
5th European Plant Science Retreat, Ghent University, Ghent, Belgium	23-26 Jul 2013
EPS PhD student day, Leiden, NL	29 Nov 2013
6th European Plant Science Retreat, UvA, Amsterdam, Nl	01-04 Jul 2014
EPS PhD student day (GET2GETHER) Soest, NL	29-30 Jan 2015
▶ **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day, Wageningen, NL	10 Feb 2012
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day, Utrecht, NL	24 Jan 2013
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day, Amsterdam, NL	25 Feb 2014
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day, Utrecht, NL	20 Feb 2015
▶ **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	02-03 Apr 2012
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22-23 Apr 2013
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	13 Apr 2015

▶	**Seminars (series), workshops and symposia**	
	Workshops:	
	KLV speedreading course	08 Feb 2012
	Lunchlezing "Initiatief nemen en assertiviteit - Hoe assertief kun je zijn?"	24 Sep 2014
	Lunchlezing "Van spanning naar ontspanning, spelen met de dialoog"	18 Nov 2015
	Seminars:	
	Monique van Oers 'Insect viruses and their applications'	23 Feb 2012
	Ralph Panstruga 'Comparative pathogenomics of powdery mildew fungi'.	04 Dec 2012
	Andrew Sugden (Editor of Science magazine) 'Demystifying Science'	08 Feb 2013
	Detleif Weigel 'Origin and consequence of genetic and epigenetic variation'	27 Feb 2013
	Kazuki Saito 'Metabolomics-based functional genomics - from Arabidopsis to crops and medicinal plants'	08 Apr 2013
	Brian Staskawicz "Effector-Targeted Breeding for Durable Disease Control of Xanthomonas diseases in Tomato and Cassava"	21 May 2013
	Pieter Dorrestein "A "GoogleMAP"-type molecular view of microbes - from culture to people"	22 Aug 2013
	Hanhui Kuang "Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability"	11 Sep 2013
	David Weller 'Soilborne Pathogens and their Natural Biocontrol Agents in Cereal-Based Production Systems"	25 Sep 2013
	Ross Mann "Endophytes in agriculture - evaluating their application via metabolomics and genomics"	04 Oct 2013
	Jos Raaijmakers 'Back to the roots: exploring and exploiting the plant microbiome'	07 Jan 2014
	Plant Sciences Seminar: Mutualism in Action (Toby Kiers and René Geurts)	11 Mar 2014
	Jeroen Mesters 'From protein solution to single crystal X-ray diffraction: Chitin binding by LysM domains at atomic resolution'	31 Mar 2014
	Jane Parker 'Reprogramming cells for defence in plant innate immunity'	09 Apr 2014
	Frank van Breusegem 'Plant Metacaspases'	09 Apr 2014
	Yuanchao Wang "Dissecting the interaction between Phytophthora sojae and soybean: making sense out of signalling and effectors"	16 Jul 2014
	Joy Bergelson WEES seminar "Maintaining an ancient balanced polymorphism for resistance amidst diffuse intereactions"	26 Sep 2014
	Michael Freitag "Chromatin structure controls centromeres and secondary metabolism in filamentous fungi"	21 Oct 2014
	Ortrun Mittelsten Scheid "Genetics and epigenetics: a complex relationship"	19 Nov 2014
	Kevin Foster WEES seminar "The evolution of cooperation and competition in microbes"	22 Jan 2015
	Yves van de Peer 'The evolutionary significance of gene and genome duplications'	03 Feb 2015
	Monica Höfte 'Towards understanding rice brown spot, a disease induced by physiological stress'	06 Feb 2015
	Chih-Hang Wu 'Helper NLR proteins of the NRC family in solanaceous plants'	05 Mar 2015
	Gero Steinberg 'Long-distance endosome trafficking drives fungal effector production during plant infection'	05 Jun 2015

	Gert Kema application as Special Professor in Tropical Phytopathology	17 Nov 2015
	Symposia:	
	Plantum 'Intraspecific Pathogen Variation - Implications and Opportunities'	22 Jan 2013
	Minisymposium: How to write a world-class paper	17 Oct 2013
	KNPV fall meeting 'The process to progress'	27 Nov 2013
	Minisymposium: Responses of plants to pathogenic micro-organisms and their secreted proteins	02 Dec 2013
	Farewell Address Prof. dr. ir. Pierre de Wit - Fungal plant pathogens and the plant immune system	05 Jun 2014
	All-inclusive Breeding: Integrating high-throughput science	16 Oct 2014
	Minisymposium: Phytopathology	24 Nov 2014
	Meeting BU Biointeractions & Plant Health – Phytopathology WU	10 Feb 2015
▶	**Seminar plus**	
	Michael Freitag "Chromatin structure controls centromeres and secondary metabolism in filamentous fungi"	21 Oct 2014
	Gero Steinberg 'Long-distance endosome trafficking drives fungal effector production during plant infection'	05 Jun 2015
▶	**International symposia and congresses**	
	2nd joined Wageningen - Marburg meeting on plant-fungal interactions	30-31 Jan 2012
	The 27th Fungal Genetics Conference 2013, Asilomar, California, USA	12-17 Mar 2013
	The 28th Fungal Genetics Conference 2015, Asilomar, California, USA	17-22 Mar 2015
▶	**Presentations**	
	Poster: 27th Fungal Genetics Conference 2013, Asilomar, California, USA	15 Mar 2013
	Poster: Symposium "All-inclusive Breeding: Integrating high-throughput science", Wageningen, NL	16 Oct 2014
	Poster: 28th Fungal Genetics Conference 2015, Asilomar, California, USA	17-22 Mar 2015
	Plant Science Slam: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	22 Apr 2013
	Talk: 7th International Utrecht PhD Summer School, Utrecht, NL	27 Aug 2013
	Talk: KNPV fall meeting 'The process to progress', Wageningen, NL	26 Nov 2013
	Talk: 6th European Plant Science Retreat, UvA, Amsterdam, NL	03 Jul 2014
	Flash: Symposium 'All-inclusive Breeding: Integrating high-throughput science', Wageningen, NL	16 Oct 2014
	Talk: EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day Utrecht, NL	20 Feb 2015
▶	**IAB interview**	
▶	**Excursions**	
	Company visit: Rijk Zwaan de Lier	27 Sep 2013
		Subtotal Scientific Exposure *24.9 credits**

3) In-Depth Studies	*date*
▶ **EPS courses or other PhD courses**	
7th International Utrecht PhD Summer School on Environmental Signaling, Utrecht, NL	26-28 Aug 2013
▶ **Journal club**	
Participitated in the literature discussion of the Verticillium group	2012-2015
▶ **Individual research training**	
Subtotal In-Depth Studies	*3.9 credits**

4) Personal development	*date*
▶ **Skill training courses**	
Course: PhD competence assessment	20 Mar 2012
Course: Scientific publishing	19 Jun 2012
Course: Project- and Time Management	30 Oct-11 Dec 2012
Course: Interpersonal Communication for PhD Students	12-13 Dec -2012
WGS PhD Workshop Carousel 2015	17 Apr 2015
Course: Last Stretch of the PhD Programme	25 Sep 2015
▶ **Organisation of PhD students day, course or conference**	
EPS Expectations day 2013	01 Feb 2013
Company visit: Rijk Zwaan de Lier	27 Sep 2013
EPS Expectations day 2014	28 Mar 2014
Company visit: Genetwister and In2Care Wageningen	19 Sep 2014
EPS PhD student day (GET2GETHER)	29-30 Jan 2015
▶ **Membership of Board, Committee or PhD council**	
EPS PhD council member	2012-2015
Subtotal Personal Development	*7.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**45.0**

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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