Recognition of *Verticillium* effector Ave1 by tomato immune receptor Ve1 mediates *Verticillium* resistance in diverse plant species Yin Song 2017

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Propositions

1. Tomato Ve1 has traits of a typical race-specific resistance (R) protein as well as of a typical pattern recognition receptor (PRR). (this thesis)
2. The modular composition of immune receptors offers opportunities to design tailored immune receptors. (this thesis)
3. For better understanding of biological systems, the massive amounts of genomic DNA sequence that are generated by next-generation sequencing technologies are useless without further efforts to precisely assemble and annotate genomes.
4. Challenging entrenched scientific beliefs can lead to ground-breaking research.
5. As much as how a critical spirit can improve the quality of science, it can simultaneously impair the quality of life.
6. In the age of e-commerce scientists need to be salespersons.

Propositions belonging to the thesis, entitled

"Recognition of *Verticillium* effector Ave1 by tomato immune receptor Ve1 mediates *Verticillium* resistance in diverse plant species"

Yin Song

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Chapter 1

General introduction

INTRODUCTION

Plants are constantly engaged in battles against a wide range of potential pathogens within their environment. Nevertheless, only few of these potential pathogens succeed in invading a plant to cause disease, as plants have evolved innate immunity to protect themselves against microbial attack (Dodds and Rathjen, 2010; Thomma et al., 2011). Plant innate immunity against pathogen attack is governed by immune receptors that sense pathogen(-induced) ligands to activate defense. Originally, the interaction between plant immune receptors and pathogen ligands was described as a "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 layer 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 has recently inspired the proposal of the Invasion Model, in which host receptors (termed invasion pattern receptors; IPRs) detect either an externally encoded or modified-self ligand that indicates invasion (termed invasion patterns; IPs) (Cook et al., 2015). In this model, any molecule can serve as an IP that is potentially 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.

Verticillium is a relatively small genus of ascomycete fungi that currently comprises ten species (Inderbitzin et al., 2011; Figure 1). All presently recognized *Verticillium* species are soil-borne fungi, and several of them cause so-called vascular wilt diseases in a wide range of economically important crops, including tomato, potato, tobacco, cotton, hop plants, lettuce, olive trees, oilseed rape, sunflower, sugar beet etc (Pegg and Brady, 2002; Figure 2). Although symptoms may vary considerably between plant hosts, the most frequently observed symptoms of Verticillium wilt include stunting, wilting, chlorosis, necrosis, vascular discoloration and early senescence (Fradin and Thomma, 2006). Within the *Verticillium* genus, *V. dahliae* is the most notorious species that can cause Verticillium wilt diseases in over 200 plant host species worldwide. *V. albo-atrum*, *V. longisporum, V. alfalfae* and *V. non-alfalfae* are also economically important vascular pathogens, albeit with narrower host ranges (Pegg and Brady, 2002; Agrios, 2005; Fradin and Thomma, 2006; Klosterman et al., 2009; Inderbitzin et al., 2011; Depotter et al., 2015). Polygenic resistance to *Verticillium* spp. has been described in several plant species, including potato, hop, alfalfa, cotton and strawberry (Simko et al., 2004; Bolek et al., 2005; Wang et al., 2008; Yang et al., 2008; Jakse et al., 2013; Antanaviciute et al., 2015), whereas single dominant resistance genes have been identified in tomato, potato and lettuce species only (Schaible et al., 1951; Simko et al., 2004; Hayes et al., 2011; Christopoulou et al., 2015). In tomato (*Solanum lycopersicum*), a single dominant locus that confers *Verticillium* resistance has been identified as the *Ve* locus, which controls *Verticillium* isolates that are assigned to race 1, but not to race 2 (Schaible et al., 1951; Pegg, 1974). This locus contains two closely linked and inversely oriented genes, *Ve1* and *Ve2*, that both encode extracellular leucine rich repeat (eLRR) receptor-like proteins (RLPs) (Kawchuk et al., 2001; Wang et al., 2010). Of these, only *Ve1* was found to act as a functional *V. dahliae* resistance gene in tomato (Fradin et al., 2009). Interestingly, interfamily transfer of *Ve1* from tomato to Arabidopsis resulted in *Verticillium* resistance in the latter species (Fradin et al., 2011, 2014; Zhang et al., 2014), implying that the underlying immune signalling pathway is conserved (Fradin et al., 2011; Thomma et al., 2011). So far, several *Ve1* homologs were identified within the Solanaceae family (in wild relatives of tomato and eggplant and in cultivated potato; Chai et al., 2003; Fei et al., 2004; Simko et al., 2004; Liu et al., 2012), as well as outside this

family (mint, lettuce, cotton and grape; Vining and Davis, 2009; Hayes et al., 2011; Zhang et al., 2011; Zhang et al., 2012; Yang et al., 2014; Chen et al., 2016; Chen et al., 2017). Through comparative population genomics, the *V. dahliae* effector protein that is recognized by the tomato Ve1 immune receptor was identified as Ave1 (for Avirulence on Ve1 tomato) (de Jonge et al., 2012). Intriguingly, homologs of *V. dahliae* Ave1 (VdAve1) were identified in a number of plant pathogenic microbes,

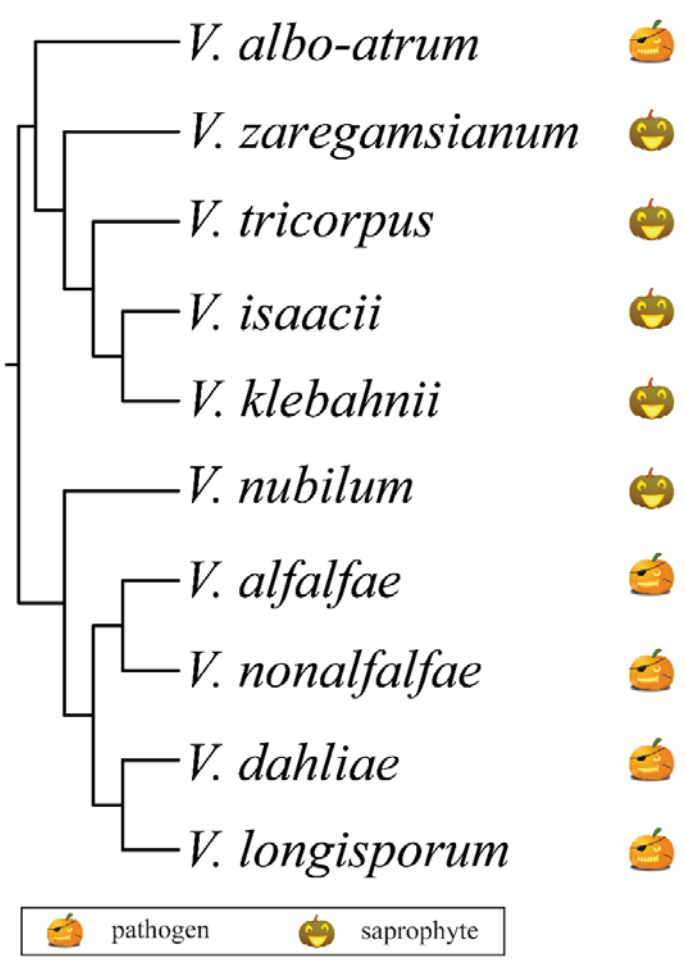

Figure 1. Phylogeny of species within the *Verticillium* genus. The phylogenetic tree of *Verticillium* species is adapted from Shi-Kunne et al. (unpublished data). *Verticillium* species marked on the right by a pirate pumpkin are pathogenic fungi on plants, while *Verticillium* species marked on the right by a smiling pumpkin are saprophytes.

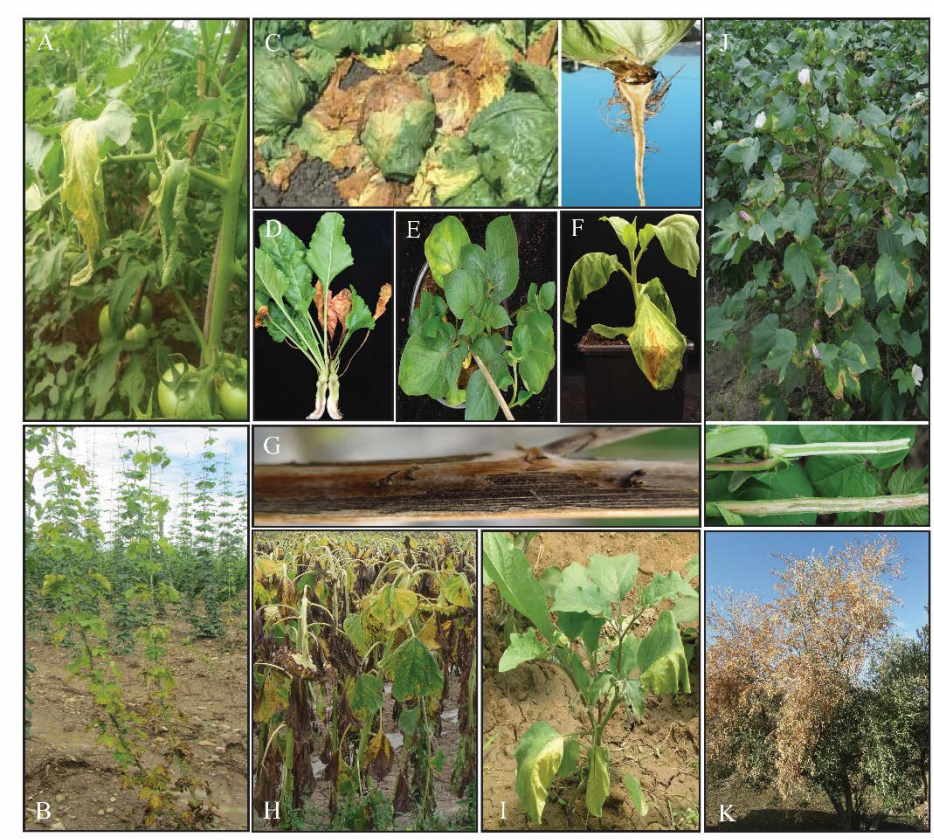

Figure 2 Disease symptoms on various host plants caused by various *Verticillium* species. A, **C-E**, **H-K**, Verticillium wilt symptoms caused by *V. dahliae* on tomato (**A**), lettuce (**C**), sugar beet (**D**), potato (**E**), sunflower (**H**), eggplant (**I**), cotton (**J**) and olive tree (**K**). **B,** Verticillium wilt of hop plants caused by *V. nonalfalfae*. **F**, Disease caused by *V. alfalfae* on tobacco. **G**, Stem striping caused by *V. longisporum* on oilseed rape. Photos are courtesy of Sebastjan Radišek and Branka Javornik (panel **B**), Krishna Subbarao (panel **C**), Jasper Depotter (panel **G**), Shuqing Yang and Jun Zhao (panel **H**), Longfu Zhu (panel **J**) and Jelle Hiemstra (panel **K**).

including the citrus bacterial canker pathogen *Xanthomonas. axonopodis* pv. *citri* (XacPNP), the fungal tomato wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), the fungal sugar beet leaf spot pathogen *Cercospora beticola* (CbAve1), the crucifer anthracnose pathogen *Colletotrichum higginsianum* (ChAve1) and the cucurbit anthracnose pathogen *Colletotrichum orbiculare* (CoAve1) (Nembaware et al., 2004; de Jonge et al., 2012; Gan et al., 2013), and a few of these homologs are differentially recognized by tomato Ve1 (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).

MAIN OBJECTIVE AND RESEARCH QUESTIONS

The main objective of my PhD research was to characterize the role of the immune receptor Ve1 in tomato and its homologs in various plant species in *Verticillium* resistance, and to mechanistically unravel the functionality of tomato Ve1. To this end, I have addressed the following research questions:

i. Can we identify a minimal motif in the effector VdAve1 that is required for its recognition by tomato immune receptor Ve1?
ii. Do homologs of tomato Ve1 that are found in other plant species similarly act as immune receptors that govern resistance against race 1 strains of *V. dahliae* in a VdAve1-dependent manner?
iii. Does ectopic expression of tomato *Ve1* in crop species confer VdAve1-dependent *Verticillium* resistance?

THESIS OUTLINE

Research on effectors secreted by pathogens during host colonization has dominated the field of molecular plant-microbe interactions over recent years. **Chapter 2** reviews the current knowledge on how soil-borne pathogens employ secreted effector molecules to support host colonization. Whereas most well-studied effectors are proteinaceous molecules, non-canonical effectors, such as metabolites and small RNAs, have also been described. Soil-borne pathogens live in a complex and sophisticated belowground environment. In addition to attenuation of host immunity and roles in self-defense, the potential role of effectors in interactions with other microbiome inhabitants, including competitors and mycoparasites, is discussed.

Effectors are molecules secreted by plant pathogens to facilitate infection, often through deregulation of plant immune responses. During host colonization, race 1 strains of *V. dahliae* secrete the effector protein VdAve1 that activates Ve1-mediated immunity. Homologs of VdAve1 were identified in the bacterial plant

pathogen *X. axonopodis* pv. *citri* (XacPNP) and in the plant pathogenic fungi *F. oxysporum* f. sp. *lycopersici* (FoAve1), *C. beticola* (CbAve1), and *C. higginsianum* (ChAve1). These homologs are differentially recognized by Ve1, with the most efficient recognition of VdAve1 and no recognition of XacPNP. **Chapter 3** aims to identify a minimal motif of VdAve1 that is required to activate Ve1-mediated immunity. The approach was based on epitope prediction, guided by the alignment of differentially recognized VdAve1 homologs, followed by functional analyses of a combination of serial deletions assays, domain swaps, synthetic peptides, three-dimensional structural prediction and chimeric proteins.

So far, several *Ve1* homologs were identified within and outside the Solanaceae family. However, functionality of these homologs against Verticillium wilt remained obscure. In **Chapter 4**, I report the cloning and functional characterization of *Ve1* homologs from tobacco (*Nicotiana glutinosa*), potato (*Solanum tuberosum*), wild eggplant (*Solanum torvum*) and hop (*Humulus lupulus*).

The tomato immune receptor Ve1 recognizes VdAve1 and its homologs from other pathogenic microbes including the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (FoAve1) and the sugar beet pathogen *C. beticola* (CbAve1). Previous work revealed that tomato Ve1 mediates resistance against multiple fungal tomato pathogens including *V. dahliae*, *V. albo-atrum* and *F. oxysporum*, and transgenic expression of tomato *Ve1* in Arabidopsis confers *Verticillium* resistance through recognition of VdAve1. In **Chapter 5** I investigated whether the tomato immune receptor gene *Ve1* can confer Verticillium wilt resistance when transferred from tomato to the closely related crop species tobacco (*Nicotiana tobacum* cv. Samsun) and the distantly related crop species cotton (*Gossypium hirsutum*). The *Ve1*-transgenic lines were subjected to molecular characterization and disease assays using race 1 *Verticillium* strains.

Previous studies have shown that the expression of silencing constructs in host plants designed to target transcripts of pathogen genes can specifically silence these genes in invading pathogens, including fungi, which is referred as host-induced gene silencing (HIGS). HIGS was shown to operate against the fungal pathogens *Blumeria graminis*, *Puccinia* spp., *Fusarium* spp., *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. In **Chapter 6** I assessed the effect of HIGS on Verticillium wilt disease. This was done by transient assays in tomato based on virus-induced

gene silencing, followed by experiments with stable transgenic *Arabidopsis thaliana* plants expressing hairpin constructs targeting transcripts of three *V. dahliae* genes.

In the final chapter of the thesis, **Chapter 7**, the major results described in the previous chapters are discussed and placed in a broader perspective.

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Chapter 2

Biological functions of effectors secreted by soil-borne plant pathogens

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ABSTRACT

Soil-borne phytopathogenic microbes live in complex and sophisticated below-ground environments. To establish themselves within their host plants, these pathogens have to overcome plant defense mechanisms, ranging from preformed barriers to activated defenses. To this end, they typically secrete effector molecules that play important roles to support host colonization through a wide range of mechanisms. Whereas most well-studied effectors are proteinaceous molecules, non-canonical effectors, such as metabolites and small RNA molecules, have also been described. In addition to targeting plant components, part of the effector catalogue may be required for self-defense against, and targeting of, other microbiome partners, including antagonists and competitors.

INTRODUCTION

Soil-borne pathogenic microorganisms cause significant yield and quality losses in crops worldwide. They reside in the soil for brief or extended periods of time, where they survive on plant residues or as resting structures such as melanised mycelium, chlamydospores, oospores, or (micro)sclerotia, until root exudates trigger them to become active and invade the roots of plants (Agrios, 2005). Once inside, they may be confined to the root system, or invade above-ground tissues, or again move outside to infect other parts of the root system or roots from neighbouring plants (Agrios, 2005). Plants that are infected by soil-borne pathogens suffer from seedling damping-off, root rot, root blackening, wilting, stunting or plant death (Agrios, 2005). Importantly, since damage to plants by soil-borne pathogens may result from below-ground infection, crop yield and quality losses caused by soil-borne pathogens are often underestimated and sometimes even remain unnoticed (De Coninck et al., 2015).

Soil-borne pathogens are notoriously difficult to control due to their general persistence in the soil for long periods of time through the formation of resilient survival structures (Koike et al., 2003; De Coninck et al., 2015). Soil fumigants that were widely used for controlling soil-borne diseases have nowadays largely been banned due to their extremely hazardous effects on the environment as well as on human health. Since many soil-borne pathogens have a wide range of hosts, crop rotation is often ineffective (Reddy, 2012).

The most important soil-borne microbial pathogens belong to three taxonomic groups: fungi, oomycetes and bacteria. The most prominent soil-borne fungal pathogens comprise *Fusarium oxysporum* (Michielse and Rep, 2009), *Verticillium* spp. (Fradin and Thomma, 2006; Klosterman et al., 2009; Klimes et al., 2015) and *Rhizoctonia solani* (Gonzalez et al., 2011), whereas the most notorious oomycetes include *Phytophthora* spp. (van West et al., 2003; Kamoun et al., 2015) and *Pythium* spp. (van West et al., 2003). Collectively, these filamentous pathogens are responsible for diseases in hundreds of plant species. Fewer diseases are caused by soil-borne bacterial pathogens, as there are only few soil-borne bacteria infecting roots, such as *Agrobacterium* spp. (Otten et al., 2008) and *Ralstonia solanacearum* (Peeters et al., 2013). In addition, soil-borne microbial pathogens occur in other kingdoms, such as Plasmodiophoromycete protists in the eukaryotic kingdom of

Rhizaria, including *Plasmodiophora brassicae* that is the causal agent of club-root disease of crucifers (Hwang et al., 2012; Schwelm et al., 2015) and *Spongospora subterranea* that causes powdery scab on potato (Harrison et al., 1997). Here, we summarize the current knowledge on the biological functions of effectors secreted by soil-borne pathogens.

EFFECTOR DISCOVERY

Effectors are defined as molecules that are secreted by phytopathogenic microbes in order to manipulate host physiology and support host colonization. Although the vast majority of effector activities and host targets presently still remains unknown, at least a subset is experimentally found to suppress immune responses (de Jonge et al., 2011; Rovenich et al., 2014; Cook et al., 2015). Importantly, this host immunity suppression activity implies that effectors can be used as probes to identify vital components of host immune systems and can be used for effector-informed crop improvement (Vleeshouwers and Oliver, 2014).

Recent advances in next-generation sequencing technologies, genome assembly, gene annotation, and effector identification methods hold promise to fully disclose pathogen effector repertoires through computational prediction (Sperschneider et al., 2015; Gibriel et al., 2016). Many bacterial pathogens directly inject effectors into the host cytoplasm via a specialized secretion machinery, namely the type III secretion (T3S) system (Cambronne and Roy, 2006; Galán and Wolf-Watz, 2006). N-terminal sequence signals direct bacterial effectors to the T3S system and thus machine learning approaches have been developed to predict bacterial T3S effectors from sequenced genomes based on N-terminal protein sequence information (McDermott et al., 2011). In oomycetes, consensus N-terminal amino acid motifs, namely the RxLR (Arg-x-Leu-Arg) motif and the LxLFLAK motif, have been proposed to facilitate host cell translocation (Whisson et al., 2007; Schornack et al., 2010). Thus, these motifs can be queried to identify RxLR and Crinkler (CRN) effectors encoded within oomycete genomes (Tyler et al., 2006; Jiang et al., 2008; Haas et al., 2009).

Prediction of proteinaceous effectors of fungal pathogens is less straightforward, as fungal effectors generally lack significant sequence similarity and consensus motifs (Sperschneider et al., 2015). To date, most characterized fungal effectors are small in size (typically less than 300 amino acids) and rich in cysteine residues (four

or more) (Stergiopoulos and de Wit, 2009). However, these features cannot strictly be used as criteria to identify effector repertoires, as some well-characterized fungal effectors lack these properties. For example, the effectors Cmu1 and ApB73 from the fungal pathogen *Ustilago maydis* are much larger in size (Djamei et al., 2011; Stirnberg and Djamei, 2016). Thus, to describe potential effector repertories of individual fungal pathogens only rather universal features, such as their secretion and expression *in planta*, can be considered (Sperschneider et al., 2015). Recently, the first machine learning tool called "EffectorP" was introduced which is trained to improve the prediction of fungal effector proteins from secretomes based on sequence-derived properties, such as sequence length, molecular weight and protein net charge, as well as cysteine, serine and tryptophan content (Sperschneider et al., 2016). When combined with *in planta* expression data, "EffectorP" is proving useful for prioritizing putative effector candidates (Sperschneider et al., 2016). Although the computational effector prediction has great potential to identify putative effectors, further experimental studies are required to validate their role in plant-pathogen interactions.

EFFECTOR EVOLUTION

In general, effectors are expected to exhibit accelerated evolution, enabling pathogens to escape or overcome recognition, evade or suppress host immunity and support colonization of specific hosts or adapt to new hosts (Stergiopoulos and de Wit, 2009; Raffaele and Kamoun, 2012; Karasov et al., 2014; Seidl and Thomma, 2014; Dong et al., 2015). Effector genes are often found in dynamic genomic regions that show increased rates of recombination, and possibly also mutation, in the genome (Raffaele and Kamoun, 2012; Karasov et al., 2014; Seidl and Thomma, 2014; Dong et al., 2015). Sometimes these regions even concern separate chromosomes that can be transferred between pathogens. In bacteria, pathogenicity islands are clusters of genes that contain an abundance of genes involved in host associations that are located either on the chromosome or on accessary plasmids. These genomic regions contain sequences associated with flanking repeats or transposons as well as with tRNA genes, which are targets for genetic integration and excision (Hacker et al., 1997; McCann and Guttman, 2008).

In filamentous plant pathogens, effector genes are often embedded within highly variable lineage-specific (LS) genomic regions. For example, comparative analysis of the genomes of *Fusarium* species revealed that *Fusarium* pathogens carry LS genomic regions that are found as small conditionally dispensable chromosomes (CDCs) containing effector genes (Ma et al., 2010). Further genomic comparisons between CDCs from legume-infecting *F. oxysporum* strains revealed small conserved genomic regions that contain *in planta* expressed genes encoding secreted effector proteins (Williams et al., 2016). Similarly, genomic comparisons of multiple strains of *V. dahliae* revealed that all *V. dahliae* strains carry LS genomic regions that are significantly enriched for *in planta* expressed effector genes (de Jonge et al., 2013; Faino et al., 2015; 2016). Genetic flexibility of such dynamic genomic regions is governed by various mechanisms including recombination and activity of transposable elements (TEs) (Raffaele and Kamoun, 2012; Seidl and Thomma, 2014). Genomic rearrangements induce duplications, deletions and translocations, leading to the gain, or loss of genomic material, while TEs can generate a local genomic environment that facilitates genomic rearrangements (Raffaele and Kamoun, 2012; Seidl and Thomma, 2014). For *V. dahliae* it has been shown that LS regions evolved by genomic rearrangements that are mediated by erroneous double-strand repair, often utilizing TEs as a substrate for repair (de Jonge et al., 2013; Faino et al., 2016). TEs seem to contribute to effector evolution in oomycete genomes as well (Haas et al., 2009; Raffaele et al., 2010). These findings illustrate that soil-borne plant pathogens deploy various mechanisms to establish variable genomic regions that are enriched for effectors. Ultimately, these mechanisms maintain effector catalogues that impact pathogen lifestyle and host colonization.

EFFECTORS WITH APOPLASTIC FUNCTIONS

The initial contact between host plants and soil-borne pathogens is often established in the apoplast, the extracellular spaces of plant tissues. The apoplast is an environment with a relatively low pH (Felle, 1998) that is hostile for microbes because of various plant-secreted hydrolytic enzymes, including chitinases and glucanases, that affect microbial cell wall integrity and release microbial cell wall fragments (Sánchez-Vallet et al., 2015; Rovenich et al., 2016). These released microbial cell wall fragments may activate host immune receptors, leading to

production of plant-derived proteases, antimicrobial compounds, and lignins for thickening of the plant cell walls that constitute the next host barrier for the pathogen (Hückelhoven, 2007; Sánchez-Vallet et al., 2015; Rovenich et al., 2016). To overcome these biochemical and physical barriers in the apoplast, soil-borne pathogens require effector molecules.

Extracellular alkalinisation of host tissue is thought to contribute to fungal virulence (Prusky and Yakoby, 2003). To thrive in the acidic apoplast, some fungal pathogens secrete effectors to modulate the extracellular pH (Prusky and Yakoby, 2003). The *F. oxysporum* genome encodes a peptide that is homologous to the plant peptide hormone RALF (rapid alkalinisation factor), which is known to increase extracellular alkalinisation (Murphy and De Smet, 2014). Genetic analysis revealed that functional *Fusarium* RALF (F-RALF) is required for efficient host alkalinisation and contributes to the virulence of *F. oxysporum* in tomato plants (Masachis et al., 2016). F-RALF homologues are widely distributed in several bacterial species and various fungi, many of which infect plants, including *V. dahliae* and *V. alfalfae* (Masachis et al., 2016; Thynne et al., 2016), implying that other phytopathogenic microbes may use RALF peptides to efficiently induce extracellular alkalinisation in the host, thereby enabling infection.

To protect hyphae from plant-secreted chitinases, fungal pathogens secrete effectors to prevent cell wall hydrolysis by plant chitinases and interfere with host immune receptor activation (Sánchez-Vallet et al., 2015). Vd2LysM is a chitin-binding LysM-containing effector from *V. dahliae* that protects fungal hyphae from degradation by plant hydrolytic enzymes (Kombrink et al., 2017). Similar protective activity mediated by LysM effectors was found in other fungal pathogens (Kombrink and Thomma, 2013; Sánchez-Vallet et al., 2015). Although the molecular mechanism by which LysM effectors protect hyphae against hydrolysis by plant chitinases presently remains elusive, they may form chitin-dependent oligomeric chains to cover fungal hyphae and prevent access of plant chitinases to the chitin in the fungal cell wall (Sánchez-Vallet et al., 2015). This mechanism was previously demonstrated for the *Cladosporium fulvum* effector Avr4 and the *Pseudocercospora fuligena* orthologue PfAvr4 that bind to fungal cell walls through an invertebrate chitin-binding domain in order to protect hyphae from host chitinases (van den Burg et al., 2006; Kohler et al., 2016). Alternatively, Vd2LysM protects hyphae through indirect

or direct inhibition of host chitinase activity. It has been proposed that direct inhibition of plant chitinases is a general strategy for fungal pathogens to protect fungal cell walls (Sánchez-Vallet et al., 2015). Indeed, *F. oxysporum* f. sp. *lycopersici* secretes a metalloprotease and a serine protease that synergistically cleave extracellular tomato chitinases, leading to compromised antifungal activity and enhanced disease in tomato (Jashni et al., 2015). Similar activities occur in other fungal pathogens, including *V. dahliae* (Jashni et al., 2015). In addition to protecting the fungal cell walls from hydrolysis by plant chitinases, LysM-containing effectors outcompete host immune receptors for chitin fragment binding to interfere with chitin-triggered immunity. The *C. fulvum* LysM effector extracellular protein 6 (Ecp6) binds to chitin fragments with high specificity through its LysM domains to prevent chitin-triggered immunity (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). Similarly, the Vd2LysM effector deregulates chitin-triggered immunity through chitin fragment binding, and contributes to virulence of *V. dahliae* during infection (de Jonge et al., 2013; Kombrink et al., 2017).

In addition to the ability to prevent hydrolysis by plant chitinases and interfere with chitin-triggered immunity, effectors have been shown to inhibit plant glucanases in order to avoid host immune receptor activation (Rose et al., 2002; Sánchez-Rangel et al., 2012). A glucanase inhibitor protein 1 (GIP1) from *Phytophthora sojae* was shown to directly inhibit a soybean endoglucanase (EGaseA), thus preventing release of oligosaccharides and activation of the corresponding, yet enigmatic, host immune receptor (Rose et al., 2002).

Plants secrete proteases, such as papain-like cysteine proteases (PLCPs), serine and aspartic proteases into the apoplast (Jashni et al., 2015; Misas-Villamil et al., 2016). Several plant proteases are induced in the presence of pathogens, are able to induce expression of plant defense genes, and degrade effectors secreted by pathogens (Jashni et al., 2015; Misas-Villamil et al., 2016). Thus, plant proteases acting as important components of the plant defense system could be targeted by effectors. The tomato serine protease P69B is inhibited by two serine proteases, EPI1 and EPI10 (extracellular proteinase inhibitor 1 and 10), from *Phytophthora infestans* (Tian et al., 2004; 2005). Similarly, PLCP C14 of tomato and potato is inhibited by two cystatin-like effectors EPIC1 and EPIC2B from *P. infestans* (Kaschani et al., 2010). Moreover, the tomato protease C14 is also targeted by another *P. infestans* effector,

AVRblb2, which prevents C14 secretion into the apoplast (Bozkurt et al., 2011). The finding that *P. infestans* evolved distinct and structurally divergent protease inhibitors to target the same plant protease indicates that inhibition of that particular plant protease is important for *P. infestans* infection. An additional example for an important plant protease targeted by different phytopathogens is provided by inhibitors of tomato PLCP Rcr3 (Required for *Cladosporium* resistance-3) found in unrelated pathogens (Rooney et al., 2005; Song et al., 2009; Lozano-Torres et al., 2012). The sequence-unrelated effectors EPIC1 and EPIC2B from *P. infestans* (Song et al., 2009), Avr2 from *C. fulvum* (Rooney et al., 2005), and Gr-VAP1 from the nematode *Globodera rostochiensis* (Lozano-Torres et al., 2012) all inhibit tomato Rcr3. Both effectors Avr2 and EPIC2B also inhibit Pip1 (*Phytophthora* inhibited protease 1), which is related to tomato Rcr3 (Tian et al., 2007; van Esse et al., 2008). However, the role of these particular plant proteases in plant defense remains unknown.

The necrosis and ethylene-inducing peptide 1 (NEP1) was originally identified in culture filtrates from *F. oxsporum* (Bailey, 1995), and NEP1 homologues named as NLPs (NEP1-like proteins) found in many bacteria, fungi, and oomycetes that are generally associated with necrotic activity in dicotyledonous plants through plasma membrane permeabilization and cytolysis of plant cells (Ottmann et al., 2009). With the increasing availability of bacterial, fungal, and oomycete genomes, it has become apparent that NLPs are widely distributed in microorganisms (Gijzen and Nürnberger, 2006; Oome and Van den Ackerveken, 2014), and that the NLP family is significantly expanded in several oomycetes (Gijzen and Nürnberger, 2006; Cabral et al., 2012; Dong et al., 2012; Oome and Van den Ackerveken, 2014). Most identified NLPs not only trigger cell death but also elicit strong immune responses in dicots (Gijzen and Nürnberger, 2006; Qutob et al., 2006). There is accumulating evidence that NLP effectors exhibit functional diversification in various pathogens (Dong et al., 2012; Zhou et al., 2012; Santhanam et al., 2013; Oome and Van den Ackerveken, 2014). Characterization of a set of *NLP*s in *V. dahliae* indicated that the expanded *V. dahliae NLP* family displays functional diversification, with differential cytotoxicity among the NLP family members (Zhou et al., 2012; Santhanam et al., 2013). Another observation for functional diversification in the *V. dahliae* NLP family comes from the differential contributions of cytotoxic NLP members NLP1 and NLP2 to virulence on

different host plants. Both NLP1 and NLP2 are required for full virulence of *V. dahliae* strain JR2 on tomato and *Arabidopsis*, while neither of these two is involved in virulence of *V. dahliae* strain V592 on cotton (Zhou et al., 2012; Santhanam et al., 2013). Moreover, NLP1 is required for virulence of *V. dahliae* strain JR2 on *Nicotiana benthamiana*, whereas *NLP2* is not even expressed during *N. benthamiana* infection (Santhanam et al., 2013). One out of 3 *P. infestans* NLPs tested causes necrosis (Kanneganti et al., 2006), whereas eight out of 19 *P. sojae* NLPs examined are able to induce necrosis (Dong et al., 2012).

Cell wall-degrading enzymes (CWDEs) that are capable of degradation of cell wall polysaccharides to break down the physical barrier of the plant cell walls during infection can be considered as effectors. Functional redundancy has complicated investigations into the role of individual CWDEs in virulence (Kubicek et al., 2014). However, the importance of CWDEs acting as virulence factors was demonstrated through disruption of the *sucrose nonfermenting 1* gene (*SNF1*) in *F. oxysporum* and *V. dahliae*. *SNF1* encodes part of a kinase complex regulating the expression of CWDEs and *SNF1* mutants show compromised virulence (Ospina-Giraldo et al., 2003; Tzima et al., 2011). Other examples showing a positive correlation between individual CWDEs and virulence include studies on *R. solanacearum*, *F. oxysporum* f. sp. *lycopersici*, *V. dahliae*, *Phytophthora parasitica* and *P. sojae* (Novo et al., 2006; Wu et al., 2008; Poueymiro and Genin, 2009; Maruthachalam et al., 2011; Ma et al., 2015; Bravo Ruiz et al., 2016). These studies conclude that CWDEs are important for virulence. Genome analyses revealed that *V. dahliae*, *V. albo-atrum* and *F. oxsporum* have an expanded arsenal of particular CWDE families compared with other fungi, including a significant expansion of pectin-degrading enzymes (Klosterman et al., 2011; Zhao et al., 2013). It has been hypothesized that expansion of pectinolytic enzymes in *V. dahliae*, *V. albo-atrum* and *F. oxsporum* may facilitate host colonization (Klosterman et al., 2011; Yadeta and Thomma, 2013; Zhao et al., 2013). Genomic analysis revealed that high numbers of pectin-degrading enzymes were also observed in the genome of the broad host-range fungal pathogen *R. solani* (Hane et al., 2014). Enhanced numbers of pectinolytic enzymes in this pathogen facilitate to break down pectinaceous host barriers, presumably providing also this pathogen the capacity to colonize a broad range of plants.

EFFECTORS WITH CYTOPLASMIC FUNCTIONS

Some effectors are translocated across the plant cell membrane into the host cytoplasm, where they target plant components in order to benefit pathogen proliferation in the host.

Plant hormones are key signaling molecules that regulate multiple aspects of plant growth, development, and defense (Kazan and Lyons, 2014). Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are considered as primary defense phytohormones that act in response to pathogen infection. SA is important for resistance to biotrophic and hemibiotrophic pathogens while JA and ET are associated with resistance to necrotrophic microbes (Pieterse et al., 2012; Fu and Dong, 2013). Components of SA, JA and ET biosynthesis and signaling pathways are targeted by pathogen effectors to promote infection (Kazan and Lyons, 2014). Effectors VdIsc1 and PsIsc1, which are secreted by *V. dahliae* and *P. sojae*, respectively, are isochorismatases that hydrolyse the SA precursor isochorismate to interfere with the plant SA metabolism pathway and thus promote infection (Liu et al., 2014). Similarly, the effector PbBSMT of the protist *P. brassicae* disrupts SA signalling through decreasing the accumulation of SA in *A. thaliana* plants and promote tissue colonization (Ludwig-Muller et al., 2015). These examples indicate that reducing SA accumulation level is an important strategy for pathogens to establish infection. Pathogens also secrete effectors to interfere with phytohormone perception and signaling through mimicking of plant hormones. For example, some strains of the bacterial pathogen *Pseudomonas syringae* produce phytotoxin coronatine (COR), a structural mimic of the bioactive JA hormone JA-Ile (Geng et al., 2012). COR is perceived by the plant JA receptor COI1, activating JA-dependent responses that, in turn, attenuate SA-dependent responses involved in *P. syringage* resistance (Geng et al., 2012). Notably, COR is more active than the host JA-Ile in triggering the JA pathway, indicating that COR acts as a potent and highly specific mimic of JA-Ile perception in plants (Geng et al., 2012). In fact, bioactive JAs are produced by some *F. oxysporum* strains (Brodhun et al., 2013; Cole et al., 2014). JA-insensitive *Arabidopsis* mutants display enhanced resistance against *Arabidopsis*-infecting *F. oxysporum* strains that produce detectable JAs (Cole et al., 2014). In contrast, JA-insensitive *Arabidopsis* and tomato mutants do not show altered resistance against *Arabidopsis*- and tomato-infecting *F. oxsporum* strains that do not

produce JAs, respectively (Cole et al., 2014). Thus, some strains of *F. oxsporum* appear to secrete JAs as effectors, but the mechanism how *F. oxsporum*-secreted JAs promote infection is unknown.

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), play an essential role in plant defense against pathogens (Heller and Tudzynski, 2011). Some effectors have been shown to interfere with production of ROS in order to promote virulence. Plant catalases are localized to the peroxisome and convert H_2O_2 into H_2O and O_2, thus contributing to ROS homeostasis in plant cells (Mhamdi et al., 2010). Two CRN effectors, PsCRN63 and PsCRN115, from *P. sojae* interact with plant catalases to regulate plant cell death and H_2O_2 homeostasis to promote infection (Zhang et al., 2015). PsCRN63 promotes plant cell death through interacting with and destabilizing plant catalases, thus increasing H_2O_2 accumulation. PsCRN115, however, counteracts these processes to decrease H_2O_2 levels and inhibit plant cell death (Zhang et al., 2015). Another CRN effector, PsCRN70 from *P. sojae*, suppresses plant cell death, H_2O_2 accumulation and expression of defense-related genes, to enhance pathogen infection (Rajput et al., 2014). However, the mechanism how PsCRN70 regulates plant ROS production remains unknown. Recently, the type III effector RipAK from *R. solanacearum* has also been shown to interact with and inhibit plant catalases and suppresses plant immunity, thus resulting in enhanced bacterial infection (Sun et al., 2017). These findings clearly show that modulation of plant ROS production by pathogens through effectors is important for disease development.

The plant ubiquitin-proteasome system plays a central role in many cellular processes including defense responses and hormone signaling, and thus is an essential target of effectors (Banfield, 2015). GALA proteins (also named RipG [Ralstonia injected proteins G proteins]) are secreted by the T3S system of *R. solanacearum*, which contain a conserved GAxALA amino acid motif in the C-terminal leucine-rich repeat region and a plant-type F-box domain in the N-terminal region (Cunnac et al., 2004). The GALA effector is able to interact with the *A. thaliana* SKP1-like protein (ASK), a component of the plant SCF (SKP1-CULLIN1-F-box protein) E3 ubiquitin ligase, suggesting that the GALA effector may interfere with the ubiquitin-proteasome pathway and promote infection (Angot et al., 2006). GALA7 was shown to be a host-specificity factor required for disease promotion on *Medicago truncatula* plants and its F-box domain was found to be essential for its virulence function

(Angot et al., 2006). Mutants lacking all seven *GALA* genes in *R. solanacearum* strains GMI1000 display compromised virulence on diverse plants (Angot et al., 2006; Remigi et al., 2011). However, it is yet unknown whether GALAs contribute to degradation of host targets. Similar to GALAs, the F-box domain containing VirF, which is secreted by the type IV secretion system of *A. tumefaciens*, was previously shown to interact with *A. thaliana* ASKs and promote virulence during *A. tumefaciens* infection (Schrammeijer et al., 2001). VirF interacts with plant VIP1 (VirE2-interacting protein 1) and targets VIP1 into the host ubiquitin-proteasome for proteolysis (Tzfira et al., 2004). In *P. infestans*, the RxLR effector Avr3a was shown to interact with and stabilize the host ubiquitin E3 ligase CMPG1 for suppression of INF1-induced host cell death during biotrophic growth (González-Lamothe et al., 2006; Bos et al., 2010). These examples illustrate that the host ubiquitin-proteasome system can be exploited by pathogens through effectors for their own benefit.

Plant perception of microbial pathogens leads to transcriptional reprogramming towards host defense against pathogens. In response, pathogens secrete effectors to reprogram host gene expression by targeting defense-related transcription factors, activating the expression of host susceptibility genes and interfering with host post-transcriptional gene silencing in order to establish infection (Toruño et al., 2016). A number of host transcription factors that regulate the expression of the host defense-related genes appear to be targets of effectors. Effectors can directly target host transcription factors in order to downregulate host genes involved in defense responses. Plant WRKY transcription factors, known for the canonical WRKYGQK DNA-binding motif, are key components that regulate host defense responses against several pathogens (Rushton et al., 2010; Chi et al., 2013). The acetyltransferase PopP2 (RipP2) from *R. solanacearum* localizes to the plant cell nucleus and directly acetylates lysine residues in the WRKYGQK DNA-binding motif of WRKY transcription factors (Deslandes et al., 2003; Le Roux et al., 2015; Sarris et al., 2015). Acetylation by PopP2 interferes with the DNA binding of WRKY transcription factors and thus with the transcriptional activation of WRKY transcription factor-regulated immune genes, leading to enhanced virulence (Le Roux et al., 2015; Sarris et al., 2015). In addition to WRKY transcription factors, PopP2 interacts with and acetylates two lysine residues in the C-terminal WRKYGQK domain of the *A. thaliana* intracellular immune receptor RRS1-R (Resistance to

Ralstonia solanacearum 1). Acetylation of RRS1-R by PopP2 disrupts the binding of RRS1-R to W-box DNA sequences, leading to the activation of the RRS1-R-associated immune receptor RPS4 (Resistance to *Pseudomonas syringae* 4) (Le Roux et al., 2015; Sarris et al., 2015). *P. infestans* secretes the RxLR effector Pi03192 that interacts with two plant NAC transcription factors NTP1 and NTP2 (NAC Targeted by *Phytophthora* 1 and 2) at the endoplasmic reticulum (ER) membrane and prevents their localization from ER to the nucleus to increase host susceptibility (McLellan et al., 2013). Finally, the *Phytophthora capsici* CRN effector CRN12-997 was shown to directly bind to the tomato immune-related transcription factor SITCP14-2, resulting in compromised immunity mediated by SITCP14-2 (Stam et al., 2013). CRN12-997 affects SITCP14-2 association with nuclear chromatin and SITCP14-2 sub-nuclear localization, preventing SITCP14-2 from positively regulating defense against *P. capsici* (Stam et al., 2013). These examples illustrate show how effectors prevent the activation of host defense through modification of transcription factor DNA binding site or mislocalization of transcription factors during host colonization.

Pathogen effectors can directly act as plant transcription factors and induce the expression of host susceptibility genes. Ralstonia transcription activator-like effectors (TALEs), termed RipTALEs that are found in many *R. solanacearum* strains are structurally homologous to *Xanthomonas* TALEs (Lange et al., 2013; Li et al., 2013). *Xanthomonas* TALEs are injected into host cells and enable to directly activate host target genes through a repeat structure domain involved in the binding to specific DNA sequences, known as effector binding elements (EBEs), at promoters of host target genes (Boch and Bonas, 2010). Among the plant genes targeted by TALEs are those encoding proteins involved in development, stress responses and sugar transport. Rice *SWEET* genes are involved in sugar transport and activated by TALEs from the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, presumably facilitating sugar export for bacterial consumption and promoting infection (Boch et al., 2014; Chen, 2014). Characterization of *Xanthomonas* TALE DNA-binding specificity has uncovered several examples of host target genes that confer bacterial susceptibility as well as target genes present in resistant plants that induce disease resistance (Boch et al., 2014). It was shown that RipTALEs can also act as transcription activators in plant cells (Lange et al., 2013; Li et al., 2013). However, host target genes of RipTAL effectors remain unknown.

RNA silencing (or post-transcriptional gene silencing) serves as a major defense mechanism against viruses in plants. To counter host antiviral responses, viruses employ suppressors of RNA silencing that interfere with the host RNA silencing machinery and favour viruses to proliferate within the host (Vance and Vaucheret, 2001). However, plant viruses are not the only microorganisms to interfere with the plant RNA silencing machinery. *P. sojae* delivers two RxLR effectors, PSR1 and PSR2 (*Phytophthora* suppressor of RNA silencing 1 and 2), that act as suppressors of plant RNA silencing and enhance susceptibility to *P. sojae* (Qiao et al., 2013). Further, PSR1 was shown to interact with an evolutionarily conserved nuclear protein known as the PSR1-interacting protein 1 (PINP1) which regulates accumulation of small RNAs. Thus, PSR1 affects the biogenesis of plant small RNAs to promote infection (Qiao et al., 2015). Fungal pathogens can also manipulate RNA silencing machinery. *V. dahliae* secretes small RNAs that target the *Arabidopsis* RNA silencing component Argonaute (AGO1), downregulate the expression of host defense genes and thus promote fungal infection (Wang et al., 2016). A similar mechanism was previously demonstrated for the fungal pathogen *Botrytis cinerea*-derived small RNAs target the *Arabidopsis* AGO1 to impair the expression of host defense genes for promoting fungal virulence (Weiberg et al., 2013). These examples illustrate that the host RNA silencing machinery can be exploited by soil-borne pathogens through effectors to establish infection.

EFFECTORS PLAY ROLES IN SELF-DEFENSE AND COMPETITION

Before establishing themselves within a host plant, soil-borne pathogens need to interact with numerous microbial competitors, antagonists, or hyperparasites within the microbe-rich belowground environment. Likely, antagonists and hyperparasites produce a range of antimicrobial compounds and lytic enzymes to attack soil-borne pathogens. Consequently, soil-borne pathogens require molecules for self-defense and interaction with other microbes. For example, the phenolic antibiotic 2,4-diacetylphloroglucinol (DAPG) from Gram-negative antagonistic bacteria is toxic towards a wide range of pathogenic microbes, including fungal plant pathogens (Raaijmakers et al., 2009). It was shown that DAPG tolerance is positively correlated with the ability of *F. oxysporum* strains to degrade this antimicrobial metabolite via deacetylation into the less fungitoxic derivatives monoacetylphloroglucinol and

phloroglucinol (Schouten et al., 2004). This finding suggests that degradation of antimicrobial compounds produced by antagonistic microorganisms is an important self-protection strategy for DAPG-tolerant *F. oxysporum* strains, although the exact molecules secreted by tolerant *F. oxysporum* isolates that metabolizes DAPG remain to be identified. Moreover, *F. oxysporum* produces the secondary metabolite fusaric acid that appears to specifically repress the expression of DAPG biosynthetic genes in the antagonistic bacterium *Pseudomonas fluorescens* CHA0 through increasing the binding characteristics of the bacterial repressor-promotor complex (Schnider-Keel et al., 2000; Notz et al., 2002). In addition to self-defense, effectors secreted by soil-borne pathogens act in interaction with microbial competitors. *A. tumefaciens* uses the type VI secretion (T6S) system to secrete a DNase effector Tde that exhibits a potent antibacterial DNase activity and confer a competitive advantage to *A. tumefaciens* during host colonization (Ma et al., 2014).The toxic activity of the Tde DNase is counteracted by a cognate immunity protein, termed Tdi, protecting the toxin DNase-producing bacterium from self-killing. The *A. tumefacines* uses Tde to attack both intra- and inter-species bacterial competitors inside a plant host (Ma et al., 2014). The Tde and Tdi couples are broadly conserved among Gram-negative bacteria, suggesting a widespread antibacterial weapon beneficial for niche colonization (Ma et al., 2014).

EFFECTOR RECOGNITION

Plant immunity has been described as a multi-layered recognition system to prevent microbial infections (Jones and Dangl, 2006). The first layer involves the perception of microbe-associated molecular patterns (MAMPs), which are conserved across classes of microbes, by cell surface-localized pattern recognition receptors (PRRs) (Jones and Dangl, 2006). Activation of PRRs leads to MAMPs-triggered immunity (MTI) and acts as an early warning system against a wide range of potential pathogens (Jones and Dangl, 2006). Adapted pathogens are able to subvert these early defense responses by escaping or suppressing PTI through the activity of secreted effector molecules, resulting in effector-triggered susceptibility (Jones and Dangl, 2006). In turn, particular plant genotypes have evolved resistance (R) proteins that recognize particular effectors and activate effector-triggered immunity (ETI), which is recognized as a second layer of plant innate immunity (Jones and

Dangl, 2006). Although described 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). For example, tomato Ve1 recognizes the effector protein Ave1 that is secreted by race 1 strains of *Verticillium* spp. and contributes to fungal virulence on susceptible plant genotypes, leading to race-specific *Verticillium* resistance in tomato (Kawchuk et al., 2001; Fradin et al., 2009; de Jonge et al., 2012). Interestingly, homologues of Ave1 were found in plants but also in a number of plant pathogenic microbes. Ave1 is recognized not only by tomato Ve1, but also by a homologue from *Nicotiana glutinosa* (Zhang et al., 2013). Furthermore, Ve1 also mediates resistance against *F. oxysporum* f. sp. *lycopersici* in tomato (de Jonge et al., 2012), demonstrating involvement of this race-specific Ve1 protein in resistance against multiple fungal pathogens. Heterologous expression of tomato *Ve1* in *Arabidopsis* leads to race-specific *Verticillium* resistance triggered by the effector Ave1 (Fradin et al., 2011; de Jonge et al., 2012). These findings, combined with the observation that Ve1 mediates recognition of Ave1 homologues from multiple plant pathogens (de Jonge et al., 2012), imply that Ve1 has traits of a typical race-specific R protein as well as of a typical PRR, and indicate that R proteins and PRRs exist on a continuum in plant innate immunity (Fradin et al., 2011; Thomma et al., 2011).

NLPs act as effectors that positively contribute to pathogen virulence (Gijzen and Nürnberger, 2006; Ottmann et al., 2009), indicating that NLP effector-triggered immunity could be considered as belonging to ETI. On the other hand, the widespread distribution of NLPs among diverse microbes and perception of the conserved motif, nlp20 or nlp24, by a typical cell surface-localized complex (RLP23-SOBIR1-BAK1 complex) (Böhm et al., 2014; Oome et al., 2014; Albert et al., 2015) appears as a feature of pattern recognition in PTI. Thus, also plant detection of NLP effectors blurs the PTI-ETI dichotomy and illustrates a continuum between PTI-ETI. These examples, and the conceptual conflict that MAMPs are defined from the perspective of the host whereas effectors are defined from the perspective of the invader, recently inspired the proposal of the Invasion Model, in which host immune receptors (termed invasion pattern receptors; IPRs) detect either an externally encoded or modified-self ligand that betrays invaders (termed invasion patterns; IPs) (Cook et al., 2015). This model recognizes that ultimately any immune receptor can

be effective as long as it accurately betrays pathogen presence and elicits an appropriate response, irrespective of whether it recognizes a pathogen- or host-derived ligand that is either conserved or lineage-specific.

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Chapter 3

Tomato immune receptor Ve1 recognizes co-localized N- and C-termini of *Verticillium dahliae* effector Ave1

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ABSTRACT

Effectors are secreted by plant pathogens to facilitate infection, often through deregulation of plant immune responses. During host colonization, race 1 strains of the soil-borne vascular wilt fungus *Verticillium dahliae* secrete the effector protein Ave1 that triggers immunity in tomato genotypes that encode the cell surface-localized immune receptor Ve1. Homologs of *V. dahliae* Ave1 (VdAve1) are found in plants and in few plant pathogenic microbes, and are differentially recognized by Ve1. However, how VdAve1 is recognized by Ve1 remained unknown. Interestingly, C-terminally affinity-tagged versions of VdAve1 failed to activate Ve1-mediated immunity, suggesting that exposure of the C-terminus of VdAve1 is required for Ve1-mediated recognition. This was confirmed by subsequent analyses of C-terminal deletion mutants, and by amino acids swap experiments. Although required, only the C-terminus of VdAve1 is not sufficient to activate Ve1-mediated immunity. Further analyses of N-terminal deletion mutants revealed that also the N-terminus of VdAve1 is required to activate Ve1-mediated immunity. Intriguingly, a three-dimensional structural model of VdAve1 revealed that the N- and C-termini co-localize on a surface-exposed patch of the VdAve1 protein. Thus, we propose that a surface-exposed patch of the VdAve1 protein that is composed of co-localized N- and C-termini is recognized by the tomato cell-surface immune receptor Ve1.

INTRODUCTION

Plants are constantly engaged in battles against diverse groups of microbes within their environment. However, only few of these actually become pathogens to cause disease, as plants have developed innate immunity to protect themselves against microbial attack (Dodds and Rathjen, 2010; Thomma et al., 2011; Cook et al., 2015). In its simplest form, plant immunity against pathogen attack is governed by immune receptors that sense pathogen-derived(induced) 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 the corresponding resistance proteins (R proteins), resulting in effector-triggered immunity (ETI). Although initially portrayed as separate layers, 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; Cook et al., 2015). Moreover, the fact 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 externally encoded or modified-self ligands (termed invasion patterns; IPs) that betray invasion (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 (*Solanum lycopersicum*), a single dominant locus that confers *Verticillium* resistance has been identified as the *Ve* locus, which controls *Verticillium* isolates that are assigned to race 1 (Schaible et al., 1951). The *Ve* locus comprises two closely linked and inversely oriented genes, *Ve1* and *Ve2*, that both encode extracellular leucine-rich repeat (eLRR) receptor-like proteins (RLPs) (Kawchuk et al., 2001; Wang et al., 2010). Of these, only *Ve1* was found to confer resistance against race 1 isolates of *Verticillium* in tomato (Fradin et al., 2009). Intriguingly, interfamily transfer of *Ve1* from tomato to *Arabidopsis thaliana* has resulted in race-specific *Verticillium* resistance in the latter species (Fradin et al., 2011, 2014; Zhang et al., 2014), implying that the underlying immune signalling cascade across plant taxonomy is evolutionarily conserved (Fradin et al., 2011; Thomma et al., 2011). Moreover, homologs of tomato Ve1 that have the potential to recognize race 1 strains of *V. dahliae* have been characterized in other plant species, including tobacco, potato, wild eggplant and hop, suggesting an ancient origin of the tomato immune receptor Ve1 (Song et al., 2017).

Through comparative population genomics, the *V. dahliae* effector protein that is recognized by the tomato cell surface-localized immune receptor Ve1 was identified as Ave1 (for Avirulence on Ve1 tomato) (de Jonge et al., 2012). Interestingly, homologs of *V. dahliae* Ave1 (VdAve1) 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 crucifer pathogen *Colletotrichum higginsianum* (ChAve1) (de Jonge et al., 2012). Strikingly, however, most VdAve1 homologs were found in plants, with the most closely related homologs derived from tomato (*S. lycopersicum*; SlPNP) and grape (*Vitis vinifera*; VvPNP) (de Jonge et al., 2012). Finally, a more distantly related homolog was identified in the plant pathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (XacPNP) (de Jonge et al., 2012). Co-expression of *VdAve1*, *SlPNP*, *FoAve1*,

and *CbAve1* with tomato *Ve1* in tobacco triggers a hypersensitive response (HR), whereas co-expression of *ChAve1* with tomato *Ve1* does not lead to an HR (de Jonge et al., 2012). Moreover, Ve1 was found to mediate resistance towards *F. oxysporum* in tomato, demonstrating involvement of the tomato immune receptor Ve1 in resistance against multiple fungal pathogens (de Jonge et al., 2012).

Many 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 receptor-like kinase (RLK)-type immune receptor FLS2 (Zipfel et al., 2004), while the *Arabidopsis* RLK-type EFR immune receptor was shown to recognize elf18, an 18-amino-acid peptide derived from bacterial EF-Tu (Zipfel et al., 2006). Similarly, a highly conserved 22-amino-acid sequence derived from the bacterial cold shock protein, named csp22, is perceived by the tomato RLK-type immune receptor CORE (Felix and Boller, 2003; Wang et al., 2016), while a surface-exposed pentapeptide TKLGE of the 22 kDa ethylene-inducing xylanase (EIX) from the biocontrol fungus *Trichoderma viride* determines recognition by the RLP-type receptor LeEIX2 in tomato (Rotblat et al., 2002; Ron and Avni, 2004). Furthermore, the tyrosine-sulfated 21-amino-acid peptide RaxX21-sY of *Xanthomonas oryzae* pv. *oryzae* RaxX is sufficient to activate XA21-mediated immunity in rice (Pruitt et al., 2015). Finally, a conserved 20-amino-acid fragment present in most Nep1-like proteins (NLPs) (nlp20) is sufficient to activate RLP23-mediated immunity in *Arabidopsis* (Böhm et al., 2014; Oome et al., 2014; Albert et al., 2015). Here, we aimed to identify a minimal motif in the *V. dahliae* effector protein VdAve1 that is necessary and sufficient for recognition by the tomato cell-surface immune receptor Ve1. Our approach was based on epitope prediction, guided by the alignment of differentially recognized VdAve1 homologs, followed by functional analyses of a combination of serial deletion assays, amino acids swaps, synthetic peptides, chimeric proteins and three-dimensional modelling.

RESULTS

Sequence 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 derived from *V. dahliae* and *V.*

albo-atrum (de Jonge et al., 2012). *Ave1* alleles were not identified in any of the *V. dahliae* and *V. albo-atrum* race 2 strains analysed, nor in 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 presence in a collection of 129 *Verticillium* strains isolated from various host plants and different geographical locations, resulting in the identification of 22 novel *Ave1* alleles (Table S1). 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 species that have recently been recognized as novel species distinct from *V. albo-atrum* (Inderbitzin et al., 2011). However, a *VdAve1* homolog was identified in four isolates of *V. nubilum* (*VnAve1*), a species that is known as a saprophyte 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 and its homologs from plants (SlPNP and VvPNP) and plant pathogens (VnAve1, FoAve1, CbAve1, ChAve1 and XacPNP) revealed blocks of highly conserved amino acids that are alternated with more variable regions (Figure 1A). Based on prediction by SignalP 4.1 (Petersen et al., 2011), all VdAve1 homologs contain a predicted N-terminal signal peptide that directs secretion 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 homologs (Figure 1A), and *in silico* analysis using DISULFIND (Ceroni et al., 2006) suggests the formation of disulphide bridges between Cys^{35} and Cys^{63}, as well as between Cys^{71} and Cys^{79}. From the alignment it is apparent that XacPNP is the most divergent, while all other VdAve1 homologs are relatively similar (Figure 1A).

Comparison of the necrosis-inducing activity of VdAve1 homologs

It was previously demonstrated that tomato Ve1 recognizes not only VdAve1, but also SlPNP, FoAve1 and CbAve1 (de Jonge et al., 2012). We now also tested the necrosis-inducing capacity of VnAve1, VvPNP 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*

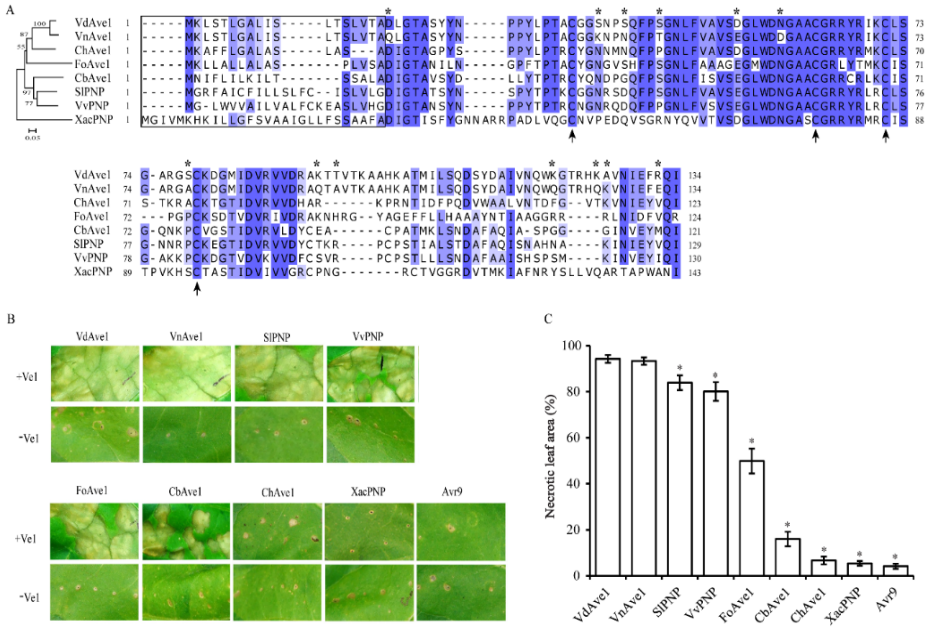

Figure 1. Distinct necrosis induced by *VdAve1* homologs through co-expression with tomato *Ve1* in *Nicotiana tabacum*. (A) Amino acid sequence alignment of VdAve1 homologs from *Verticillium dahliae* (VdAve1), *V. nubilum* (VnAve1), *Solanum lycopersicum* (SlPNP), *Vitis vinifera* (VvPNP), *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), *Cercospora beticola* (CbAve1), *Colletotrichum higginsianum* (ChAve1), and *Xanthomonas axonopodis* pv. *citri* (XacPNP). Phylogeny of VdAve1 and its homologs described above is shown. The bootstrap percentage support for each branch is indicated. The scale bar represents 5% weighted amino acid sequence divergence. Blue shade background 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. The N-terminal amino acids in the frame denote the predicted signal peptides (SP) of the VdAve1 homologs. **(B)** Coexpression of *Ve1* and *Ave1* homologs VdAve1, VnAve1, SlPNP, VvPNP, FoAve1, CbAve1, ChAve1, and XacPNP in *N. tabacum*. Expression of the sequence-unrelated effector *Avr9* from the tomato leaf mold fungus *Cladosporium fulvum* in combination with *Ve1* or absence of *Ve1* are shown as negative controls. Pictures were taken at five days post infiltration (dpi). **(C)** Quantification of necrosis resulting from recognition of VdAve1 homologs by Ve1 at 5 dpi ($n > 20$). Bars represent the average percentage of necrotic leaf area of infiltration zones with standard deviations. Asterisks above the top of the bars represent statistically significant differences when compared with VdAve1-indcued necrosis ($P < 0.05$).

(van Kan et al., 1991) with *Ve1* served as a negative control. Whereas expression of *VnAve1* or *VvPNP* 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 necrosis induced by the various VdAve1 homologs, they were co-expressed with *Ve1* in *N. tabacum* and HR development was measured by quantification of the leaf area that developed necrosis at five days post infiltration (dpi). Importantly, none of the VdAve1 homologs induced necrosis in the 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 (Figure 1B, C). 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*, necrosis did not extend beyond the wounded infiltration sites (Figure 1B, C). Upon agroinfiltration of the tomato and grape *VdAve1* homologs, *SlPNP* and *VvPNP*, most of the infiltrated leaf area developed necrosis (Figure 1B, C), occasionally affecting the complete infiltrated leaf sector. To confirm that variable levels of HR induced by VdAve1 homologs are not due to instability of the VdAve1 homologs *in planta*, GFP-tagged VdAve1 homologs were detected by immunoblotting. Similar to GFP-tagged VdAve1 protein or GFP-tagged VnAve1, all other GFP-tagged VdAve1 homologs accumulated to clearly detectable protein levels *in planta* (Figure S1).

The C-terminus of VdAve1 is required, but not sufficient, for recognition by tomato Ve1

In order to perform further functional analyses, a construct encoding C-terminally GFP-tagged VdAve1 (VdAve1_c GFP) was generated. However, C-terminal fusion of a GFP tag to VdAve1 resulted in loss of recognition by Ve1 (Figure 2A, C). Considering that the GFP tag (~27 kDa) is relatively large, we engineered C-terminally tagged VdAve1 fusions with smaller protein tags (<12 kDa; VdAve1_c 3xHA, VdAve1_c 6xHis, VdAve1_c 4xMyc, VdAve1_c 10xMyc and VdAve1_c FLAG; Figure 2A). Despite their smaller size, all additionally tested C-terminal tags abolished, or significantly reduced, HR development on *Ve1*-expressing tobacco leaves (Figure 2A, C). Importantly, the C-terminally GFP-tagged VdAve1 fusion was clearly detected by immunoblotting (Figure S2A), suggesting that accessibility of the VdAve1 C-terminus is important in VdAve1 recognition by Ve1.

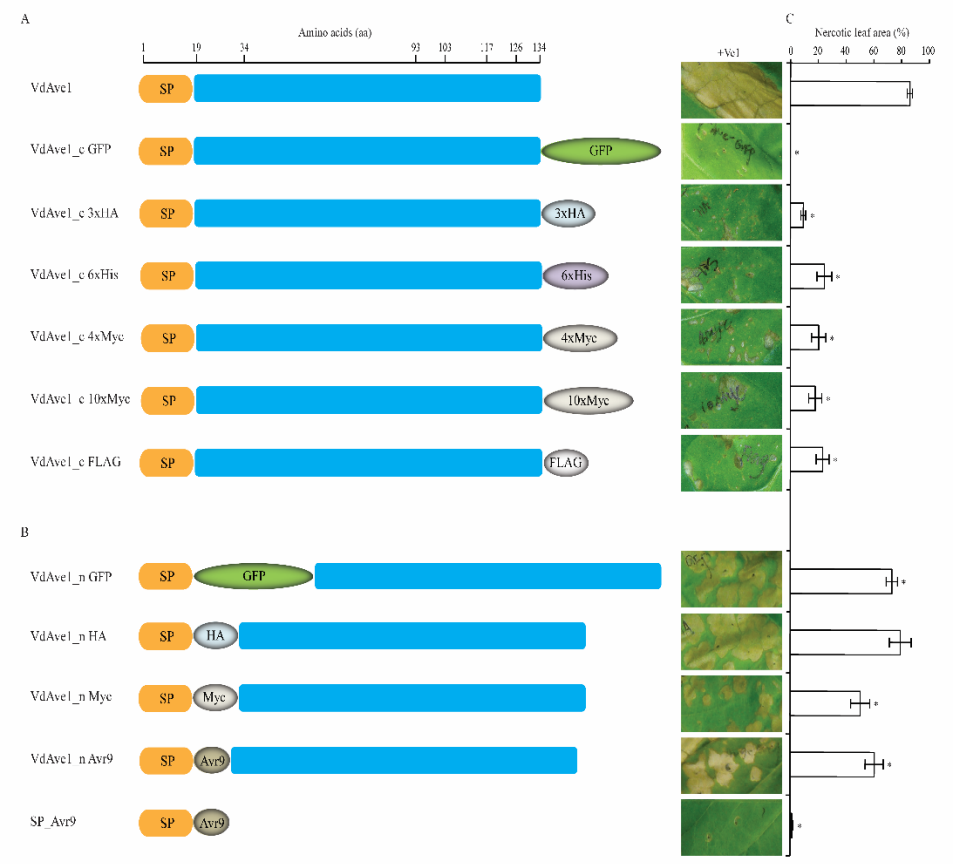

Figure 2. Effect of C- or N- terminally tagged VdAve1 versions on recognition of VdAve1 by Ve1 in tobacco. (A) Constructs encoding full-length VdAve1 (VdAve1) and C-terminally tagged VdAve1 versions (VdAve1_c GFP, VdAve1_c 3xHA, VdAve1_c 6xHis, VdAve1_c 4xMyc, VdAve1_c 10xMyc, VdAve1_c FLAG) were assayed for necrosis-inducing capability. The constructs were co-expressed with *Ve1* in tobacco respectively, and the occurrence of necrosis was monitored at 5 dpi. The feature includes a predicated signal peptide (SP) involved in effector secretion. **(B)** Constructs encoding N-terminally fused VdAve1 versions (VdAve1_n GFP, VdAve1_n HA, VdAve1_n Myc and VdAve1_n Avr9) were assayed for necrosis-inducing capability. The constructs were co-expressed with *Ve1* in tobacco respectively, and the necrosis occurrence was monitored at 5 dpi. A construct encoding mature Avr9 fused with VdAve1 signal peptide (SP_Avr9) co-expressed with *Ve1* is used as a negative control. **(C)** Quantification of necrosis resulting from recognition of tagged VdAve1 proteins by Ve1 at 5 dpi. The graph shows the average percentage of necrotic leaf area of infiltration zones at 5 dpi ($n > 5$). Data are presented as mean with standard deviations. Asterisks indicate statistically significant differences when compared with wild-type VdAve1-indcued necrosis ($P < 0.05$).

To further investigate the role of the C-terminus in recognition of VdAve1 by Ve1, a number of C-terminal truncation mutants were generated. Deletion of 42

amino acids from the C-terminus (Lys^{93} to Ile^{134}; VdAve1$^{\Delta 93\text{-}134}$) resulted in loss of VdAve1 recognition by Ve1 (Figure 3A, B). Subsequent analysis of step-wise smaller truncations revealed that a C-terminal deletion of nine amino acids (VdAve1$^{\Delta 126\text{-}134}$) resulted in loss of Ve1-mediated recognition (Figure 3A, B), even though the presence of GFP-tagged VdAve1$^{\Delta 126\text{-}134}$ protein *in planta* was verified by immunoblotting (Figure S2A). We subsequently performed complementation experiments in *V. dahliae* to confirm the importance of the C-terminus for activation of Ve1-mediated immunity. To this end, we expressed *VdAve1*$^{\Delta 126\text{-}134}$ driven by the native *VdAve1* promoter in a *VdAve1* deletion mutant (*V. dahliae* JR2Δ*VdAve1*; de Jonge et al., 2012) and inoculated *Ve1* tomato plants with the complemented strains. Plants that were inoculated with three independent *V. dahliae* strains expressing *VdAve1*$^{\Delta 126\text{-}134}$ (VdAve1$^{\Delta 126\text{-}134}$ #1, VdAve1$^{\Delta 126\text{-}134}$ #2 and VdAve1$^{\Delta 126\text{-}134}$ #3) showed a similar disease phenotype as *Ve1* plants inoculated with the *V. dahliae* JR2Δ*VdAve1* strain, whereas plants inoculated with wild-type *V. dahliae* strain JR2 and the *VdAve1* complementation strain resembled mock-inoculated plants (Figure 3C, D). Collectively, these results demonstrate that the C-terminal nine amino acids of VdAve1 are required to activate Ve1-mediated immunity.

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, C), C-terminal nine-amino-acid swaps between VdAve1 and XacPNP were performed. An expression construct encoding a chimeric VdAve1 protein was engineered in which the C-terminal nine amino acids of VdAve1 were replaced by those of XacPNP (Vd_XacPNP$^{c\ 9AA}$; Figure 4A). As expected, similar to XacPNP, co-expression of *Vd_XacPNP*$^{c\ 9AA}$ with *Ve1* in tobacco failed to induce an HR, as only minimal necrotic spots were observed (Figure 4A, B). Conversely, a construct encoding another chimeric VdAve1 protein was generated in which the last nine amino acids of XacPNP were replaced by those of VdAve1 (Xac_VdAve1$^{c\ 9AA}$; Figure 4A). Co-expression of *Xac_VdAve1*$^{c\ 9AA}$ with *Ve1* in tobacco induced a relatively strong HR when compared with XacPNP or Vd_XacPNP$^{c\ 9AA}$-induced HR, although full necrosis was not observed in the infiltrated leaf area (Figure 4A, B). Immunoblotting confirmed that the GFP-tagged Vd_XacPNP$^{c\ 9AA}$ and Xac_VdAve1$^{c\ 9AA}$ proteins were present *in planta* (Figure S2B).

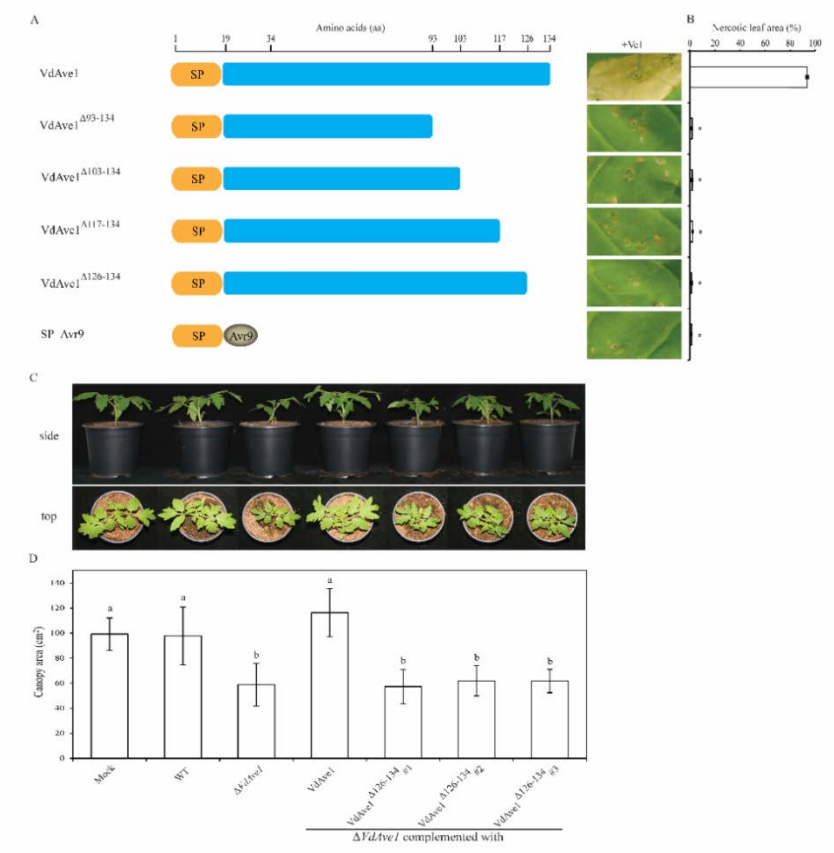

Figure 3. The C-terminal nine amino acids of VdAve1 are required to establish Ve1-mediated immunity. (A) VdAve1 C-terminal truncations result in loss of recognition by Ve1. Constructs encoding four VdAve1 truncations that lack the C-terminal 42 (VdAve1$^{\Delta 93\text{-}134}$), 32 (VdAve1$^{\Delta 103\text{-}134}$), 18 (VdAve1$^{\Delta 117\text{-}134}$) and 9 (VdAve1$^{\Delta 126\text{-}134}$) amino acids of VdAve1 co-infiltration with *Ve1* were assayed respectively, and the occurrence of necrosis was recorded at 5 dpi. Constructs VdAve1 and SP_Avr9 were used as a positive and negative control, respectively. The feature includes a predicated signal peptide (SP) involved in effector secretion. **(B)** Quantification of necrosis resulting from recognition of VdAve1 C-terminal truncations by Ve1 at 5 dpi. The graph shows the average percentage of necrotic leaf area of infiltration zones at 5 dpi (n > 5). Data are presented as mean with standard deviations. Asterisks indicate statistically significant differences when compared with wild-type VdAve1-indcued necrosis ($P < 0.05$). **(C)** Complementation assays in *Verticillium dahliae* show that the VdAve1 C-terminal nine amino acids are required to activate Ve1-mediated immunity in tomato. Three independent *V. dahliae VdAve1* deletion (Δ*VdAve1*) strains expressing a construct encoding VdAve1 lacking the C-terminal nine amino acids (VdAve1$^{\Delta 126\text{-}134}$ #1, VdAve1$^{\Delta 126\text{-}134}$ #2 and VdAve1$^{\Delta 126\text{-}134}$ #3) escape recognition by *Ve1* tomato compared with *V. dahliae* wild-type (WT) and genetic complementation strains (VdAve1) evidenced by stunted *Ve1* plants at 14 days post *Verticillium* inoculation. **(D)** Average canopy area of 8 *Ve1* tomato plants inoculated with different *V. dahliae* strains or mock-inoculation. Different letters indicate statistically significant differences ($P < 0.05$). The data shown are representative of three independent experiments.

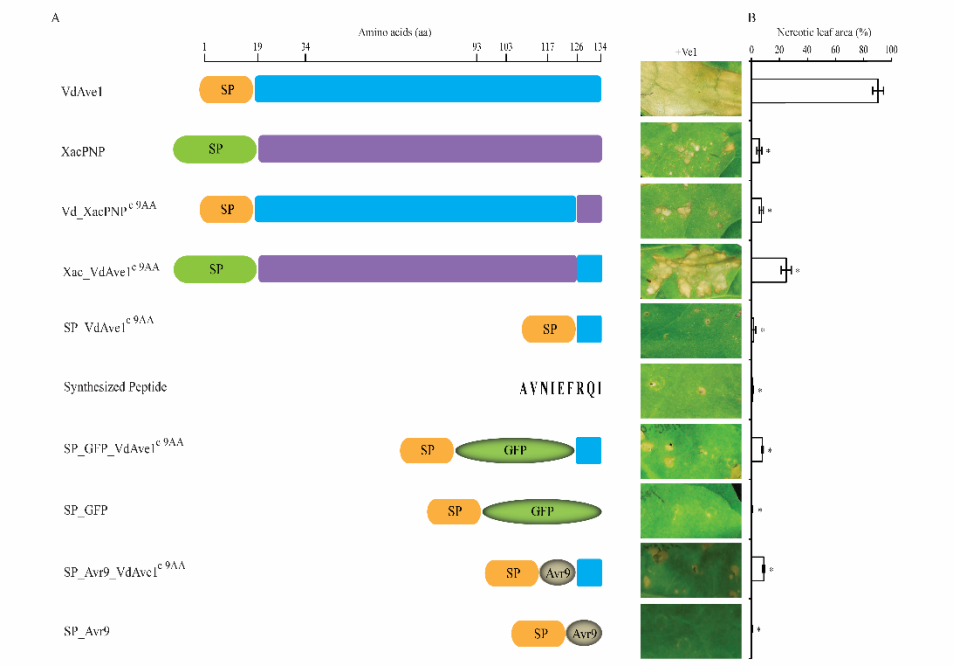

Figure 4. (A) The C-terminal nine amino acids of VdAve1 are critical but not sufficient for Ve1-mediated recognition in tobacco. Constructs encoding full-length VdAve1 and XacPNP with their own signal peptides (SP) and two chimeras in which their C-terminal nine amino acids were swapped (Vd_XacPNP$^{c\ 9AA}$ and Xac_VdAve1$^{c\ 9AA}$) were assayed. In chimera Vd_XacPNP$^{c\ 9AA}$ the C-terminal nine amino acids of VdAve1 were replaced by those of XacPNP, while in chimera Xac_VdAve1$^{c\ 9AA}$ the C-terminal nine amino acids of XacPNP were replaced by those of XacPNP. A construct encodes the C-terminal nine amino acids of VdAve1 fused to the VdAve1 signal peptide (SP_VdAve1$^{c\ 9AA}$). A construct encodes a GFP that is C-terminally fused to the C-terminal nine amino acids of VdAve1, and N-terminally fused to the VdAve1 signal peptide to establish extracellular secretion (SP_GFP_VdAve1$^{c\ 9AA}$), while a construct encoding GFP fused with the VdAve1 signal peptide (SP_GFP) and construct SP_Avr9 were used as negative controls. Furthermore, a chemically synthetized peptide encompassing VdAve1 C-terminal nine amino acids peptide (AVNIEFRQI) was used. A construct VdAve1 was used as a positive control. The feature includes a predicated signal peptide (SP) involved in effector secretion. All the constructs were co-expressed with *Ve1* in tobacco respectively, and the occurrence of necrosis was monitored at 5 dpi. **(B)** Quantification of necrosis resulting from recognition of VdAve1 chimeras by Ve1 at 5 dpi. The graph shows the average percentage of necrotic leaf area of infiltration zones at 5 dpi (n > 10). Data are presented as mean with standard deviations. Asterisks indicate statistically significant differences when compared with wild-type VdAve1-indcued necrosis ($P < 0.05$).

These results confirm that the C-terminal nine amino acids of VdAve1 are critical for recognition by Ve1, and furthermore suggest that these are sufficient for recognition.

To determine whether the C-terminal nine amino acids are indeed 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_VdAve1$^{c\ 9AA}$; Figure 4A). This construct was co-expressed with *Ve1* in tobacco, but no necrosis was observed in the infiltrated leaf (Figure 4A, B). Additionally, infiltration of a chemically synthetized peptide encompassing the C-terminal nine amino acids of VdAve1 (peptide sequence: AVNIEFRQI) did not induce an HR in *Ve1*-expressing tobacco up to a concentration of 1 mg/mL (Figure 4A, B). However, possibly the nine amino acid peptide is not stable in the apoplast. In an attempt to overcome such complications, we generated two 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_VdAve1$^{c\ 9AA}$ and SP_Avr9_VdAve1$^{c\ 9AA}$; Figure 4A). As negative controls, two 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 4A) were generated. All constructs were co-expressed with *Ve1* in tobacco and HR development was monitored at 5 dpi. However, only slight necrosis was observed following infiltration of *Ve1* with *SP_GFP_VdAve1$^{c\ 9AA}$* or *SP_Avr9_VdAve1$^{c\ 9AA}$* in the infiltrated sector (Figure 4A, B), despite detection of SP_GFP_VdAve1$^{c\ 9AA}$ protein *in planta* by immunoblotting (Figure S2A). These data reveal that the C-terminal nine amino acids of VdAve1 are not sufficient to activate Ve1-mediated immunity.

To test whether longer stretches of the C-terminus of VdAve1 can be used to trigger Ve1-mediated recognition, we generated a construct encoding the C-terminal 18 amino acids of VdAve1 fused to the VdAve1 signal peptide (SP_VdAve1$^{c\ 18AA}$; Figure 5A). This construct was co-expressed with *Ve1* in tobacco, but only slightly increased necrosis was observed in the infiltrated sector (Figure 5A, B). We further generated two 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 18 amino acids of VdAve1 (SP_GFP_VdAve1$^{c\ 18AA}$ and SP_Avr9_VdAve1$^{c\ 18AA}$; Figure 5A). Co-infiltrations of *Ve1* with *SP_GFP* or *SP_Avr9* were used as negative controls. Co-expression of *Ve1* with *SP_GFP_VdAve1$^{c\ 18AA}$* or *SP_Avr9_VdAve1$^{c\ 18AA}$* in tobacco induced similar necrosis formation as SP_VdAve1$^{c\ 18AA}$ protein at 5 dpi

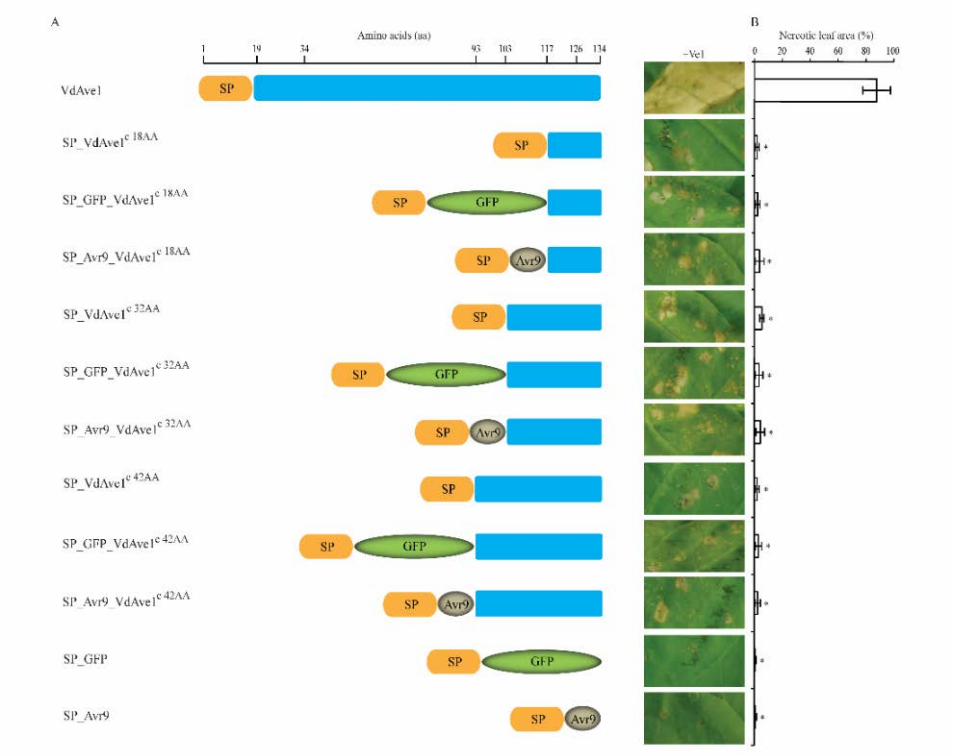

Figure 5. VdAve1 C-terminus is not sufficient for VdAve1 recognition by Ve1. (A) Occurrence of the necrosis in tobacco upon co-expression of VdAve1 C-terminal chimeras with *Ve1*. Three constructs encoding the VdAve1 signal peptide (SP) fused to the VdAve1 C-terminal 18-amino-acid (SP_VdAve1$^{c\ 18AA}$), or the GFP and VdAve1 C-terminal 18-amino-acid (SP_GFP_ VdAve1$^{c\ 18AA}$) , or the mature Avr9 and VdAve1 C-terminal 18-amino-acid (SP_Avr9_ VdAve1$^{c\ 18AA}$) were generated. Furthermore, constructs encoding the extended VdAve1 C-termini (SP_VdAve1$^{c\ 32AA}$ and SP_VdAve1$^{c\ 42AA}$), or N-terminally GFP-tagged VdAve1 C-terminal extensions (SP_GFP_VdAve1$^{c\ 32AA}$ and SP_GFP_VdAve1$^{c\ 42AA}$), or N-terminally Avr9-fused VdAve1 C-terminal extensions (SP_Avr9_VdAve1$^{c\ 32AA}$ and SP_Avr9_VdAve1$^{c\ 42AA}$) were assayed too. Constructs VdAve1, SP_GFP and SP_Avr9 were used as controls. The feature includes a predicated signal peptide (SP) involved in effector secretion. The constructs were co-expressed with *Ve1* in tobacco respectively, and the necrosis occurrence was recorded at 5 dpi. **(B)** Quantification of necrosis resulting from recognition of VdAve1 C-terminal chimeras by Ve1 at 5 dpi. The graph shows the average percentage of necrotic leaf area of infiltration zones at 5 dpi (n > 10). Data are presented as mean with standard deviations. Asterisks indicate statistically significant differences when compared with wild-type VdAve1-indcued necrosis ($P < 0.05$).

(Figure 5A, B). Immunoblotting confirmed that the chimeric SP_GFP_VdAve1$^{c\ 18AA}$ protein was present *in planta* (Figure S3). Subsequent analyses showed that also the C-terminal 32 amino acids and the C-terminal 42 amino acids of VdAve1 induce signs of a weak HR at 5 dpi (Figure 5A, B), although their presence was verified by

immunoblotting (Figure S3). Taken together, these results reveal that the C-terminus of VdAve1 is required, but not sufficient, to activate Ve1-mediated recognition.

The N-terminus of VdAve1 is required, but not sufficient for Ve1-mediated recognition

We previously observed that affinity tags that were C-terminally fused to VdAve1 compromised recognition by Ve1, resulting in the finding that the C-terminus of VdAve1 is required for recognition. Accordingly, when we fused various tags (GFP, HA, Myc and Avr9) to the N-terminus of mature VdAve1, we similarly observed significantly compromised Ve1-mediated recognition upon use of the GFP (VdAve1_n GFP), or Myc (VdAve1_n Myc), or Avr9 (VdAve1_n Avr9) tag (Figure 2B, C), although N-terminal tagging of VdAve1 with HA (VdAve1_n HA) did not significantly affect recognition by Ve1 (Figure 2B, C). Immunoblotting confirmed that the N-terminally GFP-tagged VdAve1 protein was stably produced *in planta* (Figure S2A). These data suggest that, besides the VdAve1 C-terminus, also the N-terminus of VdAve1 is involved in recognition by Ve1.

To further investigate the importance of the N-terminus of VdAve1 in Ve1-mediated recognition, recognition of VdAve1 upon deletion of the N-terminal 16 amino acids (from Asp^{19} to Ala^{34}; $VdAve1^{\Delta 19\text{-}34}$) by Ve1 was tested in tobacco. Indeed, deletion of the 16 N-terminal amino acids of the mature VdAve1 abolished recognition by Ve1 (Figure 6A, B). Furthermore, we engineered two 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 mature VdAve1 lacking the N-terminal 16-amino-acid of mature VdAve1 (SP_GFP_$VdAve1^{\Delta 19\text{-}34}$ and SP_Avr9_ $VdAve1^{\Delta 19\text{-}34}$; Figure 6A). Co-expression of *Ve1* with *SP_GFP_ $VdAve1^{\Delta 19\text{-}34}$* or *SP_Avr9_ $VdAve1^{\Delta 19\text{-}34}$* in tobacco did not result in Ve1-mediated recognition (Figure 6A, B), although production of SP_GFP_$VdAve1^{\Delta 19\text{-}34}$ was confirmed by immunoblotting (Figure S4). To further confirm the involvement of the VdAve1 N-terminus in Ve1-mediated recognition, we introduced the coding sequence *$VdAve1^{\Delta 19\text{-}34}$* under the control of the native *VdAve1* promoter into *V. dahliae* JR2Δ*VdAve1* (de Jonge et al., 2012) and performed disease assays on *Ve1* tomato plants. These assays showed that, similar to *V. dahliae* JR2Δ*VdAve1*, also complementation strains expressing *$VdAve1^{\Delta 19\text{-}34}$*

caused clear Verticillium wilt symptoms on *Ve1* tomato plants, while wild-type *V. dahliae* strain JR2 and the complementation strain expressing *VdAve1* caused no disease symptoms on tomato plants expressing *Ve1* (Figure 6C, D). These results reveal that the N-terminal 16 amino acids of mature VdAve1 are required to trigger Ve1-mediated immunity.

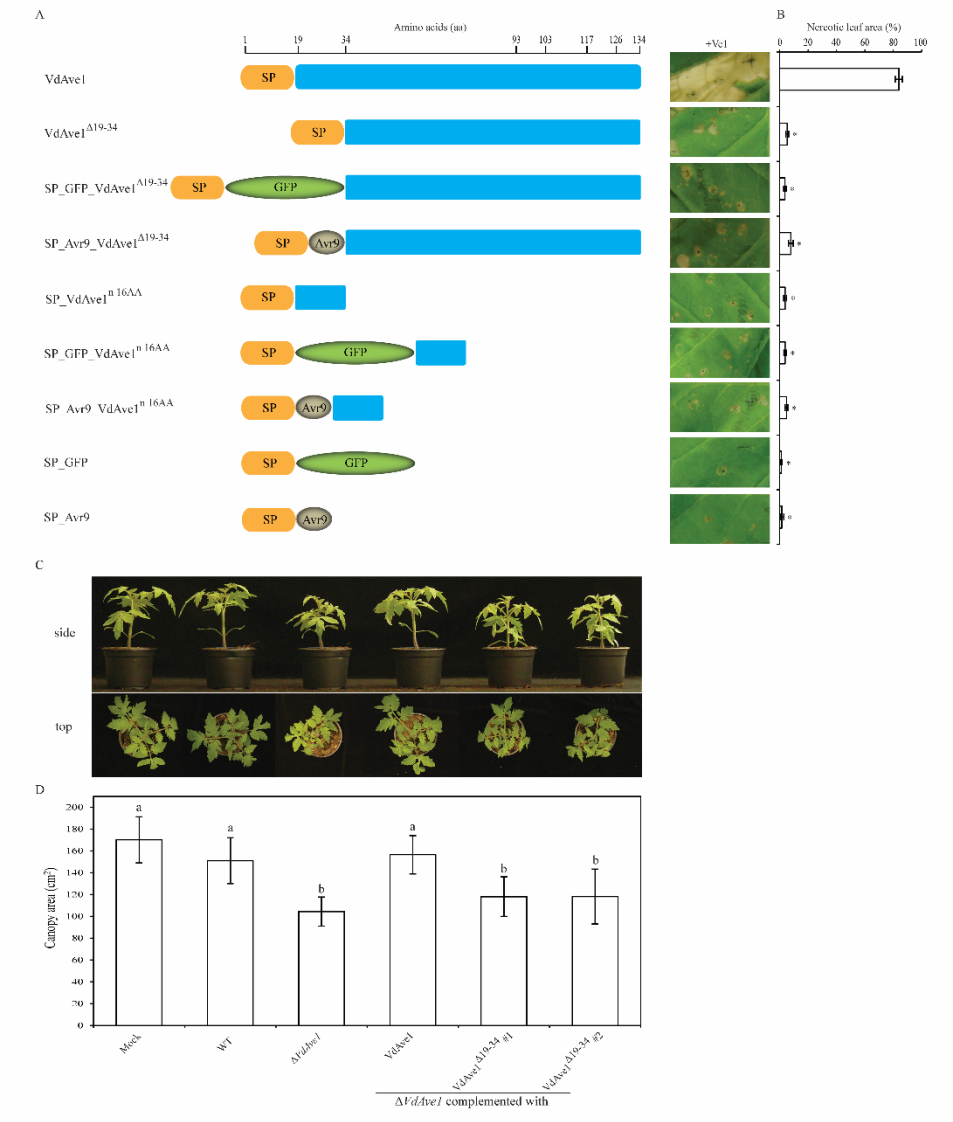

Figure 6. (A) The N-terminal sixteen amino acids of mature VdAve1 are required but not sufficient for Ve1-mediated recognition in tobacco. Occurrence of the necrosis in *N. tabacum* upon co-expression of N-terminal chimeras with *Ve1*. Constructs encoding a VdAve1 truncation that lacks the N-terminal 16-amino-acid of mature VdAve1 (VdAve1$^{\Delta 19\text{-}34}$), and the corresponding N-terminal GFP or Avr9 fusion (SP_GFP_VdAve1$^{\Delta 19\text{-}34}$ and SP_Avr9_VdAve1$^{\Delta 19\text{-}34}$) were used. Furthermore, constructs encoding the VdAve1 signal peptide (SP) fused to the N-terminal 16-amino-acid of mature VdAve1 (SP_VdAve1$^{n\ 16AA}$), or the GFP and the N-terminal 16-amino-acid of mature VdAve1 (SP_GFP_VdAve1$^{n\ 16AA}$), or the mature Avr9 and VdAve1 C-terminal 16-amino-acid (SP_GFP_VdAve1$^{n\ 16AA}$) were assayed. Constructs VdAve1, SP_GFP and SP_Avr9 were used as controls. The constructs include a predicted signal peptide (SP) to direct effector secretion. The constructs were co-agroinfiltrated with *Ve1* in tobacco respectively, and the necrosis occurrence was monitored at 5 dpi. **(B)** Quantification of necrosis resulting from recognition of VdAve1 N-terminal chimeras by Ve1 at 5 dpi. The graph shows the average percentage of necrotic leaf area of infiltration zones at 5 dpi (n > 10). Data are presented as mean with standard deviations. Asterisks indicate statistically significant differences when compared with wild-type VdAve1-indcued necrosis ($P < 0.05$). **(C)** Complementation assays in *V. dahliae* show that the N-terminal sixteen amino acids of mature VdAve1 protein are required to activate Ve1-mediated immunity in tomato. Two independent *V. dahliae VdAve1* deletion (Δ*VdAve1*) strains expressing a construct encoding VdAve1 lacking the N-terminal sixteen amino acids of mature VdAve1(VdAve1$^{\Delta 19\text{-}34}$ #1 and VdAve1$^{\Delta 19\text{-}34}$ #2) escape recognition by *Ve1* tomato compared with *V. dahliae* wild-type (WT) and genetic complementation strains (VdAve1) evidenced by stunted *Ve1* plants at 14 days post *Verticillium* inoculation. **(D)** Average canopy area of 8 *Ve1* tomato plants inoculated with different *V. dahliae* strains or mock-inoculation Different letters indicate statistically significant differences ($P < 0.05$). The data shown are representative of three independent assays.

To examine whether that the N-terminal 16 amino acids are sufficient to trigger Ve1-mediated recognition, we engineered a construct encoding the N-terminal 16-amino-acid of mature VdAve1 fused to the VdAve1 signal peptide (SP_VdAve1$^{n\ 16AA}$; Figure 6A). However, co-expression of *SP_VdAve1$^{n\ 16AA}$* with *Ve1* in tobacco was not able to induce an HR at 5 dpi (Figure 6A, B). Furthermore, two constructs were designed in which the coding sequence of GFP or Avr9 was N-terminally fused to the VdAve1 signal peptide and C-terminally tagged to the N-terminal 16-amino-acid of mature VdAve1 (SP_GFP_VdAve1$^{n\ 16AA}$ and SP_Avr9_VdAve1$^{n\ 16AA}$; Figure 6A). Co-expression of *Ve1* with *SP_GFP_VdAve1$^{n\ 16AA}$* or *SP_Avr9_VdAve1$^{n\ 16AA}$* in tobacco failed to induce necrosis in tobacco (Figure 6A, B), although immunoblotting showed that the SP_GFP_VdAve1$^{n\ 16AA}$ was present *in planta* (Figure S4). Collectively, these results demonstrate that the N-terminal 16 amino acids of mature VdAve1 are required, but not sufficient, to establish Ve1-mediated immunity.

Structural modelling reveals co-localization of VdAve1 C- and N-termini on a surface-exposed patch

In an attempt to gain a better insight in VdAve1 recognition by Ve1, a three-dimensional structural model of VdAve1 was generated. 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 VdAve1 structural model shows that the C-terminal nine amino acids (Figure 7; shown in red) are exposed at the surface of the mature VdAve1 protein. The model also predicts that the N-terminus (Figure 7; shown in orange) congregates with the C-terminus on the exposed surface of the mature VdAve1 protein (Figure 7), which suggests that Ve1 may recognize a surface-exposed patch of the VdAve1 protein that includes both the N- and C-termini. Taken together, we conclude that a surface-exposed patch of the VdAve1 protein that is composed of co-localized N- and C-termini is recognized by the tomato cell-surface immune receptor Ve1.

DISCUSSION

We have previously shown that the *V. dahliae* effector VdAve1 is recognized by the tomato cell-surface immune receptor Ve1 (de Jonge et al., 2012), and we have identified several homologs of VdAve1 that are differentially recognized by Ve1 (Figure 1B, C). In this study, we reveal that a surface-exposed patch of the VdAve1 protein that is composed of co-localized N- and C-termini of *V. dahliae* effector VdAve1 is recognized by tomato immune receptor Ve1. Our analyses revealed that the C-terminus as well as the N-terminus individually are required, but not sufficient, to activate Ve1-mediated immunity.

Plant cell surface-localized PRRs are often activated upon recognition of short peptide sequences on the surface of their ligands, such as flg22 or flgII-28 that are derived from flagellin (Felix et al., 1999; Cai et al., 2011; Clarke et al., 2013), the pentapeptide TKLGE derived from EIX (Rotblat et al., 2002), the csp22 peptide derived from the bacterial cold shock proteins (Felix and Boller, 2003), elf18 or EFa50 derived from EF-Tu (Kunze et al., 2004; Furukawa et al., 2014), and the nlp20 peptide found in most NLPs (Böhm et al., 2014; Oome et al., 2014). In our study, we attempted to identify such motif in VdAve1 for recognition by the tomato

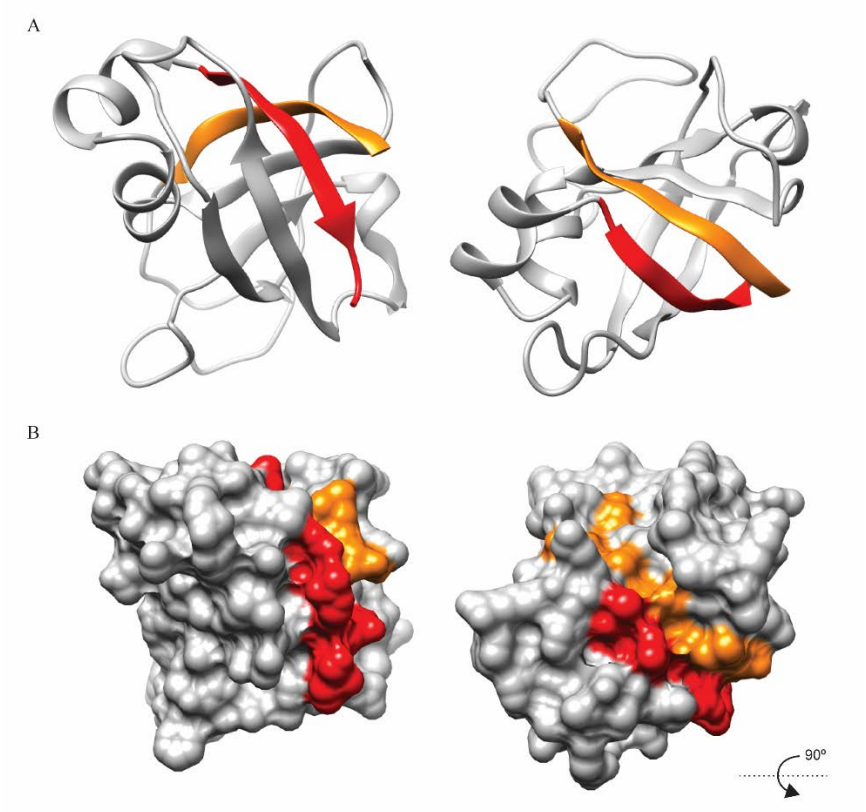

Figure 7. Three-dimensional structural model of the VdAve1 protein. The VdAve1 structure was predicted using I-TASSER (Zhang, 2008). The inferred VdAve1 structure is of high quality indicated by a confidence score (C-Score) of 1.22, and displayed as a ribbon **(A)**, and surface **(B)** model from the side (left) and the top (right). The C-terminal nine amino acids sequence of VdAve1 is indicated in red, while the N-terminal eight amino acids sequence is indicated in orange.

cell-surface immune receptor Ve1. Clearly, our results suggest that the co-localization of the two termini of the primary amino acid sequence, rather than a contiguous stretch of amino acids composes the recognition motif. Our efforts to identify an artificial minimal peptide that can be recognized by tomato Ve1 by generating chimeric peptides consisting of the N-terminal sixteen amino acids in combination with various C-terminal peptides, and fused to various tags, were fruitless (data not shown). This suggests that also the folding of the recognized sequence patch, and the spatial orientation of the two protein termini, is important for the activation of Ve1-meidated immunity.

Although several minimal motifs have been identified in ligands of cell surface

receptors, similar examples for intracellular NLR (nucleotide-binding domain leucine-rich repeat) immune receptors have not been reported. Moreover, it has suggested that simultaneous recognition of multiple epitopes within a single effector is required for NLR activation. For example, distinct regions of the *Pseudomonas syringae* effector AvrRps4 are required for the activation of the intracellular NLR receptors PRS4/RRS1-mediated immunity (Sohn et al., 2009, 2012). Similarly, multiple contact points are likely required for recognition of the flax-rust effectors AvrL567 and AvrM by the corresponding NLR receptors in flax (Wang et al., 2007; Ve et al., 2013). Likewise, multiple residues at separate locations on the surface of the *Hyaloperonospora arabidopsidis* effector ATR1 are required for recognition by the *Arabidopsis* NLR receptor RPP1 (Chou et al., 2011; Goritschnig et al., 2016). In contrast to these findings, our data suggest that, although physically separated on the primary amino acid chain, recognition converges at a single surface-exposed patch of the VdAve1 protein.

Thus far, we have failed to show a direct physical interaction between VdAve1 and Ve1. Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2, and subsequent alanine scanning mutagenesis on the solvent exposed β-strand/β-turn residues across the eLRR domain previously identified several regions of the Ve1 protein that are required for functionality (Fradin et al., 2014; Zhang et al., 2014). In these studies, Ve1 functionality was restricted to three consecutive eLRR regions, namely eLRR1-eLRR8, eLRR20-eLRR23 and eLRR32-eLRR37, of which two regions eLRR1-eLRR8 and eLRR20-eLRR23 were proposed to contribute to ligand binding, while eLRR32-eLRR37 was proposed to function in immune signalling activation (Zhang et al., 2014). Realistically, final confirmation of this model cannot be obtained through domain swaps, domain deletions, gene shuffling analyses and site-directed mutagenesis within the immune receptor or the recognized ligand, but will ultimately have to follow from structural analysis of receptor-ligand interactions, for instance through crystallography.

MATERIALS AND METHODS

Plant materials and plant growth conditions

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) and *35S::Ve1* tomato (*Solanum*

lycopersicum cv. MoneyMaker background; Fradin et al., 2009) plants were grown in the greenhouse (Unifarm, Wageningen, the Netherlands) 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.

Sequence alignments and phylogenetic analysis

Signal peptide prediction was performed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011). Multiple sequence alignments were conducted by using the Cluastal X program (http://www.clustal.org/). Phylogenetic tree was constructed by using the neighbour-joining method in MEGA7 (http://www.megasoftware.net/). Bootstrap reassembling analysis based on 1000 replicates was used to assess the confidence values indicated at the individual nodes.

Generation of binary expression vectors

Construction of all binary expression vectors is described in Methods S1.

Transient expression assays

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 *Ve1* and *VdAve1*, or *VdAve1* homologs, or *VdAve1* chimeras 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), infiltrated leaves were photographed, and necrosis was quantified by using ImageJ to measure the area of necrosis as percentage of the total infiltrated leaf area (Song et al., 2017).

Protein extracts and immunoblotting

For immunological detection of GFP-tagged VdAve1 homologs and VdAve1 chimeras, *A. tumefaciens* carrying the corresponding expression constructs was

infiltrated into mature tobacco leaves as described previously (Zhang et al., 2013). The co-immunoprecipitations and immunoblotting were performed as described previously (Zhang et al., 2014).

Generation of complementation *V. dahliae* strains

To generate *VdAve1*, *VdAve1*$^{\Delta 126\text{-}134}$ and *VdAve1*$^{\Delta 19\text{-}34}$ complementation constructs, DNA fragments containing the *Pac*I and *Not*I restriction sites were amplified by PCR from the corresponding plasmids VdAve1, VdAve1$^{\Delta 126\text{-}134}$ and VdAve1$^{\Delta 19\text{-}34}$ using primers listed in Table S2, and cloned into the vector pFBT005 which *ToxA* promoter was replaced by the native *VdAve1* promoter (~1.6 kb) and contains a nourseothricin cassette, respectively. All the constructs were confirmed by DNA sequencing (Eurofins Genomics, Ebersberg, Germany), and subsequently transformed into *A. tumefaciens* strain AGL1 by electroporation. *A. tumefaciens*-mediated transformation of *V. dahliae* strain JR2Δ*VdAve1* (de Jonge et al., 2012) was performed as previously described (Santhanam, 2012). *V. dahliae* transformants were selected on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) plates containing 50 µg/mL 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 7 to 10 days. Genomic DNA was extracted from individual transformants and PCR was performed to test presence of the inserted nourseothricin cassette and presence of the inserted chimeric *VdAve1* fragment.

Disease assays

V. dahliae was grown on PDA plates at 22 °C, and conidia were collected from 7- to 10- day-old *V. dahliae* cultures on PDA plates and washed with tap water. Disease assays on tomato plants were performed as previously described (Fradin et al., 2009). Briefly, 10-day-old tomato plants were uprooted, the roots were rinsed in water, dipped for 5 min in a suspension of 10^6 conidiospores/mL water while the roots of mock plants were dipped in tap water without conidiospores, and transplanted to fresh commercial potting soil (Horticoop, Bleiswijk, the Netherlands). Disease symptoms were scored up to 14 days post *Verticillium* inoculation, inoculated plants were photographed. 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 INFORMATION

Table S1. *Verticillium* strains analysed for presence of *VdAve1* homologs.

Species	Isolate	Original host	Origin	*Ave1*
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	-
	T9	cotton	USA	-
	Vd1795	sugar beet	USA	-
	Vd850-4	potato	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	+
	2009-605	pepper	Ukrain	+
	Vd52	pepper	Austria	+
	V4	cotton	Spain	+
	V117	cotton	Spain	-
	V991	cotton	China	-
	BP2	cotton	China	-
	CQ2	cotton	China	-
	1cd3-2	cotton	China	-
	4TM6-15	cotton	China	-
	1hn-1	cotton	China	-
	CFA3	sunflower	China	-
	GRN1	sunflower	China	-
	GYA2	sunflower	China	-
	HnA4	sunflower	China	-
	SX4	sunflower	China	-
	WYA3	sunflower	China	-

Table S1 (continued)

Species	Isolate	Original host	Origin	*Ave1*
V. dahliae	HeA4	sunflower	China	-
	12	sunflower	China	-
	77	sunflower	China	-
	89	sunflower	China	-
	VPRI 42056	tomato	Australia	-
	VPRI 42057	tomato	Australia	-
	VPRI 42058	tomato	Australia	-
	VPRI 42079	tomato	Australia	-
	VPRI 42080	tomato	Australia	-
	VPRI 42081	tomato	Australia	-
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.	-

Table S1 (continued)

Species	Isolate	Original host	Origin	*Ave1*
V. nonalfalfae	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	-
	AR01/067	tomato	U.K.	-
	AR0/140	tomato	U.K.	-
	AR01/JS1	tomato	U.K.	-
	PD83/53a	tomato	Netherlands	-
	PD2000/4186a	tomato	Netherlands	+
	VnaCBS385.91	tomato	Netherlands	+
	Vna5431	tomato	unknown	+
	Vna1	unknown	Luxembourg	-
V. alfalfae	Luc	alfalfa	U.K.	+
	41	alfalfa	Canada	-
	CBS392.91	alfalfa	Netherlands	-
	Va2	unknown	Netherlands	-
	107	alfalfa	USA	-
	PD693	potato	Iran	-
	314193	potato	Australia	-
	340646	potato	Spain	+
	11	alfalfa	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	-

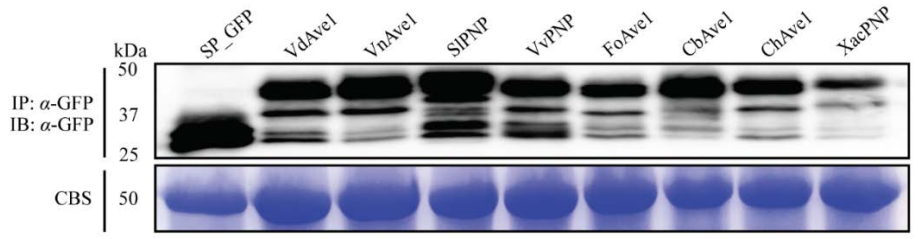

Figure S1. Stability of N-terminally GFP-tagged of mature VdAve1 homologs proteins *in planta*. Total protein extracts of transiently transformed leaf tissue were subjected to immunoprecipitation(IP) using *α*-GFP affinity beads. Immunoprecipitation proteins were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS/PAGE) and immunoblotted (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band. GFP-tagged VdAve1 signal peptide was used as a control.

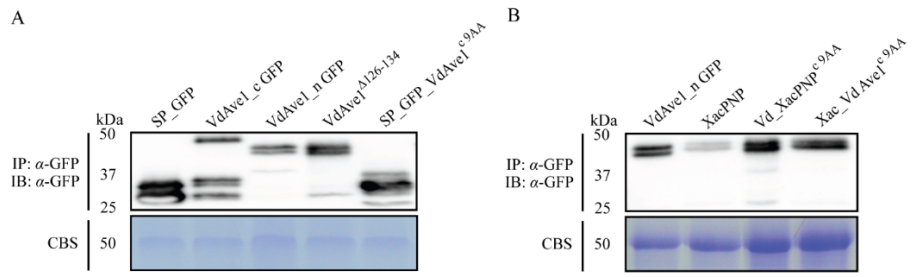

Figure S2. Presence of GFP-tagged VdAve1 chimeras proteins *in planta*. (A) VdAve1_c GFP, VdAve1_n GFP, SP_GFP_VdAve1$^{c\ 9AA}$ and N-terminally GFP-tagged VdAve1$^{\Delta 126\text{-}134}$ proteins are detected *in planta* by immunoprecipitation (IP) using *α*-GFP affinity beads, followed by immunoblotting (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band. GFP-tagged VdAve1 signal peptide was used as a control. **(B)** N-terminally GFP-tagged XacPNP, Vd_XacPNP$^{c\ 9AA}$ and XacP_VdAve1$^{c\ 9AA}$ proteins are detected *in planta* by immunoprecipitation (IP) using *α*-GFP affinity beads, followed by immunoblotting (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band. GFP-tagged VdAve1 signal peptide and VdAve1_n GFP were used as controls.

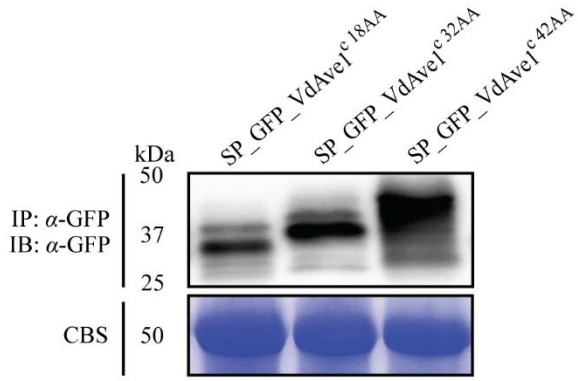

Figure S3. Stability of N-terminally GFP-tagged VdAve1 C-termini proteins *in planta*. SP_GFP_VdAve1$^{c\ 18AA}$, SP_GFP_VdAve1$^{c\ 32AA}$ and SP_GFP_VdAve1$^{c\ 42AA}$ proteins are detected *in planta* by immunoprecipitation (IP) using *α*-GFP affinity beads, followed by immunoblotting (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band.

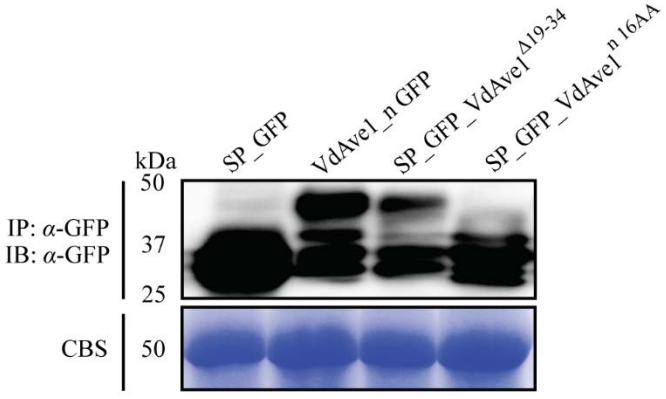

Figure S4. Presence of GFP-tagged VdAve1 N-terminal truncation proteins *in planta*. SP_GFP_VdAve1$^{\Delta 18\text{-}34}$ and SP_GFP_VdAve1$^{n\ 16AA}$ proteins are detected *in planta* by immunoprecipitation (IP) using *α*-GFP affinity beads, followed by immunoblotting (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band. GFP-tagged VdAve1 signal peptide and VdAve1_n GFP were used as controls.

Table S2. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
VdAve1-F (attB1)	ggggacaagtttgtacaaaaaagcaggctATGAAGCTTTCTACGCTT	VdAve1 and VdAve1 chimeras
VdAve1-R (attB2)	ggggaccactttgtacaagaaagctgggtTTATATCTGTCTAAATTC	VdAve1 and VdAve1 chimeras
VnAve1-F (attB1)	ggggacaagtttgtacaaaaaagcaggctATGAAGCTTTCTACGCTT	VnAve1
VnAve1-R (attB2)	ggggaccactttgtacaagaaagctgggtTTATATCTGTTCAAACTC	VnAve1
SlPNP-R (attB2)	ggggaccactttgtacaagaaagctgggtTCAAATCTGGACATATTC	SlPNP
VvPNP-R (attB2)	ggggaccactttgtacaagaaagctgggtTCAAATCTGTATGTACTC	VvPNP
FoAve1-R (attB2)	ggggaccactttgtacaagaaagctgggtTCATCTTTGTACAAAATCG	FoAve1
CbAve1-R (attB2)	ggggaccactttgtacaagaaagctgggtCTATATCTGCATATACTC	CbAve1
ChAve1-R (attB2)	ggggaccactttgtacaagaaagctgggtTCAAATTTGTACGTACTC	ChAve1
XacPNP-R (attB2)	ggggaccactttgtacaagaaagctgggtTTAAATATTTGCCCAGGG	XacPNP
VdAve1(ΔSC)-R (attB2)	ggggaccactttgtacaagaaagctgggtcTATCTGTCTAAATTC	VdAve1 (C-terminal tag)
VdAve1$^{\Delta 93\text{-}134}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTACTTATGCCTCGTTCCCTT	VdAve1$^{\Delta 93\text{-}134}$
VdAve1$^{\Delta 103\text{-}134}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTAAACAATGGCATCATATGAGT	VdAve1$^{\Delta 103\text{-}134}$
VdAve1$^{\Delta 117\text{-}134}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTACTTGTGTGCTGCTTTGGTAA	VdAve1$^{\Delta 117\text{-}134}$
VdAve1$^{\Delta 126\text{-}134}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTAAGCTCTGTCAACCACCCGCA	VdAve1$^{\Delta 126\text{-}134}$
GFP-VdAve1$^{c\ 9AA}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTATATCTGTCTAAATTCGATGTT GACCGCTTTGTATAGTTCATCCATGC	SP_GFP_VdAve1$^{c\ 9AA}$
Avr9-VdAve1$^{c\ 9AA}$-R	TTATATCTGTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCT TCCACTGATTATGTACACATTGGAGCTTA	SP_Avr9_VdAve1$^{c\ 9AA}$
GFP-VnAve1-F	GCATGGATGAACTATACAAACAATTAGGGACCGCATCC	VnAve1_n GFP
GFP-VnAve1-R	GGATGCGGTCCCTAATTGTTTGTATAGTTCATCCATGC	VnAve1_n GFP
GFP-SlPNP-F	GCATGGATGAACTATACAAAGATATTGGCACGGCTAC	SlPNP_n GFP
GFP-SlPNP-R	GTAGCCGTGCCAATATCTTTGTATAGTTCATCCATGC	SlPNP_n GFP
GFP-VvPNP-F	GCATGGATGAACTATACAAAGACATTGGCACTGCAAAC	VvPNP_n GFP
GFP-VvPNP-R	GTTTGCAGTGCCAATGTCTTTGTATAGTTCATCCATGC	VvPNP_n GFP
GFP-FoAve1-F	GCATGGATGAACTATACAAAGATATCGGAACTGCAAATATTC	FoAve1_n GFP
GFP-FoAve1-R	GAATATTTGCAGTTCCGATATCTTTGTATAGTTCATCCATGC	FoAve1_n GFP
GFP-CbAve1-F	GCATGGATGAACTATACAAAGACATCGGCACCGCCGTC	CbAve1_n GFP
GFP-CbAve1-R	GACGGCGGTGCCGATGTCTTTGTATAGTTCATCCATGC	CbAve1_n GFP
GFP-ChAve1-F	GCATGGATGAACTATACAAAGATATCGGAACAGCAGGC	ChAve1_n GFP
GFP-ChAve1-R	GCCTGCTGTTCCGATATCTTTGTATAGTTCATCCATGC	ChAve1_n GFP
GFP-XacPNP-F	GCATGGATGAACTATACAAAGACATCGGTACAATTAG	XacPNP_n GFP
GFP-XacPNP-R	CTAATTGTACCGATGTCTTTGTATAGTTCATCCATGC	XacPNP_n GFP
GFP-Vd_XacPNP$^{c\ 9AA}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTAAATATTTGCCCAGGGCGCTGT TCTTGCCTTATGCCTCGTTCCCTT	Vd_XacPNP$^{c\ 9AA}$_ n GFP
GFP-VdAve1$^{c\ 18AA}$-R	TTATATCTGTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCT TCCACTGATTTTTGTATAGTTCATCCATGC	SP_GFP_VdAve1$^{c\ 18AA}$
Avr9_VdAve1$^{c\ 18AA}$-R	TTATATCTGTCTAAATTCGATGTTGACCGCCTTATGCCTCGTTCCCT TCCACTGATTATGTACACATTGGAGCTTA	SP_Avr9_VdAve1$^{c\ 18AA}$

Table S2 (continued)

Primer name	Oligonucleotide sequence (5′→3′)	Description[a]
VdAve1$^{c\ 32AA}$-F	ATGAAGCTTTCTACGCTTGGAGCCCTCATTTCATTGACTTCACTGG TCACTGCCGCGACCATGATCTTATCT	SP_ VdAve1$^{c\ 32AA}$
GFP-VdAve1$^{c\ 32AA}$-F	GCATGGATGAACTATACAAAGCGACCATGATCTTATCT	SP_GFP_VdAve1$^{c\ 32AA}$
GFP-VdAve1$^{c\ 32AA}$-R	AGATAAGATCATGGTCGCTTTGTATAGTTCATCCATGC	SP_GFP_VdAve1$^{c\ 32AA}$
Avr9-VdAve1$^{c\ 32AA}$-F	TAAGCTCCAATGTGTACATGCGACCATGATCTTATCT	SP_Avr9_VdAve1$^{c\ 32AA}$
Avr9-VdAve1$^{c\ 32AA}$-R	AGATAAGATCATGGTCGCATGTACACATTGGAGCTTA	SP_Avr9_VdAve1$^{c\ 32AA}$
VdAve1$^{c\ 42AA}$-F	ATGAAGCTTTCTACGCTTGGAGCCCTCATTTCATTGACTTCACTGG TCACTGCCAAGACAACTGTTACCAAAGC	SP_ VdAve1$^{c\ 42AA}$
GFP-VdAve1$^{c\ 42AA}$-F	GCATGGATGAACTATACAAAAAGACAACTGTTACCAAAGC	SP_GFP_VdAve1$^{c\ 42AA}$
GFP-VdAve1$^{c\ 42AA}$-R	GCTTTGGTAACAGTTGTCTTTTTGTATAGTTCATCCATGC	SP_GFP_VdAve1$^{c\ 42AA}$
Avr9-VdAve1$^{c\ 42AA}$-F	TAAGCTCCAATGTGTACATAAGACAACTGTTACCAAAGC	SP_Avr9_VdAve1$^{c\ 42AA}$
Avr9-VdAve1$^{c\ 42AA}$-R	GCTTTGGTAACAGTTGTCTTATGTACACATTGGAGCTTA	SP_Avr9_VdAve1$^{c\ 42AA}$
GFP-VdAve1$^{\Delta 19\text{-}34}$-F	GCATGGATGAACTATACAAATGCGGCGGCAGCAATCCC	SP_GFP_VdAve1$^{\Delta 19\text{-}34}$
GFP-VdAve1$^{\Delta 19\text{-}34}$-R	GGGATTGCTGCCGCCGCATTTGTATAGTTCATCCATGC	SP_GFP_VdAve1$^{\Delta 19\text{-}34}$
Avr9-VdAve1$^{\Delta 19\text{-}34}$-F	TAAGCTCCAATGTGTACATTGCGGCGGCAGCAATCCC	SP_Avr9_VdAve1$^{\Delta 19\text{-}34}$
Avr9-VdAve1$^{\Delta 19\text{-}34}$-R	GGGATTGCTGCCGCCGCAATGTACACATTGGAGCTTA	SP_Avr9_VdAve1$^{\Delta 19\text{-}34}$
VdAve1$^{n\ 16AA}$-R (attB2)	ggggaccactttgtacaagaaagctgggtTTAGGCAGTGGGAAGGTA	SP_ VdAve1$^{n\ 16AA}$
GFP-VdAve1$^{n\ 16AA}$-R	GGCAGTGGGAAGGTAGGGTGGGTTATAGTAGGATGCGGTCCCTAG	SP_GFP_VdAve1$^{n\ 16AA}$
Avr9-VdAve1$^{n\ 16AA}$-R	GGCAGTGGGAAGGTAGGGTGGGTTATAGTAGGATGCGGTCCCTAGA TCATGTACACATTGGAGCTTA	SP_Avr9_VdAve1^{n16AA}
VdAve1-F (*Pac*I)	TTAATTAAAATGAAGCTTTCTACGCTTGGA	For VdAve1, VdAve1$^{\Delta 19\text{-}34}$ complementation strains
VdAve1-R (*Not*I)	GCGGCCGCTCATATCTGTCTAAATTCGATGTTGA	For VdAve1, VdAve1$^{\Delta 19\text{-}34}$ complementation strains
VdAve1$^{\Delta 126\text{-}134}$-R (*Not*I)	GCGGCCGCTCACTTATGCCTCGTTCCCTTCCAC	For VdAve1$^{\Delta 126\text{-}134}$ complementation strains

[a]The type of experiment for which the primers were used.

Methods S1. Generation of binary expression vectors

Constructs for the constitutive expression of *VdAve1*, *SlPNP*, *VvPNP*, *FoAve1*, *CbAve1*, *ChAve1* and *XacPNP* have been described previously (de Jonge et al., 2012; Song et al., 2016). *VnAve1* was amplified from *V. nubilum* cDNA by using the primers VnAve1-F (attB1) and VnAve1-R (attB2) listed in Table S2, and subsequently cloned into the entry vector pDONR 207 by using the Gateway® BP Clonase® II Enzyme Mix (Invitrogen, California, USA), and recombined into the Gateway-compatible destination vector pSol2092 (Zhang et al., 2013) to generate expression construct pSol2092::VnAve1.

Constructs encoding C-terminally tagged VdAve1 versions. To generate VdAve1 fused at the C-terminus to the green fluorescent protein (GFP), the VdAve1 coding sequence without stop codon was amplified by using primers VdAve1-F(attB1) and VdAve1(ΔSC)-R(attB2) listed in Table S2. The PCR-amplified fragment was cloned into pDONR207 by using the Gateway® BP Clonase® II Enzyme Mix (Invitrogen, California, USA) to generate entry vector pDONR207::VdAve1 (ΔSC) verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany). Subsequently, pDONR207::VdAve1 (ΔSC) was transferred into the Gateway-compatible destination vector pSol2095 (C-terminal GFP tag) (Zhang et al., 2013) by using Gateway® LR Clonase® II Enzyme Mix (Invitrogen, California, USA) to generate C-terminally GFP-tagged VdAve1 (VdAve1_c GFP; Figure 2A). Similarly, pDONR207::VdAve1 (ΔSC) was recombined into the Gateway-compatible destination vectors pGWB14, pGWB8, pGWB17, pGWB20 and pGBW11 (Nakagawa et al., 2007) to generate C-terminally affinity-tagged VdAve1 versions VdAve1_c 3xHA, VdAve1_c 6xHIS, VdAve1_4xMyc, VdAve1_c 10xMyc, VdAve1_c FLAG (Figure 2A), respectively.

Constructs encoding N-terminally fused VdAve1 versions. To fuse different tags (GFP, HA, Myc and mature Avr9) to the N-terminus of mature VdAve1, the corresponding tag 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 mature VdAve1 protein. DNA fragments for constructs VdAve1_n GFP, VdAve1_n HA, VdAve1_n Myc, VdAve1_n Avr9, SP_GFP and SP_Avr9 (Figure 2C) were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany), and cloned into the entry vector pDONR 207 and subsequently transferred into the Gateway-compatible destination vector pSol2092 (Zhang et al., 2013).

Constructs encoding VdAve1 C-terminal deletions. To generate VdAve1 truncations VdAve1$^{\Delta 93\text{-}134}$, VdAve1$^{\Delta 103\text{-}134}$, VdAve1$^{\Delta 117\text{-}134}$, VdAve1$^{\Delta 126\text{-}134}$ and VdAve1$^{n\ 16AA}$, these DNA fragments were amplified from plasmid pSol2092::VdAve1 (Zhang et al., 2013) by using the forward primer VdAve1-F (attB1) in combination with the reverse primers VdAve1$^{\Delta 93\text{-}134}$-R (attB2), VdAve1$^{\Delta 103\text{-}134}$-R (attB2), VdAve1$^{\Delta 117\text{-}134}$-R (attB2), VdAve1$^{\Delta 126\text{-}134}$-R (attB2) and VdAve1$^{n\ 16AA}$-R (attB2) (Table S2), respectively. A fragment *SP_GFP_VdAve1*$^{c\ 9AA}$ was amplified by PCR from plasmid SP_GFP using the primers VdAve1-F (attB1) and

GFP-VdAve1$^{c\ 9AA}$-R (attB2) (Table S2). *Vd_XacPNP$^{c\ 9AA}$*, *Xac_VdAve1$^{c\ 9AA}$*, *SP_VdAve1$^{c\ 9AA}$*, *SP_VdAve1$^{c\ 18AA}$*, and *VdAve1$^{\Delta 19\text{-}34}$* were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany), and cloned into the entry vector pDONR 207 and subsequently transferred into the vector pSol2092 (Zhang et al., 2013). The AVNIEFRQI peptides were synthesized by GenScript corporation (GenScript, New Jersey, USA).

Constructs encoding N-terminally GFP-tagged VdAve1 homologs and chimeras. To generate VnAve1, SlPNP, VvPNP, FoAve1, CbAve1, ChAve1, XacPNP and Xac_VdAve1$^{c\ 9AA}$ fused GFP tag to the N-terminus, the GFP coding sequence lacking the stop codon was fused at the N-terminus to the sequence encoding the signal peptide of VdAve1, and at the C-terminus to the mature VdAve1 homologs. The *SP_GFP* fragment and DNA fragments for the mature VdAve1 homologs were separately amplified and fused by the overlapping PCR. Seven *SP_GFP* fragments were amplified from plasmid SP_GFP by using the forward primer VdAve1-F (attB1) in combination with the reverse primers GFP-VnAve1-R, GFP-SlPNP-R, GFP-VvPNP-R, GFP-FoAve1-R, GFP-CbAve1-R, GFP-ChAve1-R and GFP-XacPNP-R (Table S2). Eight fragments *VnAve1*, *SlPNP*, *VvPNP*, *FoAve1*, *CbAve1*, *ChAve1*, *XacPNP* and *Xac_VdAve1$^{c\ 9AA}$* were amplified from plasmids VnAve1, SlPNP, VvPNP, FoAve1, CbAve1, ChAve1, XacPNP and Xac_VdAve1$^{c\ 9AA}$ by using the forward primers GFP-VnAve1-R, GFP-SlPNP-R, GFP-VvPNP-R, GFP-FoAve1-R, GFP-CbAve1-R, GFP-ChAve1-R, GFP-XacPNP-R, GFP-XacPNP-R in combination with the corresponding reverse primers VnAve1-R (attB2), SlPNP-R (attB2), VvPNP-R(attB2), FoAve1-R (attB2), CbAve1-R (attB2), ChAve1-R (attB2) and XacPNP-R (attB2), VdAve1-R (attB2) (Table S2). Subsequently, eight desired DNA fragments were obtained by overlapping PCR by using the forward primer VdAve1-F (attB1) in combination with the corresponding reverse primers VnAve1-R (attB2), SlPNP-R (attB2), VvPNP-R (attB2), FoAve1-R (attB2), CbAve1-R (attB2), ChAve1-R (attB2), XacPNP-R (attB2), and XacPNP-R (attB2) (Table S2), and cloned into the entry vector pDONR 207 and subsequently recombined into the vector pSol2092 (Zhang et al., 2013). To generate VdAve1$^{\Delta 126\text{-}134}$, Vd_XacPNP$^{c\ 9AA}$ fused GFP tag to the N-terminus, the GFP coding sequence lacking the stop codon was fused at the N-terminus to the sequence encoding the signal peptide of VdAve1, and at the C-terminus to the VdAve1 chimeras. Two desired DNA fragments were amplified from plasmid VdAve1_n GFP by using the forward primer VdAve1-F (attB1) in combination with the reverse primers VdAve1$^{\Delta 126\text{-}134}$-R (attB2) and GFP-Vd_XacPNP$^{c\ 9AA}$-R (attB2) (Table S2), and cloned into the vector pDONR 207 and subsequently transferred into the vector pSol2092 (Zhang et al., 2013).

Constructs encoding C- or N-terminus. To generate constructs SP_VdAve1$^{c\ 32AA}$, SP_VdAve1$^{c\ 42AA}$, DNA fragments were amplified by the 1st round PCR using plasmid VdAve1 as templates and the reverse primer VdAve1-R (attB2) in combination with the forward primers VdAve1$^{c\ 32AA}$-F, VdAve1$^{c\ 42AA}$-F (Table

S2), and followed by the 2nd round PCR using primers VdAve1-F (attB1) and VdAve1-R (attB2) (Table S2). The resulting PCR fragments were cloned into the entry vector pDONR 207, sequenced and subsequently recombined into the vector pSol2092 (Zhang et al., 2013).

Constructs encoding N-terminally GFP or Avr9-fused C- or N-terminus. To generate SP_GFP_VdAve1$^{c\ 18AA}$, SP_GFP_VdAve1$^{n\ 16AA}$, DNA fragments were amplified by the 1st round PCR using plasmid SP_GFP as templates and the forward primer VdAve1-F (attB1) in combination with the forward primers GFP-VdAve1$^{c\ 18AA}$-R and GFP-VdAve1$^{n\ 16AA}$-R (Table S2), respectively, and followed by the 2nd round PCR using primers VdAve1-F (attB1) and VdAve1-R (attB2) (Table S2). To generate constructs SP_Avr9_VdAve1$^{c\ 9AA}$, SP_Avr9_VdAve1$^{c\ 18AA}$, SP_Avr9_VdAve1$^{n\ 16AA}$, DNA fragments were amplified by the 1st round PCR using plasmid SP_Avr9 as template and the forward primer VdAve1-F (attB1) in combination with the forward primers Avr9-VdAve1$^{c\ 9AA}$-R, Avr9-VdAve1$^{c\ 18AA}$-R and Avr9-VdAve1$^{n\ 16AA}$-R (Table S2), respectively, and followed by the 2nd round PCR using primers VdAve1-F (attB1) and VdAve1-R (attB2) (Table S2). The resulting PCR fragments were cloned into the vector pDONR 207, sequenced and subsequently transferred into the vector pSol2092 (Zhang et al., 2013). To construct SP_GFP_VdAve1$^{c\ 32AA}$, SP_GFP_VdAve1$^{c\ 42AA}$, SP_GFP_VdAve1$^{\Delta 19\text{-}34}$, SP_Avr9_VdAve1$^{c\ 32AA}$, SP_Avr9_VdAve1$^{c\ 42AA}$ and SP_Avr9_VdAve1$^{\Delta 19\text{-}34}$, DNA fragments *SP_VdAve1$^{c\ 32AA}$*, *SP_VdAve1$^{c\ 42AA}$*, *VdAve1$^{\Delta 19\text{-}34}$*, *SP_GFP* and *SP_Avr9* were separately amplified and fused by the overlapping PCR. Fragments *SP_GFP* and *SP_Avr9* were amplified from plasmids SP_GFP and SP_Avr9 by using the forward primer VdAve1-F (attB1) in combination with the reverse primers GFP-VdAve1$^{c\ 32AA}$-R, GFP-VdAve1$^{c\ 42AA}$-R, GFP-VdAve1$^{\Delta 19\text{-}34}$-R, Avr9-VdAve1$^{c\ 32AA}$-R, Avr9-VdAve1$^{c\ 42AA}$-R and Avr9-VdAve1$^{\Delta 19\text{-}34}$-R (Table S2). Fragments *SP_VdAve1$^{c\ 32AA}$*, *SP_VdAve1$^{c\ 42AA}$* and *VdAve1$^{\Delta 19\text{-}34}$* were amplified from plasmid VdAve1 using the reverse primer VdAve1-R (attB2) and in combination with the forward primers GFP-VdAve1$^{c\ 32AA}$-F, GFP-VdAve1$^{c\ 42AA}$-F, GFP-VdAve1$^{\Delta 19\text{-}34}$-F, Avr9-VdAve1$^{c\ 32AA}$-F, Avr9-VdAve1$^{c\ 42AA}$-F and Avr9-VdAve1$^{\Delta 19\text{-}34}$-F (Table S2). Subsequently, six desired DNA fragments *SP_GFP_VdAve1$^{c\ 32AA}$*, *SP_GFP_VdAve1$^{c\ 42AA}$*, *SP_GFP_VdAve1$^{\Delta 19\text{-}34}$*, *SP_Avr9_VdAve1$^{c\ 32AA}$*, *SP_Avr9_VdAve1$^{c\ 42AA}$* and *SP_Avr9_VdAve1$^{\Delta 19\text{-}34}$* were obtained by overlapping PCR using the primers VdAve1-F (attB1) and VdAve1-R (attB2) (Table S2), and cloned into the entry vector pDONR 207 and subsequently recombined into the vector pSol2092 (Zhang et al., 2013).

All the destination vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

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Chapter 4

Broad taxonomic characterization of Verticillium wilt resistance genes reveals ancient origin of the tomato Ve1 immune receptor

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ABSTRACT

Plant pathogenic microbes secrete effector molecules to establish themselves on their hosts, whereas plants use immune receptors to try and intercept such effectors in order to prevent pathogen colonization. The tomato cell surface-localized receptor Ve1 confers race-specific resistance against race 1 strains of the soil-borne vascular wilt fungus *Verticillium dahliae* that secrete the Ave1 effector. Here, we describe the cloning and characterization of *Ve1* homologues from tobacco (*Nicotiana glutinosa*), potato (*Solanum tuberosum*), wild eggplant (*Solanum torvum*) and hop (*Humulus lupulus*), and demonstrate that particular Ve1 homologues govern resistance against *V. dahliae* race 1 strains through recognition of the Ave1 effector. Phylogenetic analysis shows that Ve1 homologs are widely distributed in land plants. Thus, our study suggests an ancient origin of the Ve1 immune receptor in the plant kingdom.

INTRODUCTION

In order to activate immune responses that ward off invading microorganisms, plants employ immune receptors that detect pathogen(-induced) ligands of various nature (Boller and Felix, 2009; Thomma et al., 2011). The recognition of such ligands by immune receptors results in the activation of defense responses, which are often accompanied by a hypersensitive response (HR) in which necrosis of plant tissue surrounding the site of attempted penetration is sacrificed to restrict further pathogen invasion.

Verticillium wilts are vascular wilt diseases that are caused by soil-borne fungal pathogens that belong to the *Verticillium* genus. *Verticillium dahliae* is the most notorious species that can infect hundreds of dicotyledonous hosts (Fradin and Thomma, 2006; Inderbitzin et al., 2011). In tomato (*Solanum lycopersicum*), the *Ve* locus that confers race-specific resistance against *Verticillium* has been characterized (Fradin et al., 2009; Kawchuk et al., 2001). This locus contains two closely linked and inversely oriented genes, *Ve1* and *Ve2*, which encode extracellular leucine-rich repeat receptor-like proteins (eLRR-RLPs) (Kawchuk et al., 2001; Wang et al., 2008, 2010). Of these, only *Ve1* was found to provide *V. dahliae* resistance in tomato (Fradin et al., 2009). Interestingly, interfamily transfer of *Ve1* from tomato to Arabidopsis resulted in *Verticillium* resistance in the latter species (Fradin et al., 2011, 2014; Zhang et al., 2014), implying that the underlying immune signaling pathway is conserved (Fradin et al., 2011; Thomma et al., 2011).

Comparative genomics of *V. dahliae* race 1 and race 2 strains identified the Ave1 effector that activates Ve1-mediated immunity (de Jonge et al., 2012). Interestingly, Ave1 homologs were found in the bacterial plant pathogen *Xanthomonas axonopodis* pv. *citri* (XacPNP) and in the plant pathogenic fungi *Colletotrichum higginsianum* (ChAve1), *Cercospora beticola* (CbAve1), and *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), and these homologs are differentially recognized by Ve1 (de Jonge et al., 2012). During optimization of an agroinfiltration assay in tobacco for functional analysis of Ve1 signaling, we found that expression of *Ave1* in leaves of *Nicotiana glutinosa* triggered an HR, suggesting that this species contains an endogenous *Ve1* allele (Zhang et al., 2013a). Indeed, inoculation experiments revealed that *N. glutinosa* is resistant to race 1 *V. dahliae*, while an

Ave1 deletion strain was able to cause Verticillium wilt disease on these plants (Zhang et al., 2013a).

So far, several *Ve1* homologs were identified within and outside the Solanaceae family, such as *SlVe1* from *Solanum lycopersicoides* (Chai et al., 2003), *StVe1* from *S. tuberosum* (Simko et al., 2004a), *StVe* and *StoVe1* from *S. torvum* (Fei et al., 2004; Liu et al., 2012), *mVe1* from *Mentha longifolia* (Vining and Davis, 2009), *Vr1* from *Lactuca sativa* (Hayes et al., 2011), *VvVe* from *Vitis vinifera* (Tang et al., 2016) and *GbVe*, *Gbve1*, *Gbvdr5* and *Gbvdr3* from *Gossypium barbadense* (Chen et al., 2016; Yang et al., 2014; Zhang et al., 2011; Zhang et al., 2012). However, functionality of these homologs against Verticillium wilt often remains obscure. Here, we describe the cloning and functional characterization of Verticillium wilt resistance genes from tobacco (*N. glutinosa*), potato (*S. tuberosum*), wild eggplant (*S. torvum*) and hop (*Humulus lupulus*), and demonstrate that particular Ve1 homologs govern resistance against *V. dahliae* race 1 strain through the recognition of the Ave1 effector.

RESULTS

Isolation of *NgVe1* from *Nicotiana glutinosa*

In our first attempt to clone the previously identified *Ve1* homolog from *N. glutinosa* (Zhang et al., 2013a), a single cDNA fragment of ~2800 bp was obtained using primers that were designed on the tomato *Ve1* sequence (Table S1). To obtain the full-length *N. glutinosa Ve1* (*NgVe1*) transcript sequence, 3' rapid amplification of cDNA end (RACE) polymerase chain reaction (PCR) was performed, resulting in a single fragment of approximately 1200 bp. Likewise, a fragment of approximately 640 bp was amplified with 5' RACE (Methods S1). The sequences of the three fragments were aligned to deduce the full-length *NgVe1* cDNA sequence. Subsequently, a pair of *NgVe1*-specific primers (NgVe1-F and NgVe1-R; Table S1) was designed and amplicons amplified from *N. glutinosa* cDNA and genomic DNA were sequenced, indicating that both amplicons are identical (GenBank accession: KT895339) and that *NgVe1* is an intronless gene.

The full-length cDNA of *NgVe1* is 3225 bp, and contains a predicted translation initiation site (ATG) at nucleotide position 34 and a stop codon (TGA) at nucleotide position 3178, resulting in a single open reading frame of 3147 bp. The predicted

NgVe1 protein comprises 1048 amino acids (GenBank accession: ALK26499), and shares an overall identity of 76% with tomato Ve1 (Figure S1). Immunoblotting analysis using GFP antibody displayed clear signals for NgVe1-GFP and Ve1-GFP in transiently transformed tobacco leaves (Figure S2).

Co-expression of *Ave1* and *NgVe1* induces an HR in *N. tabacum*

Recently, an optimized agroinfiltration assay has been developed for Ve1-mediated immune signaling in *N. tabacum*, revealing a swift HR upon co-expression of tomato *Ve1* with *V. dahliae Ave1* (Zhang et al., 2013a). To test functionality of *NgVe1*, co-expression with *Ave1* upon agroinfiltration in *N. tabacum* was performed. At 5 days post infiltration, the infiltrated leaves developed clear necrosis, and the HR induced upon co-expression of *NgVe1* and *Ave1* was as strong as HR induced upon co-infiltration of tomato *Ve1* and *Ave1*, for which the complete infiltrated areas became fully necrotic (Figure 1A). In contrast, agroinfiltration of *NgVe1* or *Ave1* alone did not induce necrosis (Figure 1A). These data strongly suggest that *NgVe1* is a functional homolog of tomato *Ve1*.

Targeting of *NgVe1* expression in *N. glutinosa* compromises Ave1-induced HR, but not *Verticillium* resistance

To investigate the role of *NgVe1* in *N. glutinosa Verticillium* resistance, we used virus-induced gene silencing (VIGS). *Tobacco rattle virus* (TRV)-based VIGS is a well-established method for gene functional analysis in several Solanaceae species, also for investigation of Ve1-mediated *Verticillium* resistance (Fradin et al., 2009; Senthil-Kumar et al., 2007; Zhang et al., 2013a). In an attempt to establish VIGS in *N. glutinosa*, a 1:1 mixture of *Agrobacterium tumefaciens* cultures carrying *pTRV1* and *pTRV2::PDS* to target the *phytoene desaturase* (*PDS*) gene was infiltrated into cotyledons of *N. glutinosa* plants. Visible photobleaching symptoms were observed in all agroinfiltrated *N. glutinosa* plants by 4 weeks post infiltration (Figure S3A), albeit that a strongly varying degree of photobleaching was observed (Figure S3A). Nevertheless, a recombinant TRV vector was designed to target *NgVe1* expression (*pTRV2::NgVe1*). As a negative control, a construct (*pTRV2::GUS*) containing a fragment of the *β-glucuronidase* (*GUS*) gene was used. At 4 weeks after TRV infection, mature leaves were agroinfiltrated to express *Ave1*, with *VdNLP1* as a

positive control (Santhanam et al., 2013) and the functionally and structurally unrelated effector *Avr9* from the tomato leaf mould pathogen *Cladosporium fulvum* as a negative control (van Kan et al., 1991; Van der Hoorn et al., 2000). Agroinfiltration of *Ave1* in *N. glutinosa* upon *GUS* targeting resulted in a clear HR within 5 days, confirming that TRV infection did not compromise Ve1-mediated HR (Figure 1B). However, targeting of *NgVe1* expression in *N. glutinosa* significantly compromised HR upon expression of *Ave1* (Figure 1B). As expected, VdNLP1-mediated cell death was not compromised upon targeting of *NgVe1* expression whereas Avr9 expression never triggered HR (Figure 1B).

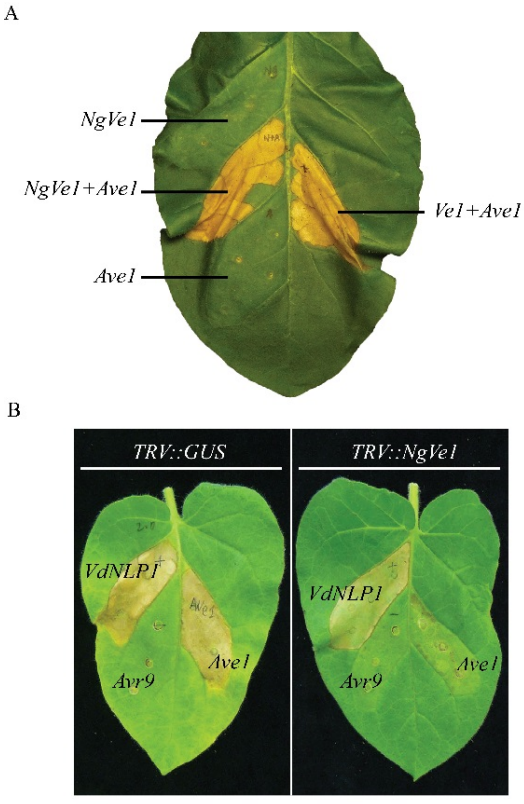

Figure 1. Expression of *NgVe1* in tobacco mediates the Ave1-triggered hypersensitive response (HR). (A) Co-expression of *NgVe1* and *Ave1* in tobacco results in an HR. Pictures were taken at 5 days post infiltration, and show representative leaves of at least three independent assays. As a positive control, HR was induced upon co-infiltration of *Ve1* and *Ave1*. As a negative control, *NgVe1* and *Ave1* were expressed separately. **(B)** Ave1-triggered HR, but not the VdNLP1-mediated cell death is attenuated in *NgVe1*-silenced *Nicotiana glutinosa* plants, while Avr9 does not trigger cell death.

To test the role of *NgVe1* in *Verticillium* resistance, 3 weeks after TRV inoculated plants were challenged either with *V. dahliae* race 1 strain JR2 (Faino et al., 2015), or a transformant from which the *Ave1* gene had been deleted (*V. dahliae* JR2⊿*Ave1*; de Jonge et al., 2012), and monitored for disease development (stunting, wilting, chlorosis and necrosis) up to 14 days post inoculation (dpi). As expected, no disease symptoms were observed in *N. glutinosa* plants upon *GUS* targeting and subsequent mock-inoculation or upon inoculation with the *V. dahliae* JR2, whereas the *Ave1* deletion strain caused clear Verticillium wilt disease (Figure. S3B). However, unexpectedly, in repeated assays also no Verticillium wilt symptoms were observed upon *NgVe1* targeting and subsequent inoculation with *V. dahliae* (Figure S3B). However, in line with the extremely variable photobleaching (Figure S3A), assessment of the silencing efficiency revealed only a slight reduction in *NgVe1* expression in *NgVe1*-targeted *N. glutinosa* plants when compared with *GUS*-silenced plants (Figure S3C), and attempts to increase the silencing efficiency were unsuccessful. These results confirm previous observations that *N. glutinosa* is not very amenable to TRV-based VIGS (Senthil-Kumar et al., 2007; Zhang et al., 2013a), and, furthermore, suggest that the moderate silencing efficiency that we obtained in our experiments is sufficiently high to compromise the NgVe1-mediated HR, but insufficient to compromise NgVe1-mediated resistance.

Expression of *NgVe1* in Arabidopsis confers *Verticillium* resistance

As TRV-based VIGS appeared not to be very suitable for gene functional analysis in *N. glutinosa*, we pursued other strategies to functionally characterize *NgVe1*. We have previously shown that interfamily transfer of tomato *Ve1* to Arabidopsis resulted in resistance against race 1 *Verticillium* strains, providing a relatively fast method to assess Ve1 functionality (Fradin et al., 2011; 2014; Zhang et al., 2014). To further confirm functionality of *NgVe1* in *Verticillium* resistance, heterologous expression of *NgVe1* in Arabidopsis was obtained (Figure S4). No obvious developmental alterations were observed in transgenic plants when compared with wild-type plants (Figure 2A) and, subsequently three independent *NgVe1*-transgenic lines (*NgVe1-1*, *NgVe1-2* and *NgVe1-3*) as well as transgenic plants expressing tomato *Ve1* (Fradin et al., 2011) and non-transgenic controls were inoculated with *V. dahliae* JR2. Interestingly, like tomato *Ve1*-expressing plants,

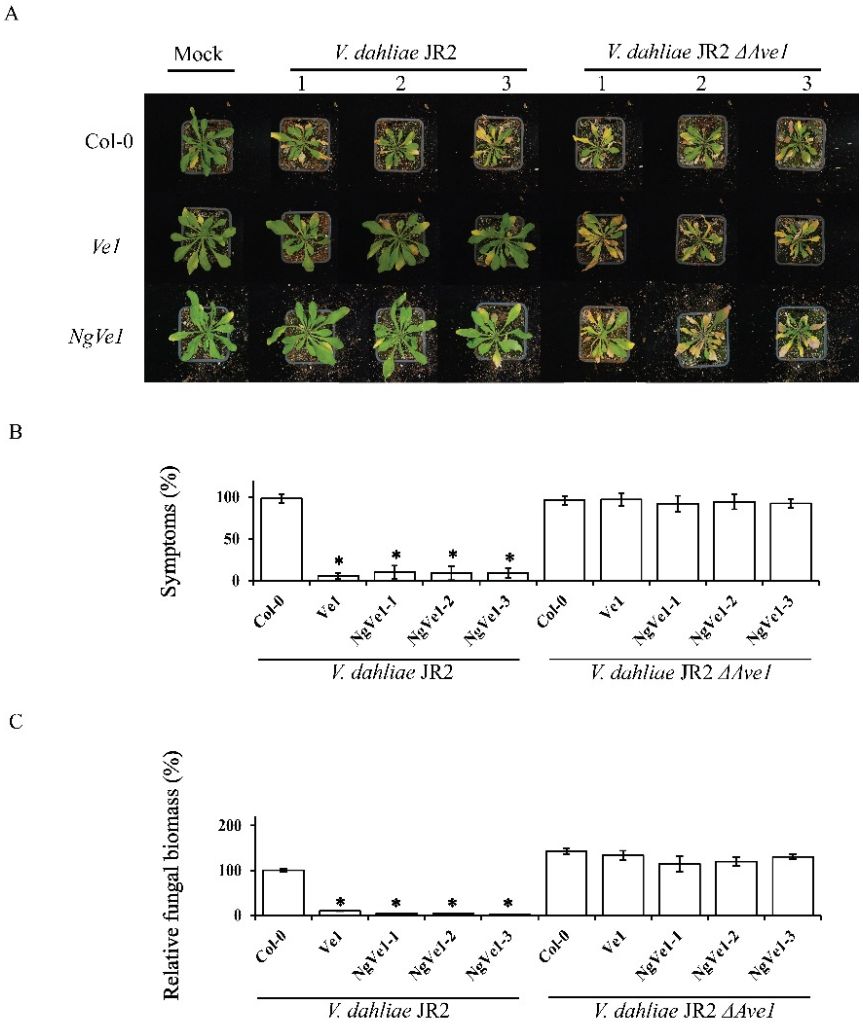

Figure 2. Expression of *NgVe1* in Arabidopsis mediates resistance against race 1 *Verticillium dahliae*. (A) Typical appearance of non-transgenic Arabidopsis and transgenic lines that constitutively express *NgVe1* upon mock-inoculation or inoculation with *V. dahliae* strain JR2 or *V. dahliae* JR2*ΔAve1* at 21 days post inoculation (dpi). **(B)** Quantification of Verticillium wilt symptoms in Arabidopsis Col-0 and transgenic plants at 21 dpi. Bars represent quantification symptom development as percentage of diseased rosette leaves with standard deviation, with Col-0 (control) set to 100%. **(C)** Fungal biomass determined by quantitative PCR (qPCR) in Arabidopsis Col-0 and transgenic plants at 21 dpi. Bars represent *Verticillium* internal transcribed spacer (*ITS*) transcript levels relative to *AtRuBisCo* (RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase) transcript levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in Col-0 (control) is set to 100%. Three independent lines are shown (1, 2 and 3). Asterisks indicate significant differences when compared with Col-0 ($P < 0.05$). *Ve1* transgenic plants were used as a positive control. The data shown are representative of at least three independent experiments.

NgVe1-transgenic plants were clearly resistant to race 1 *V. dahliae* as significantly less Verticillium wilt symptoms were observed when compared with non-transgenic control plants (Figure 2A, B). In contrast, *NgVe1* and *Ve1* transgenic plants were as diseased as non-transgenic controls upon inoculation with the *Ave1* deletion strain (Figure 2A, B). These data are further supported by fungal biomass quantifications that revealed significantly reduced fungal accumulation in *NgVe1*-transgenic and *Ve1*-expressing Arabidopsis plants for *V. dahliae* carrying *Ave1*, but not for the *Ave1* deletion mutant, when compared with wild-type Arabidopsis plants (Figure 2C). Collectively, these data confirm that NgVe1 acts as a functional homologue of tomato Ve1 that recognizes race 1 *V. dahliae*.

Cloning and functional analysis of *Ve1* homologues from potato and wild eggplant

Ve gene homologues occur in the solanaceous species *S. lycopersicoides* (*SlVe1*; Chai et al., 2013) and the wild eggplant species *S. torvum* (*StVe* and *StoVe1*; Fei et al., 2004; Liu et al., 2012). Moreover, in tetraploid potato (*S. tuberosum*), a quantitative trait locus (QTL) for *Verticillium* resistance was identified using the tomato *Ve1* gene as a probe. This locus was found to contain at least 11 genes, all putatively encoding LRR-type receptor-like proteins (Simko et al., 2004a). The tomato and potato genomes are highly collinear and the QTL locus was mapped to a region on potato chromosome 9 that is syntenic to the short arm of the tomato chromosome 9 that carries *Ve1* and *Ve2* (Diwan et al., 1999; Simko et al., 2004b). Subsequently, this *Verticillium* resistance QTL locus was annotated and found to contain two predicted *receptor-like protein 12-like* genes [National Center for Biotechnology Information (NCBI): XM_006362308 and XM_006362309] in the genome sequence of *S. tuberosum* group Phureja DM 1-3 516 R44 (Xu et al., 2011). Here, the coding sequences (CDSs) of *Ve* gene homologues were amplified from cDNA of the heterozygous diploid potato breeding line *S. tuberosum* group Tuberosum RH 89-039-16 (Xu et al., 2011), sequenced and submitted to NCBI as *StuVe1* and *StuVe2* (GenBank accessions: KT946795 and KT946797) (Methods S1). The predicted StuVe1 and StuVe2 proteins are composed of 1053 and 1138 amino acids (GenBank accessions: ALK26501 and ALK26503), respectively, and share 87% and 84%

identity with tomato Ve1 and 81% and 91% identity with tomato Ve2, respectively, and 82% identity with each other (Figure S1).

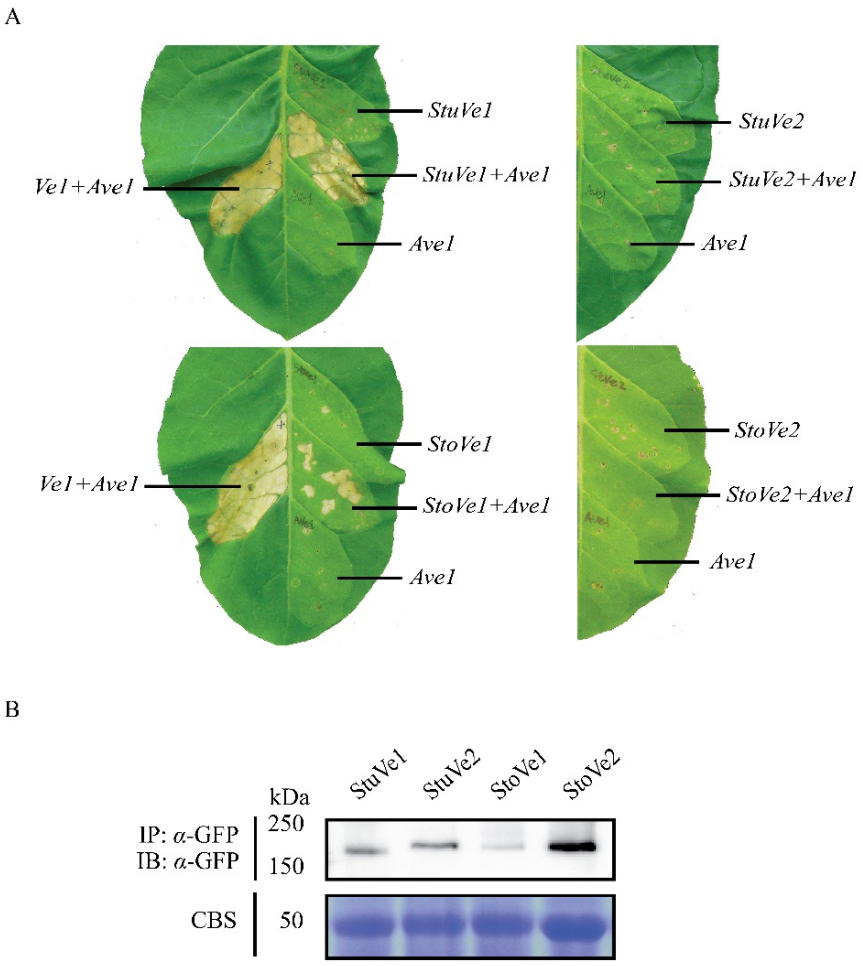

Figure 3. StuVe1 and StoVe1, but not StuVe2 and StoVe2, recognize Ave1 in *Nicotiana tabacum.* (A) Co-expression of *StuVe1* and *StoVe1*, but not *StuVe2* and *StoVe2*, with *Ave1* in tobacco induces signs of a relatively weak hypersensitive response (HR). Pictures were taken at 5 days post infiltration, and show representative leaves of at least three independent assays. As a positive control, HR was induced upon co-infiltration of *Ve1* and *Ave1*. As a negative control, *StuVe1*, *StuVe2*, *StoVe1*, *StoVe2*, and *Ave1* were expressed separately. **(B)** Green fluorescent protein (GFP)-tagged StuVe1, StuVe2, StoVe1, and StoVe2 proteins are present *in planta* by immunopurification (IP) using GFP-affinity beads, followed by immunoblotting (IB) using α-GFP antibody. Coomassie Blue Staining (CBS) of the blotting containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band.

To study the composition of the *Ve* locus in wild eggplant, the CDSs of *Ve* gene homologs were cloned from the cDNA of the *Verticillium*-resistant *S. torvum* genotype Tuolubamu, sequenced and deposited at NCBI as *StoVe1* and *StoVe2* (GenBank accessions: KT946794 and KT946796) (Methods S1). The predicted StoVe1 and StoVe2 proteins comprise 1051 and 1135 amino acids (GenBank accessions: ALK26500 and ALK26502), respectively, and share 83% and 80% identity with tomato Ve1 and 81% and 85% identity with tomato Ve2, respectively, and 92% identity with each other (Figure S1).

To check functionality of *StuVe1*, *StuVe2*, *StoVe1* and *StoVe2*, mature tobacco leaves were co-infiltrated with a 1:1 mixture of *A. tumefaciens* cultures carrying *Ave1* and the various *Ve1* homologues. Intriguingly, agroinfiltration in at least three independent assays revealed that expression of *Ave1* together with *StuVe1* or *StoVe1* induced signs of a weak HR at 5 days post infiltration (Figure 3A). However, when compared with the HR induced upon co-agroinfiltration of tomato *Ve1* and *Ave1*, only a minor part of the infiltrated region developed necrosis (Figure 3A). Nevertheless, agroinfiltration of *Ave1* with *StuVe2* or *StoVe2* induced no such responses at all (Figure 3A). Immunoblotting confirmed that the StuVe1, StuVe2, StoVe1, and StoVe2 fusion proteins were expressed (Figure 3B).

As VIGS-based gene silencing in potato genotype Tuberosum RH 89-039-16 and wild eggplant genotype Tuolubamu has not been established, we did not attempt VIGS-based assays to test the role of these *Ve1* homologuess in *Verticillium* resistance. Rather, heterologous expression in Arabidopsis was pursued (Figure S4). No developmental alterations were observed in transgenic plants when compared with *Ve1*-expressing and wild-type plants (Figure 4A) and three independent transgenic lines expressing *StuVe1*, *StuVe2*, *StoVe1* or *StoVe2* were assayed for *V. dahliae* resistance. Intriguingly, despite the weak HR observed upon agroinfiltration together with *Ave1* in *N. tabacum*, *StoVe1*- and *StuVe1*-expressing plants were clearly resistant to race 1 *V. dahliae* strain JR2, similar to *Ve1*-transgenic plants (Figure 4A, B). In contrast, *StuVe2*- and *StoVe2*-transgenic plants were as diseased as non-transgenic controls (Figure 4A, B). Importantly, all genotypes were equally susceptible to the *V. dahliae Ave1* deletion mutant (Figure 4A, B), suggesting that all these *Ve1* alleles recognize the Ave1 effector. The phenotypes correlated with the degree of *V. dahliae* colonization as determined by real-time PCR (Figure 4).

Collectively, these data confirm that StuVe1 and StoVe1, but not StuVe2 and StoVe2, act as functional homologues of tomato Ve1 that confer resistance to race 1 *V. dahliae*.

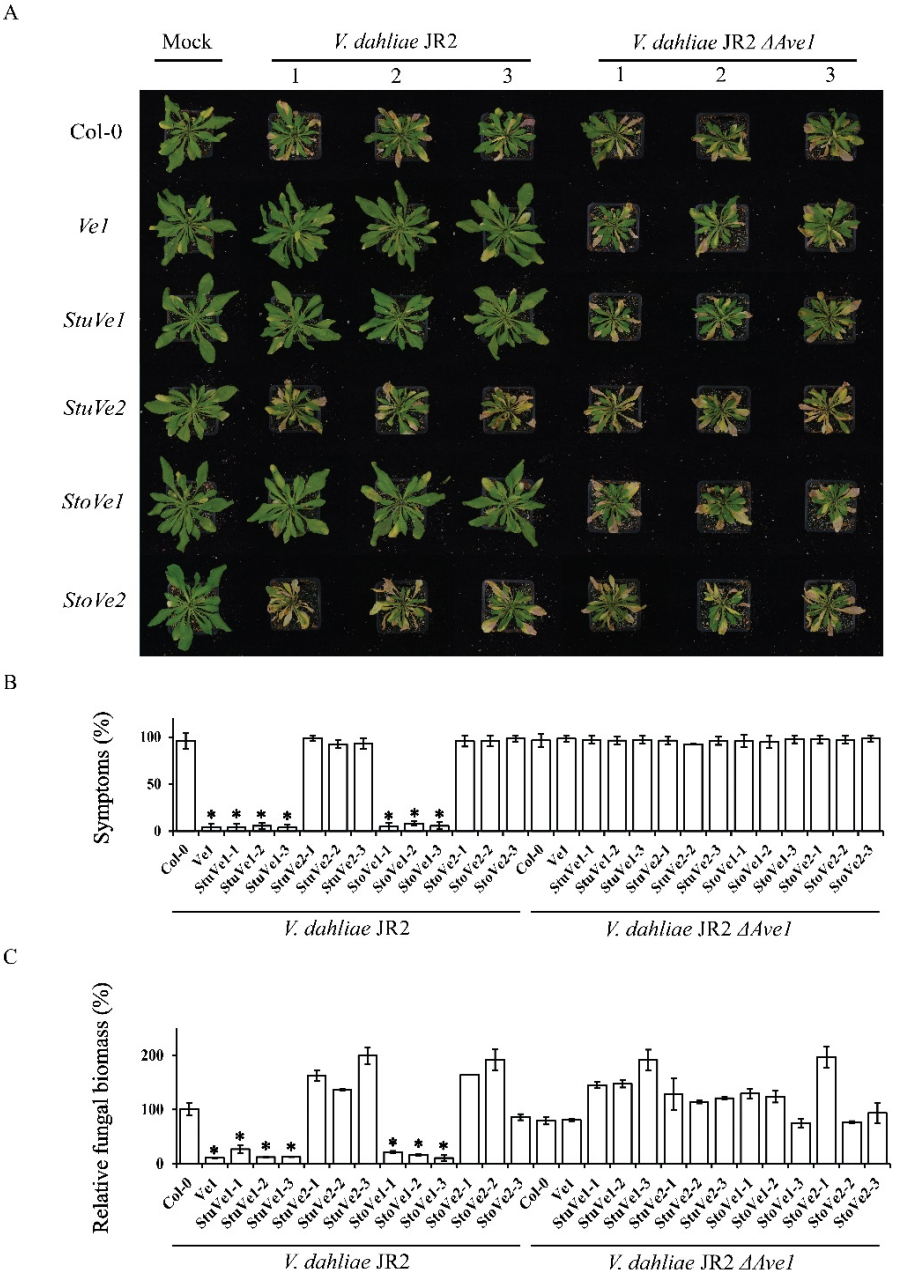

Figure 4. StuVe1 and StoVe1, but not StuVe2 and StoVe2, provide resistance against race 1 *Verticillium dahliae* in Arabidopsis. (A) Typical appearance of non-transgenic Arabidopsis and transgenic plants that engineered to express 35S-driven *StuVe1*, *StuVe2*, *StoVe1* or *StoVe2* upon mock-inoculation or inoculation with *V. dahliae* JR2 or *V. dahliae* JR2*ΔAve1* at 21 days post inoculation (dpi). **(B)** Quantification of Verticillium wilt symptoms in Arabidopsis Col-0 and transgenic plants at 21 dpi. Bars represent quantification symptom development as percentage of diseased rosette leaves with standard deviation, with Col-0 (control) set to 100%. **(C)** Fungal biomass determined by quantitative PCR (qPCR) in Arabidopsis Col-0 and transgenic plants at 21 dpi. Bars represent *Verticillium* internal transcribed spacer (*ITS*) transcript levels relative to *AtRuBisCo* (RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase) transcript levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in Col-0 (control) is set to 100%. Three independent lines are shown (1, 2 and 3). Asterisks indicate significant differences when compared with Col-0 ($P < 0.05$). *Ve1* transgenic plants were used as a positive control. The data shown are representative of at least three independent experiments.

HLVe1-2A, but not HLVe1-2B, recognizes *Verticillium* effector Ave1

Polygenic resistance to *Verticillium* spp. has also been described in several non-solanaceous species, including hop, alfalfa, cotton and strawberry (Antanaviciute et al., 2015; Bolek et al., 2005; Jakse et al., 2013; Wang et al., 2008; Yang et al., 2008). Genetic resistance against Verticillium wilt in hop (*Humulus lupulus*) was introduced into breeding programs from American wild hop (*H. lupulus* var. *neomexicanus*) and is still used today as the main resistance source (Darby, 2001). Genetic analysis identified a single significant QTL for this resistance, suggesting that Verticillium wilt resistance in hop is conferred by more than a single gene (Jakse et al., 2013; Majer et al., 2014). To investigate the presence of *Ve*-like sequences in hop, Southern blotting with the tomato *Ve1* gene as probe was performed, revealing low copy numbers of *Ve*-like sequences in hop cultivars (Figure S5 and Methods S1). With thermal asymmetric interlaced (TAIL)-PCR (Terauchi and Kahl, 2000), several *Ve*-like sequences were identified (Methods S1). Further analysis revealed two *Ve1* alleles in the *Verticillium*-resistant hop cultivar 'Wye Target', designated *HLVe1-2A* (GenBank accession: KJ647426) and *HLVe1-2B* (GenBank accession: KJ647427), which both encode 1039-amino-acid proteins (GenBank accessions: AIE39594 and AIE39595) sharing 52% and 51% identity with tomato Ve1 and Ve2, respectively, and 98% identity with each other (Figure S1). To investigate the functionality of *HLVe1-2A* and *HLVe1-2B*, co-agroinfiltration with *Ave1* in *N. tabacum* was performed. When mature tobacco leaves were co-infiltrated with a 1:1 mixture of *A. tumefaciens* cultures carrying *Ave1* and *HLVe1-2A*,

signs of a weak HR were observed at 5 days post infiltration with a minor part of the infiltrated region developing necrosis (Figure 5A). However, in contrast, co-expression of *Ave1* and *HLVe1-2B* in tobacco induced no such response, similar to co-agroinfiltration of *Ve1*, *HLVe1-2A,* and *HLVe1-2B* with *Avr9* (Figure 5A). To test whether failure of HLVe1-2B to induce an HR is the result of the instability of the protein, the coding regions of *HLVe1-2A* and *HLVe1-2B* were cloned to generate C-terminally GFP-tagged expression constructs, and the stability of both proteins was verified by immunoblotting (Figure 5B).

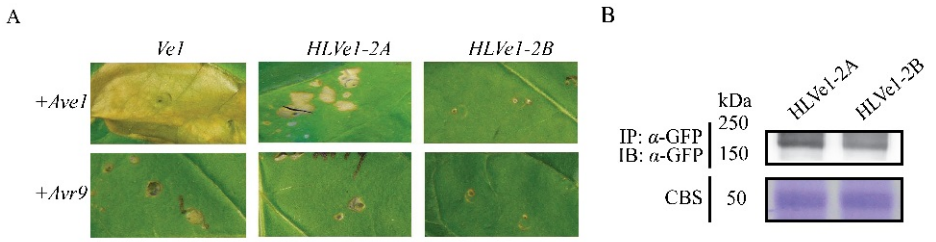

Figure 5. Co-expression of *HLVe1-2A*, but not *HLVe1-2B*, with *Ave1* in *Nicotiana tabacum* activates a hypersensitive response (HR). (A) *HLVe1-2A* or *HLVe1-2B* was transiently co-expressed with *Ave1* in *N. tabacum*, respectively. As a negative control, *Avr9* was co-expressed with *Ve1* homologues. As a positive control, HR was induced upon co-expression of *Ve1* and *Ave1*. **(B)** Green fluorescent protein (GFP)-tagged HLVe1-2A and HLVe1-2B proteins are detected *in planta* by immunopurification (IP) using GFP-beads, followed by immunoblotting (IB) using *α*-GFP antibody. Coomassie Blue Staining (CBS) of the blotting containing total protein extracts showed equal loading in each lane based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band.

To further assess the role of *HLVe1-2A* and *HLVe1-2B* in resistance to *V. dahliae*, heterologous expression in Arabidopsis was pursued (Figure S4). No phenotypic alterations were observed in plants that expressed *HLVe1-2A* or *HLVe1-2B* when compared with *Ve1*-transgenic or non-transgenic plants (Figure 6A), and three independent transgenic lines for *HLVe1-2A* and *HLVe1-2B* were assayed for *V. dahliae* resistance. Interestingly, despite the weak HR observed upon agroinfiltration together with *Ave1* in *N. tabacum*, *HLVe1-2A*-expressing plants were clearly resistant to *V. dahliae* race 1 strain JR2 as few, if any, symptoms were observed (Figure 6A, B). In contrast, *HLVe1-2B* transgenic plants were as susceptible as non-transgenic controls (Figure 6A, B), and all genotypes were

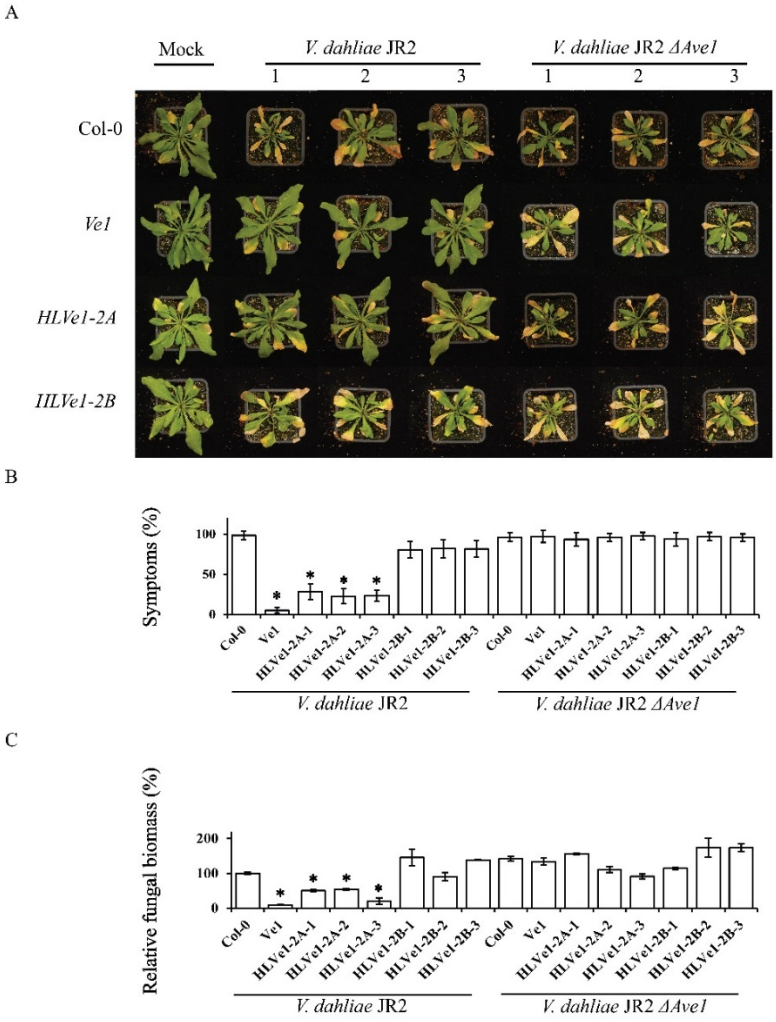

Figure 6. *HLVe1-2A*, but not *HLVe1-2B*, confers resistance to race 1 *Verticillium dahliae* in Arabidopsis. (A) Typical appearance of non-transgenic Arabidopsis and transgenic lines that constitutively express *HLVe1-2A* or *HLVe1-2B* upon mock-inoculation or inoculation with *V. dahliae* strain JR2 or *V. dahliae* JR2*ΔAve1* at 21 days post inoculation (dpi). **(B)** Quantification of Verticillium wilt symptoms in Arabidopsis Col-0 and transgenic lines at 21 dpi. Bars represent quantification symptom development as percentage of diseased rosette leaves with standard deviation, with Col-0 (control) set to 100%. **(C)** Fungal biomass determined by quantitative PCR (qPCR) in Arabidopsis Col-0 and transgenic plants at 21 dpi. Bars represent *Verticillium* internal transcribed spacer (*ITS*) transcript levels relative to *AtRuBisCo* (RuBisCo, ribulose-1,5-bisphosphate carboxylase/oxygenase) transcript levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in Col-0 (control) is set to 100%. Three independent lines are shown (1, 2 and 3). Asterisks indicate significant differences when compared with Col-0 ($P < 0.05$). *Ve1* transgenic plants were used as a positive control. The data shown are representative of at least three independent experiments.

equally susceptible to the *V. dahliae Ave1* deletion mutant (Figure 6A, B). The phenotypes correlated with the level of *V. dahliae* biomass as determined by real-time PCR (Figure 6). Collectively, these data verify that HLVe1-2A, but not HLVe1-2B, acts a functional Ve1 homologue that provides resistance to race 1 *V. dahliae*.

Functional Ve1 homologues mediate recognition of Ave1 homologues from multiple plant pathogens

We have previously shown that tomato Ve1 recognizes not only *V. dahliae* and *V. albo-atrum* Ave1, but also homologues derived from *F. oxysporum* f. sp. *lycopersici* (FoAve1) and *C. beticola* (CbAve1) (de Jonge et al., 2012). To investigate whether the newly identified functional Ve1 homologs similarly recognize these Ave1 homologues, co-expression of the five functional *Ve1* homologs with a series of *Ave1* homologs [*FoAve1*, *CbAve1*, *ChAve1*, *CoAve1* (*Colletotrichum orbiculare*; Gan et al, 2013) and *XacPNP* (Gottig et al., 2008)] was performed (Figure 7A). Co-expression of the effector *Avr9* (van Kan et al., 1991; Van der Hoorn et al., 2000) in combination with the Ve1 homologues was used as a negative control. To compare the HR induced upon co-expression of *Ave1* homologues and functional Ve1 homologues in tobacco, HR development was measured by quantification of the leaf area that developed necrosis at 5 days post infiltration (Figure 7B). Co-expression of *Ve1* with *FoAve1* and *CbAve1*, but not with *ChAve1*, *CoAve1* and *XacPNP*, in *N. tabacum* resulted in HR (Figure 7). *StuVe1* seems to recognize a wider panel of Ave1 homologues, as co-expression with *Ave1*, *FoAve1*, *CbAve1*, *ChAve1*, *CoAve1* and *XacPNP* induced HR (Figure 7). For *NgVe1* and *StoVe1*, co-infiltration with *Ave1* and *FoAve1* induced HR, whereas infiltration with *CbAve1*, *ChAve1*, *CoAve1* and *XacPNP* failed to induce HR (Figure 7). Finally, the most narrow recognition spectrum is observed for HLVe1-2A that recognizes none of the Ave1 homologues apart from that from *V. dahliae* (Figure 7). These data demonstrate that the newly identified functional Ve1 homologues, similar to tomato Ve1, differentially recognize Ave1 homologues from different plant pathogens.

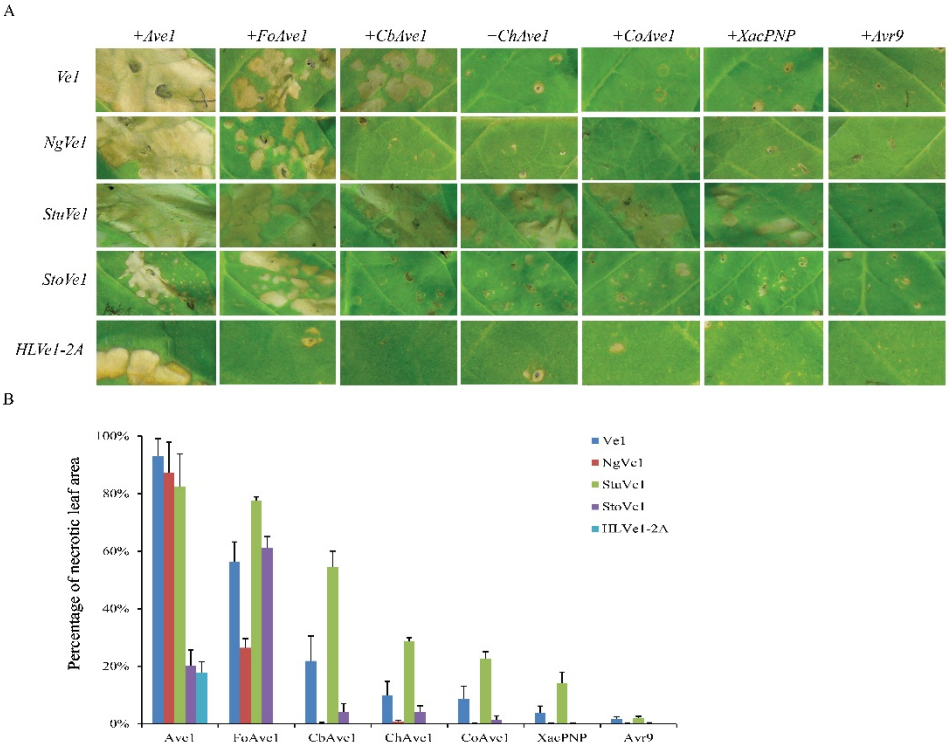

Figure 7. Distinct necrosis induced by *Ave1* homologues through co-expression with functional *Ve1* orthologues in *Nicotiana tabacum*. **(A)** Co-expression of functional *Ve1* orthologues with *Ave1* homologues (*Ave1*, *FoAve1*, *CbAve1*, *ChAve1*, *CoAve1* and *XacPNP*) in *N. tabacum*. Expression of *Avr9* in combination with *Ve1* homologues is shown as negative controls. Leaves were photographed at 5 days post infiltration to visualize necrosis resulting from recognition by functional Ve1 homologues. **(B)** Quantification of necrosis resulting from recognition of Ave1 homologues by functional Ve1 orthologues at 5 days post infiltration. Bars represent the average percentage of necrotic leaf area of infiltration zones with standard deviation.

Comparison of the protein sequences of Ve1 homologs

Tomato Ve1 is predicted to contain a signal peptide, an eLRR domain composed by two eLRR regions, separated by a non-LRR island domain (also referred as C1, C3 and C2, respectively; Figure S6 and S7), a transmembrane domain, and a short cytoplasmic tail that lacks obvious motifs for intracellular signaling (Kawchuk et al., 2001; Wang et al., 2010; Zhang and Thomma, 2013). Alignment of the functional Ve1 protein sequences identified in this study clearly shows the typical eLRR-RLP domain architecture (Figure S6). All Ve1 homologues contain 37 eLRR repeats in two eLRR regions that are interrupted by a non-LRR island domain (Figure S6).

Previously, we have determined that three eLRR regions are crucial for Ve1 functionality; eLRR_1 to eLRR_8, eLRR_20 to eLRR_23 and eLRR_32 to eLRR_37 (Zhang et al., 2014). A comparison of the functional Ve1 homologues studied here shows that the eLRR_1 to eLRR_8 (44.2% identity) and eLRR_20 to eLRR_23 (46.5% identity) regions of the C1 domain are only slightly more conserved than the eLRR_9 to eLRR_19 (40.2% identity) and eLRR_24 to eLRR_31 (45.0% identity) regions of the C1 domain, respectively (Figure S6 and S7). A similar comparison of the C1 domains among the non-functional Ve1 homologues studied here shows that the eLRR_1 to eLRR_8 (50.2% identity) region of the C1 domain is slightly more conserved that the eLRR_9 to eLRR_19 (45.5% identity) region of the C1 domain, whereas the eLRR_20 to eLRR_23 (50.0% identity) region of the C1 domain is conserved to a similar extent to the eLRR_24 to eLRR_31 (50.3% identity) region of the C1 domain (Figure S7). Further comparison among the functional Ve1 homologues shows that the C3 domain (eLRR_32 to eLRR_37, 48.4% identity) is more conserved than the C1 domain (43.3% identity), C2 domain (8.0% identity) and C-terminal eLRR-flanking domain (9.2% identity) (Figure S6 and S7). This result is consistent with a previous comparison of tomato eLRR-RLPs, which showed that the C3 domain is more conserved than the C1 domain (Fradin et al., 2014). Finally, a comparison of the non-functional Ve1 homologues studied here shows that the C1 domain (48.7% identity) and C3 domain (eLRR_32 to eLRR_37, 53.3% identity) are more conserved than the C2 domain (8.0% identity) and C-terminal eLRR-flanking domain (7.2% identity) (Figure S7). Collectively, these findings do not point towards a particular conservation of the three LRR regions that were previously implicated in Ve1 functionality.

Phylogenetic analysis of *Ve1* homologues in the plant kingdom

To determine the phylogenetic breadth among *Ve1* homologues in plants, we systematically queried the available genomes of 41 plant species for the occurrence of *Ve1* homologues. In these genomes, we identified 1361 *bona fide Ve1* homologues, all of which occur in land plants (embryophytes) and none in green algae (Figure 8A). To further analyze the phylogenetic relationship of tomato Ve1 and its close homologues, we used a neighbor-joining phylogeny to guide the extraction of the tomato Ve1 clade and the relevant sister clades (Figure 8B and S8). These

sequences were used to infer a refined maximum likelihood phylogeny containing 608 *Ve1* homologues that encompasses monocots and dicots. This phylogeny revealed a *Ve1* orthologous group, defined at the last common ancestor of monocots and dicots, which contains all functional *Ve1* homologues that have been described so far (Figure 8B and S8). The broad phylogenetic distribution, with homologues present in all land plants, establishes that *Ve1* is an ancient immune receptor (Figure 8 and S8), and that the last common ancestor contained at least a single, but more likely several, *Ve1*-like genes. Moreover, we inferred a *Ve1* orthologous group that comprises both monocots and dicots and includes all functional *Ve1* genes, suggesting the conservation of function within this group of genes.

DISCUSSION

In this article, we describe the cloning and characterization of *Ve1* homologues within and outside the Solanaceae family, and demonstrate that Ve1 homologues of tobacco (NgVe1), potato (StuVe1), wild eggplant (StoVe1) and hop (HLVe1-2A) act as functional homologues of tomato Ve1 by providing resistance to race 1 *V. dahliae* strain, mediated through the recognition of the Ave1 effector, implying that functional Ve1 homologues are conserved across plant species within and outside the Solanaceae. We further show that all functional Ve1 proteins contain a conserved domain architecture with 37 eLRR repeats (Figure S6). It has been previously determined that the two regions of the C1 domain, namely eLRR_1 to eLRR_8 and eLRR_20 to eLRR-23, are required for Ve1 functionality, probably because they contribute to ligand binding (Zhang et al., 2014). Here, these regions appear to be only slightly more conserved than other regions within the functional Ve1 homologues (Figure S6 and S7). In addition, the C3 domain (eLRR_32 to eLRR_37) was shown to be critical for Ve1 functionality (Zhang et al., 2014; Figure S6), potentially through interaction with common factors required for downstream signalling (Fradin et al., 2014; Fritz-Laylin et al., 2005; Zhang and Thomma, 2013)). As expected, this region is most conserved among the functional Ve1 homologues (Figure S6 and S7). Previously, Ve1 homologues from other plant species have also been associated with Verticillium wilt resistance, although conclusive evidence for a causal role in resistance has mostly been lacking. For example, Vining and Davis (2009) showed that the mint *Ve1* homologue *mVe1* associates with Verticillium wilt

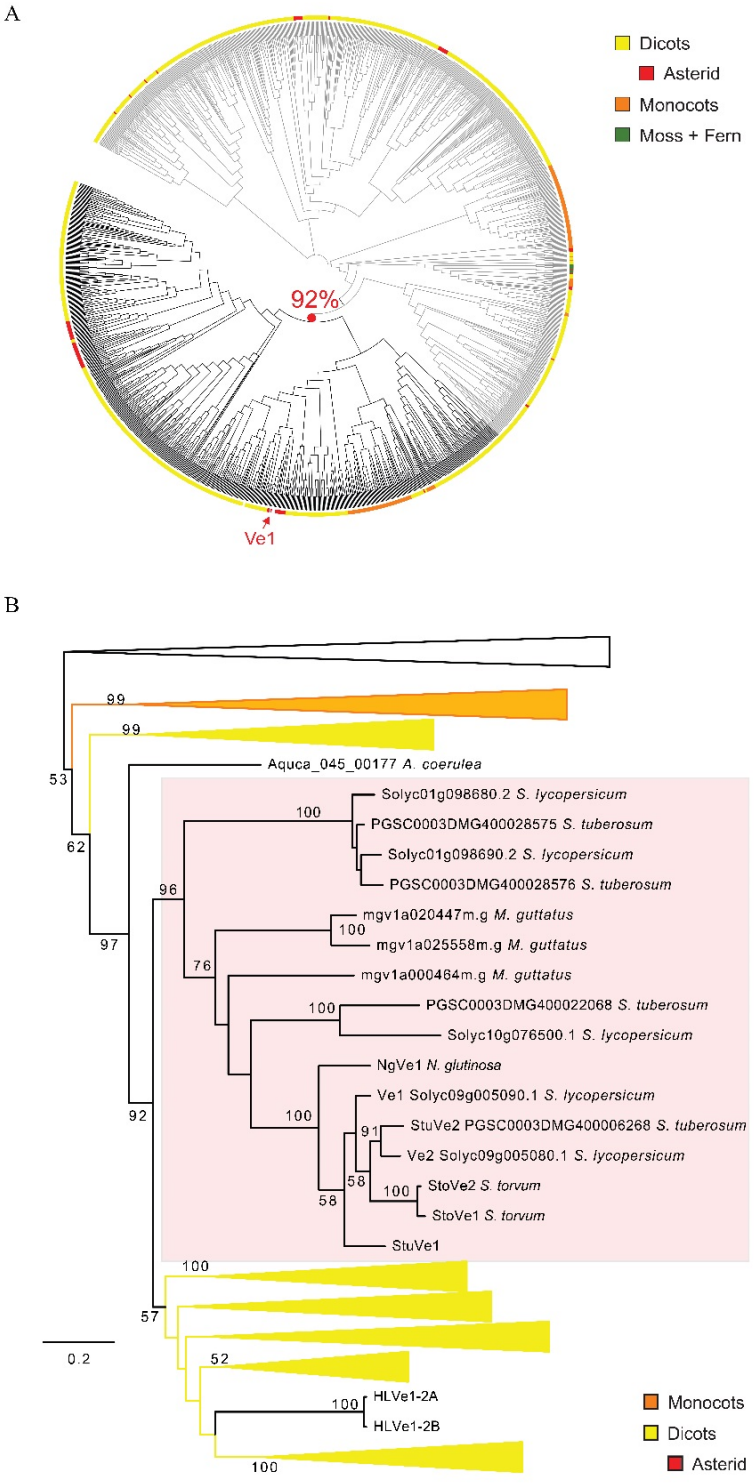
A
Dicots
Asterid
Monocots
Moss + Fern
92%
Ve1
B
99
99
53
Aquca_045_00177 A. coerulea
100
Solyc01g098680.2 S. lycopersicum
PGSC0003DMG400028575 S. tuberosum
62
Solyc01g098690.2 S. lycopersicum
PGSC0003DMG400028576 S. tuberosum
96
mgv1a020447m.g M. guttatus
100
97
mgv1a025558m.g M. guttatus
76
mgv1a000464m.g M. guttatus
100
PGSC0003DMG400022068 S. tuberosum
Solyc10g076500.1 S. lycopersicum
NgVe1 N. glutinosa
92
Ve1 Solyc09g005090.1 S. lycopersicum
100
91
StuVe2 PGSC0003DMG400006268 S. tuberosum
Ve2 Solyc09g005080.1 S. lycopersicum
58
100
StoVe2 S. torvum
58
StoVe1 S. torvum
StuVe1
100
57
0.2
52
100
HLVe1-2A
HLVe1-2B
100
Monocots
Dicots
Asterid

Figure 8. Phylogenetic analysis of Ve1 homologs indicates that Ve1 homologues are widely distributed in land plants. (A) Phylogeny of Ve1 proteins from 41 plant species from the Phytozome database. Tomato Ve1 is indicated with red arrow. **(B)** Maximum likelihood phylogenetic tree of selected Ve1 homologues (dark grey clades in A) displaying the tomato Ve1 clade and the relevant sister clades. Tomato Ve1 clade is indicated by highlighted background. Official gene identifiers and species name are indicated. Bootstrap values are shown in the tree. The scale represents branch length expressed as the relative number of amino acid substitutions.

resistance. Genome-wide analysis of disease resistance genes in lettuce, in combination with QTL mapping, showed that three *Ve* homologues, including *Vr1*, are located within a 100-kb region on chromosome 9 that co-segregates with resistance to race 1 *V. dahliae* (Christopoulou et al., 2015; Hayes et al., 2011). The grapevine *Ve1* homologue *VvVe* has been shown to enhance defense against *V. dahliae* in *N. benthamiana* (Tang et al., 2016). Remarkably, cotton *Ve1* homologues *GbVe*, *Gbve1*, *Gbvdr5* and *Gbvdr3* have been shown to confer *Verticillium* resistance upon ectopic expression in Arabidopsis or cotton (Chen et al., 2016; Yang et al., 2014; Zhang et al., 2011; Zhang et al., 2012), although this concerns *V. dahliae* isolates that do not carry the *Ave1* effector gene, and thus Verticillium wilt resistance cannot be mediated by Ave1 recognition in these cases (Song et al., unpublished data).

Previously, we have noted the absence of correlation between Ave1-induced HR and resistance through Ve1, as treatment with Ave1 leads to HR in tomato and tobacco plants that express *Ve1*, but not in *N. benthamiana* or Arabidopsis, whereas *Ve1*-transgenic Arabidopsis shows Ave1-triggered resistance (de Jonge et al., 2012; Zhang et al., 2013a, b). Similarly, in the present study, we observed robust resistance mediated by the *S. torvum* and hop *Ve1* homologues *StoVe1* and *HLVe1-2A*, whereas only a weak HR is observed on co-expression of *Ave1*. These findings suggest that the HR may occur as a consequence of Ve1/Ave1-induced immune signaling under particular conditions, but is not required for *V. dahliae* resistance (Zhang et al., 2013b). This finding may also explain that we were unable to compromise *NgVe1*-mediated *V. dahliae* resistance on VIGS in *N. glutinosa*, but were able to compromise Ave1-mediated HR.

Phylogenetic analysis revealed that *Ve1* homologues are widely distributed in phylogenetically distant plant species, implying an ancient origin of the Ve1 immune receptor. Nevertheless, this origin does not imply functionality in *V.*

dahliae resistance. The most obvious example is the close tomato homologue *Ve2* which, despite its homology, does not act as a functional *V. dahliae* resistance gene. Similarly, in this study, we identified the non-functional *Ve1* homologues *StuVe2*, *StoVe2* and *HLVe1-2B*. The *Ve* locus as it occurs in tomato, with two homologues *Ve1* and *Ve2*, appears to originate before speciation, as clustered *Ve1* family members also appear in potato and wild eggplant. Furthermore, a functional study addressing Ave1 recognition in the genus *Nicotiana* only identified Ave1 recognition, and thus presence of a potentially functional *Ve1* homologue, in the species *N. glutinosa* (Zhang et al., 2013a). Considering the extremely wide host range of *V. dahliae*, and the general occurrence of strains that carry *Ave1*, the question arises whether the ancient progenitor of the currently functional Ve1 orthologues already functioned as a *V. dahliae* resistance gene, and thus that several species/homologues lost the capacity to recognize Ave1 as a result of adverse effects associated with Ve1 functionality, or whether several species/homologues evolved the capacity to recognize Ave1 after the occurrence of speciation events. The latter hypothesis would imply that Ave1 recognition evolved multiple times in the plant kingdom through parallel evolution. Our present data do not allow verification or disqualification of either hypothesis.

Plants and animals employ germline-encoded pattern recognition receptors (PRRs) to detect broadly distributed microbe-associated molecular patterns (MAMPs) and to activate antimicrobial defense (Macho and Zipfel, 2014). We have previously noted that tomato Ve1 is an ancient pathogen receptor with traits of typical PRRs. This finding was based on the transferability of *Ve1* across plant species and the observation that Ve1 resistance affected three fungal species; *V. dahliae, V. albo-atrum* and *F. oxysporum* (Fradin et al., 2011; de Jonge et al., 2012; Thomma et al., 2011). We have now demonstrated that members of the *Ve1* gene family in *N. glutinosa*, *S. tuberosum*, *S. torvum* and *H. lupulus* encode receptors that recognize Ave1 and are able to mediate *V. dahliae* resistance in Arabidopsis. As our findings are based on stable expression in a heterologous host, we realize that definitive evidence for the role of the *Ve1* homologues in disease resistance in the endogenous hosts from which the *Ve1* homologues are derived needs to be provided through targeted gene deletion in these hosts, or stable expression in susceptible genotypes of these species. We also discovered that the functional Ve1

homologues have divergent recognition specificities, suggesting that some of them recognize an even wider spectrum of plant pathogens than tomato Ve1 (Figure 7). Collectively, these findings mean that tomato Ve1 has traits of a typical race-specific resistance protein as well as of a typical PRR. Similarly, Arabidopsis RLP23 recognizes an epitope of Nep-like effector proteins (NLPs) that are widely distributed among bacteria, fungi and oomycetes (Gijzen and Nürnberger, 2006) to induce immune responses (Albert et al., 2015). Thus, it is becoming apparent that MAMP receptor systems are more dynamic than generally appreciated and are conditioned similar to prototypical resistance genes (Albert et al., 2015; Cook et al., 2015; Shibuya and Desaki, 2015). Findings like these have inspired the proposal of the "Invasion Model", which describes plant immunity as a surveillance system to detect invasion, in which host receptors, termed invasion pattern receptors (IPRs), detect either externally encoded or modified-self ligands that indicate invasion, termed invasion patterns (IPs) (Cook et al., 2015).

MATERIALS AND METHODS

Plant growth conditions and manipulations

Plants were grown at 21°C/19°C during 16 h/8 h light/dark photoperiods, respectively, in the climate chamber or the greenhouse with a relative humidity of ~75%, and 100 $W \cdot m^{-2}$ supplemental light when light intensity dropped below 150 $W \cdot m^{-2}$. Arabidopsis transformations were performed as described previously (Clough and Bent, 1998).

Isolation of *Ve1* homologues

The isolation of *Ve1* homologues from *N. glutinosa*, *S tuberosum*, *S. torvum* and *H. lupulus* is described in Methods S1 and Tables S2-S4.

Binary over-expression constructs and transient expression *in planta*

For *NgVe1*, *StuVe1, StuVe2*, *StoVe1*, *StoVe2 HLVe1-2A* and *HLVe1-2B* constructs, CDS regions were amplified from *N. glutinosa*, *S. tuberosum* and *S. torvum* cDNA, respectively, while the CDS regions of *HLVe1-2A* and *HLVe1-2B* were amplified from the corresponding plasmids (Table S1). The CDS fragments were cloned into pDONR207 by using Gateway® BP Clonase® II Enzyme Mix (Invitrogen, Carlsbad,

USA). All pDONR207 clones were sequenced, and fragments were subsequently transferred to pEarleyGate 100, pSol2095 (C-terminal GFP tag) (Zhang et al., 2013a), or to pFAST_R02 as described (Shimada et al., 2010) by using Gateway® LR Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA). Constructs for constitutive expression of *Ave1*, *FoAve1*, *CbAve1*, *ChAve1* were described previously (de Jonge et al., 2012). *CoAve1* (Gan et al., 2013) and *XacPNP* (Gottig et al., 2008) were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany), and subsequently recombined into the destination vector pSol2092 (Zhang et al., 2013a) to generate expression constructs pSol2092_CoAve1 and pSol2092_XacPNP. All constructs were transformed to *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

A. tumefaciens carrying expression constructs were infiltrated into tobacco plants as described previously (Zhang et al., 2013a). *A. tumefaciens* cultures containing constructs to express Ave1 and Ve1 homologues were mixed in a 1:1 ratio and infiltrated into leaves of 5- to 6-week-old tobacco plants. At 5 days post infiltration, pictures were taken, and necrosis was quantified by using ImageJ to measure the area of necrosis as percentage of the total infiltrated leaf area.

Protein extracts and immunoblotting

For detection of GFP-tagged Ve1 homologs, *A. tumefaciens* carrying the corresponding expression constructs was infiltrated into mature tobacco leaves as described previously (Zhang et al., 2013a). The co-immunopurifications and immunoblotting were performed as described previously (Zhang et al., 2014).

Reverse transcription-PCR (RT-PCR) and reverse transcription-quantitative PCR (RT-qPCR)

Target specificity of the constructs *TRV::GUS* and *TRV::NgVe1* was determined in the TRV-infected *N. glutinosa* plants. Four weeks after TRV inoculation, whole tobacco plants were collected, and frozen in liquid nitrogen, and stored at -80°C for total RNA isolation.

For the expression of *Ve1* homologues in the corresponding transgenic plants, 2-week-old Arabidopsis seedlings were harvested and ground into a powder in liquid nitrogen. Total RNA extraction, cDNA synthesis and RT-PCR were performed

as described earlier (Zhang et al., 2013b; Table S1). To analyse expression of *NgVe1* expression in TRV-targeted *N. glutinosa* plants, RT-qPCR was conducted using primers NgVe1-F(qRT) and NgVe1-R(qRT) with tobacco *Actin* as an endogenous gene (Table S1) using the qPCR Core Kit for SYBR Green I (Eurogentec Nederland BV, Maastricht, NL) as described earlier (Fradin et al., 2009).

VIGS

Constructs *pTRV2::PDS* and *pTRV2::GUS* were used as controls. To silence *NgVe1* in *N. glutinosa*, the construct *pTRV2::NgVe1* was generated. TRV vectors were agroinfiltrated as described before (Liu et al., 2002; Zhang et al., 2013a). Briefly, cotyledons of 10- to 15-day-old *N. glutinosa* seedlings were infiltrated with 1:1 mixtures of *pTRV1* and *pTRV2* constructs. Photobleaching was observed at 4 weeks after agroinfiltration of *pTRV2::PDS*. For HR assays, 4 weeks after virus inoculations, mature leaves were agroinfiltrated to individually express *Ave1*, *VdNLP1* (Santhanam et al., 2013) and *Avr9* (van Kan et al., 1991; Van der Hoorn et al., 2000). *VdNLP1* and *Avr9* were used as positive and negative controls, respectively. Five days after agroinfiltration, leaves were examined for the development of HR. For Verticillium wilt disease assays, 3 weeks after TRV infection, the TRV-infected plants were inoculated with race 1 *V. dahliae* strain JR2 (Faino et al., 2015), and the corresponding *Ave1* deletion mutant (*V. dahliae* JR2*ΔAve1*; de Jonge et al., 2012) and tap water as control. The inoculated plants were evaluated by the observation of disease symptoms at 14 dpi.

Disease assays

Verticillium dahliae was grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) at 22°C, and conidia were collected from 7- to 10-day-old plates and washed with tap water. *Verticillium* disease assays on *N. glutinosa* plants were performed as described previously (Zhang et al., 2013a). Briefly, 5-week-old plants were uprooted, the roots were rinsed in water, dipped for 3 min in a suspension of 10^6 conidiospores/mL water, and transferred to commercial potting soil (Horticoop, Bleiswijk, the Netherlands). Verticillium wilt disease assays on *Arabidopsis*, as well as *Verticillium* biomass quantification in infected *Arabidopsis* plants were performed as described before (Ellendorff et al., 2009).

Phylogenetic identification

To obtain the phylogenetic relationship of tomato Ve1 and its homologues, we identified similar sequences in 41 plant species acquired from Phytozome (v9.1) (Goodstein et al., 2012) and manually added Ve1 homologues of tobacco, potato, wild eggplant and hop. Sequence similarity was established using BLAST (version 322.28+), applying a conservative e-value cutoff of 1e-50. To prevent spurious hits, we removed sequences where the matching area was < 75% or the 'actual-matching' was < 50% of either Ve1 or the subject. The matching area is defined as the area from the start position of the first segment to the end position of the last segment, and the 'actual-matching' area is defined as the sum of the covered area by each individual segment. Moreover, sequences that deviate in length (< 80% or > 120% of the length of Ve1) or contain protein domains other than leucine-rich repeats, as predicted by HMMER3 (version 3.0)(Finn et al., 2011) on a local PFAM database (version 27), were discarded. Subsequently, all protein sequences were aligned using MAFFT (version 7.047b) (Katoh et al., 2002) and the most consistent alignment (LINSI) was chosen using trimAl (version 1.2) (Capella-Gutiérrez et al., 2009), after which the heuristic method of trimAl was applied to trim the alignment. This cleaned alignment was used to construct an initial phylogenetic tree using quick tree (version 1.1; 1000 bootstraps). The clade of interest (with tomato Ve1) and surrounding sequences were manually gathered and realigned. The final phylogenetic tree was inferred using RAxML (version 7.6.3) (Stamatakis, 2006), with the gamma model of rate heterogeneity and the Whelan and Goldman amino acid substitution matrix.

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SUPPLEMENTAL INFORMATION

	Ve1	NgVe1	StuVe1	StuVe2	StoVe1	StoVe2	HLVe1-2A	HLVe1-2B
Ve1	-	76%	87%	84%	83%	80%	52%	51%
Ve2	84%	74%	81%	91%	81%	85%	52%	51%
NgVe1	76%	-	78%	74%	74%	73%	50%	50%
StuVe1	87%	78%	-	82%	82%	81%	51%	51%
StuVe2	84%	74%	82%	-	81%	85%	51%	51%
StoVe1	83%	74%	82%	81%	-	92%	51%	51%
StoVe2	80%	73%	81%	85%	92%	-	51%	51%
HLVe1-2A	52%	50%	51%	51%	51%	51%	-	98%
HLVe1-2B	51%	50%	51%	51%	51%	51%	98%	-

Figure S1. Percentage of amino acid identity shared between nine Ve1 homologues. The highest percentage of homology between two Ve1 homologues is indicated in red. Dashes (-) represent identical sequences.

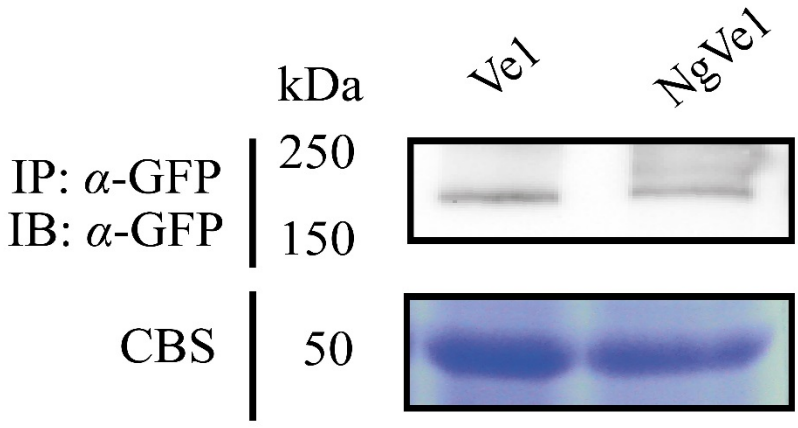


Figure S2. Stability of green fluorescent protein (GFP)-fused NgVe1 protein *in planta*. Total protein extracts of transiently transformed leaf tissue were subjected to immunopurification (IP) by using GFP-affinity beads. Immunopurified proteins were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblotted (IB) using *α*-GFP antibody. Coomassie Blue Staining (CBS) of the blot containing total protein extracts showed equal loading in each lane based on the 50 kDa RuBisCo (ribulose-1,5-bisphospate carboxylase/oxygenase) band. GFP-tagged Ve1 protein was used as a control.

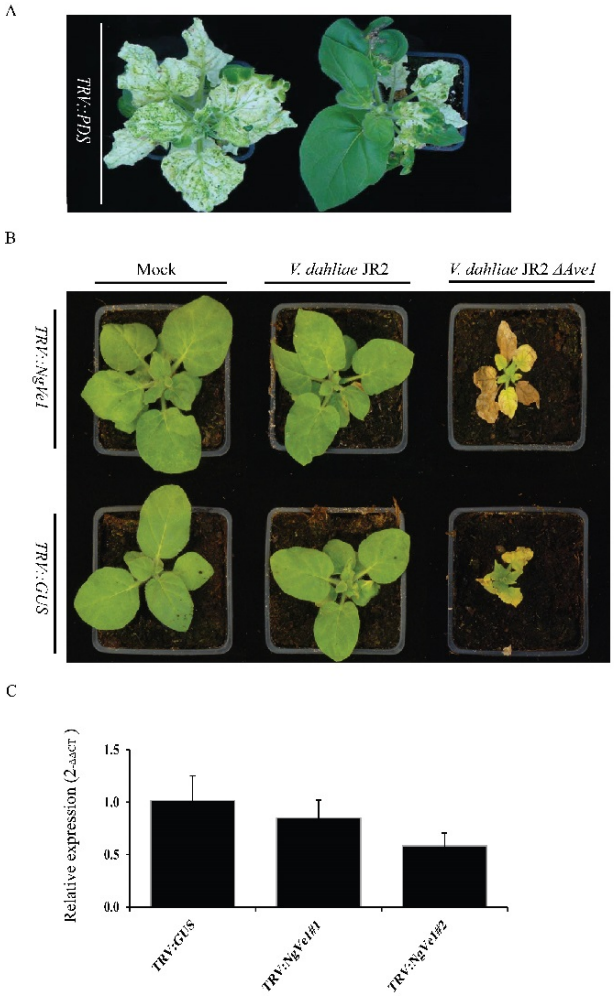


Figure S3. ***Tobacco rattle virus*** **(TRV)-mediated gene silencing in** ***Nicotiana glutinosa*****. (A)** Virus-induced gene silencing of the phytoene desaturase (*PDS*) gene results in patchy photobleaching in leaves of *N. glutinosa*. Pictures were taken at 4 weeks post *TRV::PDS* infiltration, and show representative infected plants of at least three independent assays. **(B)** *TRV::NgVe1*-inoculated plants show resistance against *Verticillium dahliae* strain JR2, but not *V. dahliae* JR2*ΔAve1*. *N. glutinosa* plants were inoculated with a recombinant TRV targeting the *β-glucuronidase* (*GUS*) gene as a control (*TRV::GUS*) or recombinant TRV targeting the *NgVe1* gene (*TRV::NgVe1*). At 3 weeks after TRV infiltration, TRV-inoculated plants were inoculated either with *V. dahliae* strain JR2, or with *V. dahliae* JR2*ΔAve1* mutant. Pictures were taken at 2 weeks after *V. dahliae* inoculation, and show representative inoculated plants of at least three independent assays. **(C)** Silencing efficiency was determined by using reverse transcription-quantitative PCR (RT-qPCR) at 28 days post infiltration in *TRV::NgVe1*- and *TRV::GUS*-inoculated *N. glutinosa* plants. Bars represent levels of *NgVe1* transcripts relative to the transcript levels of *N. glutinosa actin* (for normalization) with standard deviation of a sample of three pooled plants. *NgVe1* expression in the *TRV::GUS*-infected plants is set to 1.

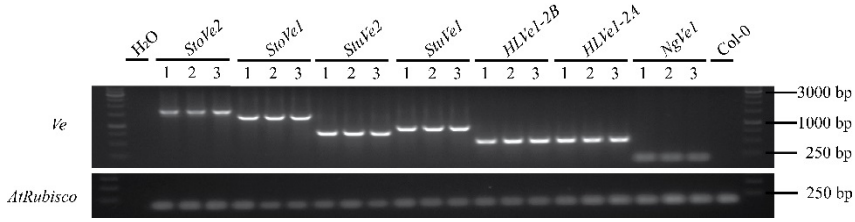


Figure S4. Expression of *NgVe1, HLVe1-2A, HLVe1-2B, StuVe1, StuVe2, StoVe1* and *StoVe2* in transgenic plants was detected by reverse transcription-PCR (RT-PCR). As an endogenous control, a fragment of the *AtRuBisCo* (RuBisCo, ribulose-1,5-bisphospate carboxylase/oxygenase) gene was amplified from cDNA. For each construct, three independent transgenic lines are shown (1, 2 and 3). Water is used as a control.

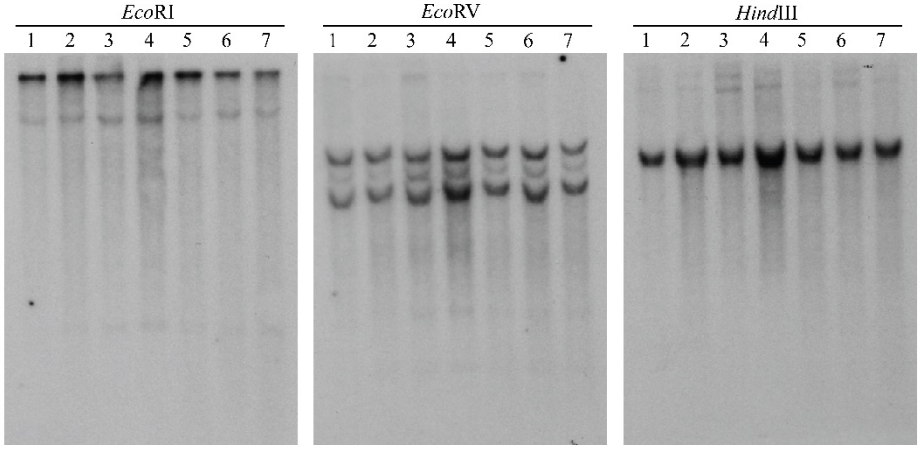
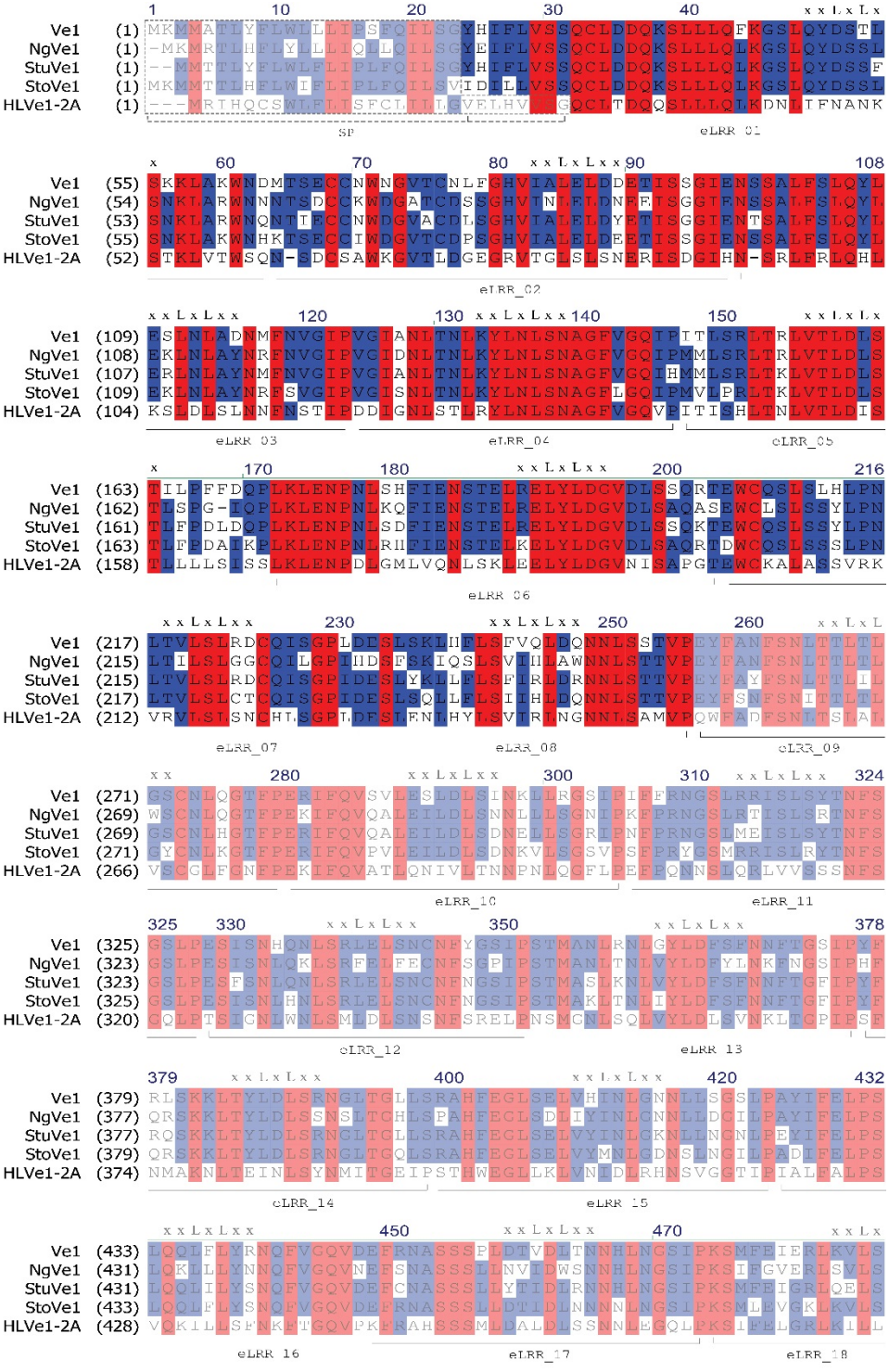
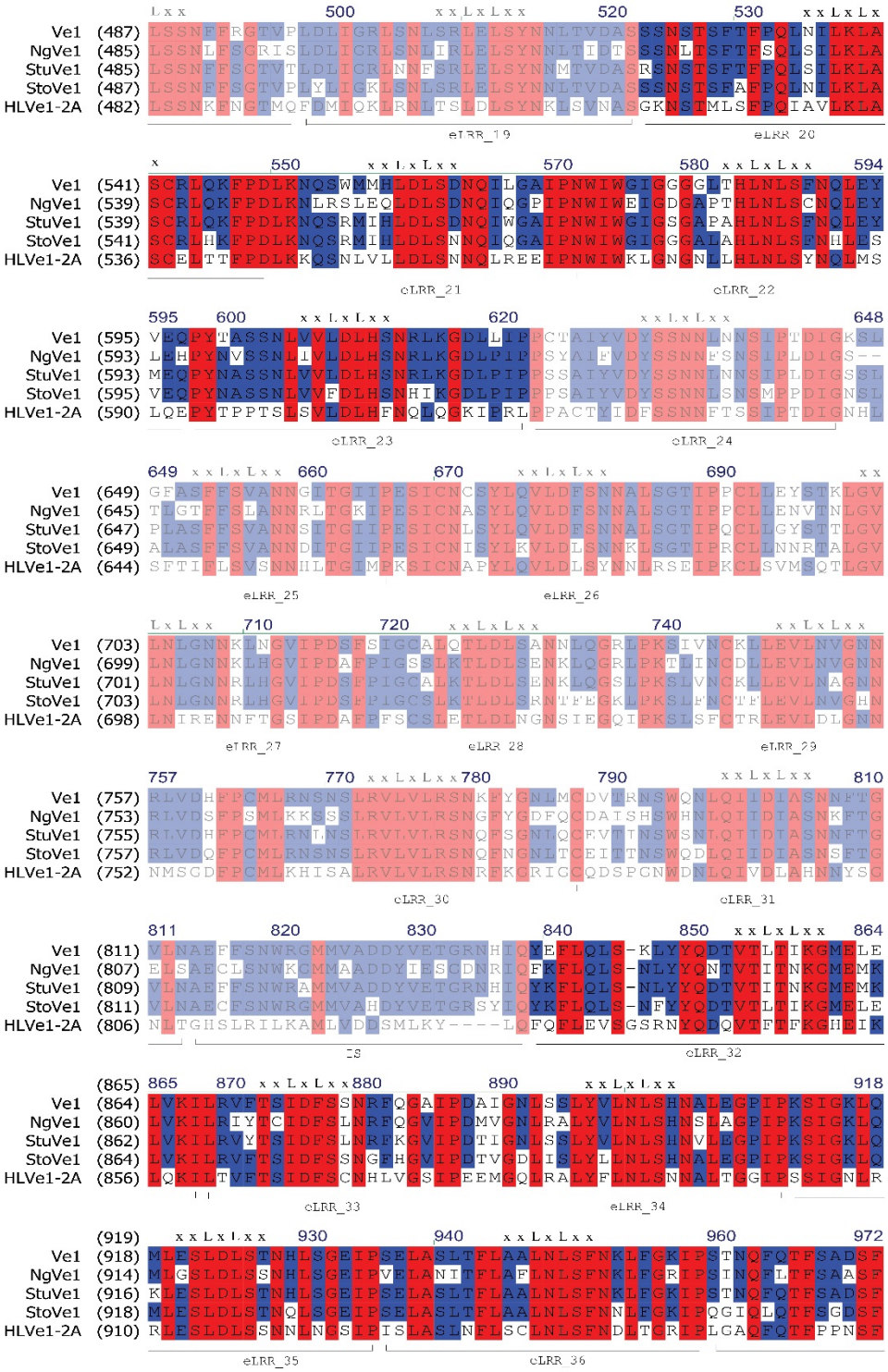


Figure S5. Sothern blotting of seven hop cultivars with the tomato *Ve1* gene as a probe reveals the presence of *Ve*-like sequences in the hop genome. Seven hop cultivars are indicated as: 1, 'Wye target'; 2, 'Fuggle'; 3, 'Wye Challanger'; 4, 'Savinjski Golding'; 5, 'Aurora'; 6, 'Celeia'; 7, 'Yeoman'. Hop genomic DNA was digested with the restriction enzymes *Eco*RI, *Eco*RV and *Hind*III, separated on 0.8% agarose gel and blotted on nylon membranes with the P^{32}-labelled tomato *Ve1* gene sequence as a probe.


Ve1 (1) MKMMATLYFLWLLLIPSFQILSGYHIFLVSSQCLDDQKSLLLQFKGSLQYDSTL
NgVe1 (1) -MKMRTLHFLYLLLIQLLQILSGYEIFLVSSQCLDDQKSLLLQLKGSLQYDSSL
StuVe1 (1) --MMTTLYFLWLFLIPLFQILSGYHIFLVSSQCLDDQKSLLLQLKGSLQYDSSF
StoVe1 (1) MKMMTTLHFLWIFLIPLFQILSVIDILLVSSQCLDDQKSLLLQLKGSLQYDSSI
HLVe1-2A (1) ---MRTHQCSWLFLISFCLILLGVELHVLGQCITDQQSLLLQLKDNLTFNANK
SP
eLRR 01
Ve1 (55) GKKLAKWNDMTSECCNWNGVTCNLFGHVIALELDDETISSGIENSSALFSLQYL
NgVe1 (54) SNKLARWNNNTSDCCKWDGATCDSSGHVTNLFLDNFETSGGIFNSSALFSLQYL
StuVe1 (53) SNKLARWNQNTTFCCNWDGVACDLSGHVTALFLDYETTSGGIFNTSALFSLQYL
StoVe1 (55) SNKLAKWNHKTSECCIWDGVTCDPSGHVIALFLDEETISSGIENSSALFSLQYL
HLVe1-2A (52) STKLVTWSQN-SDCSAMKGVTLDGEGRVTGLSLSNERISDGIHN-SRLFRLQHL
eLRR_02
Ve1 (109) FSLNLADNMFNVGIPVGIANLTNLKYLNLSNAGFVGQIPITLSRLTRLVTLDLS
NgVe1 (108) EKLNLAYNRFNVGIPVGIDNLTNLKYLNLSNAGFVGQIPMMLSRLTRLVTLDLS
StuVe1 (107) ERLNLAYNMFNVGIPVGIANLTNLKYLNLSNAGFVGQIHMMLSRLTKLVTLDLS
StoVe1 (109) EKLNLAYNRFSVGIPVGISNLTNLKYLNLSNAGFLGQIPMVLPRLTKLVTLDLS
HLVe1-2A (104) KSLDLSLNNFNSTIPDDIGNLSTLRYLNLSNAGFVGQVPITISHLTNLVTLDIS
eLRR_03
eLRR_04
eLRR_05
Ve1 (163) TILPFFDQPLKLENPNLSHFIENSTELRELYLDGVDLSSQRTEWCQSLSLHLPN
NgVe1 (162) TLSPG-IQPLKLENPNLKQFIENSTELRELYLDGVDLSTVPEYFANFSNLTTLTL
StuVe1 (161) TLFPDLDQPLKLENPNISDFIENSTELRELYLDGVDLSSQKTEWCQSLSSYLPN
StoVe1 (163) TLFPDATKPLKLENPNLRHFIENSTELKELYLDGVDLSAQRTDWCQSLSSSLPN
HLVe1-2A (158) TLLLLSISSLKLENPDLGMLVQNLSKLEELYLDGVNISAPGTEWCKALASSVRK
eLRR_06
eLRR_07
eLRR_08
eLRR_09
Ve1 (217) LTVLSLRDCQISGPLDFSLSKLIFLSFVQLDQNNLSSTVPEYFANFSNLTTLTL
NgVe1 (215) LTILSLGGCQILGPIHDGFSKIQSLSVIILAWNNLSTTVPEYFANFSNLTTLTL
StuVe1 (215) LTVLSLRDCQISGPIDESLYKLLFLSFIRLDRNNLSTTVPEYFAYFSNLTTLIL
StoVe1 (217) LTVLSLCTCQISGPIDESLSQLLFLSIIHLDQNNLSTTVPEYFSNFSNITTLTL
HLVe1-2A (212) VRVLSLSNCHLSGPLDFSLFNLHYLSVIRLNGNNLSAMVPQWFADFSNLTSLAL
eLRR_10
eLRR_11
Ve1 (271) GSCNLQGTFPERIFQVSVLESLDLSINKLLRGSIDIFFRNGSLRRISLSYTNFS
NgVe1 (269) WSCNLQGTFPEKIFQVQALEILDLSNNLLLSGNIPKFFRNGSLRTISLSRTNFS
StuVe1 (269) GSCNLHGTFPERIFQVQALEILDLSDNELLSGRLPNFPRNGSLMEISLSYTNFS
StoVe1 (271) GYCNLKGTFPERIFQVPVLEILDLSDNKVLSGSVPSFPRYGSMRRISLRYTNFS
HLVe1-2A (266) VSCGLFGNFPEKIFQVATLQNIVLTNNPNLQGFLPEFPQNNSLQRLVVSSSNFS
eLRR_12
eLRR_13
Ve1 (325) GSLPESISNHQNLSRLELSNCNFYGSIPSTMANLRNLGYLDFSFNNFTGSIPYF
NgVe1 (323) GSLPESISNLQKLSRFELFECNFSGPIPSTMANLTNLVYLDFYLNKFNGSIPHF
StuVe1 (323) GSLPESFSNLQNLSRLELSNCNFNGSIPSTMASLKNLVYLDFSFNNFTGFIPYF
StoVe1 (325) GSLPESISNLHNLSRLELSNCNFNGSIPSTMAKLTNLIYLDFSFNNFTGFIPYF
HLVe1-2A (320) CQLPTSIGNLWNLSMLDLSNSNFSRELPNSMCNLSQLVYLDLSVNKLTGPIPSF
eLRR_14
eLRR_15
Ve1 (379) RLSKKLTYLDLSRNGLTGLLSRAHFEGLSFLVHINLGNNLLSGSLPAYIFELPS
NgVe1 (377) QRSKKLTYLDLSSNSLTGHLSPAHFEGLSDLIYINLGNNLLDGILPAYIFELPS
StuVe1 (377) RQSKKLTYLDLSRNGLTGLLSRAHFEGLSELVYINLGKNLLNGNLPEYIFELPS
StoVe1 (379) QRSKKLTYLDLSRNGLTGQLSRAHFEGLSELVYMNLGDNSLNGILPADIFELPS
HLVe1-2A (374) NMAKNLTEINLSYNMITGEIPSTHWEGLLKLVNIDLRHNSVGGTIPIALFALPS
eLRR_16
eLRR_17
eLRR_18
Ve1 (433) LQQLFLYRNQFVGQVDEFRNASSSPLDTVDLTNNHLNGSIPKSMFEIERLKVLS
NgVe1 (431) LQKLLLYNNQFVGQVNEFSNASSSLLNVIDWSNNHLNGSIPKSIFGVERLSVLS
StuVe1 (431) LQQLILYSNQFVGQVDEFCNASSSLLYTIDLRNNHLNGSIPKSMFEIGRLQELS
StoVe1 (433) LQQLFLYSNQFVGQVDEFRNASSSLLDTIDLNNNNLNGSIPKSMLEVGKLKVLS
HLVe1-2A (428) VQKILLSFNKFTGQVPKFRAHSSSMLDALDLSSNNLEGQLPKSIFELGRLKILLL


Ve1 (487)
NgVe1 (485)
StuVe1 (485)
StoVe1 (487)
HLVe1-2A (482)
eLRR_19
eLRR_20
Ve1 (541)
NgVe1 (539)
StuVe1 (539)
StoVe1 (541)
HLVe1-2A (536)
cLRR_21
cLRR_22
Ve1 (595)
NgVe1 (593)
StuVe1 (593)
StoVe1 (595)
HLVe1-2A (590)
cLRR_23
cLRR_24
Ve1 (649)
NgVe1 (645)
StuVe1 (647)
StoVe1 (649)
HLVe1-2A (644)
eLRR_25
eLRR_26
Ve1 (703)
NgVe1 (699)
StuVe1 (701)
StoVe1 (703)
HLVe1-2A (698)
eLRR_27
eLRR_28
eLRR_29
Ve1 (757)
NgVe1 (753)
StuVe1 (755)
StoVe1 (757)
HLVe1-2A (752)
cLRR_30
cLRR_31
Ve1 (811)
NgVe1 (807)
StuVe1 (809)
StoVe1 (811)
HLVe1-2A (806)
cLRR_32
Ve1 (864)
NgVe1 (860)
StuVe1 (862)
StoVe1 (864)
HLVe1-2A (856)
cLRR_33
eLRR_34
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eLRR_35
cLRR_36

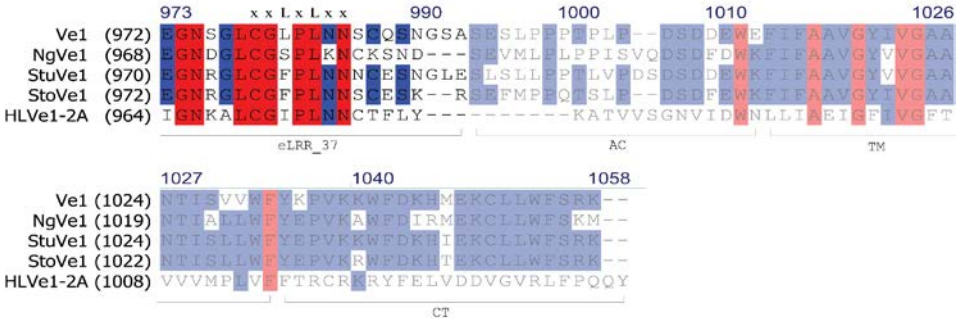

Figure S6. Primary structure and protein sequence alignment of functional Ve1 proteins from tomato, tobacco, potato, wild eggplant and hop. The N-terminal amino acids in the dashed frame denote the predicted signal peptides (SPs) of the functional Ve1 homologues. eLRR, extracelluar leucine-rich repeat (C1 domain and C3 domain); IS, non-LRR island domain (C2 domain); AC, acidic domain; TM, transmembrane domain; CT, cytoplasmic domain. The locations of the predicted solvent-exposed β-sheet (xxLxLxx) on the concave surface of the receptor are indicated above the eLRR domains. Identical amino acid residues are highlighted in red, whereas conserved amino acid residues are highlighted in blue. Three consecutive eLRR regions required for the functionality of Ve1 are indicated by bold colors, whereas other regions that could not be implicated in Ve1 functionality are indicated in light colors. Ve1 homologue sequences can be found in the GenBank database using the following GenBank accessions: ACR33105 (Ve1); ALK26499 (NgVe1); ALK26501 (StuVe1); ALK26500 (StoVe1); AIE39594 (HLVe1-2A).

(A high resolution image of the **Figure S6** can be downloaded from the link: http://onlinelibrary.wiley.com/doi/10.1111/mpp.12390/full).

	Protein sequence region		Protein sequence identity	
Functional Ve1 homologs	C1 domain (eLRR_1 to eLRR_31)	eLRR_1 to eLRR_8	43.3%	44.2%
		eLRR_9 to eLRR_19		40.2%
		eLRR_20 to eLRR_23		46.5%
		eLRR_24 to eLRR_31		45.0%
	C2 domain		8.0%	
	C3 domain (eLRR_32 to eLRR_37)		48.4%	
	C-terminal eLRR-flanking domain		9.2%	
Non-functional Ve1 homologs	C1 domain (eLRR_1 to eLRR_31)	eLRR_1 to eLRR_8	48.7%	50.2%
		eLRR_9 to eLRR_19		45.5%
		eLRR_20 to eLRR_23		50.0%
		eLRR_24 to eLRR_31		50.3%
	C2 domain		8.0%	
	C3 domain (eLRR_32 to eLRR_37)		53.3%	
	C-terminal eLRR-flanking domain		7.2%	

Figure S7. Percentage of amino acid identity shared between the C1 domain, C2 domain, C3 domain and C-terminal extracelluar leucine-rich repeat (eLRR)-flanking domain of the functional and non-functional Ve1 homologues from tomato, tobacco, potato, wild eggplant and hop.

Figure S8. An unrooted phylogenetic tree based on protein sequences of Ve1 homologues from the tomato clade and the relevant sister clades. The Tomato Ve1 clade is indicated by the highlighted background. Official gene identifiers and species name are indicated. Bootstrap values are shown in the tree. The scale represents branch length expressed as the relative number of amino acid substitutions.

(A high resolution image of the **Figure S8** can be downloaded from the link: http://onlinelibrary.wiley.com/doi/10.1111/mpp.12390/full).

Methods S1. Isolation of *Ve1* homologues

A ~1300 bp fragment was amplified from *Nicotiana glutinosa* cDNA using primers combination (Ve1SeqF1 and NgVe1-GSPR). To obtain the full-length *NgVe1* sequence, 5' and 3' rapid amplification of cDNA ends (RACE) reactions were performed by using the GeneRace® Core Kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions with minor modifications. Forward gene-specific primers NgVe1-GSPF1 and NgVe1-GSPF2 were used for 3' RACE, while reverse gene-specific primers NgVe1-GSPR1 and NgVe1-GSPR2 were used for 5' RACE (Table S1). The first round of PCR was followed by a nested PCR. The RACE products were purified and cloned into the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Carlsbad, USA) and sequenced. The full-length *NgVe1* was determined by aligning 5' and 3' RACE product sequences with the partial cDNA fragment cloned earlier (Zhang et al., 2013). Primers NgVe1-F and NgVe1-R (Table S1) were designed based on sequence information of inserts from multiple clones derived from 5' RACE and 3' RACE products, and were utilized to amplify the full-length cDNA and genomic DNA (gDNA) from *N. glutinosa*. PCR reactions were performed using the Thermo Scientific Phusion High-Fidelity PCR Kit (Thermo Scientific, Massachusetts, USA). The amplicons of full-length *NgVe1* from *N. glutinosa* cDNA and gDNA were sequenced using pre-mixed sequencing primers (Table S1). The sequence of full-length *NgVe1* is deposited at NCBI under accession number: KT895339.

To amplify the coding sequences (CDSs) of *Ve* gene homologues in heterozygous diploid potato, the primer pairs StuVe1-attB1/StuVe1-attB2 and StuVe2-attB1/StuVe2-attB2, respectively (Table S1), were designed based on the sequences of the *Ve* locus (NCBI reference sequence: XM_006362308 and XM_006362309) from a doubled monoploid potato genotype Phureja DM 1-3 516 R44 (Xu et al., 2011). To obtain the CDSs of *StoVe1* and *StoVe2,* the primer pairs StoVe1-attB1/StoVe1-attB2 and StoVe2-attB1/StoVe2-attB2, respectively (Table S1), were designed based on the sequence of *StoVe1* (GenBank accession: DQ020574; Liu et al., 2012) and *StVe* (GenBank accessions: AY311527; Fei et al., 2004) from wild eggplant. All PCR products were cloned into pDONR207 by using Gateway® BP Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA), and subsequently sequenced using pre-mixed sequencing primers (Table S1), and named *StuVe1* (GenBank accession: KT946795) and *StuVe2* (GenBank accession: KT946797), *StoVe1* (GenBank accession: KT946794) and *StoVe2* (GenBank accession: KT946796).

Southern blotting was performed to query for *Ve* homologues in hop. 5 µg of hop genomic DNA (seven hop cultivars: 'Wye target', 'Fuggle', 'Wye Challanger', 'Savinjski Golding', 'Aurora', 'Celeia' and 'Yeoman') was digested with *Eco*RI, *Eco*RV and *Hind*III, separated on 0.8% agarose gel and transferred to nylon membranes. Southern blotting was performed with the P^{32}-labelled tomato *Ve1* gene sequence as a probe and washing was performed with 0.5X SSC (Maniatis et al., 1982). A pair of specific and degenerative primers HLVe(deg)-F and HLVe(deg)-R (Table S1) was constructed based on the protein

ClustalW alignment of tomato Ve1 (GenBank accession: AAK58682), mint *Verticillium* wilt resistance-like protein (GenBank accession: ACB99689), three vine grape proteins (GenBank accessions: CAO63881, CAN76702 and CAO63885) and a hop EST sequence (GenBank accessions: GD247683), which showed similarity to *Ve1*. A *Ve*-like sequence from hop cultivar 'Wye Target' with a length of ~1300 bp was amplified. Its alignment showed similarity to *Ve1* from amino acid 142 forward. This part of the sequence was the basis for isolation of the flanking sequences of *HLVe* using TAIL-PCR combined six degenerate primers (Table S2) with designed hop sequence-specific primers (Table S3) from the hop cultivar 'Wye target'. A primary TAIL-PCR reaction was performed in 20 µl containing 20 ng of hop genomic DNA, 1X Dream *Taq* Buffer, 0.8 mM of dNTPs, 0.8 U of Dream *Taq* enzyme (Thermo Scientific, Massachusetts, USA), 0.2 mM of primary reaction hop specific primer and one of the following arbitrary primers: AD1, AD6 (2 µM), AD2 (3 µM), AD3, AD4 or AD5 (4 µM) (Table S2; S3). The reaction was diluted 50 times and 1 µl was used in the secondary TAIL-PCR reaction (20 µl) with a changed amount of Dream *Taq* polymerase (0.6 U), secondary reaction hop specific primer (0.2 mM) and a different amount of AD primers (AD1 and AD6 1,5 µM, AD2, AD3, AD4 or AD5 2 µM) (Table S2; S3). The reaction mixture of the tertiary TAIL-PCR was identical to the secondary one, with the exception that 1 µl of diluted secondary reaction diluted 1:50 was added as template and tertiary reaction hop specific primer was used (Table S2; S3). The amplification protocols are listed as Table S4. Amplicons of tertiary TAIL-PCR reactions were cleaned and sequenced and the obtained sequences were assembled in CodonCode Aligner (version 3.7.1); contigs were further checked for similarity with *Ve1* using blast X algorithm. Based on the assembled sequences, two full-length *Ve1*-like sequences were amplified from hop cultivar 'Wye Target' genomic DNA using primers HLVe-F and HLVe-R (Table S1). Two sequences were deposited at NCBI as *HLVe1-2A* (GenBank accession: KJ647426) and *HLVe1-2B* (GenBank accession: KJ647427).

Table S1. Primers used for amplification, sequencing and expression of *Ve1* homologs.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
Ve1SeqF1	TTCAATGTTGGCATACCAGTTGG	Fradin et al., 2011
NgVe1-GSPR (Nested)	ACTTGCATTCCAACAGGCTTAAGGGT	5′ RACE
GeneRacer™ 3′ Primer	GCTGTCAACGATACGCTACGTAACG	3'RACE (provided in RACE Kit)
GeneRacer™ 3′ Nested Primer	CGCTACGTAACGGCATGACAGTG	3'RACE (provided in RACE Kit)
GeneRacer™ 5′ Primer	CGACTGGAGCACGAGGACACTGA	5'RACE (provided RACE Kit)
GeneRacer™ 5′ Nested Primer	GGACACTGACATGGACTGAAGGAGTA	5'RACE (provided in RACE Kit)
NgVe1-GSPF1	CAATGCCTTGAGTGGCACAATACCA	3'RACE
NgVe1-GSPF2 (Nested)	TCTTGGAGTGCTGAATCTTGGGAAC	3'RACE
NgVe1-GSPR1	CCAAGCAAGATGGATGACAGAAAGAG	5'RACE
NgVe1-GSPR2 (Nested)	TCGCTCGCCTGAGCTGAAAGATCAA	5'RACE
NgVe1-F	GTCATATACCTATACAAGTTTGCATG	*NgVe1* transcription sequence
NgVe1-R	GTACTCCTCATTTATTGGTTTAAGC	*NgVe1* transcription sequence
NgVe1-attB1	ggggacaagtttgtacaaaaaagcaggctATGAAAATGAGAACTCTACA	*NgVe1* coding sequence
NgVe1-attB2	ggggaccactttgtacaagaaagctgggtTCACATCTTTGAAAACCA	*NgVe1* coding sequence
NgVe1-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtcCATCTTTGAAAACCAAAG	GFP-tagged NgVe1
NgVe1-F(TRV)	ggggacaagtttgtacaaaaaagcaggctGACTCTCAGACCTCATCTACAT	*NgVe1* (VIGS)
NgVe1-R(TRV)	ggggaccactttgtacaagaaagctgggtCTGGGAAAAAGTGAATGAC	*NgVe1* (VIGS)
NgVe1-F(qRT)	TCTTTCTGTCATCCATCTTGC	*NgVe1* (RT-qPCR & RT-PCR)
NgVe1-R(qRT)	TGTCTCCTGACTCAATGTAATC	*NgVe1* (RT-qPCR & RT-PCR)
HLVe(deg)-F	ACGCCAGTAAATCGACGAAG	Degenerative primer for hop *Ve*-like sequence
HLVe(deg)-R	CKRTTISWRTGIARRTCIAHAVE	Degenerative primer for hop *Ve*-like sequence
HLVe-F	TTCGTTTGCCCATTTTGTTT	Full length hop *Ve1*-like sequence
HLVe-R	TGAACGCCTTCTTGGTCT	Full length hop *Ve1*-like sequence
HLVe1-attB1	ggggacaagtttgtacaaaaaagcaggctATGAGAATTCATCAGTGT	*HLVe1-2A & HLVe1-2B* coding sequence
HLVe1-attB2	ggggaccactttgtacaagaaagctgggtTTAATACTGTTGAGGAAA	*HLVe1-2A & HLVe1-2B* coding sequence
HLVe1-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtcATACTGTTGAGGAAAGAG	GFP-tagged HLVe1-2A & HLVe1-2B
HLVe1-F	TTGCCTCGTGTGAACTAACTAC	*HLVe1-2A & HLVe1-2B* (Seq & RT-PCR)
HLVe1-R	CCTGTGAAGTTGTTTTCTCGTA	*HLVe1-2A & HLVe1-2B* (Seq & RT-PCR)
StuVe1-attB1	ggggacaagtttgtacaaaaaagcaggctATGATGACAACTCTGTACTTTCT	*StuVe1* coding sequence
StuVe1-attB2	ggggaccactttgtacaagaaagctgggtTCACTTTCTTGAAAACCAAA	*StuVe1* coding sequence
StuVe1-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtaCTTTCTTGAAAACCAAAG	GFP-tagged StuVe1

Table S1 (continued)

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
StuVe2-attB1	ggggacaagtttgtacaaaaaagcaggctATGAGATTTTTACACTTTCTATGG	*StuVe2* coding sequence
StuVe2-attB2	ggggaccactttgtacaagaaagctgggtTCAAAACTTTTTGTGATATATGAC	*StuVe2* coding sequence
StuVe2-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtaAAACTTTTTGTGATATATGACT	GFP-tagged StuVe2
StuVe1-F1(Seq)	CTTGTCAGATAACGAGTTGCTT	Potato *StuVe1* (Seq)
StuVe1-R1(Seq)	GAAATGAATCTGGGATAACACC	Potato *StuVe1* (Seq)
StuVe1-F2(Seq)	CTTTCACATTTCCCCAGTTG	Potato *StuVe1* (seq)
StuVe1-R2(Seq)	CGTAATCATCAGCAACCATC	Potato *StuVe1* (Seq)
StuVe2-F1(Seq)	TTCCACAATGGCAAACCTTA	Potato *StuVe2* (Seq)
StuVe2-R1(Seq)	CCTTCAGGCTGGTTGAGTTC	Potato *StuVe2* (Seq)
StuVe2-F2(Seq)	ACTTTCTCTCTTCCATCCGT	Potato *StuVe2* (Seq)
StuVe2-R2(Seq)	GAAGTTGGAAGAAAGTGAGAG	Potato *StuVe2* (Seq)
StoVe1-attB1	ggggacaagtttgtacaaaaaagcaggctATGAAAATGATGACAACTCTCC	*StoVe1* coding sequence
StoVe1-attB2	ggggaccactttgtacaagaaagctgggtTCACTTCCTTGAAAACCAAA	*StoVe1* coding sequence
StoVe1-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtcCTTCCTTGAAAACCAAAGC	GFP-tagged StoVe1
StoVe2-attB1	ggggacaagtttgtacaaaaaagcaggctATGAGATTTTTACACTTTCTATGG	*StoVe2* coding sequence
StoVe2-attB2	ggggaccactttgtacaagaaagctgggtTCAAAACTTTTTGTGACATATGAC	*StoVe2* coding sequence
StoVe2-attB2(ΔSC)	ggggaccactttgtacaagaaagctgggtcAAACTTTTTGTGACATATGACC	GFP-tagged StoVe2
StoVe1-F(Seq)	AGTGGTAGTGTTCCGAGTTT	Wild eggplant *StoVe1* (Seq)
StoVe1-R(Seq)	ACATTCAGGACCTCCAAAAA	Wild eggplant *StoVe1* (Seq)
StoVe2-F(Seq)	AAGTTGCTTAGTGGTAGTATTC	Wild eggplant *StoVe2* (Seq)
StoVe2-R(Seq)	TGGAGGAACTTATACTGGAT	Wild eggplant *StoVe2* (Seq)
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	pCR blunt II TOPO (Seq)
M13-R	TCACACAGGAAACAGCTATGAC	pCR blunt II TOPO (Seq)
pENTRattL1	TCGCGTTAACGCTAGCATGGATCTC	pDONR207 (Seq)
pENTRattL2	ACATCAGAGATTTTGAGACACGGGC	pDONR207 (Seq)
NgActin-F	TATGGAAACATTGTGCTCAGTGG	Tobacco actin (RT-qPCR)
NgActin-R	CCAGATTCGTCATACTCTGCC	Tobacco actin (RT-qPCR)
AtRubisco-F	GCAAGTGTTGGGTTCAAAGCTGGTG	Arabidopsis Rubisco
AtRubisco-R	CCAGGTTGAGGAGTTACTCGGAATGCTG	Arabidopsis Rubisco
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	*V. dahliae*
STVe1-R	CTTGGTCATTTAGAGGAAGTAA	*V. dahliae*

[a]The type of experiment for which the primers were used is indicated in brackets (RACE: rapid amplification of cDNA ends, VIGS: Virus-induced gene silencing, Seq: sequencing, RT-PCR: Reverse transcription-PCR, RT-qPCR: reverse transcription-quantitative PCR).

Table S2. Degenerative primers used for TAIL-PCR amplification of the flanking regions of putative hop *Ve1* homologues.

Primer name	Oligonucleotide sequence (5'→3')	Level of degeneracy	Reference
AD1	NTCGASTWTSGWGTT	64X	Liu et al., 1995
AD2	NGTCGASWGANAWGAA	128X	Liu et al., 1995
AD3	WGTGNAGWANCANAGA	256X	Liu et al., 1995
AD4	STTGNTASTNCTNTGC	256X	Qiu et al., 2010 (AD8)
AD5	WCAGNTGWTNGTNCTG	256X	Qiu et al., 2010 (AD9)
AD6	TTGIAGNACIANAGG*	16X	Qiu et al., 2010 (AD11)

*I-inosine

Table S3. Specific primers used for the TAIL-PCR based isolation of hop *Ve1* sequences.

1° TAIL PCR		2° TAIL PCR		3° TAIL PCR	
Name	Oligonucleotide sequence (5'→3')	Name	Oligonucleotide sequence (5'→3')	Name	Oligonucleotide sequence (5'→3')
VeT1F1	TTTGGGAACTTGGCAATGGGAATCTTATC	VeT1F2	GATGAGCCTGCAGGAACCATATTCTC	VeT1F3	GCCTGCAGGAACCATATTCTCCTC
VeT1R1	GTTTTGAACCAGCATCCCCAAATCC	VeT1R2	ATCCCCAAATCCGGGTTCTCAAGTTTC	VeT1R3	GTGGAGATATCAAGAGTGACCAAGTTTGTC
Ve1T2F1	CACATGGAGCTCAAATTCAGATTG	Ve1T2F2	AGTTTAGATGGCGAGGGACGT	Ve1T2F3	GCTTGAGCAGTGAGTGGATCTCT
Ve2T2F1	TCACATGGAGTCAAAATTCAGATTG	Ve2T2F2	TAACTTTGGATGGTGAGGGACG	Ve2T2F3	TTGAGCAATGAGCGGATCTCT
K1T2F1	ATTCCCGATGCCTTTCCG	K1T2F2	TTGGGAACAATGATATGAGTGGTG	K1T2F3	TGGTGATTTTCCATGCTTGTTG
K1T2R1	AGCACTTGAAGATAGGGTGCATTA	K1T2R2	TTACATATGGATTTTGGCACAATACC	K1T2R3	GTCTGCGGGAATTGAGGAAGT
K2T2F1	GCTCAATTCATGATGCCTTTCC	K2T2F2	CTTGGGAACAATCATATGAGTGGTAC	K2T2F3	GTGGTACTTTTCCTTGCTTGTTGA
K2T2R1	TCAAGCACTTGAAGGTAGGTTGC	K2T2R2	CATATGGATTTTGGCATAGTTCCTATAA	K2T2R3	ATGTCTGTGGGAATTGAGCAAGA
Ve1T3F1	CAACTTCATGGGGAAATACCTTATTG	Ve1T3F2	CCATCTAAATCTTTCCCACAATCAT	Ve1T3F3	CTAACCAGCTCCAAGGGAAGATT
Ve1T4F1	TCACAGGCAGAATCCCATCAT	Ve1T4F2	ACACCTGGAGTCCTTAGACCTCTC	Ve1T4F3	AATCCCATCAACCCTTGCAA
Ve2PR1	CTGCAATCTGAATTTTGACTCCAT	Ve2PR2	TGTCCTTCAGTTGAAGCAACAAAG	Ve2PR3	GTAAGGCATTGACCAGAGACGA

Primers are listed according to the TAIL-PCR procedure, consisting of three steps (1° – primary, 2° – secondary, 3° – tertiary) in which each was utilized.

Table S4. Amplification conditions used for TAIL-PCR (Liu et al., 1995).

Level of TAIL-PCR	No. of cycles	Step in amplification
1°	1	1 min 93°C, 1 min 95°C
	5	1 min 94°C, 1 min 62°C, 2.5 min 72 °C
	1	1 min 94°C, 2 min cooling to 72 °C, 2.5 min 72°C
	15	30 s 94°C, 1 min 68°C, 2.5 min 72°C
		30 s 94°C, 1 min 68°C, 2.5 min 72°C
		30 s 94°C, 1 min 44°C, 2.5 min 72°C
	1	5 min 72°C
2°	12	30 s 94°C, 1 min 68°C, 2.5 min 72°C
		30 s 94°C, 1 min 68°C, 2,5 min 72°C
		30 s 94°C, 1 min 44°C, 2,5 min 72°C
	1	5 min 72°C
3°	20	1 min 94 °C, 1 min 44°C, 2.5 min 72°C
	1	5 min 72°C

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Chapter 5

Transfer of tomato immune receptor Ve1 confers Ave1-dependent *Verticillium* resistance in tobacco and cotton

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ABSTRACT

Verticillium wilts caused by soil-borne fungal species of the *Verticillium* genus are economically important plant diseases that affect a wide range of host plants, and are notoriously difficult to combat. Perception of pathogen(-induced) ligands by plant immune receptors is a key component of plant innate immunity. In tomato, race-specific resistance to Verticillium wilt is governed by the cell surface-localized immune receptor Ve1 through recognition of the effector protein Ave1 that is secreted by race 1 strains of *Verticillium* spp. It was previously demonstrated that transgenic expression of tomato *Ve1* in the model plant *Arabidopsis thaliana* leads to Verticillium wilt resistance. Here, we investigated whether tomato *Ve1* can confer *Verticillium* resistance when expressed in the crop species tobacco (*Nicotiana tabcum*) and cotton (*Gossypium hirsutum*). We show that transgenic tobacco and cotton plants constitutively expressing tomato *Ve1* exhibit enhanced resistance against Verticillium wilt in an Ave1-dependent manner. Thus, we demonstrate that the functionality of tomato Ve1 in Verticillum wilt resistance through recognition of the *Verticillium* effector Ave1 is retained after transfer to tobacco and cotton, implying that the Ve1-mediated immune signalling pathway is evolutionary conserved across these plant species. Moreover, our results suggest that transfer of tomato *Ve1* across sexually incompatible plant species can be exploited in breeding programmes to engineer Verticillium wilt resistance.

INTRODUCTION

In order to activate immune responses to ward off invading microorganisms, plants deploy immune receptors that detect pathogen invasion through sensing pathogen(-induced) ligands (Dodds and Rathjen, 2010; Thomma et al., 2011; Cook et al., 2015). The recognition of such ligands results in the activation of defense responses, which are sometimes accompanied by a hypersensitive response (HR) in which plant tissue surrounding the site of attempted penetration is sacrificed to restrict further pathogen invasion. Based on structure and subcellular location, immune receptors fall into two major classes; cell surface localized receptors that comprise receptor kinases (RKs) and receptor-like proteins (RLPs) that monitor the extracellular space, and cytoplasm-localized nucleotide-binding domain leucine-rich repeat receptors (NLRs) that survey the intracellular environment (Rodriguez-Moreno et al., 2017).

Verticillium wilts are vascular wilt diseases caused by soil-borne fungal pathogens that belong to the *Verticillium* genus (Fradin and Thomma, 2006; Klimes et al., 2015). Although Verticillium wilt symptoms may vary considerably between plant hosts, the most frequently observed symptoms of Verticillium wilt include stunting, wilting, chlorosis, necrosis, vascular discoloration and early senescence (Fradin and Thomma, 2006). Within the *Verticillium* genus, *V. dahliae* is the most notorious pathogenic species that can infect hundreds of dicotyledonous hosts, including ecologically important plants and many high-value crops worldwide (Fradin and Thomma, 2006; Klosterman et al., 2009). *V. albo-atrum*, *V. alfalfae*, *V. nonalfalfae* and *V. longisporum* are also economically important vascular pathogens, albeit with narrower host ranges (Pegg and Brady, 2002; Agrios, 2005; Fradin and Thomma, 2006; Klosterman et al., 2009; Inderbitzin et al., 2011; Depotter et al., 2016). Verticillium wilt diseases are difficult to control due to the long viability of the resting structures, the wide host range of the pathogens, and the inability of fungicides to affect the pathogen once in the plant vascular system. Thus, the most sustainable manner to control Verticillium wilt diseases is the use of resistant cultivars (Fradin and Thomma, 2006; Klosterman et al., 2009).

In tomato (*Solanum lycopersicum*), a single dominant locus that confers *Verticillium* resistance has been identified as the *Ve* locus, which controls *Verticillium* isolates that are assigned to race 1 whereas race 2 strains of

Verticillium escape recognition (Schaible et al., 1951; Pegg, 1974). The *Ve* locus contains two closely linked and inversely oriented genes, *Ve1* and *Ve2*, both of which encode extracellular leucine-rich repeat (eLRR) RLPs (Kawchuk et al., 2001; Wang *et al*., 2010). Of these, only *Ve1* was found to confer resistance against race 1 isolates of *Verticillium* in tomato (Fradin et al., 2009). Interestingly, interfamily transfer of *Ve1* from tomato to *Arabidopsis thaliana* leads to race-specific *Verticillium* resistance in the latter species (Fradin et al., 2011, 2014; Zhang et al., 2014), implying that the underlying immune signalling cascade is evolutionary conserved (Fradin et al., 2011; Thomma et al., 2011). Moreover, homologs of tomato Ve1 that have the potential to recognize race 1 strains of *V. dahliae* have been characterized in other plant species, suggesting an ancient origin of the tomato immune receptor Ve1 (Song et al., 2017a).

Through comparative population genomics of race 1 and race 2 strains of *V. dahliae*, the effector protein that activates Ve1-mediated immunity was identified as Ave1 (for Avirulence on Ve1 tomato) (de Jonge et al., 2012). No allelic variation was found among the identified *Ave1* alleles from *V. dahliae* as well as from *V. alfalfae* and *V. nonalfalfae* (de Jonge et al., 2012; Song et al., 2017b). Interestingly, homologues of Ave1 were found in the bacterial plant pathogen *Xanthomonas axonopodis* pv. *citri* (XacPNP) and in the plant-pathogenic fungi *Colletotrichum higginsianum* (ChAve1), *Cercospora beticola* (CbAve1), *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), as well as in plants (de Jonge *et al*., 2012). A few of these homologues are differentially recognized by tomato Ve1 in *Nicotiana tabacum* (de Jonge et al., 2012; Song et al., 2017b). Although the intrinsic function of Ave1 remains unknown, it is clear that Ave1 contributes to fungal virulence on susceptible plant genotypes (de Jonge et al., 2012).

Plant immune receptors are pivotal elements of the plant immune system that act as sentinels against pathogens. Engineering plants via transfer of immune components, such as plant immune receptors, has the potential to improve disease resistance in crops (Rodriguez-Moreno et al., 2017). Previous reports showed that the transfer of individual cell surface immune receptors into crops confers enhanced disease resistance against diverse pathogens, including bacteria, fungi and oomycetes. For example, transfer of the *Arabidopsis* cell surface immune receptor EFR results in responsiveness to bacterial elongation factor Tu and

bacterial resistance in tomato (Lacombe et al., 2010), rice (Lu et al., 2015; Schwessinger et al., 2015) and wheat (Schoonbeek et al., 2015). Similarly, introduction of the rice Xa21 confers bacterial resistance in sweet orange (Mendes et al., 2010), tomato (Afroz et al., 2011) and banana (Tripathi et al., 2014). Heterologous expression of the *Nicotiana benthamiana FLS2* in citrus leads to increased disease resistance to citrus canker (Hao et al., 2016). Moreover, the *Arabidopsis* DORN1/LecRK-I.9 enhances resistance to *Phytophthora infestans* in potato (Bouwmeester et al., 2014). Finally, ectopic expression of the *Arabidopsis RLP23* in potato plants enhances immunity to the oomycete and fungal plant pathogens *P. infestans* and *Sclerotinia sclerotiorum* (Albert et al., 2015). In this study, we investigated whether the immune receptor gene *Ve1* can confer Verticillium wilt resistance when transferred from tomato to the closely related crop species tobacco (*N. tabacum* cv. Samsun) and the distantly related crop species cotton (*Gossypium hirsutum*).

RESULTS

Generation of *Ve1*-expressing *Nicotiana tabacum* plants

Previously, it was shown that co-expression of *Ve1* and *Ave1* by agroinfiltration induces an HR in *N. tabacum* (Zhang et al., 2013a), suggesting that required signalling components acting downstream of tomato Ve1 are functionally conserved in tobacco. To further test whether tomato Ve1 can confer resistance to race 1 *Verticillium* spp., transgenic tobacco lines expressing tomato *Ve1* were generated. The binary plasmid pSol2095_Ve1 encoding C-terminally eGFP-tagged Ve1 (Zhang et al., 2013a; Figure 1A) was transferred to *N. tabacum* cv. Samsun via *Agrobacterium*-mediated transformation. Primary transformants were selected in tissue culture by their ability to regenerate in the presence of kanamycin, and eight independent T0 transformation events expressing *Ve1* were obtained (Figure S1A).

Intriguingly, 45 out of 56 progeny (T1 plants) derived from the eight T0 transformation events were significantly smaller in size when compared to the parental line, while 11 plants displayed a normal stature (Figure S1B and C). We assumed these 45 are *Ve1*-transgenic plants while the 11 correspond to segregating wild-type plants. To assay whether Ve1 protein accumulated in these plants, we isolated proteins from a line (Ve1 #0) that exhibited normal growth and three lines

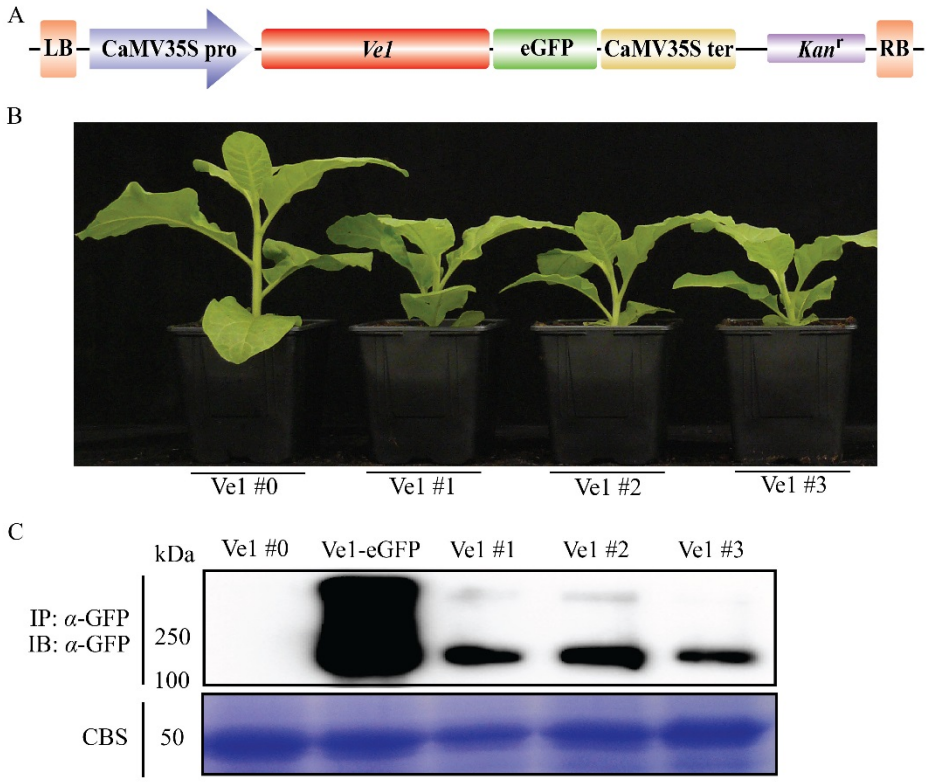

Figure 1. Generation and characterisation of *Ve1*-transgenic *Nicotiana tabacum* lines. (A) Schematic representation of the T-DNA region of the binary vector pSol2095_Ve1 used for tobacco transformation. CaMV35Spro: CaMV35S promoter, eGFP: enhanced green fluorescent protein, CaMV35Ster: CaMV35S terminator; *Kan*^{r}: kanamycin resistance gene, LB and RB: left and right T-DNA borders, respectively. **(B)** Typical appearance of 6-week-old plants of four independent tobacco lines (Ve1 #0, #1, #2 and #3). **(C)** Accumulation of eGFP-tagged Ve1 protein (~144 kDa) in leaves of the four independent *Ve1* transgenic tobacco lines and wild-type tobacco cv. Samsun transiently expressing the eGFP-tagged Ve1 fusion protein (Ve1-eGFP). Total protein extracts of transformed leaf tissue were subjected to immunoprecipitation (IP) using *α*-GFP affinity beads. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS/PAGE) and immunoblotted (IB) using *α*-GFP antibody. Coomassie blue staining (CBS) of the blot containing total protein extracts is shown as a loading control based on the 50-kDa RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) band.

(Ve1 #1, #2 and #3) that showed a dwarfed phenotype (Figure 1B), and performed immunoblotting analysis using anti-GFP-HRP antibody. This analysis showed that eGFP-tagged Ve1 protein was clearly detected in the three dwarfed lines, but not in the line that displays a normal stature (Figure 1C), suggesting that dwarfing of these tobacco lines is due to Ve1 expression. Nevertheless, the progeny of these

three dwarfed lines was used for further assays to assess the contribution of Ve1 expression to Verticillium wilt resistance.

Generation of isogenic *Verticillium* genotypes lacking or expressing *Ave1*

To identify *Verticillium* strains that can be used for inoculation assays on tobacco, six strains, comprising *V. dahliae* strains JR2 and St14.01, *V. nonalfalfae* strains Vna5431, CBS385.91 and Vna1, and *V. alfalfae* strain Va2 (Table S1) were inoculated onto wild-type tobacco cv. Samsun plants, and Verticillium wilt symptoms were scored up to 14 days post inoculation (dpi). As anticipated, the various *Verticillium* strains caused different degrees of Verticillium wilt symptoms on these plants (Figure S2A). Among these six *Verticillium* genotypes, *V. alfalfae* strain Va2 and *V. nonalfalfae* strain Vna5431 caused the most severe disease symptoms (Figure S2A and B) and were selected for Verticillium wilt disease assays on tobacco. As *V. nonalfalfae* Vna5431 carries the *Ave1* gene, it belongs to race 1, while *V. alfalfae* Va2 belongs to race 2 as it lacks *Ave1* (Figure S2C).

In order to thoroughly investigate Ve1-mediated Verticillium wilt resistance in tobacco, isogenic *Verticillium* genotypes lacking or expressing *Ave1* are required. To this end, we deleted *Ave1* from the genome of *V. nonalfalfae* Vna5431, and simultaneously introduced *Ave1* into *V. alfalfae* Va2 genome. Subsequently, the wild-type *V. nonalfalfae* strain Vna5431 and two independent *Ave1* deletion mutants were used to inoculate *Ve1* tomato plants and tomato plants that lack *Ve1* (Figure S3A). As expected, targeted deletion of *Ave1* resulted in gain of virulence on *Ve1* tomato plants (Figure S3B and C). Moreover, these *Ave1* deletion mutants displayed reduced virulence on tomato plants lacking *Ve1* when compared to the corresponding wild-type *V. nonalfalfae* strain Vna5431 (Figure S3B and D). These results show that, also for *V. nonalfalfae* strain Vna5431 Ave1 acts as a virulence factor on tomato, and confirm that deletion of *Ave1* leads to escape of Ve1-mediated resistance.

Simultaneously, the wild-type *V. alfalfae* strain Va2 and two independent *Ave1* expression strains were inoculated onto tomato plants that express or lack *Ve1* (Figure S4A). However, the wild-type *V. alfalfae* strain Va2, as well as the two *Ave1*-expressing *V. alfalfae* strains, failed to cause visible disease symptoms on tomato plants (Figure S4B, D and E), suggesting that the *V. alfalfa*e strain Va2 does not have

the capacity to infect tomato. Subsequently, we inoculated these strains on *N. glutinosa*, which is resistant to race 1 strains of *Verticillium* due to the occurrence of an endogenous *Ve1* allele (Zhang et al., 2013a; Song et al., 2017a). As expected, *V. alfalfae* strain Va2 was able to infect *N. glutinosa* plants, while the *Ave1* expression strains failed to cause infection (Figure S4C and F). These results reveal that ectopic expression of *Ave1* in *V. alfalfae* strain Va2 can activate Ve1-mediated resistance against Verticillium wilt.

Ave1 acts as a virulence factor on tobacco

It was previously determined that Ave1 acts as a virulence factor of *V. dahliae* on tomato and *A. thaliana* (de Jonge et al., 2012). To investigate the contribution of *Ave1* to *Verticillium* virulence on tobacco, isogenic *Ave1* mutants and the corresponding wild-type *Verticillium* strain were inoculated onto tobacco cv. Samsun plants. Interestingly, *Ave1* deletion strains of *V. nonalfalfae* Vna5431 displayed significantly reduced virulence on tobacco plants when compared with the corresponding wild-type *V. nonalfalfae* strain Vna5431 at 21 dpi (Figure S5A), as inoculation with the *Ave1* deletion strains resulted in reduced stunting (Figure S5B) and compromised fungal colonization (Figure S5C). Conversely, the two *Ave1* expression strains in *V. alfalfae* Va2 showed clearly increased aggressiveness on tobacco plants when compared with the corresponding wild-type strain (Figure S5D-F). These experiments show that Ave1 acts as a virulence factor on tobacco plants.

Tomato Ve1 confers Ave1-dependent Verticillium wilt resistance in tobacco

To test if constitutive *Ve1* expression in tobacco confers resistance against Verticillium wilt in an Ave1-dependent manner, three independent *Ve1*-transgenic lines (Ve1 #1, #2 and #3) as well as non-transgenic control plants were challenged with either the wild-type race 1 *V. nonalfalfae* strain Vna5431, or an *Ave1* deletion mutant (*V. nonalfalfae* Vna5431Δ*Ave1*) and inspected for Verticillium wilt symptoms up to 21 dpi. Interestingly, *Ve1*-transgenic tobacco plants were clearly more resistant to the race 1 *V. nonalfalfae* strain Vna5431, as significantly fewer Verticillium wilt symptoms developed when compared with non-transgenic controls (Figure 2A and B). Importantly, despite the fact that the *Ave1* deletion

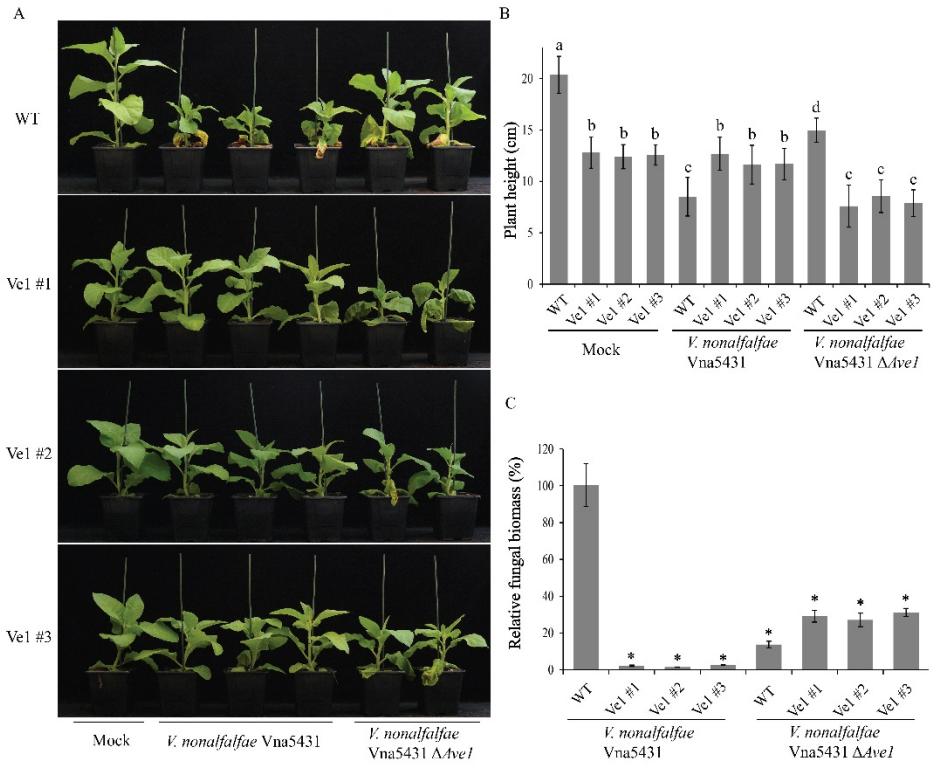

Figure 2. Tobacco plants expressing *Ve1* show Ave1-triggered resistance against *Verticillium nonalfalfae*. (A) Typical appearance of wild-type tobacco cultivar Samsun plants (WT) and three independent *Ve1* transgenic tobacco plants (#1, #2 and #3) that were engineered to express tomato *Ve1* upon mock-inoculation, inoculation with *Ave1*-carrying *V. nonalfalfae* Vna5431, or an *Ave1* deletion strain of *V. nonalfalfae* Vna5431 (Δ*Ave1*) at 21 days post inoculation (dpi). Inoculation experiments were performed with at least 16 plants for each fungal strain and independently repeated three times. **(B)** Quantification of *Verticillium*-induced plant stunting at 21 dpi. Bars represent averages with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *Verticillium ITS* levels relative to tobacco *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tobacco cv. Samsun plants upon inoculation with the wild-type *V. nonalfalfae* strain Vna5431 is set to 100%. Asterisks indicate statistically significant differences when compared with tobacco cv. Samsun plants upon inoculation with the wild-type *V. nonalfalfae* strain Vna5431 (Student's *t*-test; $P < 0.05$). The data shown are representative of three independent experiments.

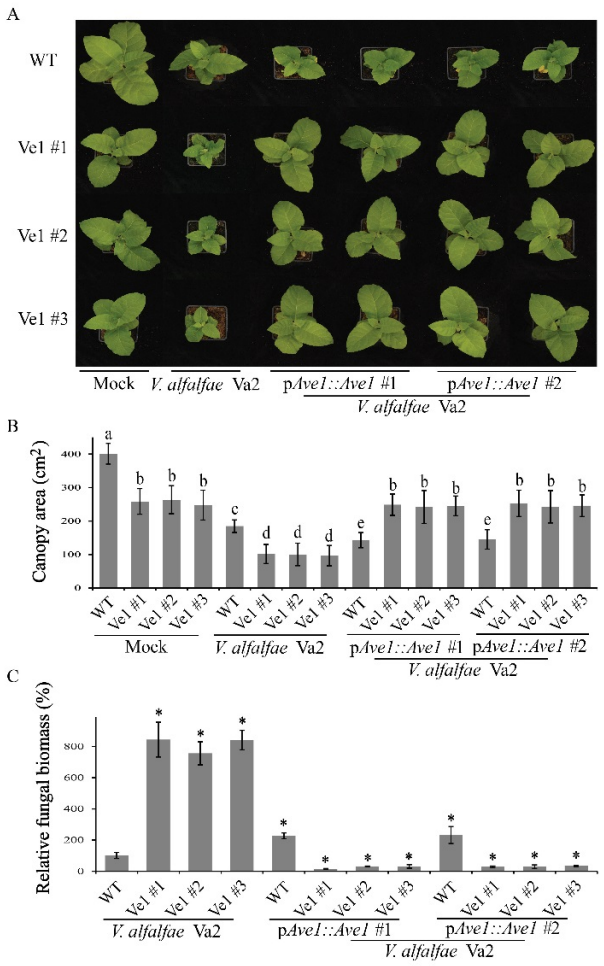

Figure 3. Tobacco plants expressing *Ve1* exhibit Ave1-triggered resistance against *Verticillium alfalfae*. (A) Typical appearance of wild-type tobacco cultivar Samsun plants (WT) and three independent *Ve1* transgenic tobacco plants (#1, #2 and #3) that were engineered to express tomato *Ve1* upon mock-inoculation, inoculation with *V. alfalfae* Va2, or two *Ave1*-experssing strains of *V. alfalfae* Va2 (p*Ave1::Ave1* #1 and #2) at 14 dpi. Inoculation experiments were performed with at least 16 plants for each fungal strain and independently repeated three times. **(B)** Quantification of the canopy area of tobacco plants at 14 dpi. Bars represent averages with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 14 dpi. Bars represent *Verticillium ITS* levels relative to tobacco *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tobacco cv. Samsun plants upon inoculation with the wild-type *V. alfalfae* strain Va2 is set to 100%. Asterisks indicate statistically significant differences when compared with tobacco cv. Samsun plants upon inoculation with the wild-type *V. alfalfae* strain Va2 (Student's *t*-test; $P < 0.05$). The data shown are representative of three independent experiments.

mutant of *V. nonalfalfae* Vna5431 displays compromised virulence on wild-type tobacco plants, *Ve1*-transgenic tobacco plants were clearly susceptible to this *Ave1* deletion mutant (Figure 2A and B). The phenotypes correlated with the degree of fungal colonization as determined by real-time PCR (Figure 2C). Additionally, the three independent *Ve1*-transgenic tobacco lines and non-transgenic controls were inoculated with either the wild-type *V. alfalfae* strain Va2, or the two independent *Ave1* expression strains, and monitored for the development of Verticillium wilt symptoms at 14 dpi. Intriguingly, upon inoculation with the *Ave1* expression strains, no symptoms of Verticillium wilt were observed on the *Ve1*-expressing tobacco plants, whereas the non-transgenic controls displayed clear symptoms of Verticillium wilt (Figure 3). Importantly, all *Ve1*-transgenic lines were susceptible to the wild-type *V. alfalfae* strain Va2. Collectively, these data show that tobacco plants expressing *Ve1* display enhanced Verticillium wilt resistance in an Ave1-dependent manner.

Generation of isogenic *V. dahliae* strains lacking *Ave1*

Verticillium wilt of cotton is mostly caused by *V. dahliae*, and thus far effective tools to control Verticillium wilt in cotton are lacking (Cai et al., 2009). In a previous attempt to investigate whether tomato *Ve1* can confer resistance against Verticillium wilt in cotton, transgenic cotton (*G. hirsutum* cv. YZ-1) lines that express tomato *Ve1* were generated, but no increased Verticillium wilt resistance was observed (Liu et al., 2014). However, it was realized later on that the *V. dahliae* strains used in this study did not contain *Ave1* (Liu et al., 2014).

To re-address the potential value of *Ve1* to engineer Verticillium wilt resistance in cotton, we pursued *Ave1*-carrying *V. dahliae* that can cause clear Verticillium wilt symptoms on *G. hirsutum* cv. YZ-1 plants. To this end, we tested *V. dahiae* strains JR2, V4, V991 and V117 (Table S1) on cotton cultivar YZ-1, and the development of Verticillium wilt symptoms was monitored at 21 dpi. As expected, differential degrees of Verticillum wilt symptoms were observed on these cotton plants (Figure S6A). Whereas *V. dahliae* strain JR2 that carries *Ave1* only induced mild symptoms on cotton, *V. dahliae* strain V4 that similarly carries *Ave1*, and *V. dahliae* strains V991 and V117 that both lack *Ave1* induced considerably stronger

Verticillium wilt symptoms (Figure S6). Thus, race 1 *V. dahliae* strain V4 was selected for further assays on cotton.

In order to obtain an isogenic line that lacks *Ave1*, targeted replacement of *Ave1* in *V. dahliae* strain V4 through homologous recombination was pursed (Figure S7A). To test whether the *Ave1* deletion strains of *V. dahliae* V4 indeed overcome recognition by Ve1, two independent *Ave1* deletion strains were inoculated onto tomato plants that express or lack *Ve1* (Figure S7A). As expected, *Ve1* tomato plants that were inoculated with two independent *Ave1* deletion strains of *V. dahliae* V4 showed a similar disease phenotype as *Ve1* tomato plants inoculated with the *V. dahliae* JR2Δ*Ave1* strain (Figure S7B and C; de Jonge et al., 2012), whereas *Ve1* tomato plants inoculated with wild-type *V. dahliae* strains V4 and JR2 resembled mock-inoculated *Ve1* tomato plants (Figure S7B and C). Moreover, the *Ave1* deletion strains of *V. dahliae* strain V4 displayed significantly reduced virulence on susceptible tomato plants when compared with the corresponding wild-type race 1 *V. dahliae* strain V4 (Figure S7B and D). These results are in line with previous results show that Ave1 acts as a virulence factor on tomato, and confirm that deletion of *Ave1* leads to escape of Ve1-mediated Verticillium wilt resistance (de Jonge et al., 2012).

Ave1 acts as a virulence factor on cotton

To investigate whether Ave1 acts as a virulence factor on cotton, two independent *Ave1* deletion strains and the corresponding wild-type strain V4 were used to inoculate cotton cv. YZ-1 plants. Interestingly, the *Ave1* deletion strains of *V. dahliae* V4 displayed clearly reduced virulence on wild-type cotton plants when compared with the corresponding wild-type strain up to 28 dpi (Figure S8A), as inoculation with *Ave1* deletion mutants resulted in significantly reduced stunting (Figure S8B) and compromised fungal colonization (Figure S8C). This assay demonstrates that Ave1 acts as a virulence factor also on cotton.

Cotton plants expressing *Ve1* exhibit enhanced Verticillium wilt resistance in an Ave1-dependent manner

To investigate if cotton plants constitutively expressing tomato *Ve1* display enhanced resistance against race 1 *V. dahliae*, two *Ve1*-trangenic lines (Ve1-4 and

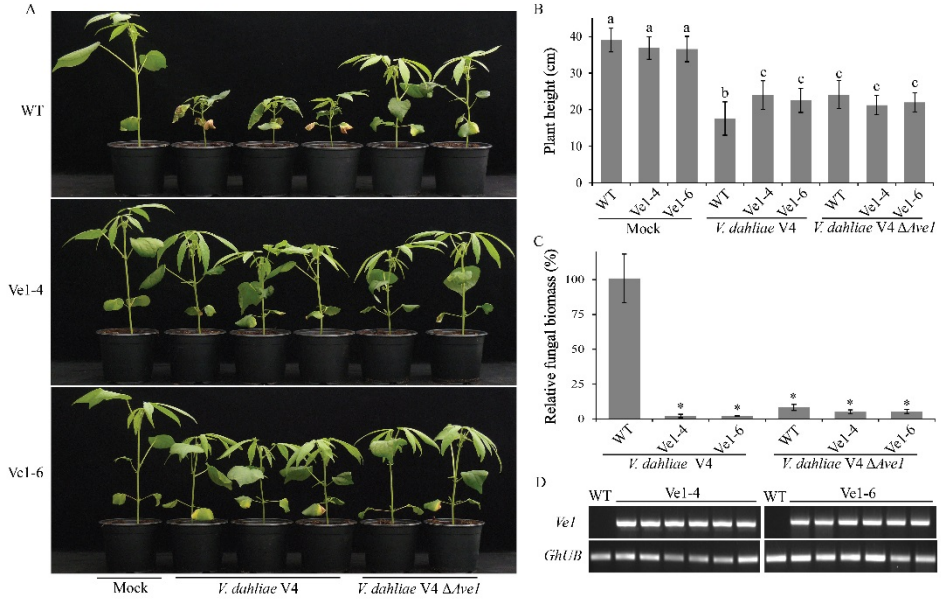

Figure 4. Cotton plants expressing *Ve1* display Ave1-triggerd resistance against *Verticillium dahliae*. (A) Typical appearance of wild-type cotton cultivar YZ-1 plants (WT) and two independent *Ve1* transgenic cotton plants (Ve1-4 and Ve1-6) upon mock inoculation, inoculation with *Ave1*-carrying *V. dahliae* V4, or an *Ave1* deletion strain of *V. dahliae* V4 (Δ*Ave1*) at 28 dpi. Inoculation experiments were performed with at least 20 plants for each fungal strain and independently repeated three times. **(B)** Quantification of *Verticillium*-induced plant stunting at 28 dpi. Bars represent averages with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 28 dpi. Bars represent *Verticillium ITS* levels relative to cotton *ubiquitin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in cotton cv. YZ-1 plants upon inoculation with the wild-type *V. dahliae* strain V4 is set to 100%. Asterisks indicate statistically significant differences when compared with cotton cv. YZ-1 plants upon inoculation with the wild-type *V. dahliae* strain V4 (Student's *t*-test; $P < 0.05$). **(D)** Expression of tomato *Ve1* in individual transgenic cotton plants and non-transgenic controls of cotton cv. YZ-1 (WT) as detected with reverse transcription-PCR (RT-PCR). As an endogenous control, a fragment of the cotton *ubiquitin* gene (*GhUB*) was amplified. The data shown are representative of three independent experiments.

Ve1-6) as well as non-transgenic control plants were inoculated with either the race 1 *V. dahliae* strain V4 or an *Ave1* deletion mutant (*V. dahliae* V4Δ*Ave1*), and monitored for Verticillium wilt symptoms up to 28 dpi. As expected, clear Verticillium wilt symptoms were observed on non-transgenic plants upon inoculation with *V. dahliae* strain V4 and with the corresponding *Ave1* deletion mutant (Figure 4A-C), despite the observation that *Ave1* deletion compromises

virulence on cotton. Interestingly, *Ve1*-expressing cotton plants exhibited significantly enhanced resistance against *V. dahliae* strain V4, as less Verticillium wilt symptoms were observed when compared with non-transgenic controls (Figure 4). When the two *Ve1*-transgenic lines and non-transgenic controls were challenged with *V. dahliae* strain V991 that does not carry *Ave1*, *Ve1*-expressing cotton lines were as susceptible as non-transgenic controls (Figure 5), confirming that the enhanced Verticillium wilt resistance upon *Ve1* expression is Ave1-dependent. Taken together, these data demonstrate that transfer of tomato immune receptor Ve1 into cotton confers Ave1-dependent Verticillium wilt resistance.

DISCUSSION

Major management strategies for Verticillium wilt diseases in crops include chemical and biological control, cultivation practices, and for the use of disease-resistant cultivars (Fradin and Thomma, 2006; Klosterman et al., 2009). Although chemical control has been proven to be successful for many diseases and pests, no truly effective fungicides are commercially available to control Verticillium wilt diseases once plants have been infected (Fradin and Thomma, 2006; Klosterman et al., 2009). Biocontrol measures and cultivation practices for controlling Verticillium wilts are time-consuming and laborious, and control effectiveness largely depends on the field conditions. Therefore, breeding for disease-resistant cultivars has been considered as the most sustainable approach to control Verticillium wilt diseases in crops (Fradin and Thomma, 2006; Klosterman et al., 2009). Presently, genetic resistance against *Verticillium* spp. has been described in several plant species, including tomato, potato, hop, alfalfa, cotton, strawberry, sunflower and lettuce (Schaible et al., 1951; Putt, 1964; Barrow, 1970; Lynch et al., 1997; Simko et al., 2004; Bolek et al., 2005; Mert et al., 2005; Wang et al., 2008; Yang et al., 2008; Hayes et al., 2011; Jakse et al., 2013; Antanaviciute et al., 2015; Christopoulou et al., 2015). However, only tomato *Ve1* has been cloned and characterized as a dominant gene responsible for race 1 Verticillium wilt resistance (Kawchuk et al., 2001; Fradin et al., 2009). We previously reported that homologues of tomato *Ve1* occur widespread in phylogenetically distant plant species (Song et al., 2017a). However, despite being widespread, *Ve1* homologues occur in a scattered fashion throughout plant phylogeny. For instance, a functional *Ve1* allele was identified in *N. glutinosa*,

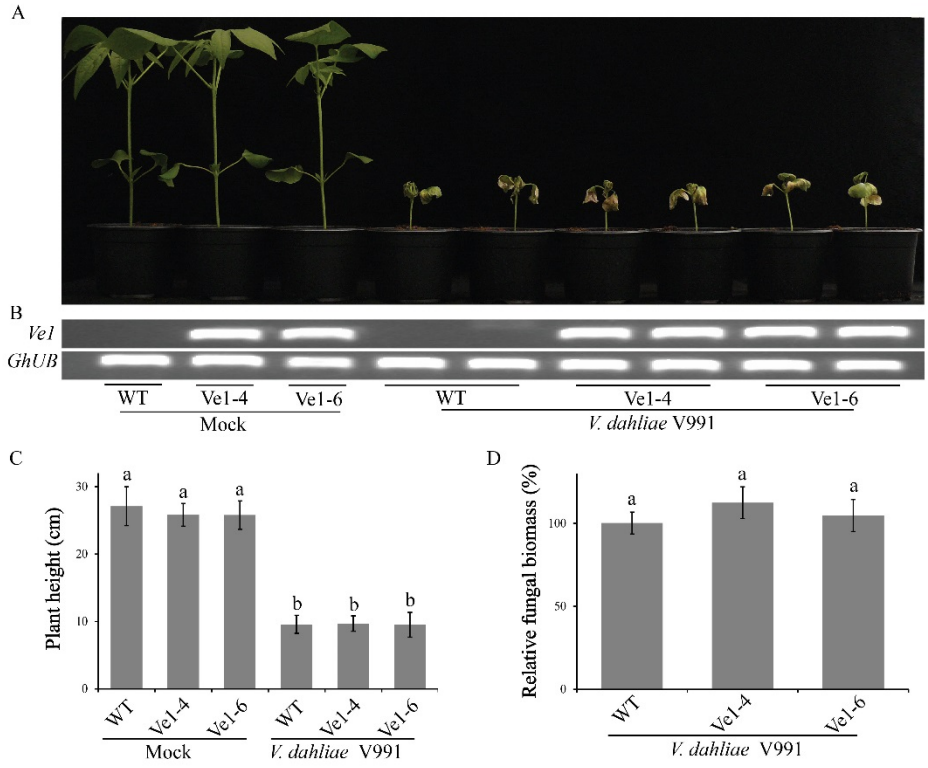

Figure 5. *Ve1*-transgenic and non-transgenic cotton plants are equally susceptible to *Verticillium dahliae* lacking *Ave1*. **(A)** Typical appearance of wild-type cotton cultivar YZ-1 plants (WT) and transgenic cotton plants expressing tomato *Ve1* upon mock inoculation or inoculation with *V. dahliae* strain V991 at 28 dpi. Inoculation experiments were performed with at least 20 plants for *V. dahliae* strain V991 and independently repeated three times. **(B)** Expression of tomato *Ve1* in individual cotton plants from wild-type controls and transgenic lines as detected with reverse transcription-PCR (RT-PCR). As an endogenous control, a fragment of the cotton *ubiquitin* gene (*GhUB*) was amplified. **(C)** Quantification of *Verticillium*-induced plant stunting at 28 dpi. Bars represent averages with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(D)** Fungal biomass as determined with real-time PCR at 28 dpi. Bars represent *Verticillium ITS* levels relative to cotton *ubiquitin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in cotton cv. YZ-1 plants upon inoculation with the *V. dahliae* strain V991 is set to 100%. Same letter labels indicate no statistically significant differences (Student's *t*-test; $P > 0.05$). The data shown are representative of three independent experiments.

but not in other species within the genus *Nicotiana* (Zhang et al., 2013a; Song et al., 2017a). This finding implies that, most likely, many plant species lost their functional *Ve1* homologues, but underlying immune signalling cascade may have been retained. Indeed, we have previously shown that heterologous expression of

tomato *Ve1* in the model plant *A. thaliana* that does not normally respond to Ave1, results in resistance against race 1 *Verticillium* spp. (Fradin et al., 2011; de Jonge et al., 2012; Zhang et al., 2013b). In this study, we investigated whether tomato *Ve1* can confer Verticillium wilt resistance when expressed in the crop species tobacco and cotton. We show that transgenic tobacco and cotton plants constitutively expressing tomato *Ve1* display enhanced resistance against Verticillium wilt in an Ave1-dependent manner. Thus, our results reveal that the functionality of tomato Ve1 in resistance against Verticillum wilt through recognition of the *Verticillium* effector Ave1 is retained after transfer to these plant species, and further support the view that the underlying immune signalling cascade mediated by Ve1 is retained in these plant species.

To date, several examples of transgenic expression of cell surface immune receptor genes resulting in enhanced disease resistance have been reported (Rodriguez-Moreno et al., 2017). Although transgenic expression of such receptors enhanced disease resistance, in some cases it also has adverse effects on plant fitness, such as growth retardation or leaf necrosis (Bouwmeester et al., 2011, 2014; Wang et al., 2016). In this study, we observed that expression of tomato *Ve1* in tobacco caused stunted growth (Figure 1 and S1). A similar growth defect has previously been observed in *N. benthamiana* plants that constitutively express *Ve1* (Fradin, 2011). In contrast, potato (Kawchuk et al., 2001), tomato (Fradin et al., 2009), *A. thaliana* (Fradin et al., 2011) and cotton (Figure 4 and 5) plants expressing *Ve1* do not suffer from such growth defects. Based on these findings, we speculate that expression of tomato *Ve1* in the genus *Nicotiana* may lead to a constitutive activation of downstream signalling cascade of tomato Ve1 that causes growth retardation. Alternatively, a ligand that is endogenous to these tobacco genotypes is recognized, leading to immune signalling activation.

Previously, we have shown that Ave1 acts as a virulence factor on tomato as well as on *A. thaliana* (de Jonge et al., 2012). In the present study we observed that targeted *Ave1* deletion results in significantly compromised virulence of *Verticillium* spp. on tobacco (Figure S5) and cotton (Figure S8), demonstrating that Ave1 acts as a virulence factor also on these plants. Previously, the bacterial homolog XacPNP from *X. axonopodis* pv. *citri* was characterized as a virulence factor on citrus trees (Nembaware et al., 2004; Gottig et al., 2008). More recently, Ave1 homologs from

the fungal tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* (FoAve1), and the fungal sugar beet leaf spot pathogen *C. beticola* (CbAve1) were characterized as virulence factors too. Although the intrinsic function of the fungal Ave1 homologs remains enigmatic, XacPNP is thought to manipulate the physiology of the host through plant natriuretic peptide activity that affects water homeostasis, stomatal opening, and photosynthesis to promote bacterial proliferation (Gottig et al., 2008; Garavaglia et al., 2010). It is tempting to speculate that the fungal homologs promote virulence through a similar activity, but this remains to be demonstrated. In any case, the finding that Ave1 promotes virulence on tomato, tobacco, cotton as well as *A. thaliana* suggests that the molecular target of the effector is widely conserved in the plant kingdom.

In summary, our data demonstrate that transfer of tomato *Ve1* into the closely related crop species tobacco and the distantly related crop species leads to enhanced resistance against Verticillium wilt in an Ave1-dependent manner. Given that Ave1 homologues were found in a number of pathogenic microbes (Nembaware et al., 2004; de Jonge et al., 2012; Gan et al., 2013), and these homologs were differentially recognized by tomato Ve1 (de Jonge et al., 2012; Song et al., 2017b), our findings may further broaden biotechnological avenues to exploit tomato Ve1 for engineering disease resistance in an Ave1(homolog)-dependent manner, for instance through transfer or artificial evolution of tomato *Ve1*.

MATERIALS AND METHODS

Plant materials and growth conditions

Nicotiana tabacum cv. Samsun, *N. glutinosa*, cotton (*Gossypium hirsutum* cv. YZ-1), and tomato (*Solanum lycopersicum* cv. Moneymaker (*ve1*) or *35S::Ve1* tomato (*Ve1*); Fradin et al., 2009) plants were used in this study and grown in commercial potting soil (Horticoop, Bleiswijk, the Netherlands) under controlled greenhouse conditions (Unifarm, Wageningen, the Netherlands).

N. tabacum cv. Samsun seeds were surface-sterilized by 70% ethanol and 1% commercial bleach, and grown on Murashige-Skoog (MS) medium (4.4 g MS salt, 20 g sucrose and 8 g agar in 1 L) or MS medium supplemented with antibiotics in a conditioned growth chamber at 21 °C/19 °C during 16 h/8 h light/dark photoperiods, respectively, and a relative humidity of ~75%.

Generation of *Ve1*-transgenic plants

Agrobacterium tumefaciens GV3101 (pMP90) carrying the binary vector pSol2095_Ve1 to encode C-terminally eGFP-tagged Ve1 (Figure 1A; Zhang et al., 2013a) was used for transformation of tobacco *N. tabacum* cv. Samsun. Transformation was performed by the leaf disk method as previously described (Wang et al., 2016). The generated plantlets were transferred to half-strength MS medium containing 200 mg/L kanamycin to allow root development. Upon root generation, plantlets were transferred into soil and grown in the greenhouse for seed production. Independent tobacco transformation lines were confirmed by PCR and reverse transcription-PCR (RT-PCR).

Two independent T0 generation cotton lines expressing tomato *Ve1* gene driven by the *cauliflower mosaic virus* 35S promoter (Ve1-4 and Ve1-6; Liu et al., 2014) were self-pollinated to generate seeds. After two generations of selfing, T2 seeds were used for further experiments.

Protein extraction, immunoprecipitation and immunoblotting

To test whether eGFP-tagged Ve1 protein accumulated in transgenic tobacco lines, leaves of six-week-old transgenic tobacco lines were harvested and ground into a fine powder in liquid nitrogen. As a positive control, *A. tumefaciens* carrying the binary vector pSol2095_Ve1 was infiltrated into mature *N. tabacum* cv. Samsun leaves as described previously (Zhang et al., 2013a). Total proteins were extracted by using extraction buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1.0% IGEPAL® CA-630 [NP-40] (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and one protease inhibitor cocktail tablet (Roche, Basel, Switzerland) per 50 mL extract buffer). Samples were centrifuged at 14,000 rpm for 20 min at 4°C, and then 2 mL of supernatant was incubated with 10 µL (50% slurry) of GFP-trap®_A beads (ChromoTek, Munich, Germany) at 4°C for 1 h. After incubation, GFP-trap®_A beads with proteins were spun down by 1,000 rpm centrifugation and subsequently washed for six times in 1 mL extraction buffer. After each wash step the GFP-trap®_A beads were collected by 1,000 rpm centrifugation. Proteins were released from GFP-trap®_A beads by boiling for 5 min, separated on a 10% SDS-PAGE gel and wet-electroblotted onto PVDF membrane (Bio-Rad, Hercules, USA). Accumulation of eGFP-tagged Ve1 was detected by immunoblotting using anti-GFP-

HRP antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientfic, Waltham, USA) was used for signal development. Coomassie blue staining was used as loading control.

Generation of *Ave1* mutant strains

Verticillium strains (Table S1) were grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) at 22°C. The *Ave1* knockout construct pRF-HU2_Ave1 that was described previously (de Jonge et al., 2012) was used to generate *Ave1* deletion mutants in *V. nonalfalfae* strain Vna 5431 and *V. dahliae* strain V4 (Table S1). The *Ave1* complementation construct pFBT 005_pAve1::Ave1 that was described earlier (Song et al., 2017b) was used to generate *Ave1* expression strains in *V. alfalfae* strain Va2 (Table S1).

Agrobacterium tumefaciens-mediated *Verticillium* transformation was performed as described previously (Santhanam, 2012), and *Verticillium* deletion transformants were selected on PDA (Oxoid, Basingstoke, UK) containing 200 µg/mL cefotaxime and 50 µg/mL hygromycin (Duchefa, Haarlem, the Netherlands). *Ave1* expression transformants were selected on PDA supplemented with 200 µg/mL cefotaxime (Duchefa, Haarlem, the Netherlands) and 50 µg/mL nourseothricin sulphate (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Putative *Verticillium* transformants were tested by PCR, and subsequent inoculation on *Ve1* tomato plants (*Ve1*) and tomato cultivar Moneymaker plants (*ve1*) (Fradin et al., 2009), or *N. glutionsa* plants carrying a functional *Ve1* homolog (Song et al., 2017a).

Disease assays

Verticillium conidiospores were collected from 7- to 10-day-old cultures on PDA plates and washed with tap water. Disease assays were performed on tomato, tobacco and cotton plants using the root-dipping inoculation method as previously described (Fradin et al., 2009). Briefly, 10-day-old *Ve1* or *ve1* tomato seedlings (for inoculation with *Verticillium Ave1* deletion strains), or four-week-old tobacco (*N. tabacum* cv. Samsun or *N. glutinosa*) plants or 10-day-old cotton seedlings were uprooted. Next, the roots were rinsed in water, dipped for 5 min in a suspension of 10^6 conidiospores/mL water while the roots of mock plants (control) were dipped

in tap water without conidiospores, and subsequently transplanted to fresh commercial potting soil (Horticoop, Bleiswijk, the Netherlands). Disease symptoms were scored up to 14 days post inoculation (dpi) (tomato, *N. glutinosa* and *N. tabacum* cv. Samsun), or 21 dpi (*N. tabacum* cv. Samsun), or 28 dpi (cotton). To this end, plants were photographed, and Image J was used to determine the canopy area (for quantification of stunting) while the rectilinear scale was used to measure the plant height (for quantification of growth). For fungal biomass quantification *in planta*, stems of three inoculated plants were harvested at 14 dpi (for *N. tabacum* cv. Samsun upon *V. alfalfae* inoculation), 21 dpi (for *N. tabacum* cv. Samsun upon *V. nonalfalfae* inoculation), or 28 dpi (cotton upon *V. dahliae* inoculation). The samples were ground into a fine powder in liquid nitrogen and genomic DNA was isolated. Real-time PCR was conducted by using the fungus-specific primers ITS-F and ITS-R (Table S2) with primers for tobacco *actin* (GenBank accession number: X69885; for *Verticillium*-infected tobacco) or cotton *ubiquitin* (GenBank accession number: DQ116441; for *Verticillium*-infected cotton) (Table S2) as an endogenous plant control, employing an ABI 7300 PCR system (Applied Biosystems, Foster City, CA, USA) with the qPCR Core kit for SYBR Green I (Eurogentec Nederland BV, Maastricht, the Netherlands).

Gene expression analysis

For the expression of *Ve1* in transgenic tobacco plants, leaves of six-week-old tobacco plants were harvested and ground into a fine powder in liquid nitrogen. Tobacco total RNA isolation and cDNA synthesis were performed as previously described (Song et al., 2017a). RT-PCR was conducted by using the primers Ve1-F(PCR) and Ve1-R(PCR) (Table S2), and *N. tabacum actin* gene (*NtACT*) (Table S2) was used as the endogenous control.

To check whether the *Ve1* gene is expressed in individual T2 cotton plants, leaves of five-week-old cotton plants were collected separately, flash frozen in liquid nitrogen and stored at -80°C for total RNA isolation. Cotton total RNA was isolated by using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) following the manufacturer's instructions. First-strand cDNA synthesis was performed by using M-MLV reverse transcriptase system (Promega, Wisconsin, USA). RT-PCR was conducted with primers Ve1-F(RT)

and Ve1-R(RT) (Table S2) in a total volume of 25 µl with 17.9 sterilized-water, 5 µl 5x PCR buffer, 0.5 µl dNTPs, 0.5 µl of each primer, 0.1 µl GoTag DNA polymerase (Promega, Wisconsin, USA) and 1.0 µl of first-strand cDNA. Primers GhUb-F and GhUb-R (Table S2) were used to amplify the cotton *ubiquitin* gene as endogenous loading control. PCR amplification consisted of an initial denaturation step of 95°C for 5 min, followed by denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s with 35 cycles. The resulting PCR products were subjected to agarose gel electrophoresis.

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SUPPLEMENTAL INFORMATION

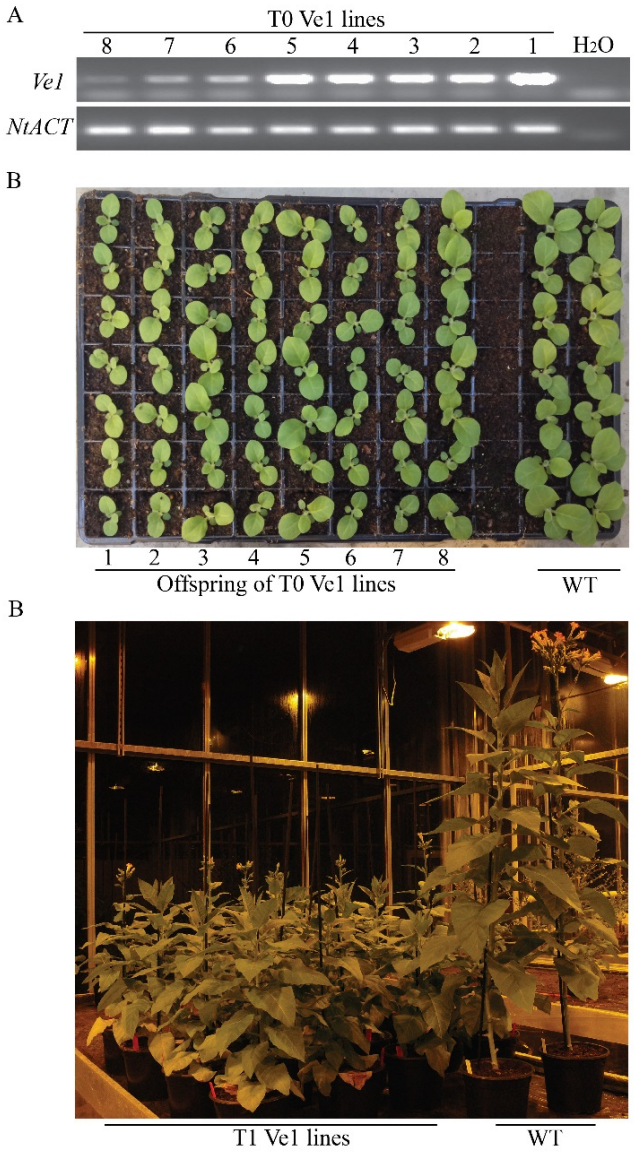

Figure S1. Characterisation of *Ve1*-transgenic *Nicotiana tabacum* cv. Samsun plants. (A) Transcripts of tomato *Ve1* in eight independent T0 transformation events were detected by reverse transcription-PCR (RT-PCR). As an endogenous control, a fragment of the *N. tabacum actin* gene (*NtACT*) was amplified. Water was used as a PCR control. **(B)** Morphology of four-week-old offspring from the eight T0 transformation events and the parental line. **(C)** Morphology of six-month-old T1 *Ve1*-transgenic tobacco lines and the recipient line tobacco cv. Samsun.

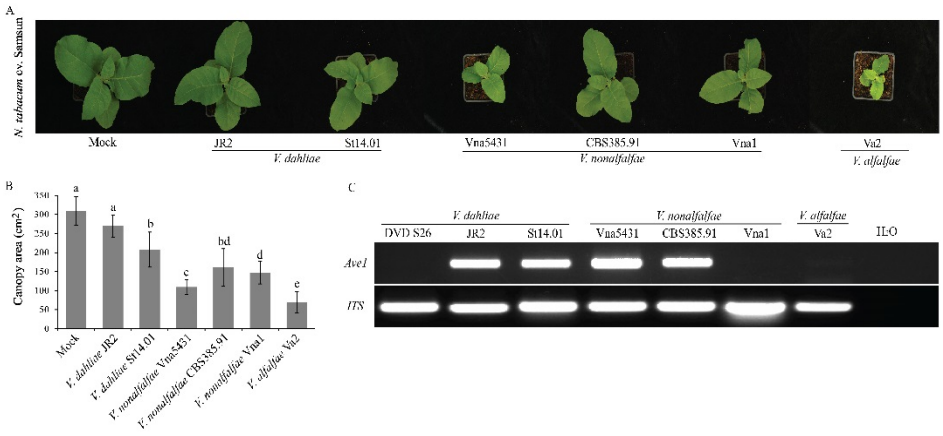

Figure S2. *Verticillium* strains induce differential degrees of Verticillium wilt symptoms on *N. tabacum* cv. Samsun plants. (A) Typical appearance of tobacco cv. Samsun plants upon mock-inoculation, inoculation with *V. dahliae* strains JR2 and St14.01, *V. nonalfalfae* strains Vna5431, CBS385.91 and Vna1, or *V. alfalfae* strain Va2 at 14 dpi. Inoculation experiments were performed with eight plants for each fungal strain and independently repeated twice. **(B)** Quantification of the canopy area of tobacco cv. Samsun plants at 14 dpi. Bars represent averages with standard deviation. Different letters indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Presence of the full-length *Ave1* coding DNA sequence in different *Verticillium* strains was determined by PCR amplification on genomic DNA. As an endogenous control, a fragment of the *Verticillium ITS* region was amplified. The race 2 *V. dahliae* strain DVD S26 that lacks *Ave1* and water were used as PCR controls. The data shown are representative of two independent experiments.

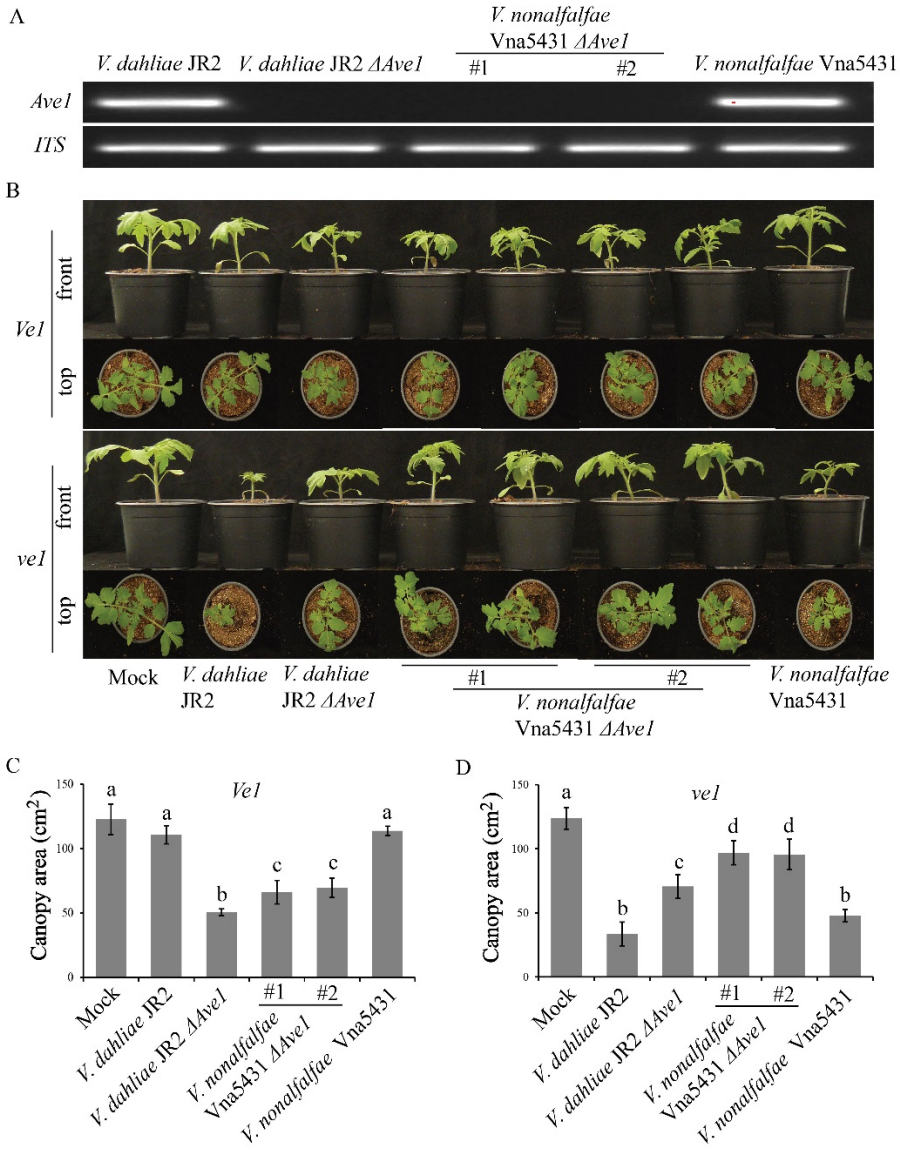

Figure S3. Analysis of *Ave1* deletion strains of *V. nonalfalfae* Vna5431. (A) Amplification of *Ave1* from genomic DNA in race 1 *V. dahliae* strain JR2, an *Ave1* deletion mutant of *V. dahliae* strain JR2 (Δ*Ave1*), race 1 *V. nonalfalfae* strain Vna5431 and two independent *Ave1* deletion strains (Δ*Ave1* #1 and #2). As an endogenous control, a fragment of the *Verticillium ITS* region was amplified. **(B)** Typical appearance of *Ve1* tomato plants (*Ve1*) and tomato plants lacking *Ve1* (*ve1*) upon mock-inoculation or inoculation with the various *Verticillium* strains at 14 days post inoculation (dpi). Average canopy area of eight *Ve1* **(C)** or *ve1* **(D)** tomato plants inoculated with the various *Verticillium* strains or mock-inoculation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). The data shown are representative of two independent assays.

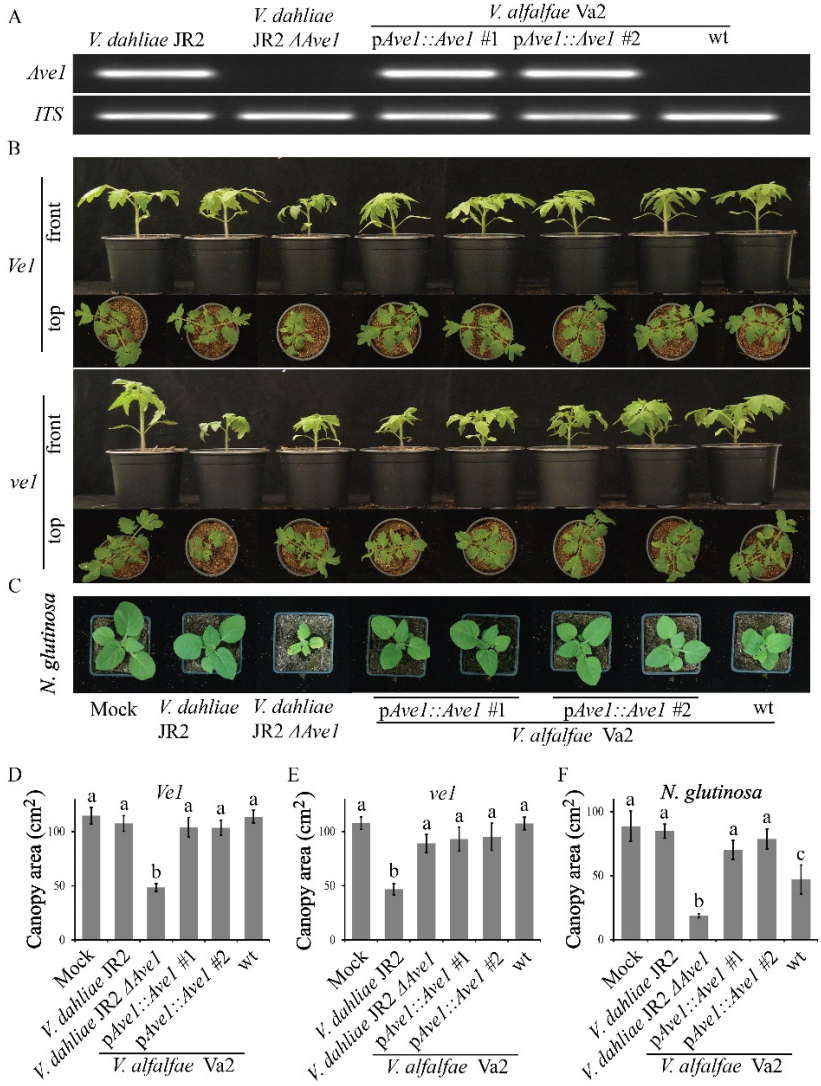

Figure S4. Analysis of ectopic expression *Ave1* strains in *V. alfalfae* Va2. (A) Amplification of *Ave1* from genomic DNA in race 1 *V. dahliae* strain JR2, an *Ave1* deletion mutant of *V. dahliae* strain JR2 (Δ*Ave1*), wild-type *V. alfalfae* strain Va2 (wt), and two independent *Ave1* expression strains (p*Ave1::Ave1* #1 and #2). As an endogenous control, a fragment of the *Verticillium ITS* region was amplified. **(B)** Typical appearance of *Ve1* tomato plants (*Ve1*) and tomato plants lacking *Ve1* (*ve1*) upon mock-inoculation or inoculation with the various *Verticillium* strains at 14 dpi. **(C)** Typical appearance of *Nicotiana glutinosa* plants upon mock-inoculation or inoculation with the various *Verticillium* strains at 14 dpi. Average canopy area of eight *Ve1* **(D)**, *ve1* **(E)** tomato plants, or *N. glutinosa* plants **(F)** inoculated with the various *Verticillium* strainsor mock-inoculation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). The data shown are representative of two independent assays.

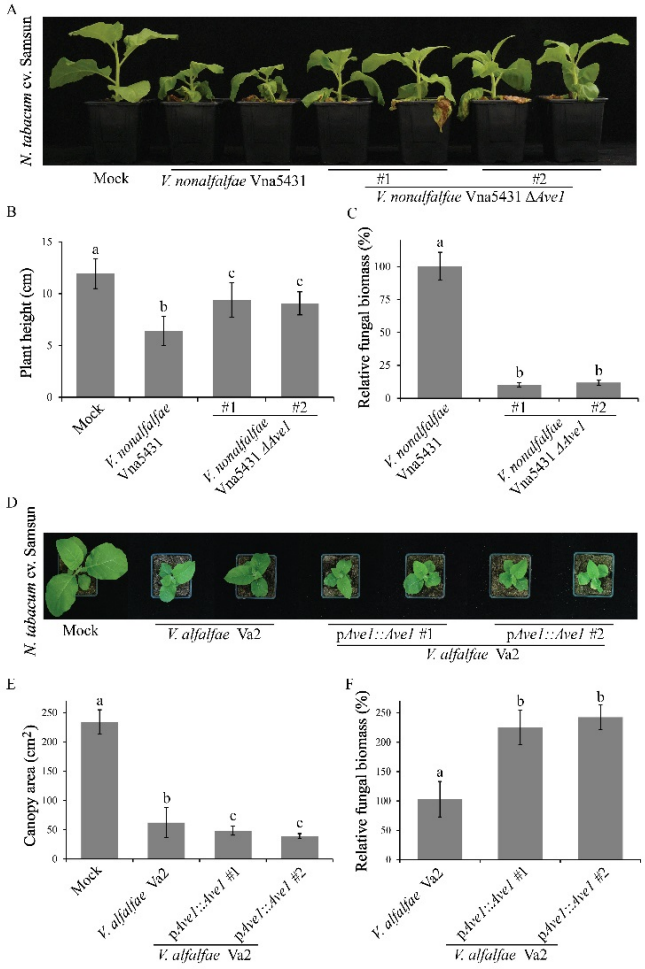

Figure S5. Ave1 acts as a virulence factor on tobacco cv. Samsun plants. (A) Typical appearance of tobacco cv. Samsun plants upon mock-inoculation, inoculation with *V. nonalfalfae* Vna5431, or two independent *Ave1* deletion strains (Δ*Ave1* #1 and #2) at 21 dpi. **(B)** Quantification of *Verticillium*-induced plant stunting at 21 dpi. Bars represent averages with standard deviation. **(C)** Fungal biomass as determined with real-time PCR at 21 dpi. Bars represent *Verticillium ITS* levels relative to tobacco *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tobacco plants upon inoculation with the wild-type *V. nonalfalfae* strain Vna5431 is set to 100%. **(D)** Typical appearance of tobacco cv. Samsun plants upon mock inoculation, inoculation with *V. alfalfae* Va2, or two *Ave1*-experssing strains of *V. alfalfae* Va2 (p*Ave1::Ave1* #1 and #2) at 14 dpi. **(E)** Quantification of in the canopy area of tobacco cv. Samsun plants at 14 dpi. Bars represent averages with standard deviation. **(F)** Fungal biomass as determined with real-time PCR at 14 dpi. Bars represent *Verticillium ITS* levels relative to tobacco *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tobacco cv. Samsun plants upon inoculation with the wild-type *V. alfalfae* strain Va2 is set to 100%. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). The data shown are representative of three independent assays.

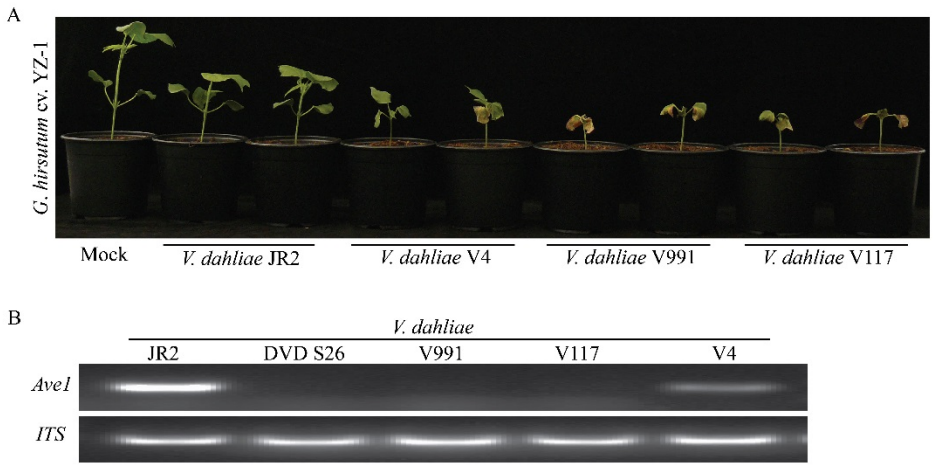

Figure S6. *V. dahliae* strains induce differential degrees of Verticillium wilt on cotton (*Gossypium hirsutum*) cv. YZ-1 plants. (A) Typical appearance of cotton cultivar YZ-1 plants upon mock-inoculation or inoculation with *V. dahliae* strains JR2, V4, V991 and V117 at 21 dpi. Inoculation experiments were performed with 10 plants for each *V. dahliae* strain and independently repeated twice. **(B)** Presence of the full-length *Ave1* coding DNA sequence was determined by PCR amplification on genomic DNA. As an endogenous control, a fragment of the *Verticillium ITS* region was amplified. The race 2 *V. dahliae* strain DVD S26 that lacks *Ave1* was used as a PCR control. The data shown are representative of two independent assays.

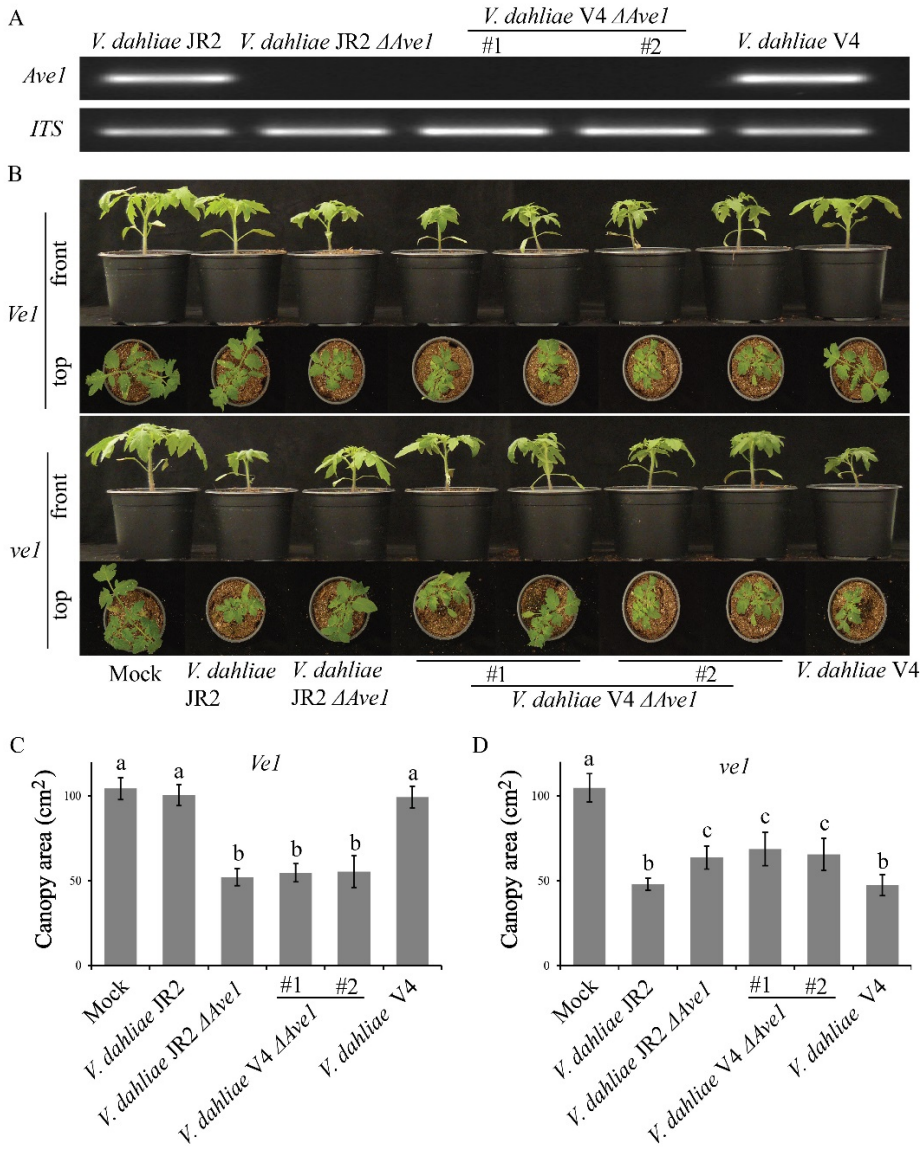

Figure S7. Analysis of *Ave1* deletion strains of *V. dahliae* V4. (A) Amplification of *Ave1* from genomic DNA in race 1 *V. dahliae* strain JR2, an *Ave1* deletion mutant of *V. dahliae* strain JR2 (Δ*Ave1*), race 1 *V. dahliae* strain V4 and two independent *Ave1* deletion strains (Δ*Ave1* #1 and #2). As an endogenous control, a fragment of the *Verticillium ITS* region was amplified. **(B)** Typical appearance of *Ve1* tomato plants (*Ve1*) and tomato plants lacking *Ve1* (*ve1*) upon mock-inoculation or inoculation with the various *V. dahliae* strains at 14 dpi. Average canopy area of eight *Ve1* **(C)** *ve1* **(D)** tomato plants inoculated with the various *V. dahliae* strains or mock-inoculation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). The data shown are representative of two independent assays.

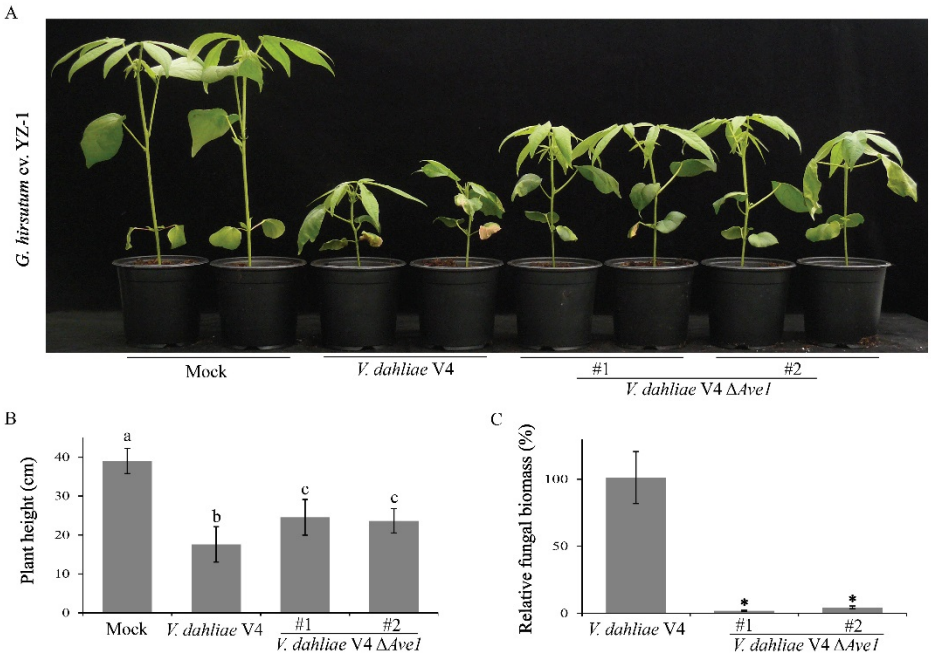

Figure S8. Ave1 acts as a virulence factor on cotton plants. (A) Typical appearance of cotton cultivar YZ-1 plants upon mock-inoculation or inoculation with *V. dahliae* V4 and two independent *Ave1* deletion strains (Δ*Ave1* #1 and #2) at 28 dpi. **(B)** Quantification of *Verticillium*-induced plant stunting at 28 dpi. Bars represent averages with standard deviation. Different letter labels indicate statistically significant differences (Student's *t*-test; $P < 0.05$). **(C)** Fungal biomass as determined with real-time PCR at 28 dpi. Bars represent *Verticillium ITS* levels relative to cotton *ubiquitin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in cotton plants upon inoculation with the wild-type *V. dahliae* strain V4 is set to 100%. Asterisks indicate statistically significant differences when compared with cotton plants upon inoculation with the wild-type *V. dahliae* strain V4 (Student's *t*-test; $P < 0.05$). The data shown are representative of three independent assays.

Table S1. *Verticillium* strains used in this study.

Strain	Description	Reference
V. dahliae JR2	Haploid, infecting tomato; *Ave1* presence	(de Jonge et al., 2012; Faino et al., 2015)
V. dahliae St14.01	Haploid, infecting tomato; *Ave1* presence	(Fradin et al., 2009; de Jonge et al., 2012)
V. dahliae DVD S26	Haploid, infecting tomato; *Ave1* absence	(de Jonge et al., 2012)
V. nonalfalfae Vna5431	Haploid, isolated from tomato; *Ave1* presence; Previously *V. albo-atrum*	(Fradin et al., 2009; Song et al., 2017);
V. nonalfalfae CBS385.91	Haploid, isolated from tomato; *Ave1* presence; Previously *V. albo-atrum*	(Fradin et al., 2009; Song et al., 2017)
V. nonalfalfae Vna1	Haploid, original host is unkown; *Ave1* absence; Previously *V. albo-atrum*	(Fradin et al., 2009; Song et al., 2017);
V. alfalfae Va2	Haploid, original host is unkown; *Ave1* absence; Previously *V. albo-atrum*	(Song et al., 2017);
V. dahliae V4	Haploid, infecting cotton; *Ave1* presence	(López-Escudero et al., 2004; Song et al., 2017)
V. dahliae V117	Haploid, infecting cotton; *Ave1* absence	(López-Escudero et al., 2004; Song et al., 2017)
V. dahliae V991	Haploid, infecting cotton; *Ave1* absence	(Xu et al., 2014)
V. dahliae JR2 Δ*Ave1*	*Ave1* deletion mutant in *V. dahliae* JR2	(de Jonge et al., 2012)
V. nonalfalfae Vna5431Δ*Ave1* #1	*Ave1* deletion mutant in *V. nonalfalfae* Vna5431, colony 1	This study
V. nonalfalfae Vna5431Δ*Ave1* #2	*Ave1* deletion mutant in *V. nonalfalfae* Vna5431, colony 2	This study
V. alfalfae Va2 p*Ave1::Ave1* #1	*V. alfalfae* strain Va2 expressing *Ave1*, colony 1	This study
V. alfalfae Va2 p*Ave1::Ave1* #2	*V. alfalfae* strain Va2 expressing *Ave1*, colony 2	This study
V. dahliae V4 Δ*Ave1* #1	*Ave1* deletion mutant in *V. dahliae* V4, colony 1	This study
V. dahliae V4 Δ*Ave1* #2	*Ave1* deletion mutant in *V. dahliae* V4, colony 2	This study

Table S2. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
Ve1-F(PCR)	CATATTGAAATTAGCGTCTTGTCGG	RT-PCR; *Ve1* expression in tobacco
Ve1-R(PCR)	ACCGAGAAAAAGGAGGCAAAAC	RT-PCR; *Ve1* expression in tobacco
Ave1-F	CACCATGAAGCTTTCTACGCTTGGAG	*Ave1*; PCR
Ave1-R	TTATATCTGTCTAAATTCGATGTTGAC	*Ave1*; PCR
ITS-F	AAAGTTTTAATGGTTCGCTAAGA	*Verticillium* ribosomal internal transcribed spacer region (ITS)
ITS-R	CTTGGTCATTTAGAGGAAGTAA	*Verticillium* ribosomal internal transcribed spacer region (ITS)
NtACT-F	CTATTCTCCGCTTTGGACTTGGCA	Tobacco *actin*
NtACT-R	AGGACCTCAGGACAACGGAAACG	Tobacco *actin*
GhUb-F	GAAGGCATTCCACCTGACCAAC	Cotton *ubiquitin*
GhUb-R	CAAAACTCCAAAATCATACCCAAAG	Cotton *ubiquitin*
Ve1-F(RT)	ATGGTTGCTGATGATTATGTGG	RT-PCR; *Ve1* expression in cotton
Ve1-R(RT)	AATCAGGCAATGGTGTAGGTG	RT-PCR; *Ve1* expression in cotton

[a]The type of experiment for which the primers were used is indicated in brackets (RT-PCR: Reverse Transcription-PCR).

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Chapter 6

Host-induced gene silencing compromises Verticillium wilt in tomato and *Arabidopsis*

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ABSTRACT

Verticillium wilt, caused by soil-borne fungi of the genus *Verticillium*, is an economically important disease that affects a wide range of host plants. Unfortunately, host resistance against Verticillium wilts is not available for many plant species, and the disease is notoriously difficult to combat. Host-induced gene silencing (HIGS) is an RNA interference (RNAi) based process in which small RNAs are produced by the host plant to target parasite transcripts. HIGS has emerged as a promising strategy for improving plant resistance against pathogens by silencing genes that are essential for these pathogens. Here, we assessed whether HIGS can be utilized to suppress Verticillium wilt disease by silencing three previously identified virulence genes of *V. dahliae* (encoding Ave1, Sge1 and NLP1) through the host plants tomato and *Arabidopsis*. In transient assays, tomato plants were agroinfiltrated with *Tobacco rattle virus* (TRV) constructs to target *V. dahliae* transcripts. Subsequent *V. dahliae* inoculation revealed suppression of Verticillium wilt disease upon treatment only with one of the three TRV constructs. Next, expression of RNAi constructs targeting transcripts of the same three *V. dahliae* virulence genes was pursued in stable transgenic *Arabidopsis thaliana* plants. In this host, *V. dahliae* inoculation revealed reduced Verticillium wilt disease in for two out of three targets. Thus, our study suggests that, depending on the target gene chosen, HIGS against *V. dahliae* is operational in tomato and *A. thaliana* plants and may be exploited to engineer resistance in Verticillium wilt-susceptible crops.

INTRODUCTION

Verticillium wilts are vascular wilt diseases that are caused by soil-borne fungi of the genus *Verticillium* (Fradin and Thomma, 2006; Klimes et al., 2015). This genus comprises ten species of soil-borne fungi that differ in their morphological features, such as resting structures, as well as in their ability to cause plant diseases (Inderbitzin et al., 2011). Within the *Verticillium* genus, *V. dahliae* is the most notorious pathogenic species that can infect hundreds of dicotyledonous hosts, including ecologically important plants and many high-value crops worldwide (Fradin and Thomma, 2006; Klosterman et al., 2009). Verticillium wilt diseases are difficult to control due to the long viability of the resting structures, the wide host range of the pathogens, and the inability of fungicides to affect the pathogen once in the plant vascular system. Thus, the most sustainable way to control Verticillium wilt diseases is the use of resistant cultivars. Polygenic resistance to *Verticillium* spp. has been described for several plant species, including potato, hop, alfalfa, cotton and strawberry (Antanaviciute et al., 2015; Bolek et al., 2005; Jakse et al., 2013; Simko et al., 2004; Wang et al., 2008; Yang et al., 2008;), whereas single dominant resistance genes have been identified only in tomato, sunflower, cotton, potato and lettuce (Barrow, 1970; Christopoulou et al., 2015; Hayes et al., 2011; Lynch et al., 1997; Mert et al., 2005; Putt, 1964; Schaible et al., 1951). In tomato (*Solanum lycopersicum*), a single dominant locus that confers *Verticillium* resistance has been identified as the *Ve* locus, which controls *Verticillium* isolates that are assigned to race 1, whereas race 2 strains escape recognition (Pegg, 1974; Schaible et al., 1951). The *Ve* locus contains two closely linked and inversely oriented genes, *Ve1* and *Ve2*, both of which encode extracellular leucine rich repeat (eLRR) receptor-like proteins (RLPs) (Kawchuk et al., 2001; Wang et al., 2010). Of these, only *Ve1* was found to confer resistance against race 1 isolates of *Verticillium* in tomato (Fradin et al., 2009). Interestingly, interfamily transfer of *Ve1* from tomato to *Arabidopsis thaliana* has resulted in race-specific *Verticillium* resistance in the latter species (Fradin et al., 2011, 2014; Zhang et al., 2014), implying that the underlying immune signaling pathway is conserved (Fradin et al., 2011; Thomma et al., 2011). Tomato Ve1 serves as an immune receptor for recognition of the effector protein Ave1 that is secreted by race 1 strains of *V. dahliae* (de Jonge et al., 2012). More recently, homologs of tomato Ve1 acting as immune receptors that govern

resistance against *V. dahliae* race 1 strains through recognition of the Ave1 effector have been characterized in other plant species including tobacco, potato, wild eggplant and hop, suggesting an ancient origin of the immune receptor Ve1 (Song et al., 2016).

Although the tomato *Ve1* gene is still currently deployed in tomato cultivars, isolates of *Verticillium* that escape Ve1-mediated recognition appeared within a few years after the introduction of the tomato *Ve1* (Pegg and Brady, 2002). These race 2 isolates of *Verticillium* steadily supplanted race 1 strains in various regions because of the extensive use of *Verticillium* race 1-resistant cultivars (Dobinson et al., 1996). Currently, no source of commercially employed resistance to *Verticillium* race 2 strains has been described.

RNA interference (RNAi) is a conserved regulatory mechanism that affects gene expression in eukaryotic organisms (Baulcombe, 2005). RNA silencing is triggered by the processing of double stranded RNA (dsRNA) precursors into short interfering RNA (siRNAs) duplexes of 21-28 nucleotides in length, and followed by the guided cleavage or translational repression of sequence-complementary single-stranded RNAs by the generated siRNAs duplexes, which are incorporated into a silencing complex called RISC (RNA-induced silencing complex) (Ruiz-Ferrer and Voinnet, 2009). Plants and other eukaryotes have evolved RNAi machineries that not only regulate developmental programs, but also provide protection from invaders, such as viruses. In plants, RNAi has been exploited extensively and has become a powerful functional genomics tool to silence the expression of genes of interest as well as to engineer viral resistance (Duan et al., 2012). Interestingly, organisms that live within, or develop intimate contact with, a host, such as bacteria (Escobar et al., 2001; 2002), nematodes (Huang et al., 2006), insects (Baum et al., 2007; Mao et al., 2007) and parasitic plants (Tomilov et al., 2008), are sensitive to small RNAs generated by the host and that are targeted to parasite transcripts. This so-called host-induced gene silencing (HIGS) has also emerged as a promising strategy against plant pathogens, including fungi and oomycetes. Initial reports of HIGS against filamentous pathogens were described for the maize kernel and ear rot pathogen *Fusarium verticillioides* (Tinoco et al., 2010) and the barley powdery mildew fungus *Blumeria graminis* (Nowara et al., 2010). Subsequent reports demonstrated the functionality of HIGS in suppressing diseases caused by the

fungal pathogens *Puccinia* spp. (Panwar et al., 2013; Yin et al., 2011; 2015; Zhang et al., 2012), *Fusarium* spp. (Koch et al., 2013; Ghag et al., 2014; Cheng et al., 2015; Hu et al., 2015; Chen et al., 2016), *Sclerotinia sclerotiorum* (Andrade et al., 2015) and *Rhizoctonia solani* (Zhou et al., 2016), as well as by the oomycete pathogens *Phytophthora infestans* (Jahan et al., 2015; Sanju et al., 2015) and *Bremia lactucae* (Govindarajulu et al., 2015). Many of these pathogens make very intimate contact with host cells, potentially facilitating the occurrence of HIGS. In this study, we assessed whether HIGS can be used to suppress Verticillium wilt disease in tomato and *A. thaliana* by targeting previously identified virulence factors of *V. dahliae.*

RESULTS

Tobacco rattle virus-based silencing in tomato compromises *V. dahliae Ave1* expression

Tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) has extensively been used in various plant species, including tomato (Liu et al., 2002; Senthil-Kumar et al., 2007), and TRV-based VIGS has successfully been used to investigate candidate genes for their involvement in Verticillium wilt resistance in tomato (Fradin et al., 2009). In order to investigate whether HIGS can be established against the xylem-colonizing fungus *V. dahliae*, we attempted to exploit TRV-based VIGS to produce dsRNAs that are targeted towards *V. dahliae Ave1* transcripts. The experiment was performed in *Ve1* tomato plants that are normally immune to infection by *Ave1*-carrying *V. dahliae* strains, such that successful HIGS would immediately result in vascular wilt disease that does not occur if *Ave1* expression is not compromised (Fradin et al., 2009; de Jonge et al., 2012). To this end, a 1:1 mixture of *Agrobacterium tumefaciens* cultures carrying *TRV1* and *TRV2::Ave1* (Figure 1A) was infiltrated into cotyledons of *Ve1* tomato plants. A recombinant construct containing a fragment of the *GUS* gene (*TRV2::GUS*) was used as a negative control (Figure 1A). At ten days after TRV treatment, plants were challenged with either the *V. dahliae* race 1 strain JR2 (Faino et al., 2015), or an *Ave1* deletion mutant (*V. dahliae* JR2⊿*Ave1*; de Jonge et al., 2012), and inspected for Verticillium wilt symptoms (stunting and wilting) up to 14 days post inoculation (dpi). As expected, no significant disease symptoms were observed on *TRV::GUS*-treated plants that were inoculated with the wild-type race 1 *V. dahliae* strain

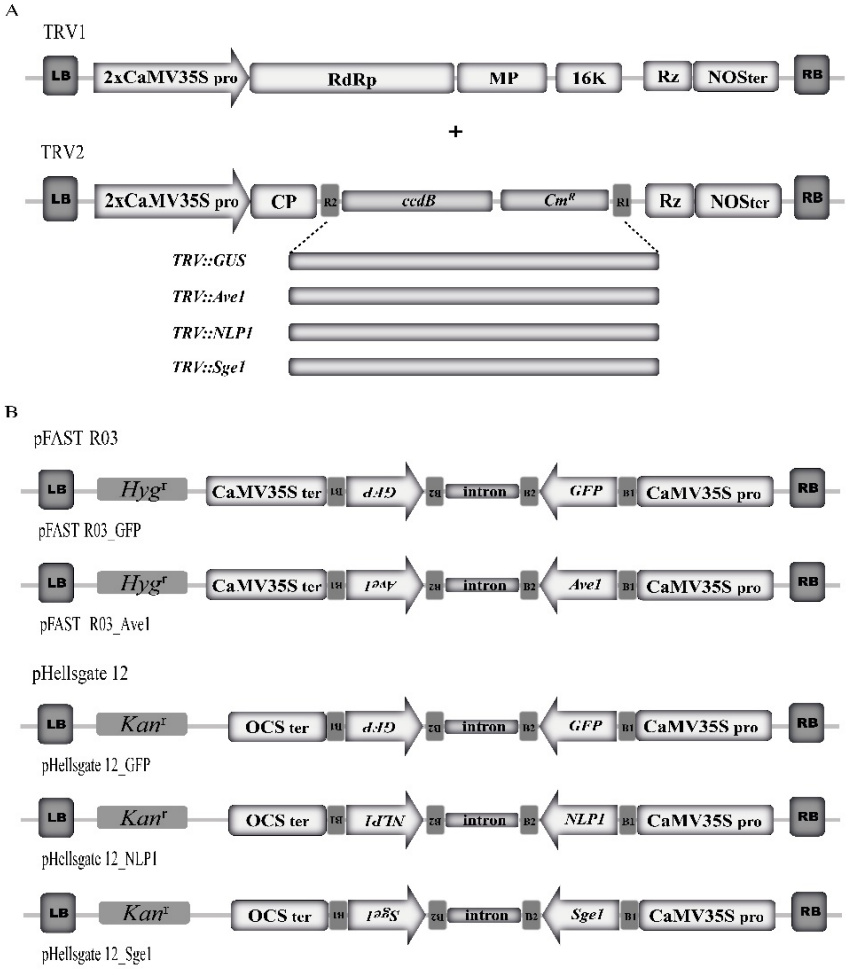

Figure 1. Schematic organization of the T-DNA region of the binary vectors used for gene silencing. (A) Schematic representation of the T-DNA region of the *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) vectors. *Verticillium dahliae Ave1*, *Sge1* and *NLP1* DNA fragments were inserted between the double CaMV35S promoter (2X35 CaMV35Spro) and the nopaline synthase gene terminator (NOSter) in the TRV2 vector to generate the TRV-based fungal gene silencing vectors *TRV::Ave1*, *TRV::Sge1* and *TRV::NLP1*, respectively. Control construct *TRV::GUS* was described earlier (Song et al., 2016). RdRp, RNA-dependent RNA polymerase; 16K, 16 kDa cysteine-rich protein; MP, movement protein; CP, coat protein; Rz, self-cleaving ribozyme; *ccdB*, negative selection marker used in bacteria; Cm^R, chloramphenicol resistance marker; R1 and R2, *att*R1 and *att*R2 sites. **(B)** Schematic diagrams of the T-DNA region of the binary vectors generated for producing a hairpin RNA of *Verticillium* genes *Ave1* (pFAST R03_Ave1), *NLP1* (pHellsgate 12_NLP1) and *Sge1* (pHellsgate 12_Sge1), as well as the *green fluorescent protein* gene (pFAST R03_GFP and pHellsgate 12_GFP) in transgenic *A. thaliana* plants. CaMV35Spro, CaMV35S promoter; CaMV35Ster, CaMV35S terminator; OCSter, octopine synthase gene terminator; Hyg^r, hygromycin resistance gene; Kan^r, kanamycin resistance gene; B1 and B2, *att*B1 and *att*B2 sites. LB and RB, left and right borders of T-DNA.

(Figure 2A), indicating that TRV treatment by itself does not compromise Ave1-triggered immunity in *Ve1* tomato plants. Furthermore, the *Ave1* deletion mutant caused clear Verticillium wilt disease, as Verticillium wilt disease developed on *Ve1* plants treated with *TRV2::Ave1* and subsequent inoculation with the *Ave1* deletion strain (Figure 2A). However, intriguingly, Verticillium wilt disease also developed on *Ve1* plants upon *TRV2::Ave1* treatment and subsequent inoculation with the wild-type race 1 *V. dahliae* strain (Figure 2A). This finding suggests that *Ave1* expression in *V. dahliae* is indeed compromised due to TRV-induced HIGS in tomato. The compromised immunity was confirmed by fungal recovery assays by plating stem sections on potato dextrose agar (PDA) plates, and by fungal biomass quantification in stem sections of the inoculated plants (Figure 2).

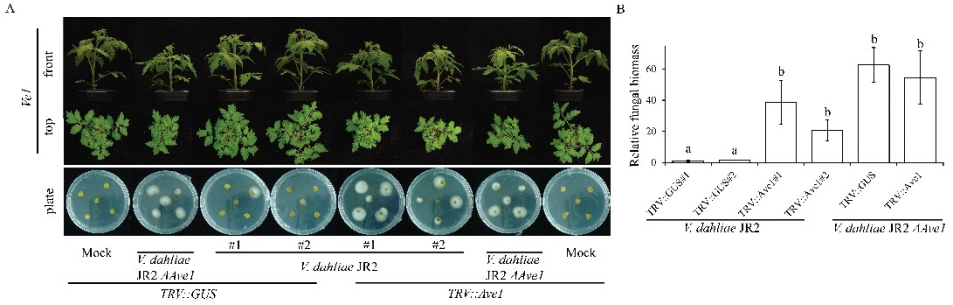

Figure 2. TRV-mediated fungal gene silencing in tomato plants compromises *Verticillium dahliae Ave1* expression. (A) Upon inoculation with the wild-type race 1 *V. dahliae* strain JR2, the impairment of Ave1-triggerred immunity in *Ve1* tomato plants treated with *TRV::Ave1* when compared with the *TRV::GUS*-treated plants evidenced by stunted *Ve1* plants at 14 days post inoculation (14 dpi) and fungal outgrowth upon plating stem sections on potato dextrose agar (PDA). The *Ave1* deletion mutant (*V. dahliae* JR2*ΔAve1*) was used as *Verticillium* inoculation control. Plants were photographed at 14 dpi. **(B)** Fungal biomass was determined by qPCR in *Verticillium*-inoculated *Ve1* plants at 14 dpi. Bars represent *Verticillium ITS* levels relative to tomato *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in *Ve1* tomato plants upon *TRV::GUS* treatment and subsequent inoculation with the wild-type race 1 *V. dahliae* strain is set to 1. Different letter labels indicate significant differences ($P < 0.05$). The data shown are representative of three independent experiments.

It was recently demonstrated that *Tobacco mosaic virus* (TMV) may infect fungi in addition to plants, remaining for up to six subcultures, and also persisted in plants infected by the virus-infected fungus (Mascia et al., 2014). This finding raises

the theoretical possibility that also TRV may infect *V. dahliae* and cause VIGS (directly) rather than HIGS from inside the tomato cells (indirectly). To exclude that the impairment of Ave1-triggered immunity in *Ve1* tomato plants is due to TRV-infection of *V. dahliae* itself, stem sections from *Verticillium*-inoculated *TRV::GUS*- and *TRV::Ave1*-treated tomato plants were placed on PDA plates. A single colony that grew from wild-type *V. dahliae*-inoculated *TRV::GUS*-treated plants (*V. dahliae* JR2$^{TRV::GUS}$) and *Ave1* deletion mutant-inoculated *TRV::Ave1*-treated plants (*V. dahliae* JR2$\Delta Ave1^{TRV::Ave1}$), and three independent colonies that grew from wild-type *V. dahliae*-inoculated *TRV::Ave1*-treated plants (*V. dahliae* JR2$^{TRV::Ave1}$) were subjected to PCR to detect a viral coat protein gene fragment of TRV (*TRV2_CP*), showing that *TRV2_CP* was not detected in all fungal isolates (Figure S1A). Furthermore, the fungal isolates were used to infect *Ve1* tomato plants and tomato plants that lack *Ve1*. This analysis showed that, similar to *V. dahliae* JR2$^{TRV::GUS}$, also *V. dahliae* JR2$^{TRV::Ave1}$ induced no disease symptoms on tomato plants expressing *Ve1*, while tomato plants lacking *Ve1* showed clear Verticillium wilt disease (Figure S1B-D). These data support the hypothesis that the impairment of Ave1-triggered immunity in *Ve1* plants is not caused by TRV-infection of *V. dahliae*, but genuinely by HIGS through TRV-treatment of tomato.

TRV-based fungal gene silencing in tomato inhibits Verticillium wilt disease

To further investigate the potential of TRV-mediated HIGS against *V. dahliae* in tomato, two previously identified virulence genes of *V. dahliae* were targeted. The first target gene is *NLP1*, encoding a member of the necrosis- and ethylene-inducing-like protein (NLP) family in *V. dahliae*, and targeted deletion of *NLP1* in *V. dahliae* significantly compromises virulence on tomato as well as on *A. thaliana* plants (Santhanam et al., 2013). The second candidate gene is *V. dahliae Sge1*, encoding a homolog of the transcription factor Sge1 (SIX Gene Expression 1) in *F. oxsporum*, and *V. dahliae* mutants of the *Sge1* are non-pathogenic on tomato (Santhanam and Thomma, 2013).

To produce dsRNAs of the gene fragments *in planta*, cotyledons of ten-day-old Moneymaker tomato plants were treated with the silencing constructs *TRV::GUS*, *TRV2::NLP1* and *TRV2::Sge1* in combination with *TRV1* (Figure 1A), respectively. At ten days after TRV treatment, plants were challenged with either the *V. dahliae*

strain JR2 (Faino et al., 2015), a *NLP1* deletion mutant (*V. dahliae* JR2⊿*NLP1*; Santhanam et al., 2013), or a *Sge1* deletion mutant (*V. dahliae* JR2⊿*Sge1*; Santhanam and Thomma, 2013), and monitored for Verticillium wilt symptoms on tomato plants at 14 dpi. As expected, significantly compromised Verticillium wilt symptoms were observed on Moneymaker tomato plants upon *TRV2::GUS* or *TRV2::NLP1*

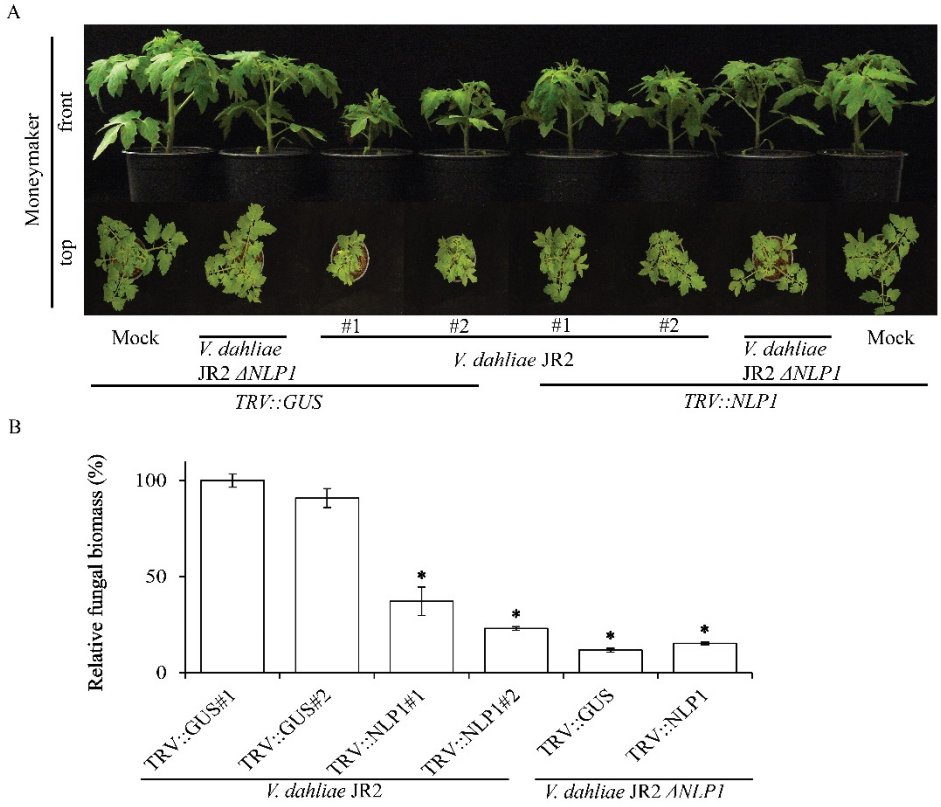

Figure 3. Effect of TRV-mediated *NLP1* silencing in Moneymaker tomato plants on *Verticillium dahliae* inoculation. (A) Agroinfiltration with the *TRV::NLP1* construct resulted in the suppression of Verticillium wilt symptoms on tomato plants, whereas no effect on disease development was observed on plants treated with the *TRV::GUS*. The *NLP1* deletion mutant (*V. dahliae* JR2*ΔNLP1*) was used as *Verticillium* inoculation control. Plants were photographed at 14 dpi. **(B)** Fungal biomass was determined by qPCR in *Verticillium*-inoculated Moneymaker tomato plants at 14 dpi. Bars represent *Verticillium ITS* levels relative to tomato *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tomato plants upon *TRV::GUS* treatment and subsequent inoculation with the wild-type *V. dahliae* strain JR2 is set to 100% (control). Asterisks indicate significant differences when compared with the *TRV::GUS*-treated plants upon inoculation with the *V. dahliae* strain JR2 ($P < 0.05$). The data shown are representative of three independent experiments.

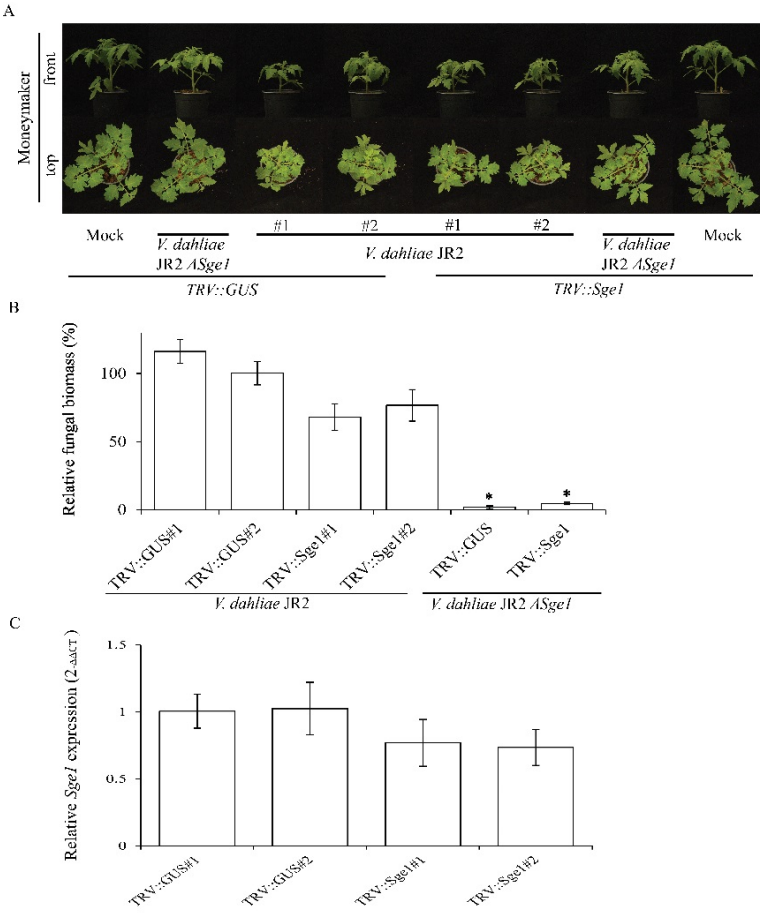

Figure 4. Effect of TRV-mediated *Sge1* silencing in tomato plants on *Verticillium dahliae* inoculation. (A) Upon inoculation with the *V. dahliae* strain JR2, no effect on disease development was observed on *TRV::Sge1*-treated plants compared to *TRV::GUS*-treated plants. The *Sge1* deletion mutant (*V. dahliae* JR2*ΔSge1*) was used as *Verticillium* inoculation control. Plants were photographed at 14 dpi. **(B)** Fungal biomass was determined by qPCR in *Verticillium*-inoculated Moneymaker tomato plants at 14 dpi. Bars represent *Verticillium ITS* levels relative to tomato *actin* levels (for equilibration) with standard deviation in a sample of three pooled plants. The fungal biomass in tomato plants upon *TRV::GUS* treatment and subsequent inoculation with the wild-type *V. dahliae* strain JR2 is set to 100% (control). Asterisks indicate significant differences when compared with the *TRV::GUS*-treated plants upon inoculation with the *V. dahliae* strain JR2 ($P < 0.05$). The data shown are representative of three independent assays. **(C)** Relative expression level for the *Sge1* gene was determined by using RT-qPCR at 14 days post inoculation with the wild-type *V. dahliae* strain on *TRV::Sge1*- and *TRV::GUS*-treated plants. Bars represent levels of *Sge1* transcripts relative to the transcript levels of *V. dahliae GAPDH* (GAPDH, glyceraldehyde-3-phosphate dehydrogenase; for normalization) with standard deviation of a sample of three pooled plants. *Sge1* expression in *V. dahliae* in the *TRV::GUS*-treated plants upon inoculation the wild-type strain *V. dahliae* is set to 1. The data shown are representative of three independent experiments.

treatment and subsequent inoculation with the *NLP1* deletion mutant of *V. dahliae* strain JR2 (Figure 3A). Interestingly, upon inoculation with the wild-type *V. dahliae* strain JR2, a moderate reduction of Verticillium wilt symptoms was observed on Moneymaker tomato plants treated with *TRV2::NLP1* when compared to *TRV::GUS*-treated plants (Figure 3A). The plants that were treated with *TRV::NLP1* and subsequent inoculation with the wild-type *V. dahliae* strain JR2 showed reduced Verticillium wilt symptoms but were not as diseased as plants upon inoculation with the *NLP1* deletion mutant or water (Figure 3A). These data are further supported by fungal biomass quantifications in stem sections of the inoculated plants (Figure 3B). In contrast, no significant Verticillium wilt disease reduction was observed in Moneymaker tomato plants upon the *TRV::Sge1* treatment and subsequent inoculation with the wild-type *V. dahliae* strain JR2, although fungal biomass quantifications revealed that less fungal biomass accumulated *in planta* in the *TRV::Sge1*-treated plants than the *TRV::GUS*-treated plants followed by inoculation with the wild-type *V. dahliae* strain JR2 (Figure 4A, B). To determine whether TRV-mediated targeting transcripts of *V. dahliae Sge1* in tomato results in complete silencing of the *V. dahliae Sge1* gene, we performed reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to measure relative expression level for the *Sge1* gene in *V. dahliae* JR2 inoculating with the *TRV::Sge1*-treated plants compared to the *TRV::GUS*-treated plants. However, only a slight reduction in *Sge1* expression in *TRV::Sge1*-targeted *V. dahliae* was monitored when compared with *TRV::GUS*-targeted *V. dahliae* (Figure 4C). In conclusion, although not all TRV-based RNAi constructs targeting *V. dahliae* transcripts in tomato suppressed Verticillium wilt disease, TRV-mediated transient HIGS against *V. dahliae* in tomato can be achieved.

HIGS in *Ve1*-transgenic *A. thaliana* does not impair Ave1-triggerred immunity

To assess whether HIGS against *V. dahliae* can be made operational in stable transgenic plants by expressing dsRNAs, we exploited hairpin RNA-based RNAi to produce dsRNAs to target *V. dahliae Ave1* transcripts in *Ve1*-expressing *A. thaliana* plants. To this end, a fragment of the *V. dahliae Ave1* gene was cloned into the

Gateway vector pFAST R03 (Shimada et al., 2010) to obtain the RNAi construct pFAST R03_Ave1 (Figure 1B) that leads to hairpin RNA formation after transcription. A recombinant RNAi construct containing a fragment of the *green fluorescent protein* (*GFP*) gene (pFAST R03_GFP) was used as a negative control (Figure 1B). Subsequently, the *Ave1* and *GFP* RNAi constructs were transformed into recipient *Ve1*-expressing *A. thaliana* plants (Fradin et al., 2011; Figure S2A). No obvious developmental alterations were observed in the transgenic plants when

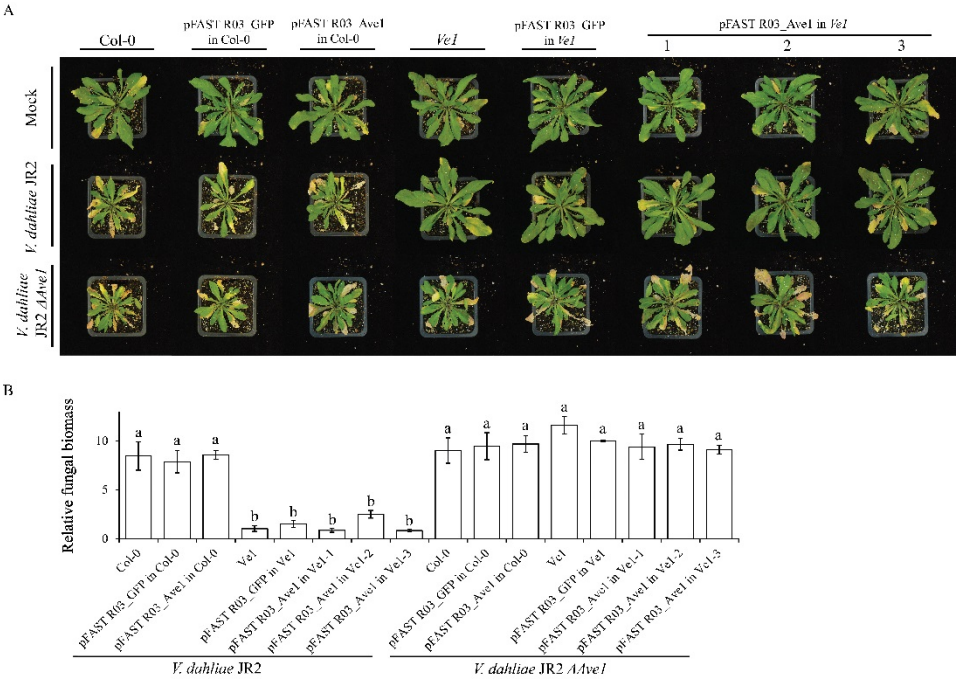

Figure 5. Analysis of *Ve1 Arabidopsis thaliana* plants expressing RNAi *Ave1* construct. (A) Upon inoculation with the race 1 *V. dahliae* strain JR2, *Ve1* plants expressing RNAi *Ave1* or *GFP* construct do not show Verticillium wilt symptoms, whereas typical Verticillium wilt symptoms are recorded on plants with or without tomato *Ve1* upon inoculation with either the *V. dahliae* JR2 or *V. dahliae* JR2*ΔAve1* at 21 dpi. Col-0 plants with or without tomato *Ve1* were used as a controls. The *V. dahliae* JR2*ΔAve1* strain was used as *Verticillium* inoculation control. **(B)** Fungal biomass was determined with qPCR in *Verticillium*-inoculated *Arabidopsis* plants at 21 dpi. Bars represent *Verticillium ITS* levels relative to *AtRuBisCo* (RuBisCo, ribulose-1, 5-bisphoshate-carboxylase/oxygenase) levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in *Ve1* plants upon inoculation with the wild-type race 1 *V. dahliae* strain JR2 is set to 1 (control). Three independent lines carrying the pFAST R03_Ave1 construct are shown (1, 2 and 3). Different letter labels indicate significant differences ($P < 0.05$). The data shown are representative of at least three independent experiments.

compared with the recipient Col-0 and *Ve1* plants (Figure 5A), and three independent *Ave1* RNAi lines expressing *Ve1* (pFAST R03_Ave1 in *Ve1*-1, pFAST R03_Ave1 in *Ve1*-2 and pFAST R03_Ave1 in *Ve1*-3) as well as transgenic and non-transgenic control lines were inoculated with either the *V. dahliae* race 1 strain JR2 or an *Ave1* deletion mutant, and monitored for Verticillium wilt symptoms up to 21 dpi. As expected, upon mock-inoculation or inoculation with the *V. dahliae* JR2, no disease symptoms were observed in *Ve1* plants and *GFP*-RNAi *Ve1* plants (Figure 5A). In contrast, *GFP*- or *Ave1*-RNAi Col-0 plants lacking *Ve1* were as diseased as non-transformed control lines (Figure 5A). However, despite transcripts for hairpin *Ave1* formation in *Ave1*-RNAi *Ve1* plants were detected (Figure S2A), Verticillium wilt symptoms were not observed in *Ave1*-RNAi *Ve1* plants upon inoculation with the wild-type race 1 *V. dahliae* strain JR2 in repeated assays, while the *Ave1* deletion strain caused clear Verticillium wilt symptoms on *Ve1* plants (Figure 5A). The phenotypes correlated with the degree of *V. dahliae* colonization as determined with qPCR (Figure 5). These data show that expression of an RNAi construct targeting *Ave1* transcripts in *A. thaliana* plants expressing *Ve1* does not compromise Ave1-triggered immunity.

HIGS in *A. thaliana* can reduce Verticillium wilt

To further investigate whether HIGS against *V. dahliae* can be established in stable transgenic *A. thaliana* plants by expressing dsRNAs, *V. dahliae NLP1* and *Sge1* were targeted. To this end, RNAi constructs pHellsgate 12_NLP1 and pHellsgate 12_Sge1 were generated (Figure 1B). The recombinant RNAi construct carrying a fragment of the *GFP* gene (pHellsgate 12 _GFP) was used as a negative control (Figure 1B). Subsequently, RNAi constructs targeting *NLP1*, *Sge1*, or *GFP* were transformed into *A. thaliana* ecotype Col-0, and independent *NLP1*-, *Sge1*, or *GFP*-RNAi lines were selected (Figure S2B, C). No phenotypic alterations were observed in *NLP1*- or *Sge1*-RNAi plants when compared with the recipient *A. thaliana* Col-0 plants or *GFP*-RNAi plants (Figure 6A, 7A). Three independent *NLP1*-RNAi lines (pHellsgate 12_*NLP1*-1, pHellsgate 12_*NLP1*-2 and pHellsgate 12_*NLP1*-3) as well as *GFP*-RNAi and non-transgenic control lines were assayed for the development of Verticillium wilt symptoms. As expected, markedly compromised Verticillium wilt symptoms were observed on *A. thaliana* plants upon inoculation with the *NLP1* deletion

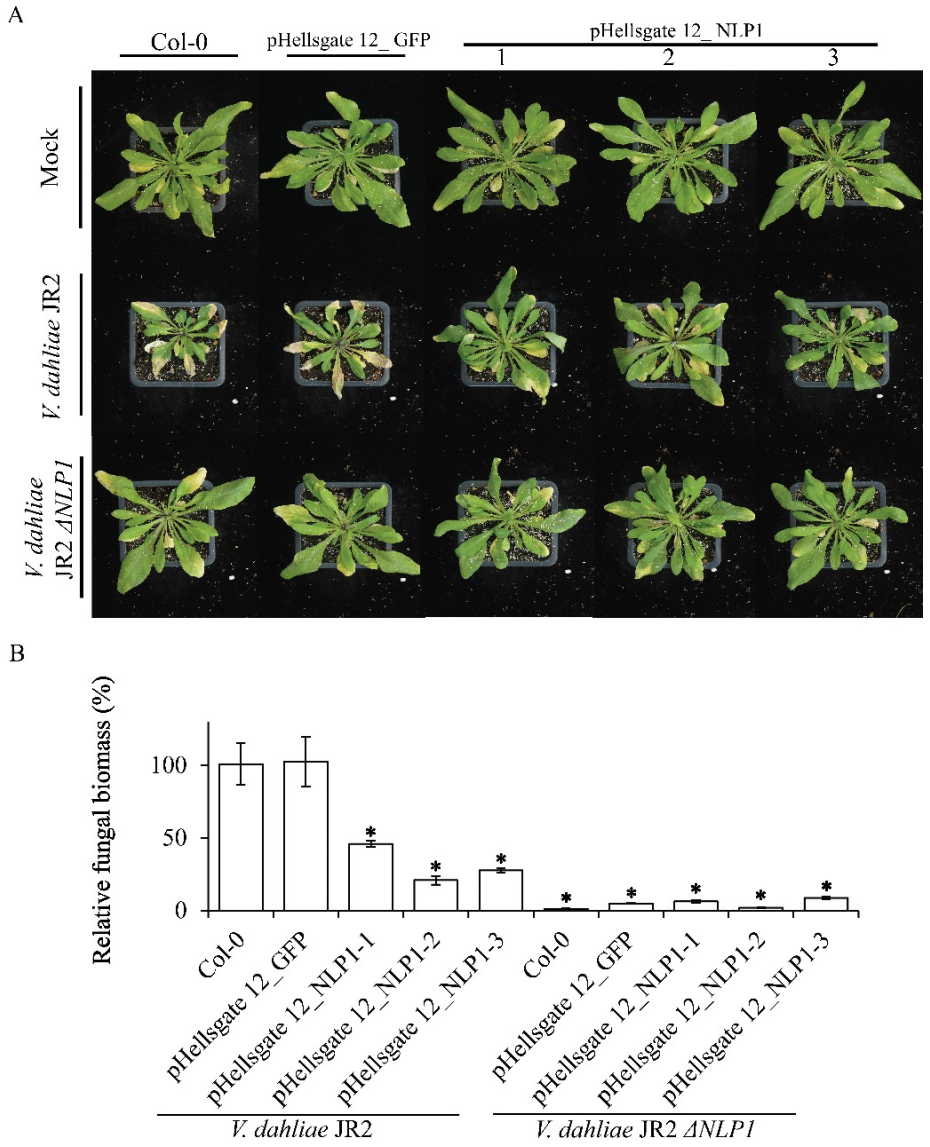

Figure 6. *Arabidopsis thaliana* Col-0 plants expressing *NLP1* RNAi construct show enhanced resistance against *Verticillium dahliae*. (A) Typical appearance of non-transgenic *A. thaliana* and transgenic lines carrying the pHellsgate 12_NLP1 construct to target *NLP1* transcripts upon mock-inoculation or inoculation with *V. dahliae* strain JR2 or *V. dahliae* JR2*ΔNLP1* at 21 dpi. **(B)** Fungal biomass was determined with qPCR in *Verticillium*-inoculated *Arabidopsis* plants at 21 dpi. Bars represent *Verticillium ITS* levels relative to *AtRuBisCo* (RuBisCo, ribulose-1, 5-bisphoshate-carboxylase/oxygenase) levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in Col-0 is set to 100% (control). Three independent lines carrying the pHellsgate 12_NLP1 construct are shown (1, 2 and 3). Asterisks indicate significant differences when compared with Col-0 ($P < 0.05$). The data shown are representative of three independent experiments.

mutant (Figure 6A). Interestingly, upon inoculation with the *V. dahliae* JR2, a significant reduction of Verticillium wilt symptoms was observed in *NLP1*-RNAi plants when compared with *GFP*-RNAi and non-transgenic controls (Figure 6A). These data are further supported by fungal biomass quantifications in stem sections of the inoculated plants (Figure 6B). Additionally, three independent *Sge1*-RNAi lines (pHellsgate 12_*Sge1*-1, pHellsgate 12_*Sge1*-2 and pHellsgate 12_*Sge1*-3) as well as *GFP*-RNAi and non-transformed control lines were assayed for the development of Verticillium wilt disease. Intriguingly, we observed a marked reduction of Verticillium wilt symptoms in *Sge1*-RNAi *A. thaliana* lines inoculated with *V. dahliae* JR2 (Figure 7A). In contrast, *GFP*-RNAi *A. thaliana* lines were as susceptible as non-transgenic control lines (Figure 7A). The phenotypes correlated with the level of *V. dahliae* biomass as determined with qPCR (Figure 7). Collectively, these results suggest that, although not all RNAi constructs targeting *V. dahliae* transcripts in *A. thaliana* induced HIGS against *V. dahliae*, hairpin RNA-mediated HIGS in *A. thaliana* can reduce Verticillium wilt disease.

DISCUSSION

In this manuscript, we show that HIGS against *V. dahliae* can be achieved through TRV-based fungal gene silencing in tomato, and through hairpin RNA-mediated fungal gene silencing in stable transgenic *A. thaliana* lines. We established the TRV-mediated HIGS assay through targeting *V. dahliae Ave1* transcripts in *Ve1* tomato plants, and further used this approach to assess whether HIGS against *V. dahliae* in tomato can be established through TRV constructs targeting previously identified *V. dahliae* virulence factors. We also investigated whether HIGS against *V. dahliae* can be established in transgenic *A. thaliana* plants through hairpin RNA-based RNAi constructs targeting transcripts of the same previously identified *V. dahliae* virulence genes. Our results clearly show that plants transiently (in tomato) or stably (in *A. thaliana*) expressing RNAi constructs targeting transcripts of genes that are essential for *V. dahliae* pathogenicity can become protected from Verticillium wilt disease. Our results are in line with, and extend beyond, recent reports on protection of cotton plants stably expressing an RNAi construct against *V. dahliae* (Zhang et al., 2016), and on bidirectional cross-kingdom RNAi and fungal uptake of external RNAs to confer plant protection (Wang et al., 2016).

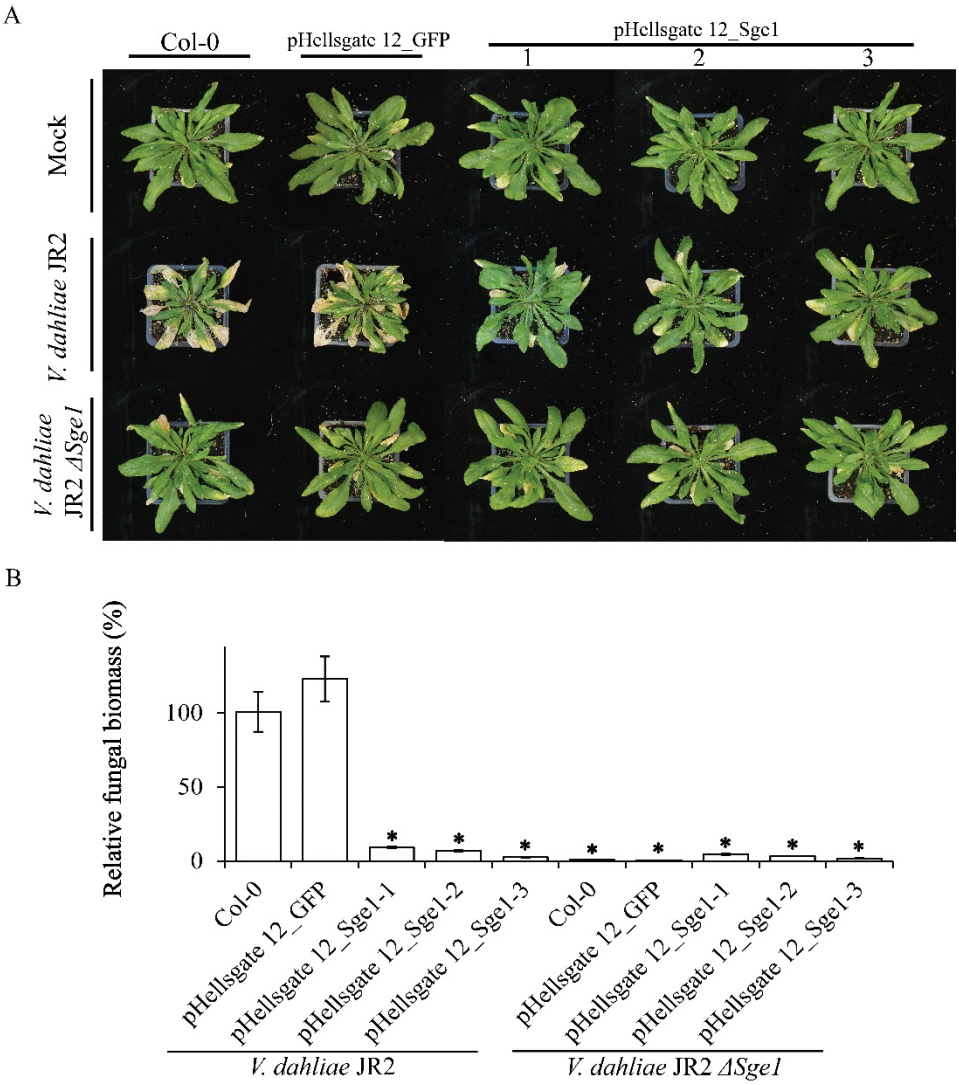

Figure 7. ***Arabidopsis thaliana*** **Col-0 plants expressing** ***Sge1*** **RNAi construct show enhanced resistance against** ***Verticillium dahliae*****. (A)** Typical appearance of non-transgenic *A. thaliana* and transgenic lines harboring the pHellsgate 12_Sge1 construct to target *Sge1* transcripts upon mock-inoculation or inoculation with *V. dahliae* strain JR2 or *V. dahliae* JR2Δ*Sge1* at 21 dpi. **(B)** Fungal biomass was determined with qPCR in *Verticillium*-inoculated *Arabidopsis* plants at 21 dpi. Bars represent *Verticillium ITS* levels relative to *AtRuBisCo* (RuBisCo, ribulose-1, 5-bisphoshate-carboxylase/oxygenase) levels (for equilibration) with standard deviation in a sample of five pooled plants. The fungal biomass in Col-0 is set to 100% (control). Three independent lines carrying the pHellsgate 12_Sge1 construct are shown (1, 2 and 3). Asterisks indicate significant differences when compared with Col-0 ($P < 0.05$). The data shown are representative of three independent experiments.

Reports on HIGS against fungal pathogen infections have accumulated over recent years (Andrade et al., 2015; Chen et al., 2016; Cheng et al., 2015; Ghag et al., 2014; Hu et al., 2015; Koch et al., 2013; Panwar et al., 2013; Yin et al., 2011; 2015; Zhang et al., 2012; Zhou et al., 2016). Among these reports, disease suppression was observed upon silencing of various types of genes, including those that encode the biosynthesis of structural components such chitin and ergosterol, but also genes involved in developmental regulation, secondary metabolism and pathogenicity. Therefore, the selection of suitable target genes is arguably the most important prerequisite for developing a successful HIGS against fungal pathogens. We selected HIGS target genes based on our previous studies of gene deletion mutants in *V. dahliae* with significantly compromised virulence (*NLP1*, Santhanam et al., 2013; *Sge1*, Santhanam and Thomma, 2013). Beforehand, it was not clear whether silencing of such genes rather than gene deletion would lead to visible virulence phenotypes, as the protein encoded by the target gene may not be completely absent. Indeed, TRV-mediated HIGS of *Sge1* in tomato did not lead to compromised Verticillium wilt symptoms, which may be explained by the incomplete silencing of the *Sge1* gene in *V. dahliae* (Figure 4C). This also explains why RNAi-mediated HIGS of *V. dahliae Ave1* did not compromise *Ve1*-mediated immunity in transgenic *A. thaliana* plants. Also in the cases where the expected visual phenotypes were obtained, fungal biomass quantifications revealed that more fungal biomass accumulated in the inoculated plants upon HIGS of the fungal target gene than upon inoculation with the corresponding *V. dahliae* deletion mutant (Figure 3B, 4B, 6B, 7B). Thus, hypothetically, RNAi may not be appropriate to target genes of which the activity is required early in the infection process when RNAi may not have taken its full effect, or genes of which a low-dose of transcripts is biologically active.

Mobility of small RNAs within organisms is a well-known phenomenon, facilitating gene silencing in adjacent cells and surrounding or even distant tissues (Weiberg et al., 2015). Over recent years, several examples of exchange of small RNAs between host plants and invading pathogens have been described, although the mechanistic details of the actual exchange remains to be elucidated (Knip et al., 2014). Nevertheless, small RNA-based bidirectional cross-kingdom gene silencing has been proposed as a common mechanism for cross-kingdom gene regulation in plant-pathogen interactions (Chaloner et al., 2016; Wang et al., 2016; Weiberg et al.,

2015). For example, endogenous small RNAs from the fungus *Botrytis cinerea* have been proposed to transfer into host plants to target defense-related plant transcripts to promote disease development (Weiberg et al., 2013). In this manner, HIGS taps into a process that naturally occurs between plants and pathogens. A search for pathogen-derived small RNAs matching transcripts of host plants or plant-derived small RNAs targeting transcripts of the invading pathogens may facilitate the development of HIGS strategies to engineer resistance in plants against pathogens for which no natural resistance sources have been identified.

MATERIALS AND METHODS

Plant growth conditions and manipulations

Plants were grown in commercial potting soil (Horticoop, Bleiswijk, the Netherlands) at 21°C/19°C during 16 h/8 h light/dark photoperiods, respectively, in the climate chamber or the greenhouse (Unifarm, Wageningen, the Netherlands) with a relative humidity of ~75%, and 100 $W \cdot m^{-2}$ supplemental light when light intensity dropped below 150 $W \cdot m^{-2}$. *A. thaliana* transformations were performed as described (Clough and Bent, 1998).

Generation of the constructs

The Gateway-compatible *Tobacco rattle virus* (TRV) two-component *Agrobacterium*-mediated expression system was used for gene silencing in tomato as previously described (Liu et al., 2002), while the Gateway-compatible vectors pFAST R03 (Shimada et al., 2010) and pHellsgate 12 (Helliwell and Waterhouse, 2003) for hairpin RNA-mediated gene silencing were used to generate stable *A. thaliana* transformants. The three *V. dahliae* genes *Ave1* (de Jonge et al., 2012), *NLP1* (Santhanam et al., 2013) and *Sge1* (Santhanam and Thomma, 2013) were selected for RNAi-based HIGS. Gene annotations for *V. dahliae Ave1*, *Sge1* and *NLP1* were obtained from Ensembl Genomes database (http://fungi.ensembl.org/Verticillium_dahliaejr2/Info/Index). Selected DNA fragments were amplified by PCR from the corresponding plasmids using gene-specific primers listed in Table S1. The DNA fragments were cloned into pDONR207 by using the Gateway® BP Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA) to generate entry vectors, and all the entry vectors were verified by DNA sequencing (Eurofins Genomics, Ebersberg,

Germany). Subsequently, the entry vector pDONR207 carrying the *Ave1* fragment was transferred to TRV2 and pFAST R03 to generate constructs *TRV2::Ave1* and pFAST R03_Ave1 (Figure 1), while pDONR207 entry vectors carrying *NLP1* or *Sge1* fragment were recombined into pTRV2 and pHellsgate 12 to generate constructs *TRV2::NLP1*, *TRV2::Sge1*, pHellsgate 12_NLP1 and pHellsgate 12_Sge1 (Figure 1) by using Gateway® LR Clonase® II Enzyme Mix (Invitrogen, Carlsbad, USA). All constructs were transformed to *Agrobacterium tumefaciens* strain GV3101 (pMP90) by electroporation.

TRV treatment

TRV vectors were agroinfiltrated as previously described (Liu et al., 2002; Fradin et al., 2011). Briefly, cotyledons of 10-day-old tomato (*Solanum lycopersicum* cv. Moneymaker (*ve1*) or *35S::Ve1* tomato (*Ve1*); Fradin et al., 2009) were infiltrated as 1:1 mixtures of *pTRV1* and *pTRV2* constructs. 10-15 days after TRV inoculation, plants were inoculated with race 1 *V. dahliae* strain JR2 (Faino et al., 2015); the corresponding mutants: *V. dahliae* JR2*ΔAve1* (de Jonge et al., 2012); *V. dahliae* JR2*ΔNLP1* (Santhanam et al., 2013); *V. dahliae* JR2*ΔSge1* (Santhanam and Thomma, 2013); or tap water as control. The inoculated plants were evaluated by observing disease symptoms up to 14 days post inoculation (dpi).

Verticillium wilt disease and fungal recovery assays

V. dahliae was grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) at 22 °C, and conidia were collected from 7- to 10-day-old PDA plates and washed with tap water. Disease assays on tomato plants were performed as previously described (Fradin et al., 2009). Briefly, twenty-day-old *Ve1* tomato plants (for *Verticillium* inoculation after TRV treatment) or ten-day-old *Ve1* and *ve1* tomato plants (for inoculation with *Verticillium* colonies re-isolated from infected tomato plants) were uprooted, the roots were rinsed in water, dipped for 5 min in a suspension of 10^6 conidiospores/mL water, and transplanted to commercial potting soil (Horticoop, Bleiswijk, the Netherlands). *Verticillium* outgrowth assays of *Ve1* tomato plants, canopy area measurement and fungal biomass quantification in tomato plants were performed as previously described (Fradin et al., 2009; Santhanam et al., 2013). *Verticillium* disease assay on *A. thaliana*, as well as fungal biomass quantification in

infected *A. thaliana* plants were performed as previously described (Ellendorff et al., 2009; Song et al., 2016). The fungus-specific primer ITS1-F, based on the internal transcribed spacer (ITS) region of the ribosomal DNA, in combination with the *V. dahliae*-specific reverse primer ST-Ve1-R (Ellendorff et al., 2009) were used to measure fungal colonization. Primers for tomato *actin* and *A. thaliana RuBisCo* (Table S1) were used as endogenous plant control as described earlier (Santhanam et al., 2013; Song et al., 2016).

Quantitative PCR (qPCR) and Reverse transcription-qPCR (RT-qPCR)

To determine expression of *V. dahliae Sge1* gene for silencing, stems of TRV-treated tomato plants were harvested at 14 days post *Verticillium* inoculation as described above, and flash frozen in liquid nitrogen, and stored at -80 °C for total RNA isolation.

To check *Ave1*, *NLP1*, *Sge1*, *GFP* DNA fragment presence in the transcripts of the corresponding transgenic *A. thaliana* lines, two-week-old transgenic and non-transgenic *A. thaliana* lines were harvested and ground into a powder in liquid nitrogen. Total RNA extraction, cDNA synthesis and RT-PCR were performed as described earlier (Song et al., 2016). Primers for hairpin expression analysis are listed in Table S1. To analyze expression of *Sge1* gene for silencing, RT-qPCR was conducted by using primers Sge1-F(qRT) and Sge1-R(qRT) with *V. dahliae GAPDH* as an endogenous control (Table S1), employing an ABI 7300 PCR system (Applied Biosystems, Foster City, USA) with the qPCR Core kit for SYBR Green I (Eurogentec Nederland BV, Maastricht, the Netherlands) as previously described (Santhanam and Thomma, 2013).

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SUPPLEMENTAL INFORMATION

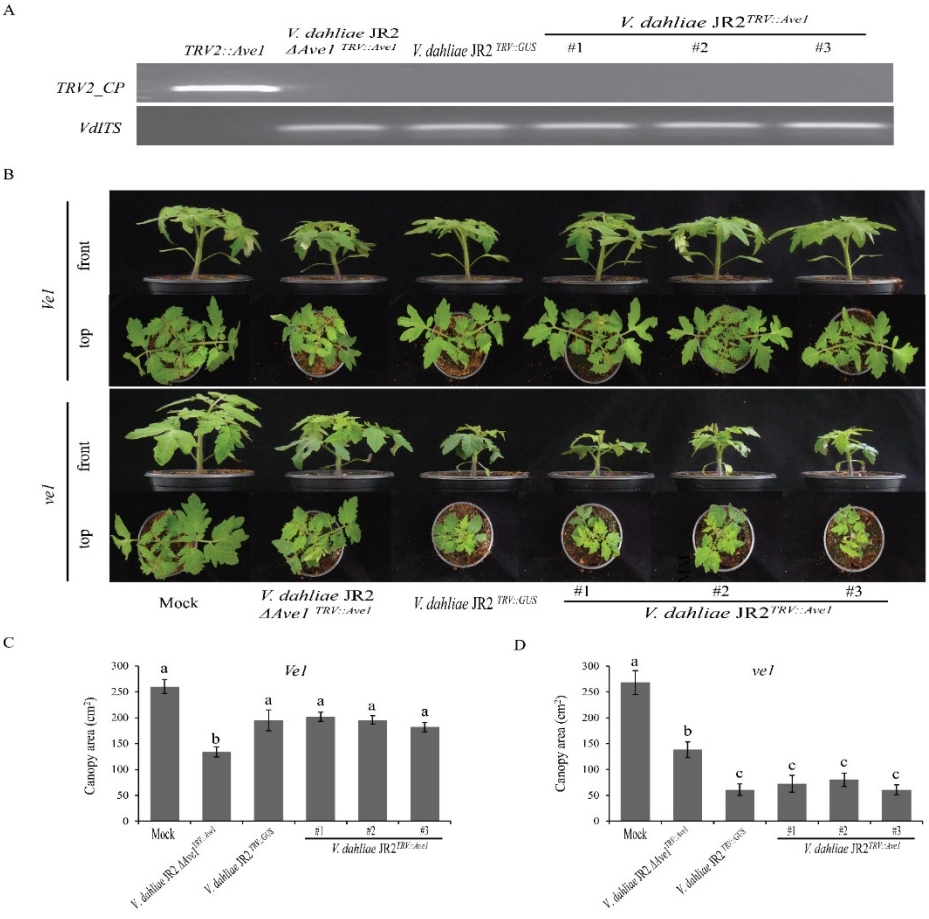

Figure S1. Analysis of *Verticillium dahliae* colonies re-isolated from TRV-treated *Ve1* tomato plants. (A) Viral coat protein gene fragment (*TRV2_CP*) was not detected by PCR in re-isolated *V. dahliae* colonies *V. dahliae* JR2*ΔAve1*$^{TRV::Ave1}$, *V. dahliae* JR2 $^{TRV::GUS}$ and *V. dahliae* JR2$^{TRV::Ave1}$ (#1, #2 and #3) that grew from wild-type *V. dahliae*-inoculated *TRV::GUS*-treated plants, *Ave1* deletion strain-inoculated *TRV::Ave1*-treated plants and wild-type *V. dahliae*-inoculated *TRV::Ave1*-treated plants, respectively. Construct *TRV::Ave1* was used as a positive control. As an endogenous control, a fragment of the *V. dahliae ITS* was amplified from all re-isolated *V. dahliae* colonies. **(B)** Typical appearance of *Ve1* tomato plants (*Ve1*) and Moneymaker tomato plants lacking *Ve1* (*ve1*) upon mock-inoculation or inoculation with re-isolated strains *V. dahliae* JR2*ΔAve1*$^{TRV::Ave1}$, *V. dahliae* JR2 $^{TRV::GUS}$, or *V. dahliae* JR2$^{TRV::Ave1}$ at 14 dpi. **(C)** Average canopy area of 10 *Ve1* plants inoculated with re-isolated strains described above or mock inoculation. (D) Average canopy area of 10 *ve1* plants inoculated with re-isolated strains described above or mock inoculation. Different letters indicate significant differences ($P < 0.05$). The data shown are representative of three independent assays.

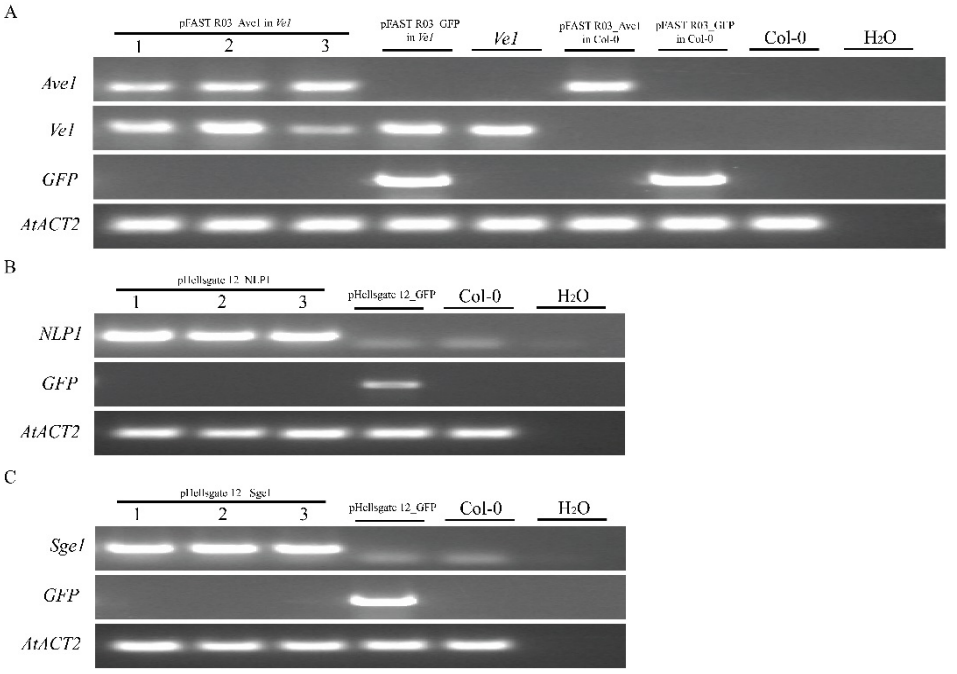

Figure S2. Polymerase chain reaction (PCR) amplification from cDNA of the corresponding transgenic *Arabidopsis thaliana* lines. (A) Transcripts for hairpin *Ave1* or *GFP* formation in transgenic lines were detected by reverse transcription-PCR (RT-PCR). For pFAST R03_Ave1 construct in *Ve1* plants three independent transgenic lines are shown (1, 2 and 3), while other lines expressing pFAST R03_Ave1 construct or pFAST R03_GFP construct are shown as controls. **(B)** Transcripts for hairpin *NLP1* or *GFP* formation in transgenic lines were PCR-detected. For pHellsgate 12_NLP1 construct in Col-0 plants three independent transgenic lines are shown (1, 2 and 3), and the corresponding control line expressing pHellsgate 12_GFP construct is shown. **(C)** Transcripts for hairpin *NLP1* or *GFP* formation in transgenic lines were PCR-detected. For pHellsgate 12_Sge1 construct in Col-0 plants three independent transgenic lines are shown (1, 2 and 3), and the corresponding control line expressing pHellsgate 12_GFP construct is shown. As an endogenous control, a fragment of the *AtACTIN2* gene was amplified from *A. thaliana* cDNA. *A. thaliana* Col-0 and water are used as RT-PCR controls.

Table S1. Primers used in this study.

Primer name	Oligonucleotide sequence (5'→3')	Description[a]
Ave1-F	ggggacaagtttgtacaaaaaagcaggctATGAAGCTTTCTACGCTT	*Ave1* (RNAi)
Ave1-R	ggggaccactttgtacaagaaagctgggtTATCTGTCTAAATTC	*Ave1* (RNAi)
Ave1-F(RT)	AGCTTTCTACGCTTGGA	RT-PCR
Ave1-R(RT)	TTGGCTGGGATTGCT	RT-PCR
NLP1-F	ggggacaagtttgtacaaaaaagcaggctGGTCTCCAAGACAGCGGTAC	*NLP1* (RNAi & RT-PCR)
NLP1-R	ggggaccactttgtacaagaaagctgggtGTTTGCGTTGTTCGAGTTGA	*NLP1* (RNAi & RT-PCR)
Sge1-F	ggggacaagtttgtacaaaaaagcaggctCCTGTCTGGCAATCTTCGTC	*Sge1* (RNAi & RT-PCR)
Sge1-R	ggggaccactttgtacaagaaagctgggtCCGTTGGTCTGCTTCTTGTT	*Sge1* (RNAi & RT-PCR)
GFP-F	ggggacaagtttgtacaaaaaagcaggctGTGGAGAGGGTGAAGGTGA	*GFP* (RNAi & RT-PCR)
GFP-R	ggggaccactttgtacaagaaagctgggtAAGGGCAGATTGTGTGGAC	*GFP* (RNAi & RT-PCR)
TRV2_CP-F	CTGCGAATCCAAACACAAC	Coat protein
TRV2_CP-R	GAACCGCCTGAACATAAAAA	Coat protein
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	*V. dahliae* (fungal biomass; qPCR)
STVe1-R	CTTGGTCATTTAGAGGAAGTAA	*V. dahliae* (fungal biomass; qPCR)
SlActin-F	CCATTCTCCGTCTTGACTTGG	Tomato *actin* (fungal biomass; qPCR)
SlActin-R	TCTTTCCTAATATCCACGTCAC	Tomato *actin* (fungal biomass; qPCR)
AtRubisco-F	GCAAGTGTTGGGTTCAAAGCTGGTG	Arabidopsis *Rubisco* (fungal biomass; qPCR)
AtRubisco-R	CCAGGTTGAGGAGTTACTCGGAATGCTG	Arabidopsis *Rubisco* (fungal biomass; qPCR)
Sge1-F(qRT)	CACCGAATTACGCTCAACCT	*V. dahliae* (RT-qPCR)
Sge1-R (qRT)	AAGTCATAGGCCGTGGAGTG	*V. dahliae* (RT-qPCR)
VdGAPDH-F	CGAGTCCACTGGTGTCTTCA	*V. dahliae* (RT-qPCR)
VdGAPDH-R	CCCTCAACGATGGTGAACTT	*V. dahliae* (RT-qPCR)
Ve1-F(RT-PCR)	CATATTGAAATTAGCGTCTTGTCGG	Tomato *Ve1* (RT-PCR)
Ve1-R(RT-PCR)	ACCGAGAAAAAGGAGGCAAAAC	Tomato *Ve1* (RT-PCR)
AtACT2-F	GATGGAGACCTCGAAAACCA	Arabidopsis *actin* (RT-PCR)
AtACT2-R	AAAAGGACTTCTGGGCACCT	Arabidopsis *actin* (RT-PCR)

[a]The type of experiment for which the primers were used is indicated in brackets (RNAi, RNA interference; RT-PCR: Reverse Transcription-PCR; qPCR, quantitative Real Time-PCR; RT-qPCR: Reverse Transcription-qPCR).

Chapter 7

General discussion

A modified version of this chapter has been published as:
Rodriguez-Moreno L[#], Song Y[#], Thomma BPHJ (2017) Transfer and engineering of immune receptors to improve recognition capacities in crops. Curr Opin Plant Biol **38**: 42-49. ([#]equal contribution)

ABSTRACT

Immune receptors are pivotal elements of the plant immune system that act as sentinels for microbial invasion. Knowingly or unknowingly, breeding for resistance has largely relied on the transfer of immune receptor recognition specificities between plant genotypes. For decades such transfers were limited to crossable species. However, advents in transgene technologies have allowed overcoming species barriers. Novel strategies for mining of recognition specificities, combined with our recently increased understanding of immune receptor functioning, allows to increase and alter recognition specificities, which should ultimately increase the spectrum of recognition specificities that are available to control plant diseases in crops.

INTRODUCTION

Plant diseases, caused by pathogens and pests, threaten food security and food safety worldwide and remain a major agricultural challenge. Like any other organism, plants evolved innate immune systems that act against pathogen attack, and which are continuously tweaked in the ongoing arms races with a wide range of microbes (Dodds and Rathjen, 2010; Thomma et al., 2011; Cook et al., 2015). Central to the immune system are receptors that detect pathogen invasion through sensing pathogen(-induced) ligands, also termed invasion patterns, to mount appropriate immune responses (Cook et al., 2015). Based on structure and subcellular location, these invasion pattern receptors fall into two major classes; cell surface localized receptors that comprise receptor kinases (RKs) and receptor-like proteins (RLPs) that monitor the extracellular space, while cytoplasm-localized nucleotide-binding domain leucine-rich repeat receptors (NLRs) survey the intracellular environment.

For decades, breeding for resistance has relied on the identification of resistance or of recognition traits in genotypes of crop species or their (wild) relatives followed by introduction into elite cultivars by crossing. For example, Verticillium wilt resistance in many modern cultivated tomato (*Solanum lycopersicum*) varieties is based on the introgression of the dominant *Ve* locus that was identified in the tomato accession Peru Wild in the 1930s (Schaible et al., 1951). Meanwhile, our mechanistic understanding of pathogen recognition has increased significantly (Zhang and Thomma, 2013; Cui et al., 2015; Li et al., 2016). For instance, research over the last decades has shown that race-specific resistance to Verticillium wilt in tomato is governed by the cell surface-localized RLP-type immune receptor Ve1 encoded by the *Ve* locus (Kawchuk et al., 2001; Fradin et al., 2009) through recognition of the effector protein Ave1 that is secreted by race 1 strains of *Verticillium* spp. (de Jonge et al., 2012). In this thesis we further propose that a surface-exposed patch of the Ave1 protein that is composed of co-localized N- and C-termini is recognized by tomato Ve1 (Chapter 3). These advances spur the identification and deployment of novel recognition specificities to enhance disease resistance in crops.

Interfamily transfer of immune receptors to confer disease resistance

Since the ground-breaking finding that introduction of the cell surface-localized elongation factor Tu receptor (EFR) from the crucifer *Arabidopsis thaliana* into the Solanaceous plants *Nicotiana benthamiana* and tomato confers responsiveness to bacterial elongation factor Tu and bacterial resistance (Lacombe et al., 2010), it has become apparent that immune receptor genes may be transferred between plant families to engineer disease resistance (Figure 1**a**; Table 1). In this thesis, we show that homologs of tomato *Ve1* are widespread in the plant kingdom (Chapter 4), and that tomato *Ve1* can be functionally transferred to crops such as tobacco and cotton (Chapter 5). Successful transfers have even been reported across the dicot and monocot clades with, for example, transfer of the barley NLR *Mildew Resistance Locus A* (*MLA1*) to *A. thaliana* induce immunity against barley powdery mildew expressing the corresponding effector AVRA1 (Maekawa et al., 2012). Arguably, examples like these imply that signalling components downstream of immune receptors are sufficiently conserved among plant species, families and even clades to mount the appropriate defence responses. Currently, most successful interfamily transfers of immune receptors have been reported for cell surface receptors (Table 1), which again also include transfer of tomato Ve1, initially to the model plant *A. thaliana* (Fradin et al., 2009), and in this thesis to the crop species tobacco and cotton (Chapter 5). Potentially, this is because this type of immune receptor participates in receptor complexes that generally require the same, highly conserved, receptor-associated kinases to mount downstream immune responses (Liebrand et al., 2014). The functionality of many, if not all, RLPs carrying extracellular leucine-rich repeats (eLRRs), requires constitutive association with the common adaptor kinase Suppressor of BAK1-Interacting Receptor-like Kinase 1 (SOBIR1) in order to constitute a bimolecular equivalent of a genuine RK (Gust and Felix, 2014). This obviously also applies to RLP-type immune receptor Ve1 (Liebrand et al., 2013). Moreover, RKs as well as RLP/SOBIR1 complexes interact in a ligand-dependent manner with the Brassinosteroid Insensitive 1 (BRI1)-Associated receptor Kinase 1 (BAK1) for immune signalling activation. Although physical recruitment of BAK1 upon Ave1-mediated activation of the Ve1-SOBIR1 complex has not yet been demonstrated, genetic requirement of *BAK1* for the Ave1-

induced Ve1-mediated immune response has previously been demonstrated in both tomato and *A. thaliana* (Fradin et al., 2009; 2011). Although BAK1 also participates in receptor complexes that mediate developmental processes, it has recently been proposed that immune and developmental signalling complexes show spatiotemporal separation in plasma membrane nanodomains, which might mechanistically explain dual BAK1 functionality (Bücherl et al., 2017). Importantly, SOBIR1 and BAK1 are widely conserved in the plant kingdom (Liebrand et al., 2014).

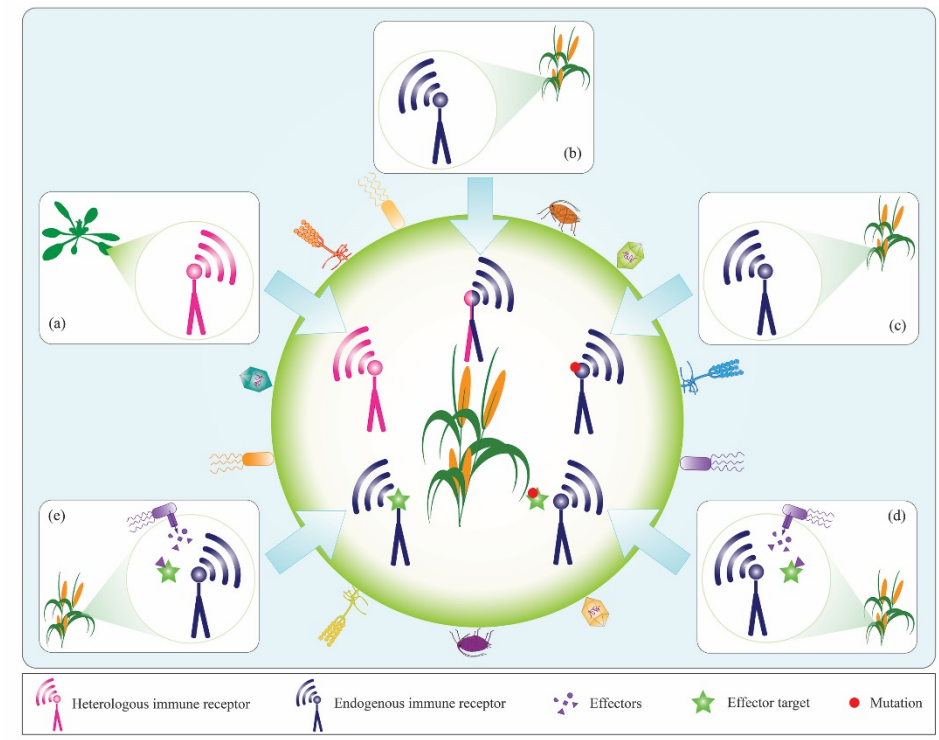

Figure 1. Strategies for plant immune receptor deployment to improve pathogen recognition capacities in crops. (a) Transfer of an immune receptor (displayed as an antenna) from another plant species to engineer disease resistance in the target crop (in the centre of the circle). **(b)** Generation of an inter-species chimeric immune receptor to engineer or alter pathogen recognition specificity. **(c)** Mutation (red dot) of an endogenous immune receptor to alter or enhance recognition specificity. **(d)** Mutation (red dot) of an effector target (green star) to expand immune receptor recognition capacity. **(e)** Integration of a decoy effector target (green star) into an immune receptor to engineer pathogen recognition.

Table 1 Examples of functional transfer of plant immune receptor genes between families.

Origin				Transferred to			Reference
Family	Species	Gene	Class[a]	Family	Species	Outcome	
Brassicaceae	*Arabidopsis thaliana*	*EFR*	RK	Solanaceae	*Solanum lycopersicum; Nicotiana benthaminana*	Multibacterial resistance	(Lacombe et al., 2010)
				Poaceae	*Oryza sativa*	Bacterial leaf blight resistance; Bacterial brown stripe resistance	(Lu et al., 2015; Schwessinger et al., 2015)
				Poaceae	*Triticum aestivum*	Bacterial halo blight resistance	(Schoonbeek et al., 2015)
	A. thaliana	*LecRK-I.9* (*DORN1*)	RK	Solanaceae	*S. tuberosum; N. benthaminana*	Late blight resistance	(Bouwmeester et al., 2014; Wang et al., 2016)
	A. thaliana	*RPS4/RRS1*	NLR	Solanaceae	*S. lycopersicum*	Bacterial speck resistance; Bacterial wilt resistance	(Narusaka et al., 2013)
				Solanaceae	*N. benthaminana*	Recognition of the two unrelated bacterial effectors PopP2 and AvrRps4	
				Cucurbitaceae	*Cucumis sativus*	Anthracnose resistance	
	A. thaliana	*RLP23*	RLP	Solanaceae	*S. tuberosum*	Oomycete and fungal resistance	(Albert et al., 2015)
	A. thaliana	*LORE*	RK	Solanaceae	*N. benthaminana; N. tabacum*	Perception of bacterial lipopolysaccharide	(Ranf et al., 2015)
Solanaceae	*S. lycopersicum*	*Ve1*	RLP	Brassicaceae	*A. thaliana*	Verticillium wilt resistance	(Fradin et al., 2011)
	N. benthamiana	*NbFLS2*	RK	Rutaceae	*Citrus sinensis*	Citrus canker resistance	(Hao et al., 2016)
	S. lycopersicum	*CORE*	RK	Brassicaceae	*A. thaliana*	Detection of bacterial cold-shock protein	(Wang et al., 2016)
	Oryza longistaminata	*XA21*	RK	Rutaceae	*Citrus sinensis*	Citrus canker resistance	(Mendes et al., 2010)
				Solanaceae	*S. lycopersicum*	Bacterial wilt resistance	(Afroz et al., 2011)
				Musaceae	*Musa sp.*	Banana Xanthomonas wilt resistance	(Tripathi et al., 2014)
				Brassicaceae	*A. thaliana*	Bacterial resistance	(Holton et al., 2015)
Poaceae	*Hordeum vulgare*	*MLA1*	NLR	Brassicaceae	*A. thaliana*	Powdery mildew resistance	(Maekawa et al., 2012)
	Oryza sativa	*RGA4/RGA5*	NLR	Solanaceae	*N. benthamiana*	Recognition of the fungal pathogen effector AVR-Pia	(Césari et al., 2014)

[a] RK, receptor kinase; RLP, receptor-like protein; NLR, nucleotide-binding domain leucine-rich repeat receptor.

Functional transfer of NLR-type immune receptors between taxonomically distinct families has met with relatively little success when compared with the transfer of cell surface receptors. Potentially this indicates that the molecular components that they need to interact with are less conserved. However, it is also increasingly becoming apparent that NLR-type immune receptors may function in heterologous pairs to activate immunity (Eitas and Dangl, 2010; Okuyama et al., 2011; Yuan et al., 2011; Brotman et al., 2013; Cesari et al., 2013; Wang et al., 2013). In support of this, transfer of the Arabidopsis NLR pair Resistance to *Pseudomonas syringae* 4 (RPS4) with Resistance to *Ralstonia solanacearum* 1 (RRS1) allows recognition of the PopP2 and AvrRps4 effectors of *Ralstonia solanacearum* and *Pseudomonas syringae*, respectively, in *N. benthamiana* and confers resistance against these bacterial pathogens in tomato (Narusaka et al., 2013). Moreover, transgenic cucumber lines expressing *RPS4-RRS1* are resistant against

Colletotrichum orbiculare, although it is not clear what effector is recognized in this case (Narusaka et al., 2013).

Exploitation of the modular nature of immune receptors to extend pathogen recognition specificities

Structurally, most immune receptors are modular proteins with differentiated domains for ligand recognition and for intracellular signalling. Conceivably, ligand perception by extracellular receptors is mostly determined by the eLRR domain, while intracellular signalling is mostly conferred by the cytoplasmic kinase domain (for RKs) or interaction with co-receptor SOBIR1 (for RLPs) (Couto and Zipfel, 2016). Such modular composition offers opportunities to engineer chimeras that are composed of domains from different receptors (Figure 1**b**). The first report of a functional chimeric plant receptor stems from the year 2000, claiming that a chimeric receptor consisting of the extracellular domain of the Arabidopsis RK BRI1, which perceives brassinosteroids to activate developmental signalling, fused to the kinase domain of the rice RK XA21, an immune receptor involved in bacterial blight resistance, mediates immune activation upon brassinosteroid treatment (He et al., 2000). Similarly, domain swaps between the tomato RLP Ve1 and its closely non-functional homolog Ve2 allowed construction of artificial receptors carrying C-terminal part of Ve2 replaced by that of Ve1 to mediate *Verticillium* resistance through perception of the effector protein Ave1 (Fradin et al., 2014). More recently, chimeras between Arabidopsis EFR and rice XA21 confirmed that the XA21 kinase domain, despite being derived from a monocot, associates with intracellular components of the EFR complex in the dicot Arabidopsis to activate immunity (Holton et al., 2015). Similarly, rice transformants expressing EFR, or EFR:XA21 chimeras, respond to *Escherichia coli* and *Xanthomonas oryzae* pv. *oryzae* elf18 (Schwessinger et al., 2015). In addition to reinforcing the notion that signalling components downstream of the receptors are widely conserved, these examples illustrate the modular nature of immune receptors.

NLRs consist of an N-terminal coiled-coil (CC) or Toll-interleukin (TIR) domain, a central ATPase nucleotide-binding site (NBS), and C-terminal LRRs. Although the high degree of polymorphism of the LRR domains suggests involvement in ligand binding (Krasileva et al., 2010), also the CC-domain was found to interact with

ligands (Chen et al., 2012). Switching from a non-active to an active state is controlled by intramolecular rearrangements within the TIR/CC and NBS subdomains associated with nucleotide exchange (Tameling et al., 2002; Bernoux et al., 2016). Domain swaps between closely related yet polymorphic proteins frequently lead to either inactive or constitutively active receptors (Slootweg et al., 2013; Steinbrenner et al., 2015; Wang et al., 2015). Thus, exploitation of the modular nature of NLRs is less straightforward than for RKs, also because the mechanism by which they exactly function is less well understood. This is illustrated by the surprising finding that the TIR-only protein "Response to the bacterial type III effector protein HopBA1" (RBA1) lacks all other canonical NLR domains, but successfully induces immunity to *P. syringae* (Nishimura et al., 2017). Nevertheless, natural chimeric immune receptors exist as so-called NLRs with integrated domains (NLR-IDs). These NLR-IDs comprise non-canonical "decoy" domains that mimic effector target proteins, and act as a trap for pathogen effectors to activate immunity (Cesari et al., 2014; Wu et al., 2015). Functional analyses of NLR-IDs suggest these receptors act in pairs with conventional NLR partners, where the NLR-ID works as pathogen "sensor" while the conventional NLR acts as immune "activator" (Cesari et al., 2014). Examples of receptor pairs containing integrated decoy domains are the Arabidopsis pair RRS1 with RPS4, where RRS1 contains a decoy WRKY domain that is targeted by two unrelated bacterial effectors PopP2 and AvrRps4 (Le Roux et al., 2015; Sarris et al., 2015), and the rice pairs Resistance Gene Analog 5 (RGA5) with RGA4 (Césari et al., 2014) and *Pyricularia oryzae* resistance-k 1 (Pik-1) with Pik-2 (Maqbool et al., 2015), where RGA5 and Pik-1 contain heavy metal-associated (HMA) domains that are targeted by the AVR1-CO39, AVR-Pik and AVR-Pia effectors of the rice blast fungus *Magnaporthe oryzae* (Cesari et al., 2013; Maqbool et al., 2015; Ortiz et al., 2017). Interestingly, the positioning of the IDs within the overall structure of NLR-IDs suggests structural plasticity, with the decoy domain integrated between the CC- and NBS domains, e.g. in Pik-1, or at the C-terminus of the NLR, e.g. in RRS1 and RGA5. NLR-IDs are widely distributed across all lineages of flowering plants (Kroj et al., 2016; Sarris et al., 2016), and recent studies on grass genomes suggest the presence of genomic hotspots containing flexible NLR-"acceptor" genes that may integrate other domains from across the genome (Bailey et al., 2017).

Artificial evolution to extend immune receptor recognition

The identification of novel pathogen recognition specificities is highly desired by plant breeders. Besides screening for novel specificities in natural collections, also artificial evolution through random or targeted mutagenesis has successfully been exploited to expand the recognition spectrum of plant immune receptors. The NLR Rx confers resistance against a single strain of potato virus X (PVX) by recognising its capsid protein (CP). Rx can recognise PVX isolates containing T and K at positions 121 and 127, respectively (CP_{TK}), but not those with K and R at these positions (CP_{KR}). Based on random mutagenesis of the LRR domain, Rx variants that also responded to CP_{KR} were generated (Farnham and Baulcombe, 2006). Furthermore, one of the selected Rx mutants, RxM1, induced systemic necrosis when plants were challenged with poplar mosaic virus (PopMV), a distant relative of PVX, due to weak recognition of the PopMV CP. In a second round of random mutagenesis, five amino acid changes near the conserved ATPase nucleotide-binding pocket increased sensitivity of RxM1 towards the PopMV-CP (Harris et al., 2013).

A similar approach was followed to alter the recognition specificities of the potato NLR R3a that only recognizes the $AVR3a^{KI}$ allele of *Phytophthora* spp., while the other major allele, $AVR3a^{EM}$, is only weakly recognized. Using random mutagenesis eight amino-acid substitutions were identified that expand R3a recognition to include $AVR3a^{EM}$. Strikingly, particular mutations in the CC ($R3a^{I148F}$), which resulted in an auto-active R3a variant, and in the NBS ($R3a^{N336Y}$) extended recognition to another AVR3a homolog from *P. capsici* (Segretin et al., 2014). The tomato R3a ortholog I2 confers resistance to *Fusarium oxysporum* f. sp. *lycopersici* through recognition of the AVR2 effector, but only weakly responds to AVR3a. Mutation of the amino acid position in I2 ($I2^{I141N}$) of which the homologous position in R3a was found to generate an auto-active phenotype not only resulted in recognition of two AVR2 effector variants that previously escaped I2 recognition, but also resulted in recognition of $AVR3a^{KI}$ and $AVR3a^{EM}$ (Giannakopoulou et al., 2015).

Rather than based on random mutagenesis, structure-guided mutation of immune receptors holds promise for extension or alteration of recognition specificities (Figure 1**c**). Presently, an increasing number of studies report on the

crystal structure of immune receptors, sometimes together with their ligands, allowing the identification of residues involved in ligand interaction (Liu et al., 2012; Sun et al., 2013; Tang et al., 2015), and thus also the rational design of receptor modifications to alter recognition specificities. For instance, analysis of the crystal structure of the ectodomain of the Arabidopsis RK Flagellin Sensing 2 (FLS2) together with the bacterial flagellin (flg22) ligand revealed that the LRR forms a solenoid structure whose inner surface interacts with both the N-terminus and C-terminus of flg22 (Sun et al., 2013). Targeted mutagenesis extended FLS2 recognition capacities towards new flg22 ligands for which the wild-type FLS2 confers little or no response (Helft et al., 2016). As CRISPR/Cas systems are further refined and developed for more cropping systems (Puchta, 2017), structure-guided engineering of immune receptors will likely become customary.

Modification of effector targets to expand receptor recognition capacities

Pathogen detection by immune receptors may occur directly by physical interaction with pathogen-derived or induced molecules, or indirectly according to the guard model by detecting effector target modifications (Van Der Biezen and Jones, 1998). The latter mechanism in particular offers the possibility to engineer host targets to be recognized by immune receptors that previously did not respond to the effector protein (Figure 1**d**). The *P. syringae* AvrPphB effector is a protease that cleaves the host kinase PBS1 (for Avirulence Protein *Pseudomonas phaseolicola* B (avrPphB) Susceptible 1) that functions in basal immunity, which is monitored by the Arabidopsis NLR RPS5 (Shao et al., 2003). Engineering of PBS1 such that it is cleaved by proteases of other pathogens results in extension of disease resistance mediated by RPS5 (Kim et al., 2016).

Members of the genus *Xanthomonas* and *Ralstonia* secrete transcription activator-like effectors (TALEs) that bind specific DNA sequences, known as effector binding elements (EBEs), to activate host target genes (Kay et al., 2007; Boch et al., 2009; Moscou and Bogdanove, 2009). In turn, plants evolved "executor" genes with EBEs embedded in the promoters that induce cell death and resistance (Römer et al., 2007). Based on “executor” sequences, novel EBEs can be engineered

that trigger immunity upon invasion of pathogens that inject TALEs (Römer et al., 2009; Hummel et al., 2012; Zeng et al., 2015).

Already 25 years ago, a so-called two-component sensor system was proposed to engineer resistance against a broad range of (obligate) biotrophic pathogens by transgenic expression of corresponding pairs of effectors and immune receptors under control of (preferably two different), pathogen-inducible promoters (de Wit, 1992). Bacterial leaf streak of rice is caused by *X. oryzae* pv. *oryzicola*, yet no resistance against this pathogen is known. Resistance was established in transgenic rice lines harbouring the *M. oryzae* AVR1-CO39 effector gene under the control of an inducible promoter with an embedded EBE when the corresponding Pi-CO39 resistance locus was present (Hutin et al., 2016). In an alternative strategy, a similar transactivation of an auto-active NLR by a TAL effector of the *X. oryzae* pv. *oryzae* triggered bacterial blight resistance (Hutin et al., 2016). Such approaches based on synthetic promoter "traps" widely expand the panel of genes that can be exploited to engineer resistance in plants that suffer from infections by TALE-injecting pathogens.

Future engineering of disease resistance in crops through the deployment of immune receptors

Securing sustainable food production remains a challenge, as pathogens continuously adapt to overcome host immunity and can emerge on novel crop species, such as the recent wheat blast outbreak in Bangladesh and India (Malaker et al., 2016). This is particularly problematic when resistance sources are scarce. The fungus *Phakopsora pachyrhizi* causes Asian soybean rust (ASR) that is one of the most economically important crop diseases and mostly controlled by chemicals as no effective genetic resistance occurs in cultivars, yet fungicide resistance is an emerging problem for this pathogen. Transgenic expression of the recently identified NLR *CcRpp1* from the legume weed pigeonpea (*Cajanus cajan*) confers full resistance to ASR in soybean (Kawashima et al., 2016), showing how (wild) relatives may provide resistance traits for crop improvement.

In addition to identification of recognition specificities based on screening for pathogen resistance, functional genomics approaches based on effector recognition (“effectoromics”) can be used for probing plant germplasm for recognition

specificities (Vleeshouwers and Oliver, 2014). This is especially powerful as it also allows screening for recognition specificities in plant species that are not hosts for the pathogen for which recognition is sought. Moreover, an effectoromics strategy allows combination with pathogenomics strategies to select the potentially most widespread or invariant effectors to, once identified and deployed, maximize durability of the recognition specificity (Bart et al., 2012). However, the subsequent cloning of immune receptor genes is typically laborious and slow, but functional genomic approaches based on NLR gene enrichment and sequencing (RenSeq), especially when combined with long-read sequencing, can accelerate mapping of functional NLRs in crop plants (Jupe et al., 2013; Witek et al., 2016), as well as in wild relatives (Stam et al., 2016).

An interesting strategy has been proposed to identify cell surface receptors particularly based on their general ligand-induced association with the "promiscuous" co-receptor BAK1. Using affinity-tagged BAK1 as molecular bait, a *N. benthamiana* eLRR-RLP-type cell surface receptor was recently identified as NbCSPR that is involved in the response to a bacterial cold-shock peptide (csp22) (Saur et al., 2016). Intriguingly, based on natural variation in csp22 perception an RK-type cell surface receptor was identified in tomato as high-affinity receptor for csp22 and named CORE, which has homologs in several other Solanaceous plants, including *N. benthamiana* (Wang et al., 2016). The latter study attempted to reconcile the role of NbCSPR and NbCORE in csp22 perception but failed to demonstrate a role for NbCSPR in csp22 responsiveness, suggesting that NbCSPR is neither sufficient nor necessary for csp22 perception. Further studies will have to clarify this apparent discrepancy, which should then also validate the strategy of using BAK1 as molecular bait to identify cell surface receptors.

A recent discovery indicated that approximately 10% of the NLRs belong to the ID-class with integrated decoys, and thus far a few hundred unique integrated domains have been identified (Kroj et al., 2016; Sarris et al., 2016). Interestingly, there is a significant overlap between integrated domains fused to plant NLRs and protein domains found to frequently interact with pathogen effectors in massive yeast two-hybrid screens against *A. thaliana* proteins (Mukhtar et al., 2011; Weßling et al., 2014; Sarris et al., 2016). Overlapping domains include putative kinase domains, DNA binding domains (e.g. the WRKY domain), and proteins

involved in redox reactions (e.g. the HMA domain) as well as hormone signalling and cytoskeleton (Sarris et al., 2016). Obviously, these molecules may be screened for activation by pathogen effectors. Indeed, this idea was tested by focussing on a particular type of NLR-ID. Multiple NLRs have integrated a predicted DNA-binding BED zinc finger domain between their CC and NBS domains (Kroj et al., 2016). The authors tested a rice NLR-type gene that encodes a ZBED protein containing three N-terminally integrated BED zinc finger domains, showing that *ZBED*-expressing rice plants were more resistant to rice blast fungus *M. oryzae* while ZBED-deficient plants were more susceptible (Kroj et al., 2016). Although recognition of pathogen effectors by BED domains in ZBED protein remains to be established, this test indicates that characterization of integrated domains in NLRs can serve as an extremely powerful way for identification of new players in plant immunity. Moreover, making use of this plasticity in NLR-IDs, novel variants may be engineered upon integration of other "pathogen-customized" domains, based on interactors that can be identified for one's favourite effector (Figure 1**e**).

Transfer and artificial evolution of plant immune receptors have successfully been deployed to improve pathogen recognition capacities (Table 1; Farnham and Baulcombe, 2006; Harris et al., 2013; Chapman et al., 2014; Segretin et al., 2014; Giannakopoulou et al., 2015; Helft et al., 2016). These strategies can also be utilized to exploit known immune receptors for engineering disease resistance in crops. In this case, homologues of *Verticillium* effector Ave1 were previously identified in a number of pathogenic microbes, including the citrus bacterial canker pathogen *Xanthomonas. axonopodis* pv. *citri* (XacPNP), the fungal tomato wilt pathogen *F. oxysporum* f. sp. *lycopersici* (FoAve1), the fungal sugar beet leaf spot pathogen *Cercospora beticola* (CbAve1), the crucifer anthracnose pathogen *Colletotrichum higginsianum* (ChAve1) and the cucurbit anthracnose pathogen *Colletotrichum orbiculare* (CoAve1) (Nembaware et al., 2004; de Jonge et al., 2012; Gan et al., 2013), and these homologs are differentially recognized by tomato Ve1 and its functional homologues (de Jonge et al., 2012; Chapter 3 and Chapter 4). It was shown that Ve1 mediates resistance against *F. oxysporum* f. sp. *lycopersici* in tomato, demonstrating involvement of this immune receptor in resistance against multiple fungal pathogens. And tomato *Ve1* can be functionally transferred to the closely related plant species (*Nicotiana tabacum*) as well as to the distantly related plant species (*A.*

thaliana and cotton), resulting in enhanced Verticillium wilt resistance in an Ave1-dependent manner (Fradin et al., 2011; de Jonge et al., 2012; Chapter 5). Thus, these findings inspire us to further exploit tomato Ve1 (homologues) for engineering disease resistance in an Ave1(homolog)-dependent manner, for example through transfer or experimental evolution of tomato *Ve1*(homologues).

Recently, Kim *et al*. (2016) took advantage of the indirection recognition mechanism to engineer a host effector target to increase novel recognition specificities by trapping unrelated pathogen-derived proteases in their act. Engineering host effector target strategy may be applicable to other immune receptors to expand their recognition capacities. Besides monitoring of host effector target cleavage, sensing of inhibition, phosphorylation and other modification of guarded effector targets through immune receptors have also been reported (Rooney et al., 2005; van Esse et al., 2008; Song et al., 2009; Liu et al., 2011; Lozano-Torres et al., 2012; Innes, 2015; Duxbury et al., 2016). For example, the tomato cell surface-localized RLP-type immune receptor Cf2 monitors the interaction of the apoplastic tomato cysteine protease Rcr3 with the effectors Avr2 and Gr-VAP1, protease inhibitors secreted by the tomato leaf mould fungus *Cladosporium fulvum* (Dixon et al., 2000; Rooney et al., 2005) and by the obligate plant-parasitic nematode *Globodera rostochiensis*, respectively (Lozano-Torres et al., 2012).

Auxiliary strategies for developing disease resistance in crops

Besides deployment of immune receptors, there are a number of other approaches for developing disease resistance in crops. Recessive disease resistance genes are commonly regarded as disease susceptibility (*S*) genes that encode host proteins required for pathogen survival and proliferation. Host genes encoding such susceptible factors are promising candidates for engineering disease resistance through loss-of-function of the host susceptible factors, as inactivation of host susceptible factors can limit pathogen survival and growth (Pavan et al., 2010; Gawehns et al., 2013; van Schie and Takken, 2014). Indeed, loss-of-function of the *Mildew resistance locus o* (*Mlo*) in barley was found to confer recessively inherited broad-spectrum resistance against powdery mildew (Jørgensen, 1992). Barley Mlo is a plasma membrane-localized protein that is required for pathogen invasion

(Humphry et al., 2006; Kusch and Panstruga, 2017). Notably, *mlo*-mediated powdery mildew resistance has also been described in other plant species, including *A. thaliana*, tomato, pea, wheat, apple, grapevine, pepper, rose, tobacco, cucumber and melon (Kusch and Panstruga, 2017). Other successful examples of recessive resistance genes are the eukaryotic translation elongation initiation factor genes *eif4e* and *eif4g*, conferring potyvirus resistance in lettuce, melon, tomato, pepper, barley, pea and rice (Wang and Krishnaswamy, 2012), and rice *xa13, xa25* and *Os11N3* (also named as *OsSWEET11*, *OsSWETT13* and *OsSWEET14*, respectively), conferring bacterial blight resistance against *X. oryzae* pv. *oryzae* (Zhang and Wang, 2013). Notably, the application of mutated alleles of host *S* genes in breeding programmes can be problematic if pleiotropic effects, such as dwarfism or spontaneous necrotic lesions, are observed in loss-of-function mutants of *S* genes (van Schie and Takken, 2014).

RNA interference (RNAi) is a widely conserved regulatory mechanism that affects gene expression in eukaryotic organisms, and cross-kingdom RNAi and small RNA (sRNA) trafficking have been described in plant-pathogen interactions (Knip et al., 2014; Weiberg and Jin, 2015). Cross-kingdom RNAi from a host plant to its interacting pathogens or pests is called host-induced gene silencing (HIGS), a phenomenon in which a host plant-generated sRNA triggers silencing of a pathogen gene (Nunes and Dean, 2012; Koch and Kogel, 2014). Although the HIGS approach has been examined in multiple crops to combat plant diseases caused by various pathogens (Nunes and Dean, 2012; Koch and Kogel, 2014; Wang et al., 2017), it has not yet been applied in breeding programmes to control plant diseases. In this thesis, we also assessed whether HIGS can be utilized to control Verticillium wilt disease by silencing virulence genes of *Verticillium dahliae* through the host plants tomato and *A. thaliana* (Chapter 6). Our results clearly show that plants transiently (in tomato) or stably (*A. thaliana*) expressing RNAi constructs targeting transcripts of genes that are essential for *V. dahliae* pathogenicity can become protected from Verticillium wilt disease, suggesting that HIGS against *V. dahliae* may be exploited to engineer Verticillium wilt resistance in crops (Chapter 6). Indeed, a recent report on HIGS protecting Verticillium wilt-susceptible cotton plants from *V. dahliae* infection has been described (Zhang et al., 2016). Considering that HIGS is limited by a ban on growing genetically modified (GM) crops in large regions of the world,

and the instability of engineered RNA silencing traits, an alternative sRNA trafficking-based disease control approach called spray-induced gene silencing (SIGS) is proposed in which spaying sRNAs that target essential pathogen genes on plant surfaces confer effective crop protection (Wang and Jin, 2017). SIGS has recently been tested for controlling *Fusarium graminearum* infection on barley (Koch et al., 2016), inhibiting *Botrytis cinerea* on vegetables, fruits and flower petals (Wang et al., 2016), and protecting cowpea and tobacco from plant viruses (Mitter et al., 2017). sRNA trafficking-based disease control approaches could be alternatives for developing disease resistance in crops for which no natural resistance source is available.

CONCLUDING REMARKS

Ultimately, once it is fully understood how ligand specificity and signal transduction activation by immune receptors is biochemically established, we may be able to design complete immune receptors *de novo*. Potentially, this allows designing a spectrum of immune receptors for any given pathogen once we know what molecules are exposed during host colonization. Presently, however, recognition specificities are scarce and should be managed wisely. Thus, strategies based on the transfer of individual immune receptor genes can be rapidly overcome under field conditions and pyramiding of immune receptors that recognize different ligands is imperative (Kim et al., 2012; Zhu et al., 2012; Jo et al., 2014; Haesaert et al., 2015; Xiao et al., 2016). Moreover, durability of the immune receptors can be further reinforced by using strategies to engineer immunity other than those exploiting immune receptors, such as editing of host susceptibility genes or exploitation of sRNA trafficking-based disease control strategies (e.g. HIGS and SIGS).

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SUMMARY

Plant-pathogenic microbes secrete effector molecules to establish disease on their hosts, whereas plants in turn employ immune receptors to try and intercept such effectors in order to prevent pathogen colonization. Based on structure and subcellular location, immune receptors fall into two major classes; cell surface-localized receptors that comprise receptor kinases (RKs) and receptor-like proteins (RLPs) that monitor the extracellular space, and cytoplasm-localized nucleotide-binding domain leucine-rich repeat receptors (NLRs) that survey the intracellular environment. **Chapter 1** describes conceptual advances explaining the plant innate immunity system, and advances in our understanding of genetic resistance against Verticillium wilt, with emphasis on *Verticillium* resistance mediated by the *Ve* locus. In tomato (*Solanum lycopersicum*), race-specific resistance to Verticillium wilt is governed by the extracellular leucine-rich repeat (eLRR)-containing RLP-type cell surface receptor Ve1, that is encoded by the *Ve* locus, through recognition of the effector protein Ave1 that is secreted by race 1 strains of *Verticillium* spp.. Homologues of *Verticillium dahliae* Ave1 (VdAve1) are found in plants and in a number of plant pathogenic microbes, and some of these VdAve1 homologues are recognized by tomato Ve1. The research described in this thesis aims to characterize the role of the tomato cell surface-localized immune receptor Ve1, and its homologues in various other plant species, in Verticillium wilt resistance.

Research on effectors secreted by pathogens during host attack has dominated the field of molecular plant-microbe interactions over recent years. **Chapter 2** reviews the current status of research on how soil-borne pathogens employ secreted effector molecules to support host colonization. Most well-studied effectors are proteinaceous molecules, but also non-canonical effectors, such as metabolites and small RNA molecules, have been described. Soil-borne pathogens live in a complex and sophisticated below-ground environment. In addition to targeting of plant components, part of the effector catalogue may be required for self-defense against, and targeting of, other microbiome partners, including antagonists and competitors.

It has previously been demonstrated that many eLRR-containing cell surface-localized immune receptors often recognize short peptide sequence stretches as epitopes of their pathogen ligands. In **Chapter 3**, we aimed to identify a minimal

motif in VdAve1 that is necessary and sufficient for recognition by the tomato cell-surface immune receptor Ve1. Epitope prediction, serial deletion mutants, domain swaps, synthetic peptides, chimeric protein and three-dimensional modelling were exploited to identify which part of the effector protein VdAve1 is recognized by Ve1. Our analyses revealed that the C-terminus as well as the N-terminus are individually required, but not sufficient, to activate Ve1-mediated immunity. A three-dimensional model of the VdAve1 protein explains why both termini are required, by showing that both termini co-localize on a surface-exposed patch of the VdAve1 protein. We propose that a surface-exposed patch of the VdAve1 protein that is composed of co-localized N- and C- termini is recognized by the tomato cell-surface immune receptor Ve1.

In **Chapter 4**, we describe the cloning and characterization of tomato *Ve1* homologues within and outside of the Solanaceae family, and demonstrate that particular Ve1 homologues from tobacco (*Nicotiana glutinosa*; NgVe1), potato (*Solanum tubersoum*; StuVe1), wild eggplant (*Solanum torvum*; StoVe1) and hop (*Humulus lupulus*; HLVe1-2A) serve as functional Ve1 homologues by conferring resistance to race 1 *V. dahliae* through recognition of VdAve1, implying that functional Ve1 homologues are conserved across plant species within and outside the Solanaceae.

It was previously demonstrated that heterologous expression of tomato *Ve1* in the model plant *Arabidopsis thaliana* that does not normally respond to the *Verticillium* effector Ave1, leads to Verticillium wilt resistance against race 1 *Verticillium* spp. In **Chapter 5** we investigated whether tomato *Ve1* can confer Verticillium wilt resistance when expressed in the crop species tobacco (*Nicotiana tabcum*) and cotton (*Gossypium hirsutum*). Stable transgenic tobacco and cotton lines constitutively expressing tomato *Ve1* were generated. Expression of tomato *Ve1* in tobacco resulted in stunted growth, while cotton plants expressing *Ve1* did not show growth retardation. *Ve1*-transgenic tobacco and cotton plants displayed enhanced resistance against Verticillium wilt in an Ave1-dependent manner. This demonstrates that the functionality of tomato Ve1 in resistance against Verticillium wilt through recognition of the *Verticillium* effector Ave1 is retained after transfer to tobacco and cotton.

Host-induced gene silencing (HIGS) is an RNA interference (RNAi)-based process in which small RNAs are produced by the host plant to target parasite transcripts. HIGS has recently emerged as a promising strategy for the improvement of plant resistance against pathogens by silencing genes that are essential for these pathogens. In **Chapter 6**, we assessed whether HIGS can be deployed to inhibit Verticillium wilt disease by silencing previously identified virulence genes of *V. dahliae* (encoding Ave1, Sge1 and NLP1 respectively) through the host plants tomato and *A. thaliana.* Firstly, we established a *Tobacco rattle virus* (TRV)-mediated HIGS assay through targeting of *V. dahliae Ave1* transcripts in *Ve1* tomato plants, and further used this approach to assess whether HIGS against *V. dahliae* in tomato could be established through TRV constructs targeting previously identified *V. dahliae* virulence factors. We also investigated whether HIGS against *V. dahliae* could be established in transgenic *A. thaliana* plants through hairpin RNA-based RNAi constructs targeting transcripts of the same previously identified *V. dahliae* virulence genes. Our results clearly show that plants transiently (in tomato) or stably (in *A. thaliana*) expressing RNAi constructs targeting transcripts of genes that are essential for *V. dahliae* pathogenicity can become protected from Verticillium wilt disease.

In **Chapter 7**, the major results described in this thesis are discussed and placed in a broader perspective. Taking the findings of this thesis into account, a current overview for the improvement of plant disease resistance through the deployment of plant immune receptors is presented, and future directions to enhance disease resistance in crops through the exploitation of immune receptors and through using other approaches, such as editing of host susceptibility genes or exploitation of sRNA trafficking-based disease control strategies, is discussed.

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Yin Song 宋银
26th September, 2017
Radix building, Wageningen

CURRICULUM VITAE

Yin Song (宋银) was born on July 16th, 1985 in Hanzhong, Shaanxi province, China. In 2008, he finished his BSc study in Plant Sciences at Southwest Forestry University, Kunming, China. One year later, Yin was enrolled in a three-year master training program at Shaanxi Normal University, Xi'an, China. He performed his MSc thesis project on metabolic engineering of pharmaceutical compounds in the medicinal plant *Salvia miltiorrhiza*, under the supervision of Prof. Zhezhi Wang. During his MSc, he worked as a volunteer research assistant in the group of Receptor-like Protein/Kinase Function and Peptide Signaling, led by Dr. Guodong Wang. In 2012 he was awarded a PhD fellowship from the China Scholarship Council (CSC) to join the Verticillium group at the Laboratory of Phytopathology, Wageningen University, the Netherlands, under the supervision of Prof. dr. Bart Thomma and Prof. dr. Pierre de Wit. His PhD research resulted in the publication of this thesis entitled: "Recognition of *Verticillium* effector Ave1 by tomato immune receptor Ve1 mediates *Verticillium* resistance in diverse plant species".

List of publications

Yin Song[#], Zhao Zhang[#], Jordi C. Boshoven, Hanna Rovenich, Michael F. Seidl, Jernej Jakše, Karunakaran Maruthachalam, Chun-Ming Liu, Krishna V. Subbarao, Branka Javornik and Bart P.H.J. Thomma (2017) Tomato immune receptor Ve1 recognizes surface-exposed co-localized N- and C-termini of *Verticillium dahliae* effector Ave1. *bioRxiv*: https://doi.org/10.1101/103473.

Jordi C. Boshoven, Malaika Ebert[$], **Yin Song**[$], Hanna Rovenich, Eduardo Rojas-Padilla, Melvin D. Bolton and Bart P.H.J. Thomma (2017) Homologs of *Verticillium dahliae* effector Ave1 contribute to virulence of various fungal plant pathogens. (submitted).

Yin Song, Linlin Liu, Yidong Wang, Dirk-Jan Valkenburg, Xianlong Zhang, Longfu Zhu and Bart P.H.J. Thomma (2017) Transfer of tomato immune receptor Ve1 confers Ave1-dependent *Verticillium* resistance in tobacco and cotton. *Plant Biotechnology Journal*. DOI: 10.1111/pbi.12804.

Luis Rodriguez-Moreno[#], **Yin Song**[#] and Bart P.H.J. Thomma (2017) Transfer and engineering of immune receptors to improve recognition capacities in crops. *Current Opinion in Plant Biology* 38: 42-49.

Yin Song, Zhao Zhang, Michael F. Seidl, Aljaz Majer, Jernej Jakse, Branka Javornik and Bart P.H.J. Thomma (2017) Broad taxonomic characterization of Verticillium wilt resistance genes reveals an ancient origin of the tomato Ve1 immune receptor. *Molecular Plant Pathology* 18: 195-209.

Yin Song and Bart P.H.J. Thomma (2016) Host-induced gene silencing compromises Verticillium wilt in tomato and *Arabidopsis*. *Molecular Plant Pathology.* DOI: 10.1111/mpp.12500.

Zhao Zhang, **Yin Song**, Chun-Ming Liu and Bart P.H.J. Thomma (2014) Mutational analysis of the Ve1 immune receptor that mediates *Verticillium* resistance in tomato. *PLoS ONE* 9:e99511. doi:10.1371/journal.pone.0099511.

Emilie F. Fradin[#], Zhao Zhang[#], Hanna Rövenich[$], **Yin Song**[$], Thomas W.H. Liebrand, Laura Masini, Grardy C.M. van den Berg, Matthieu H.A.J. Joosten and Bart P.H.J.

Thomma (2014) Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2. *PLoS ONE* 9: e88208. doi:10.1371/journal.pone. 0088208.

Donghao Wang[#], **Yin Song**[#], Yuqin Chen, Wei Yao, Zhen Li, Wenchao Liu, Sisi Yue and Zhezhi Wang (2013) Metabolic pools of phenolic acids in *Salvia miltiorrhiza* are enhanced by co-expression of *Antirrhinum majus Delila* and *Rosea1* transcription factors. *Biochemical Engineering Journal* 74: 115–120.

Donghao Wang, Wei Yao, **Yin Song**, Wenchao Liu, Zhezhi Wang (2012) Molecular characterization and expression of three galactinol synthase genes that confer stress tolerance in *Salvia miltiorrhiza*. *Journal of Plant Physiology* 169: 1838–1848.

[#,$] equal contribution

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Yin Song**
Date: **23 October 2017**
Group: **Laboratory of Phytopathology**
University: **Wageningen University & Research**

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Interactions of Ve1 and Ave1 homologues: From recognition to resistance	20 Dec 2013
► **Writing or rewriting a project proposal**	
Title: The genetics of receptor-like protein (RLP) Ve-mediated signalling in defense against *Verticillium* in *Arabidopsis* and tomato	Oct 2012
► **Writing a review or book chapter**	
Title: Transfer and engineering of immune receptors to improve recognition capacities in crops. Current Opinion in Plant Biology 2017, 38:42-49	May 2017
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student day, Amsterdam, NL**	30 Nov 2012
EPS PhD student day, Leiden, NL	29 Nov 2013
EPS PhD student day 'Get2Together', Soest, NL	28-29 Jan 2016
5th European Plant Science Retreat for PhD students, Ghent, Belgium	23-26 Jul 2013
6th European Plant Science Retreat for PhD students, Amsterdam, NL	01-04 Jul 2014
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', & Willie Commelin Scholten day, Utrecht, NL	24 Jan 2013
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', & Willie Commelin Scholten day, Amsterdam, NL	25 Feb 2014
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', & Willie Commelin Scholten day, Leiden, NL	22 Jan 2016
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', & Willie Commelin Scholten day, Wageningen, NL	23 Jan 2017
► **National meetings (e.g. Lunteren days) and other national platforms**	
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	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
► **Seminars (series), workshops and symposia**	
Mini-symposium: Frontiers in Plant Morphogenesis (Chun-Ming Liu, Sidney Shaw, Dolf Weijers, Viktor Žárský)	13 Nov 2012
EPS flying seminar Prof. Ralph Panstruga: Comparative pathogenomics of powdery mildew fungi: chasing the molecular secrets of obligate biotrophy and fungal pathogenesis	12 Dec 2012
Mini-symposium: Intraspecific pathogen variation-implications and opportunities	22 Jan 2013
EPS flying seminar Dr. Detlef Weigel: *Arabidopsis thaliana* as a model system for the study of evolutionary questions	27 Feb 2013
Flying Seminars Prof. Howard S. Judelson and Dr. Rays H. Jiang	07 May 2013

Seminar Prof. dr. Brian Staskawicz: Effector-Targeted Breeding for Durable Disease Control of *Xanthomonas* diseases in Tomato and Cassava	21 May 2013
Seminar Dr. Pieter Dorrestein: A ''GoogleAMP''-type molecular view of microbes-from culture to people	22 Aug 2013
Seminar: How to write a world-class paper	17 Oct 2013
Seminar Prof. Jiayang Li: Understanding the molecular mechanisms underlying rice tillering	15 Nov 2013
Seminar Prof. Jos Raaijmakers: Back to the Roots: exploring and exploiting the plant microbiome	07 Jan 2014
Seminar Dr. Jeroen Mesters: From protein solution to single crystal X-ray diffraction: Chitin binding by LysM domains at atomic resolution	31 Mar 2014
Seminar Prof. Jane Parker: Reprogramming cells for defence in plant innate immunity	09 Apr 2014
Seminar Dr. Frank van Breusegem: Plant metacaspases	09 Apr 2014
Seminar Prof. Sophien Kamoun: Genome and effector evolution in the Irish potato famine pathogen lineage	28 May 2014
Farewell symposium Prof. dr. Pierre de Wit: Fungal plant pathogens and the plant immune system	05 Jun 2014
Seminar Prof. Yuanchao Wang: Dissecting the interaction between *Phytophthora sojae* and soybean: making sense out of signalling and effectors	16 Jul 2014
Seminar Prof. Hanhui Kuang 'Using the *Nicotiana*-TMV system to study resistance gene evolution and plant genome stability'	11 Sep 2014
Seminar Dr. Michael Freitag: Chromatin structure controls centromeres and secondary metabolism in filamentous fungi	21 Oct 2014
Phytopathology Lab. mini-symposium (Erik Limpens, Tijs Ketelaar, Eric Schranz, Frank Takken & Weixing Shan)	24 Nov 2014
EPS symposium: Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding	11 Dec 2014
Seminar Prof. Nathalia Requena: Plant-fungal communication in the arbusucular mycorrhizal symbiosis	30 Jan 2015
Seminar Prof. Jiming Jiang: Structure and evolution of centromeres: lessions learned from plants	01 Apr 2015
Phytopathology Lab. symposium: Crop Pathology and Plant-Microbe Interactions	08 May 2015
Plant sciences seminar: Into the Battle between Plants and Viruses, but what about EVEs?	12 May 2015
EPS flying seminar Prof. Gero Steinberg: Long-distance endosome trafficking drives fungal effector production during plant infection	05 Jun 2015
EPS flying seminar Prof. Jane Parker: Plant intracellular immunity: evolutionary and molecular underpinnings	21 Jan 2016
Seminar Prof. dr. Douglas Mitchell: Genomics-enabled natural products discovery	31 Mar 2016
Seminar Dr. Pierre-Marc Delaux: Evolution of symbiotic gene networks in land plants	08 Apr 2016
Seminar Prof. Caitilyn Allen: How *Ralstonia solanacearum* succeeds in plant xylem vessels	29 Apr 2016
Seminar Prof. Wenbo Ma: Effectors as molecular probes to understand pathogenesis	20 Jun 2016
Seminars Dr. Edze Westra and Prof. Jennifer Doudna: Rewriting our genes? CRISPR-CAS systems as tools for genome editing	30 Sep 2016
1st WURomics symposium: Technology-Driven Innovation for Plant Breeding	15 Dec 2016
Farwell symposium Prof. Ton Bisseling: The Undergrond Labyrinth: Roots, Friends and Foes	08 Feb 2017
Phytopathology Lab. mini-symposium on applied Phytopathology: From the lab to the field	01 Mar 2017
► **Seminar plus**	
► **International symposia and congresses**	
COST SUSTAIN workshop: Pathogen-informed crop improvement, Wageningen, NL	08-10 Apr 2015
The 3rd COST SUSTAIN Anuual Conference, Banyus Sur Mer, France	17-19 Feb 2016
12th International Verticillium Symposium, Ljubljana, Slovenia	06-09 Oct 2016
The 5th International Conference on Biotic Plant Interactions, Xiamen, China	17-21 Aug 2017
► **Presentations**	
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	14-15 Apr 2014
Talk: COST SUSTAIN workshop, Wageningen, NL	09 Apr 2015
Talk: 12th International Verticillium Symposium, Ljubljana, Slovenia	09 Oct 2016
► **IAB interview**	
► **Excursions**	
Excursion to Rijk Zwaan, De Lier, NL	13 Sep 2013
Excursion to Enza Zaden, Enkhuizen, NL	12 Jun 2015
Subtotal Scientific Exposure	*19.7 credits**

3) In-Depth Studies		*date*
► **EPS courses or other PhD courses**		
7th Utrecht PhD summer school on Environmental Signaling, Utrecht, NL		23-26 Aug 2013
EPS Spring School "Host-Microbe Interactomics", Wageningen, NL		02-04 Jun 2014
Data analyses and visualizations in R (for biologist), Wageningen, NL		12-13 Dec 2016
► **Journal club**		
Member of literature discussion at Verticillium group in Phytopathology Lab.		2012-2016
► **Individual research training**		
	Subtotal In-Depth Studies	*5.4 credits**

4) Personal development		*date*
► **Skill training courses**		
Practical English, Wageningen, NL		Oct 2012-Feb 2013
English IETLS training, Wageningen, NL		Jun-Jul 2013
Private English training course, Wageningen, NL		Jan 2015-May 2015
Scientific Writing, Wageningen, NL		May-Jul 2016
► **Organisation of PhD students day, course or conference**		
► **Membership of Board, Committee or PhD council**		
	Subtotal Personal Development	*4.1 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.7**

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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