












# A highly polymorphic effector protein promotes fungal virulence through suppression of plant-associated Actinobacteria

Nick C. Snelders<sup>1,2\*</sup> , Jordi C. Boshoven<sup>3\*</sup>, Yin Song<sup>3,4†</sup> , Natalie Schmitz<sup>1†</sup>, Gabriel L. Fiorin<sup>3\*</sup> , Hanna Rovenich<sup>1</sup> , Grardy C. M. van den Berg<sup>3</sup> , David E. Torres<sup>2,3</sup> , Gabriella C. Petti<sup>1</sup> , Zoe Prockl<sup>1</sup> , Luigi Faino<sup>3,5</sup> , Michael F. Seidl<sup>2</sup>  and Bart P. H. J. Thomma<sup>1,3</sup> 

<sup>1</sup>Institute for Plant Sciences, Cluster of Excellence on Plant Sciences (CEPLAS), University of Cologne, 50674 Cologne, Germany; <sup>2</sup>Theoretical Biology and Bioinformatics Group, Department of Biology, University of Utrecht, 3584CH Utrecht, the Netherlands; <sup>3</sup>Laboratory of Phytopathology, Wageningen University and Research, Droevendaalsesteeg 1, 6708PB Wageningen, the Netherlands; <sup>4</sup>State Key Laboratory of Crop Stress Biology for Arid Areas and College of Agronomy, Northwest A&F University, Yangling, 712100 Shaanxi, China; <sup>5</sup>Department of Ambient Biology, Sapienza University of Rome, 00185 Rome, Italy

## Summary

Author for correspondence:  
Bart P. H. J. Thomma  
Email: [bthomma@uni-koeln.de](mailto:bthomma@uni-koeln.de)

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- Plant pathogens secrete effector proteins to support host colonization through a wide range of molecular mechanisms, while plant immune systems evolved receptors to recognize effectors or their activities to mount immune responses to halt pathogens. Importantly, plants do not act as single organisms, but rather as holobionts that actively shape their microbiota as a determinant of health. The soil-borne fungal pathogen *Verticillium dahliae* was recently demonstrated to exploit the VdAve1 effector to manipulate the host microbiota to promote vascular wilt disease in the absence of the corresponding immune receptor Ve1.
- We identify a multiallelic *V. dahliae* gene displaying *c.* 65% sequence similarity to VdAve1, named VdAve1-like (VdAve1L), which shows extreme sequence variation, including alleles that encode dysfunctional proteins, indicative of selection pressure to overcome host recognition.
- We show that the orphan cell surface receptor Ve2, encoded at the Ve locus, does not recognize VdAve1L. Additionally, we demonstrate that the full-length variant VdAve1L2 possesses antimicrobial activity, like VdAve1, yet with a divergent activity spectrum, that is exploited by *V. dahliae* to mediate tomato colonization through the direct suppression of antagonistic Actinobacteria in the host microbiota.
- Our findings open up strategies for more targeted biocontrol against microbial plant pathogens.

## Introduction

The plethora of microbes a plant associates with, its so-called microbiota, encompasses a diversity of microbes that establish a spectrum of symbiotic relationships with their host, ranging from commensalistic through endophytic to mutualistic and pathogenic (Hassani *et al.*, 2019). To survey their microbiota for potentially pathogenic invaders, plants evolved complex immune systems comprising various types of receptors that betray microbial ingress, for instance through the recognition of microbe-associated molecular patterns or microbially secreted effector proteins (Chisholm *et al.*, 2006; Jones & Dangl, 2006; Dodds & Rathjen, 2010; Thomma *et al.*, 2011; Cook *et al.*, 2015). If not recognized, microbial effectors play crucial roles during plant colonization. While they can benefit microbes in many ways, most of the effectors functionally characterized to date have been implicated in the deregulation of host immune responses or other

processes in host physiology (Rovenich *et al.*, 2014; Cook *et al.*, 2015; He *et al.*, 2020; Wang *et al.*, 2022). However, novel effector functions are still being discovered. For instance, we recently demonstrated that some effectors are secreted to manipulate plant microbiota compositions to promote host colonization (Snelders *et al.*, 2020, 2021, 2022).

Effector recognition in plants is mediated by resistance (*R*) genes, typically encoding receptors that reside on the cell surface or in the host cell cytoplasm, that detect effectors or their activities to activate effector-triggered immunity (ETI), often leading to avirulence of the pathogen (Chisholm *et al.*, 2006; Jones & Dangl, 2006; Dodds & Rathjen, 2010; Thomma *et al.*, 2011; Cook *et al.*, 2015). Consequently, effectors that are recognized by *R* proteins are referred to as avirulence factors (Avrs) (Li *et al.*, 2020). To evade ETI and restore the ability to colonize their hosts, pathogens are known to inactivate, purge or mutate their avirulence genes, or to evolve novel effectors that suppress ETI (van Kan *et al.*, 1991; Armstrong *et al.*, 2005; Gout *et al.*, 2007; Stergiopoulos *et al.*, 2007; Zhou *et al.*, 2013; Wu *et al.*, 2014; Niu *et al.*, 2016; Schmidt *et al.*, 2016; Praz *et al.*, 2017).

\*These authors contributed equally to this work.

†These authors contributed equally to this work.

*Verticillium dahliae* is a soil-borne fungal pathogen that causes vascular wilt disease in hundreds of plant species (Fradin & Thomma, 2006). The presumed asexual fungus generates genomic diversity through extensive chromosomal rearrangements and segmental duplications that gave rise to dynamic so-called lineage-specific (LS) regions, more recently referred to as adaptive genomic regions (AGRs) (Klosterman *et al.*, 2011; de Jonge *et al.*, 2013; Faino *et al.*, 2016; Shi-Kunne *et al.*, 2018; Cook *et al.*, 2020). These AGRs display extensive presence/absence variation (PAV) between *V. dahliae* strains, are rich in repeats and transposable elements, and have a distinct chromatin profile (Klosterman *et al.*, 2011; de Jonge *et al.*, 2013; Faino *et al.*, 2016; Cook *et al.*, 2020; Torres *et al.*, 2021). Additionally, AGRs are enriched in *in planta*-induced genes and harbor effector genes that are crucial for disease establishment (de Jonge *et al.*, 2012, 2013; Kombrink *et al.*, 2017). Thus, like other filamentous plant pathogens, *V. dahliae* possesses a compartmentalized genome in which (a)virulence factors locate in regions of increased plasticity when compared to the core genome, an arrangement often referred to as a 'two-speed' genome (Raffaele & Kamoun, 2012; Dong *et al.*, 2015; Torres *et al.*, 2020). Intriguingly, *V. dahliae* AGRs that are conserved between strains display enhanced sequence conservation when compared to core genomic regions (Depotter *et al.*, 2019), underscoring that accelerated evolution in these regions is predominantly mediated by presence–absence polymorphisms.

Only a few genetic resistance sources to *V. dahliae* have been identified. In tomato, the *Ve* locus provides resistance against *V. dahliae* and has been introgressed into most commercial tomato cultivars (Schaible *et al.*, 1951; Diwan *et al.*, 1999). The *Ve* locus contains two closely linked genes, *SIVe1* and *SIVe2*, that both encode extracellular leucine-rich repeat receptor-like proteins, of which only *SIVe1* was confirmed to confer *V. dahliae* resistance (Diwan *et al.*, 1999; Kawchuk *et al.*, 2001; Fradin *et al.*, 2009). Since its deployment in the 1950s, resistance-breaking strains appeared that have been assigned to race 2, whereas strains that are contained belong to race 1 (Alexander, 1962). Recently, the single dominant *SIV2* locus was shown to mediate race 2 resistance in *Solanum neorickii* and was introgressed in particular tomato rootstock cultivars (Usami *et al.*, 2017). However, resistance-breaking strains already appeared, forming race 3 (Usami *et al.*, 2017; Chavarro-Carrero *et al.*, 2021). Comparative *V. dahliae* population genomics led to the identification of the *Avrs* corresponding to *SIVe* and *SIV2* resistance, namely *VdAve1* and *VdAv2*, respectively (de Jonge *et al.*, 2012; Chavarro-Carrero *et al.*, 2021). Both effector genes are located in AGRs and display PAV between *V. dahliae* strains. While the molecular function of *VdAv2* remains unknown, we demonstrated that *VdAve1* promotes the virulence of *V. dahliae* on host plants lacking *SIVe1* through selective antimicrobial activity to manipulate host microbiota compositions. More specifically, we showed that *VdAve1* facilitates *V. dahliae* colonization of tomato and cotton through the direct suppression of associated antagonistic bacteria of the Sphingomonadales order (Snelders *et al.*, 2020). Intriguingly, race 1 strains of *V. dahliae* do not display any allelic variation for *VdAve1*, and only two

allelic *VdAv2* variants have been identified among race 2 strains that differ by one nonsynonymous single-nucleotide polymorphism (SNP) and that both activate *Av2*-mediated immunity (Chavarro-Carrero *et al.*, 2021). Hence, to date, *V. dahliae* was exclusively described to evade ETI through the loss of complete avirulence genes, and not through gene inactivation or the evolution of allelic effector variants.

In this study, we describe the identification of the *V. dahliae* effector gene *VdAve1L* that displays extensive allelic variation and demonstrate that the full-length effector variant *VdAve1L2* possesses antimicrobial activity that is exploited by the fungus to mediate tomato colonization through the direct suppression of antagonistic Actinobacteria in the host microbiota. Our findings open up strategies for improved biological control of microbial plant pathogens.

## Materials and Methods

### *Verticillium dahliae* genomics

Genome sequence data used in this study were obtained previously (Klosterman *et al.*, 2011; de Jonge *et al.*, 2012, 2013; Faino *et al.*, 2015; Chavarro-Carrero *et al.*, 2021). BLAST searches were performed with BLAST+ v.2.11.0 using standard parameters and the nucleotide sequences of *VdAve1* or each of the *VdAve1L* alleles as query. Strains lacking *VdAve1L* in their genome assemblies were further assessed for the presence of the gene by PCR using the primers listed in supporting information Table S1. Amplicons obtained from each strain were sequenced for allele determination. Discontinuity in the *VdAve1L3* allele was identified by mapping the genomic paired-end sequences to the *V. dahliae* strain JR2 reference genome using BWA-MEM (Li & Durbin, 2009; Faino *et al.*, 2015). The genetic diversity and the population structure of the sequenced *V. dahliae* strains were assessed using the reference sequence alignment-based phylogeny builder REALPHY (v.1.12) (Bertels *et al.*, 2014) and BOWTIE2 (v.2.2.6) (Langmead & Salzberg, 2012) to map genomic reads against the *V. dahliae* strain JR2 gapless genome. A maximum likelihood phylogenetic tree was built using RAXML (v.8.2.8) (Stamatakis, 2014).

### Oxford Nanopore technology sequencing

Library preparation of the PCR fragment was performed according to the protocol of Oxford Nanopore, skipping the DNA fragmentation step. The library was loaded on a Nanopore flow cell. The run yielded *c.* 870 high-quality long reads with an average length of 3869 bp with the longest read being *c.* 14 kb. Using NANOCORRECT, we used all the obtained reads to correct the 50 longest reads, of which 28 were corrected to generate a consensus. Finally, reads were used for BLAST analysis to confirm the presence of *VdAve1L3* fragments at both ends.

### Presence–absence variation analysis

Presence–absence variation was identified using whole-genome alignments of 52 *V. dahliae* strains. Paired-end short sequencing

reads were mapped to reference *V. dahliae* strain JR2 (Faino *et al.*, 2015) using BWA-MEM with default settings (Li & Durbin, 2009). Long reads were mapped to *V. dahliae* JR2 using MINIMAP2 with default settings (Li, 2018). Using the PICARD toolkit (<http://broadinstitute.github.io/picard/>), library artifacts were marked and removed with *-MarkDuplicates* followed by *-SortSam* to sort the reads. Raw read coverage was averaged per 100-bp nonoverlapping windows using the *-multicov* function of BEDTOOLS (Quinlan & Hall, 2010). Then, raw read coverage values were transformed to a binary matrix by applying a cutoff of 10 reads for short-read data;  $\geq 10$  reads indicate the presence (1), and  $< 10$  reads indicate the absence (0) of the respective genomic region. In the case of long-read data, a cutoff of 1 read was applied;  $\geq 1$  reads indicate the presence (1), and  $< 1$  reads indicate the absence (0). This matrix was further summarized to obtain the total number of presence/absence counts in each 100-bp genomic window within 50-kb upstream and downstream of the *VdAve1L* locus.

### SNP rate determination of *Verticillium dahliae* genes

To assess whether the *VdAve1L* allele indeed displays an unusual degree of sequence variation, we determined the number of SNPs in the coding sequence (CDS) of all genes in the genome of *V. dahliae* strain JR2 compared to 41 other strains that we previously sequenced using the Illumina platform (Torres *et al.*, 2021). Next, we normalized the identified number of SNPs based on the length of the corresponding CDS to determine an SNP rate.

### *VdAve1L2* gene expression

To determine *in planta* expression of *VdAve1L2*, tomato plants (*Solanum lycopersicum*) cultivar MoneyMaker were inoculated with *V. dahliae* strain DVD-S26 as described previously (Fradin *et al.*, 2009). Stems of five mock and five inoculated plants were harvested at 7, 14, and 21 d after inoculation. *In vitro* expression of *VdAve1L2* was assessed in mycelium of *V. dahliae* strain DVD-S26 grown for 5 d in triplicate on PDA plates and in soil extract (Snelders *et al.*, 2020). Total RNA of all samples was extracted using the Maxwell76 LEV Plant RNA kit (Promega), and cDNA was synthesized using M-MLV reverse transcriptase (Promega). Real-time PCR was conducted using a C1000 Touch™ Thermal Cycler (Bio-Rad) and the qPCR SensiMix kit (BioLine, GC Biotech BV, Alphen aan den Rijn, the Netherlands) using the primers listed in Table S1. Real-time PCR conditions were as follows: an initial 95°C denaturation step for 10 min followed by denaturation for 15 s at 95°C, annealing for 60 s at 60°C, and extension at 72°C for 40 cycles.

### Generation of *Verticillium dahliae* mutants

To generate *VdAve1L2* deletion lines, primers were designed to amplify *c.* 1500 bp up and downstream of the *VdAve1L2* CDS (Table S1). Both amplicons were used to generate a USER-friendly cloning construct to replace *VdAve1L2* with a hygromycin cassette through homologous recombination (Frandsen

*et al.*, 2008). To complement the *VdAve1L2* deletion mutant, a PCR fragment was amplified from genomic DNA containing the complete *VdAve1L2* CDS and *c.* 1000-bp up and downstream sequences (Table S1) and cloned into the binary vector pCG (Zhou *et al.*, 2013). To generate the *V. dahliae* DVD-S26 mutants expressing *VdAve1L2* under the control of the *VdAve1* promoter, the CDS of *VdAve1L2* was amplified and cloned into pFBT005. *Verticillium dahliae* transformations were performed as described previously (Santhanam, 2012).

### Disease assays

Inoculation of tomato plants to determine the virulence of *V. dahliae* Kleb. was performed as described previously (Fradin *et al.*, 2009). Accumulation of *V. dahliae* biomass in tomato plants was quantified with real-time PCR on genomic DNA by targeting the internal transcribed spacer region of ribosomal DNA using the primers listed in Table S1. To assess the importance of the suppression of Actinobacteria by *VdAve1L2* for tomato colonization by *V. dahliae*, we germinated tomato (*Solanum lycopersicum* L.) MoneyMaker seeds in a sealed beaker on sterile potting soil (Lentse Potgrond BV, Katwijk, the Netherlands) supplemented with water-treated tomato root microbiota or vancomycin-treated tomato root microbiota. Vancomycin treatment of the tomato root microbiota was performed as described previously, with slight modifications (Lee *et al.*, 2021). Briefly, roots with rhizosphere soil were harvested from 6-wk-old tomato MoneyMaker plants grown on potting soil (Lentse Potgrond). The material collected from four plants was pooled and ground to a fine powder in liquid nitrogen using a mortar and pestle. Subsequently, the ground material was split and transferred to 300 ml 2.5 mM MES pH 6.0 supplemented with 500  $\mu\text{g ml}^{-1}$  vancomycin or water. The suspensions were incubated for 3 h at 30°C and 120 rpm. Finally, the suspensions were divided into fractions of 50 ml, snap frozen, and stored at  $-20^\circ\text{C}$  until use. Tomato seeds were surface-sterilized by incubation for 5 min in 2% sodium hypochlorite. Next, the surface-sterilized tomato seeds were washed three times using sterile water and transferred to sealed beakers containing 60 g of sterilized potting soil and 50 ml of the water-treated or vancomycin-treated microbial suspensions. After 14 d, seedlings colonized by the microbial communities were root-dipped in spore suspensions of *V. dahliae* strain DVD-S26, a *VdAve1L2* deletion mutant or water (mock) as described previously (Fradin *et al.*, 2009) and transferred to pots containing sterile river sand soaked in Hoagland nutrient solution.

### Root microbiota analysis

Tomato inoculations were performed as described previously (Fradin *et al.*, 2009). After 10 d, plants were carefully uprooted and gently shaken to remove loosely adhering soil from the roots. Next, roots with rhizosphere soil from two tomato plants were pooled to form a single biological replicate. Alternatively, the tomato plants that received the water-treated and vancomycin-treated microbial communities were uprooted 18-d

postinoculation with *V. dahliae* and a single-root system with adhering river sand was collected as a biological control. All samples were flash-frozen in liquid nitrogen and ground using a mortar and pestle. Genomic DNA isolation was performed using the DNeasy PowerSoil Kit (Qiagen). Sequence libraries were prepared following amplification of the V4 region of the bacterial 16S rDNA (341F and 785R), and paired ends (300 bp) were sequenced using the MiSeq sequencing platform (Illumina) at Baseclear (Leiden, the Netherlands). Data analyses were performed as described previously (Snelders *et al.*, 2020).

### *Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana tabacum*

*Nicotiana tabacum* L. cv Petite Havana SR1 was infiltrated with GV3101 *A. tumefaciens* strains carrying pSOL2092 constructs for the expression of *Ve1* or *Ve2* and a *VdAve1*-like construct, as described previously (Zhang *et al.*, 2013). Plants were transferred to a climate chamber and incubated at 22°C : 19°C during 16 h : 8 h, day : night periods, respectively, with 70% relative humidity. Leaves were inspected for HR at 5 dpi.

### Protein production, purification, and refolding

Heterologous production and purification of VdAve1L2 was performed as described previously for VdAve1 (Snelders *et al.*, 2020).

### Bacterial isolates

Bacterial strains *Bacillus subtilis* AC95, *Staphylococcus xylosus* M3, *Pseudomonas corrugata* C26, *Streptomyces* sp. NE-11417C (Actinobacteria), and *Ralstonia* sp. M21 were obtained from our in-house endophyte culture collection (Snelders *et al.*, 2020). Bacterial strains *Aeromicrobium* sp. (Actinobacteria, DSM 102283), *Cellulomonas chitinilytica* (Actinobacteria, DSM 17922), *Frondehabitans peucedani* (Actinobacteria, DSM 22180), *Jatrophihabitans huperziae* (Actinobacteria, DSM 46866), *Leifsonia* sp. (Actinobacteria, DSM 102435), and *Nocardioides plantarum* (Actinobacteria, DSM 11054) were obtained from the DSMZ culture collection (Braunschweig, Germany). Bacterial strains *Novosphingobium* sp. A (NCCB 100261), *Sphingopyxis macrogoltabida* (NCCB 95163), and *Sphingobacterium* sp. (NCCB 100093) were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands).

### Antimicrobial activity assays

*In vitro* antimicrobial activity assays were performed as described previously using 0.2× tryptone soy broth (TSB) as growth medium for the bacteria (Snelders *et al.*, 2020).

### *In vitro* competition assays

Following 11 d of cultivation on PDA, the conidiospores of *V. dahliae* strains DVD-S26  $\Delta$ VdAve1L2, DVD-S26  $\Delta$ VdAve1L2 + *pVdAve1*::VdAve1L2 #1, and DVD-S26  $\Delta$ VdAve1L2 + *pVdAve1*::

VdAve1L2 #2 were harvested from plates and stored at –80°C at a concentration of  $4 \times 10^5$  spores ml<sup>-1</sup> in low-salt TSB (17 g l<sup>-1</sup> tryptone, 3 g l<sup>-1</sup> soy peptone, 0.5 g l<sup>-1</sup> NaCl, 2.5 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, and 2.5 g l<sup>-1</sup> glucose) supplemented with 10% glycerol until use. Next, bacterial isolates were grown on low-salt TSA at 28°C. Single colonies were selected and grown overnight at 28°C while shaking at 150 rpm. Overnight cultures were resuspended to an OD<sub>600</sub> = 0.02 in fresh low-salt TSB, while the fungal spore suspensions were allowed to thaw at room temperature. Finally, the bacterial and fungal spore suspensions were mixed in 500  $\mu$ l of low-salt TSB to a final concentration of OD<sub>600</sub> = 0.01 and 10<sup>3</sup> spores ml<sup>-1</sup>, respectively. Following 6 d of incubation at 22°C, the microbial suspensions were transferred to clear 24-well flat-bottom polystyrene tissue culture plates to allow imaging of fungal growth using an SZX10 stereo microscope (Olympus Co., Tokyo, Japan) equipped with an EP50 camera (Olympus).

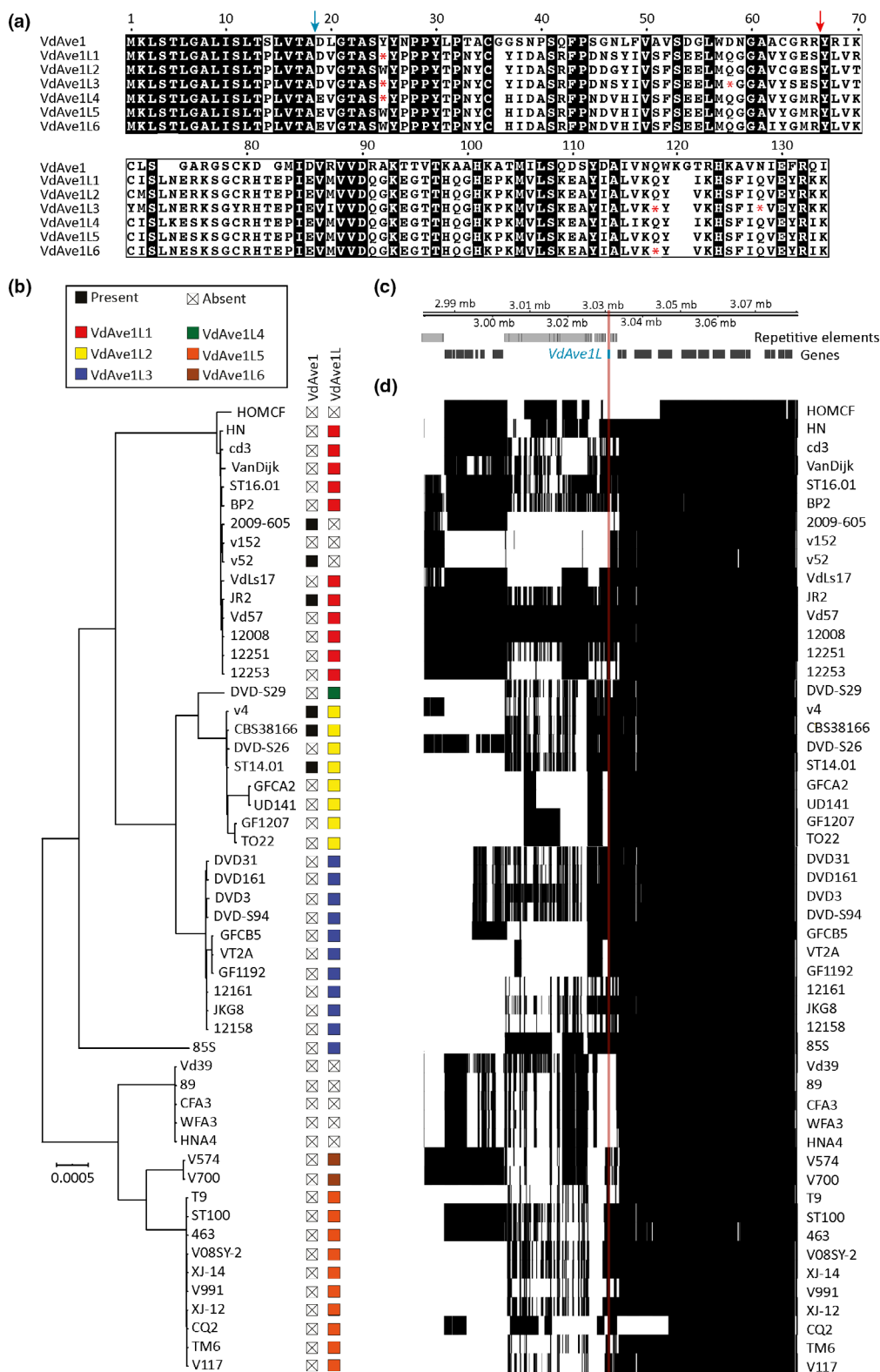
## Results

### Identification of the highly polymorphic *VdAve1*-like gene in an adaptive genomic region of *Verticillium dahliae*

Using the gapless genome assembly of *V. dahliae* strain JR2 (Faino *et al.*, 2015), a similarity search with the CDS of *VdAve1* revealed a gene with 67% nucleotide similarity that we further refer to as *VdAve1L* (for *VdAve1*-like). However, while *VdAve1* encodes a 134 amino acid protein (de Jonge *et al.*, 2012), *VdAve1L* only encodes a 24 amino acid protein due to a premature stop codon (Fig. 1a). Searches in the genomes of 51 additional *V. dahliae* isolates (Klosterman *et al.*, 2011; de Jonge *et al.*, 2012; Faino *et al.*, 2015; Chavarro-Carrero *et al.*, 2021) (Table S2) identified the gene in 42 isolates (Figs 1a,b, S1), albeit with considerable allelic variation. In total, we identified six allelic variants (*VdAve1L1*–*VdAve1L6*) that share 93–99% sequence similarity (Fig. 1a,b). Like *VdAve1L1*, also *VdAve1L3* and *VdAve1L4* encode truncated 24 amino acid proteins (Fig. 1a). By contrast, and similar to *VdAve1*, *VdAve1L2* and *VdAve1L5* encode 134 amino acid proteins including an 18 amino acid N-terminal signal peptide (Fig. 1a). Finally, *VdAve1L6* differs by only one amino acid when compared to *VdAve1L5* and is truncated after 120 amino acids (Fig. 1a).

Genome assemblies consistently assigned sections of *VdAve1L3* on separate contigs, suggesting discontinuity of this allele (Fig. S2a), which was further supported by PCR analysis on strains DVD-3, DVD-31, DVD-S94, and DVD-161 (Fig. S2b). To investigate whether the discontinuity is caused by a chromosomal rearrangement or transposable element insertion, we performed the PCR on strain DVD-3 with prolonged elongation time, yielding an amplicon of *c.* 7 kb (Fig. S2c). Subsequent sequence analysis revealed that *VdAve1L3* is interrupted by a long terminal repeat retrotransposon classified as VdLTRE3 (Faino *et al.*, 2016).

As expected based on the observed PAV among some *V. dahliae* strains (Fig. 1b), *VdAve1L* is localized in an AGR (Fig. 1c,d) (Cook *et al.*, 2020). However, the allelic variation of *VdAve1L* is unexpected given the previously observed commonly increased



**Fig. 1** *Verticillium dahliae* strains carry a multiallelic *VdAve1*-like gene. (a) Protein sequence alignment of the identified *VdAve1L* proteins and *VdAve1*. Red asterisks (\*) indicate stop codons in the *VdAve1L* alleles. The blue arrow indicates the predicted signal peptide cleavage site, and the red arrow indicates the site where discontinuity is observed in *VdAve1L3*. (b) Phylogenetic tree of sequenced *V. dahliae* strains and the presence-absence variation of *VdAve1* and *VdAve1L* alleles in the corresponding genomes. (c, d) *VdAve1L* is located in an adaptive genomic region of the *V. dahliae* genome. The red line indicates the physical position of *Ave1L2*. The matrix displays the presence-absence (black/white) variation in 100 base-pair nonoverlapping windows in the genomic region surrounding *VdAve1L*. The upper panel displays the annotated genes and repetitive elements identified in this genomic region, indicated in black and gray, respectively.

sequence conservation of AGR sequences that are shared among *V. dahliae* strains (Depotter *et al.*, 2019). Intriguingly, further analysis revealed that *VdAve1L* displays the highest SNP rate of all genes in the *V. dahliae* genome (Fig. S3) and that all of the identified SNPs, 30 in total (Fig. S4), cause protein sequence variation. Thus, *VdAve1L* is a highly polymorphic gene that displays accelerated evolution by PAV, transposon-mediated sequence disruption, and sequence variation.

### VdAve1L proteins are not recognized by SIVe2

The extreme sequence variation of VdAve1L is likely the result of selection pressure to overcome recognition by a plant immune receptor. The only *R* gene known to confer resistance to *V. dahliae* is tomato *SIVe1* (Kawchuk *et al.*, 2001; Fradin *et al.*, 2009), which resides in the *Ve* locus together with *SIVe2* that similarly encodes a receptor-like protein (Fradin *et al.*, 2009). *SIVe2* is expressed similar to *SIVe1*, yet the encoded receptor does not recognize VdAve1 and its function remains unknown (Fradin *et al.*, 2009; de Jonge *et al.*, 2012). Thus, we tested whether SIVe2 can recognize a current or predicted ancestral VdAve1L variant. To this end, we reversed disruptive mutations in *VdAve1L1*, *VdAve1L3*, and *VdAve1L4* by replacing premature stop codons with corresponding codons in *VdAve1L2* and *VdAve1L5* to yield the derivatives *VdAve1L1\**, *VdAve1L3\**, and *VdAve1L4\** (Fig. 2a,b). Similarly, we replaced stop codons in *VdAve1L3\** and removed the retrotransposon (Fig. 2a,b). Additionally, based on an alignment consensus sequence, we constructed the predicted common *VdAve1L* ancestor *VdAve1L\*\** (Fig. 2a,b). Subsequently, we co-expressed the various genes with *SIVe2* in *N. tabacum*, and with *SIVe1* as a negative control, but no hypersensitive response (HR) could be observed except upon co-expression of *VdAve1* (Fig. 2c). Consequently, it is unlikely that SIVe2 recognized VdAve1L and drove its diversification.

### VdAve1L2 is a virulence factor that functionally diverged from VdAve1

Only the alleles *VdAve1L2* and *VdAve1L5* encode full-length proteins (Fig. 1), yet none of the strains that encode VdAve1L5 is pathogenic on tomato (Li, 2019). To determine whether VdAve1L2, like VdAve1, contributes to virulence on tomato, we assessed its expression in *V. dahliae* race 2 strain DVD-S26 during host colonization, showing clear expression *in planta* (Fig. S5). Interestingly, while we previously also detected the expression of *VdAve1* during cultivation *in vitro* on PDA and in soil extract (Snelders *et al.*, 2020), we did not detect *VdAve1L2* expression under these conditions (Fig. S5).

To determine the importance of VdAve1L2 for tomato colonization, we generated deletion mutants in the race 2 strain DVD-S26 and in the race 1 strains ST14.01 and CBS38166 (Fig. S6). Inoculation of tomato plants with the deletion mutants of the race 1 strains revealed no virulence contribution of VdAve1L2 (Fig. 3a,b). Strikingly, however, we detected strongly compromised tomato colonization of the *VdAve1L2* deletion mutant in strain DVD-S26 (Fig. 3c), which was restored in a

complementation mutant (Fig. 3c). Thus, VdAve1L2 contributes to *V. dahliae* virulence on tomato in the absence of *VdAve1*. To address the hypothesis that VdAve1 and VdAve1L2 are functionally redundant, we introduced *VdAve1L2* under the control of the *VdAve1* promoter in a *VdAve1* deletion mutant of the JR2 strain and tested its virulence on tomato, yet *VdAve1L2* failed to restore the virulence penalty of *VdAve1* deletion (Fig. 3d). Collectively, our data suggest that VdAve1 and VdAve1L2 have functionally diverged.

### VdAve1L2 promotes *Verticillium dahliae* virulence through the suppression of Actinobacteria

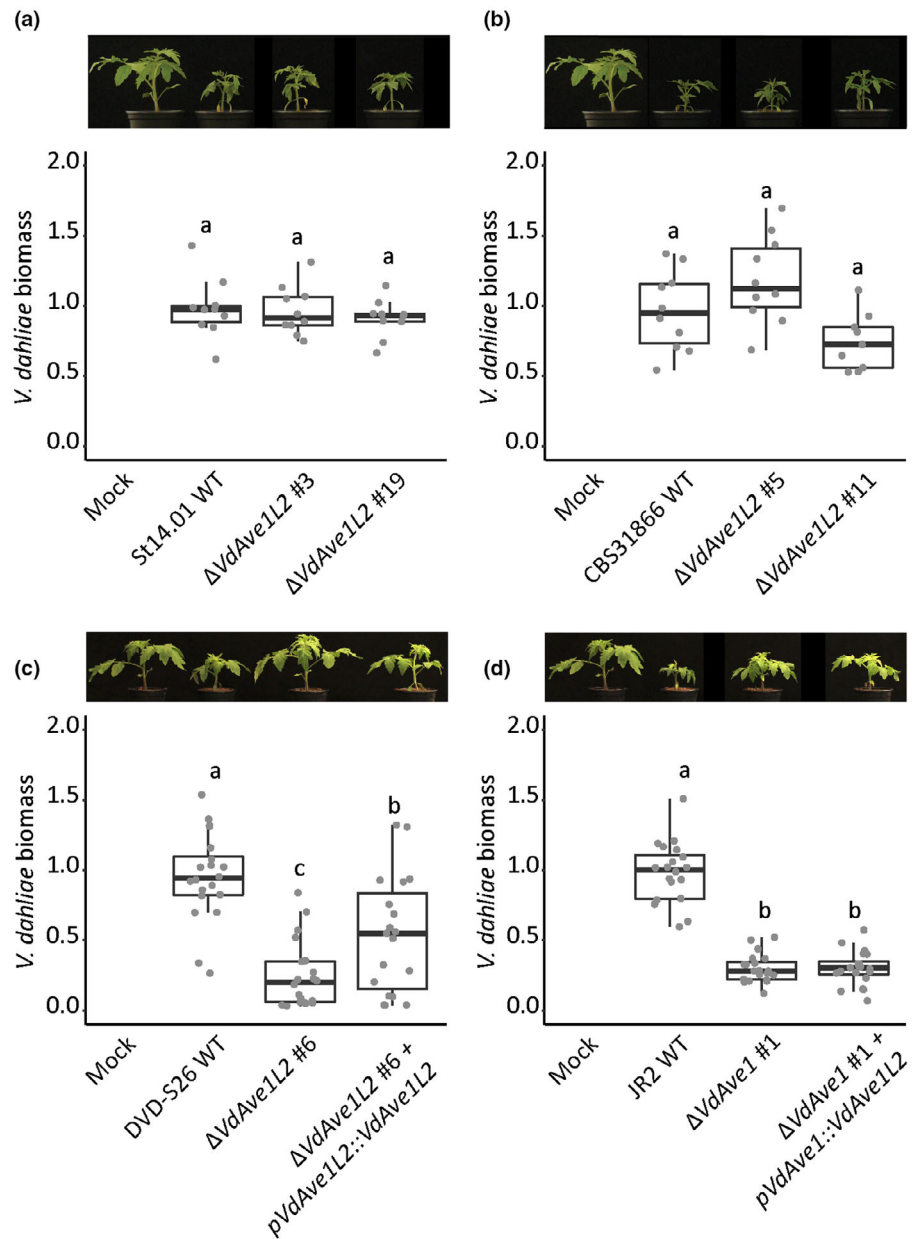
While most effector proteins functionally characterized to date act in the manipulation of host physiology, we recently showed that VdAve1 is an antibacterial effector protein that is secreted by *V. dahliae* to suppress microbial antagonists in the microbiomes of its hosts (Snelders *et al.*, 2020). Thus, we hypothesized that VdAve1L2 may similarly exert antibacterial activity. *In vitro* assays previously revealed a strong activity of VdAve1 on the gram-positive bacterium *B. subtilis* (Snelders *et al.*, 2020). Interestingly, VdAve1L2 affected *B. subtilis* growth as well, albeit markedly less effectively (Fig. 4a). Furthermore, similar to VdAve1 (Snelders *et al.*, 2020), VdAve1L2 inhibited the growth of plant-associated *Novosphingobium* sp. and *S. xylosum*, but not of *A. tumefaciens*, *P. corrugata*, and *Ralstonia* sp. However, in contrast to VdAve1 (Snelders *et al.*, 2020), VdAve1L2 did not clearly inhibit the growth of *Sphingobacterium* sp. during the exponential growth phase (Fig. S7a,b). Collectively, we conclude that VdAve1L2 is an antibacterial effector with a diverged activity spectrum when compared to VdAve1.

To determine whether VdAve1L2 secretion by *V. dahliae* impacts host microbiota, we performed bacterial community analysis based on 16S ribosomal DNA profiling on tomato roots colonized by *V. dahliae* strain DVD-S26 and the *VdAve1L2* deletion mutant. Furthermore, the *VdAve1* deletion mutant of *V. dahliae* strain JR2 and the corresponding transformant expressing *VdAve1L2* were included. In correspondence with previous observations (Snelders *et al.*, 2020), colonization by *V. dahliae* did not dramatically impact the overall composition of bacterial phyla in tomato root microbiota, and also not their  $\alpha$ -diversities (Fig. 4b,c). Importantly, however, a principal coordinate analysis based on Bray–Curtis dissimilarities ( $\beta$ -diversity) revealed separation of the bacterial communities based on *V. dahliae* genotype (Fig. 4d), suggesting that the secretion of VdAve1L2 impacts root microbiota compositions. Based on pairwise comparisons between the abundances of the bacterial phyla detected in the microbiota in the presence and absence of VdAve1L2, we identified Actinobacteria as the sole phylum that was significantly suppressed in the microbiota colonized by *V. dahliae* strains secreting VdAve1L2 (Fig. 4e).

To test whether the suppression of Actinobacteria is the direct consequence of the antimicrobial activity of VdAve1L2, we incubated representatives of three of the most abundant Actinobacterial families, Nocardioideae, Microbacteriaceae, and Cryptosporangiaceae, with VdAve1L2, and monitored their



**Fig. 3** VdAve1L2 is a virulence factor of *Verticillium dahliae* that functionally diverged from VdAve1. (a, b) VdAve1L2 does not contribute to the virulence of *V. dahliae* race 1 strains St14.01 (a) and CBS31866 (b) on tomato. Photographs display representative stunting symptoms of tomato plants 14-d postinoculation with wild-type (WT) *V. dahliae* strains and the corresponding VdAve1L2 deletion mutants. *Verticillium dahliae* biomass in tomato stems was quantified by real-time PCR. Letters represent nonsignificant biomass differences (one-way ANOVA and Tukey's *post hoc* test;  $P < 0.05$ ;  $n = 10$ ). (c, d) VdAve1L2 contributes to the virulence of *V. dahliae* race 2 strain DVD-S26 on tomato (c) but fails to restore the virulence that is lost by *V. dahliae* race 1 strain JR2 upon deletion of VdAve1. (d) Photographs display representative stunting phenotypes of tomato plants 14-d postinoculation with the WT *V. dahliae* strains, the corresponding VdAve1L2 or VdAve1 deletion mutants, and the mutant expressing VdAve1L2 under the control of the VdAve1 promoter. *Verticillium dahliae* biomass in tomato stems was quantified by real-time PCR. Letters represent nonsignificant biomass differences (one-way ANOVA and Tukey's *post hoc* test;  $P < 0.05$ ;  $n \geq 17$ ). The graphs are generated with GGPLOT2 and display all individual data points, with the box plot indicating the 25th percentile Q1, the median (thick line) and the 75th percentile Q3 of a distribution. The whiskers capture roughly 99% of the distribution.

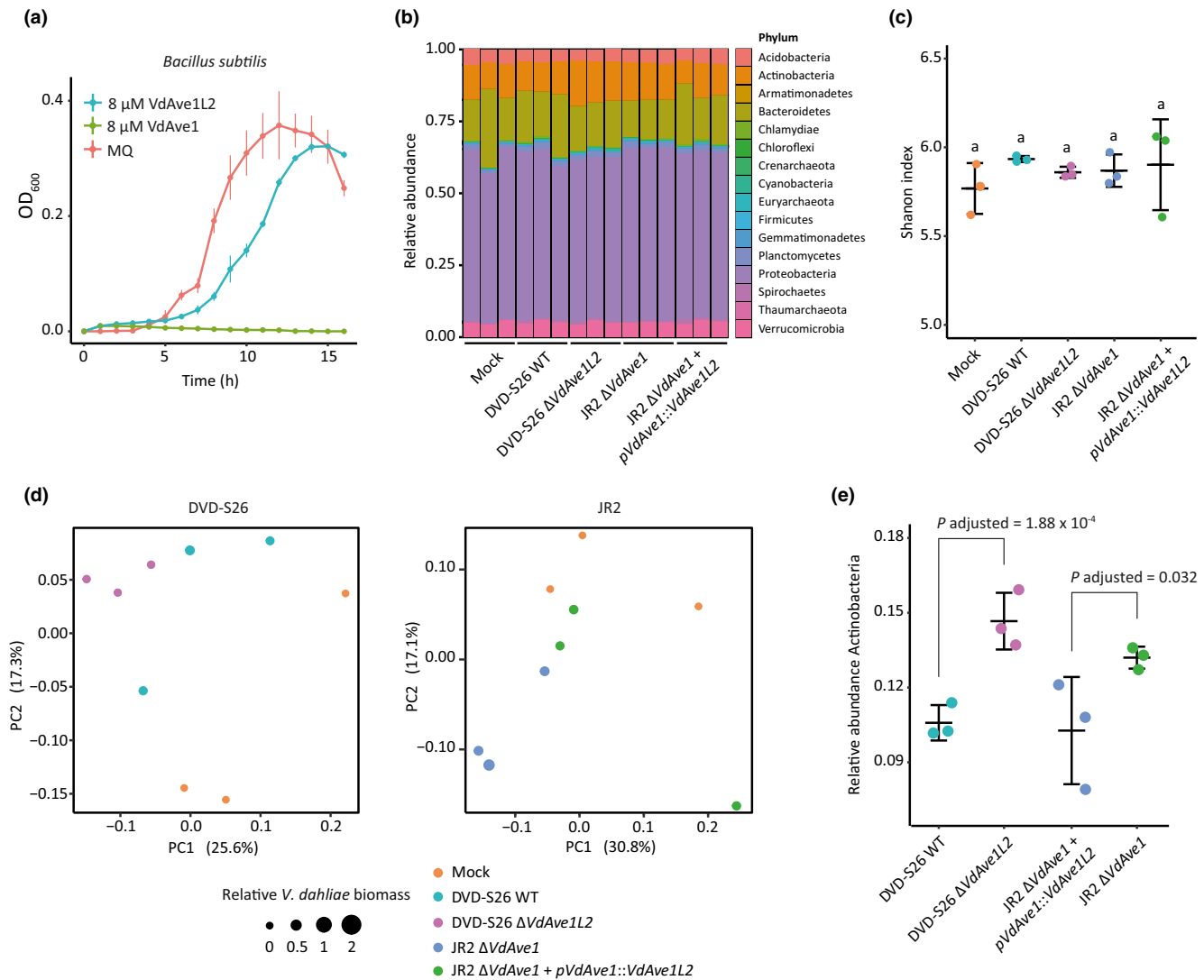


growth *in vitro*. Intriguingly, all tested Actinobacteria displayed higher sensitivity to the effector than most of the other bacteria tested thus far (Fig. 5a), suggesting that Actinobacteria are genuine and direct targets of VdAve1L2 *in planta*.

Actinobacteria are important players of plant-associated microbial communities and have repeatedly been assigned roles in disease suppression (Berendsen *et al.*, 2018; Chen *et al.*, 2020; Lee *et al.*, 2021). In accordance with their capacity to produce antimicrobial secondary metabolites, suppression of microbial pathogens by Actinobacteria often involves direct antibiosis, although they have also been implicated in the induction of systemic immunity (Conn *et al.*, 2008; Berendsen *et al.*, 2018; van Bergeijk *et al.*, 2020; Lee *et al.*, 2021). To test whether *V. dahliae* exploits VdAve1L2 to compete with Actinobacteria, we co-cultivated the VdAve1L2 deletion mutant of *V. dahliae* strain

DVD-S26 with the VdAve1L2-sensitive Actinobacteria *Frondebactria peucedani* and *C. chitinilytica* and the VdAve1L2-insensitive proteobacterium *A. tumefaciens*. Furthermore, we included transformants of the VdAve1L2 deletion mutant that express VdAve1L2 under the control of the VdAve1 promoter that is highly active during *in vitro* growth, in contrast to the VdAve1L2 promoter (Figs S5, S8). As anticipated, secretion of VdAve1L2 failed to counter the antagonistic activity of *A. tumefaciens* and did not promote *V. dahliae* growth when confronted with this bacterium (Fig. 5b). However, *V. dahliae* clearly benefited from VdAve1L2 secretion in competition with both Actinobacteria, as it mediated enhanced fungal growth and development of larger colonies (Fig. 5b). Collectively, our findings suggest that *V. dahliae* secretes VdAve1L2 to antagonize Actinobacteria in the host microbiota.

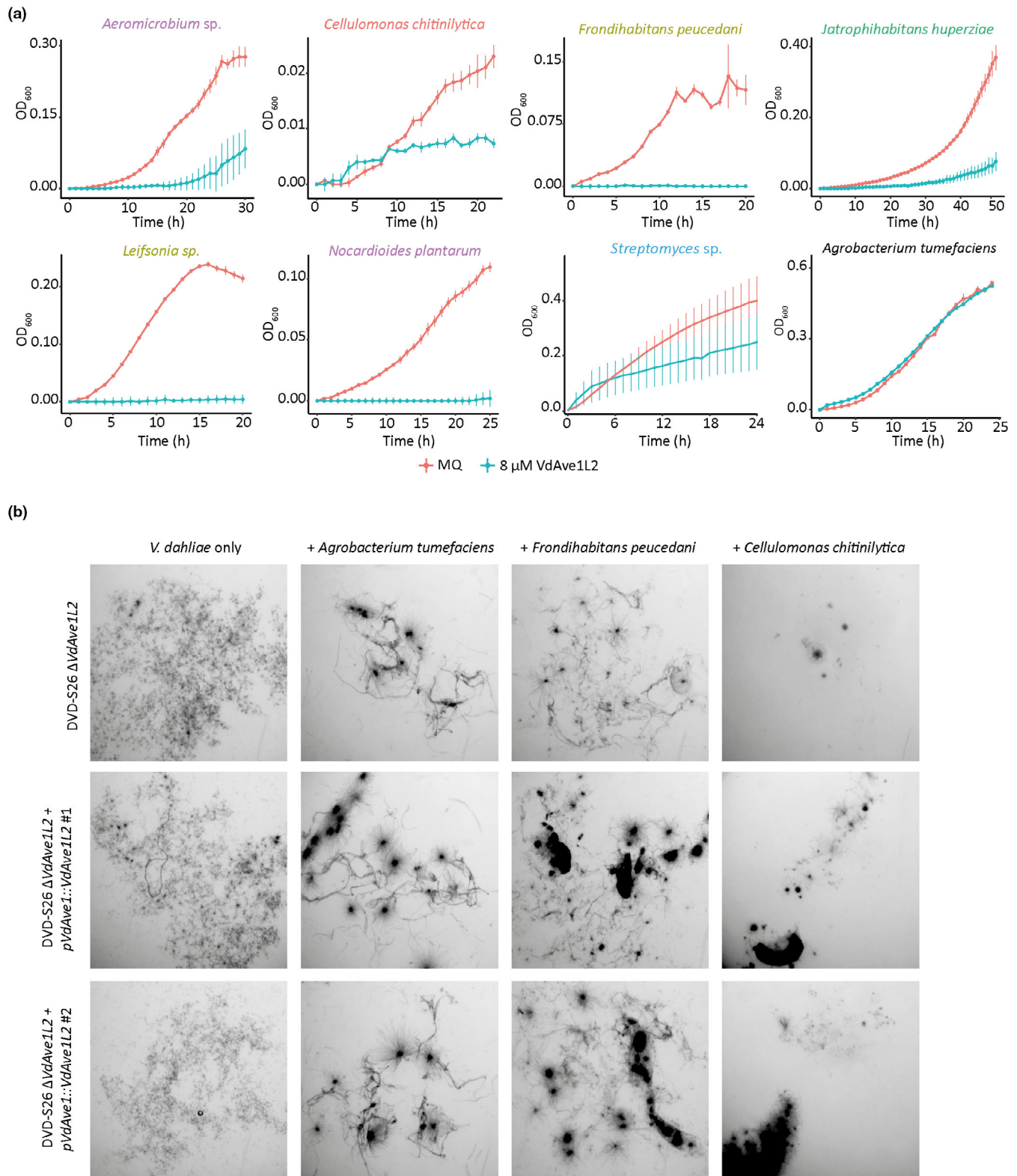




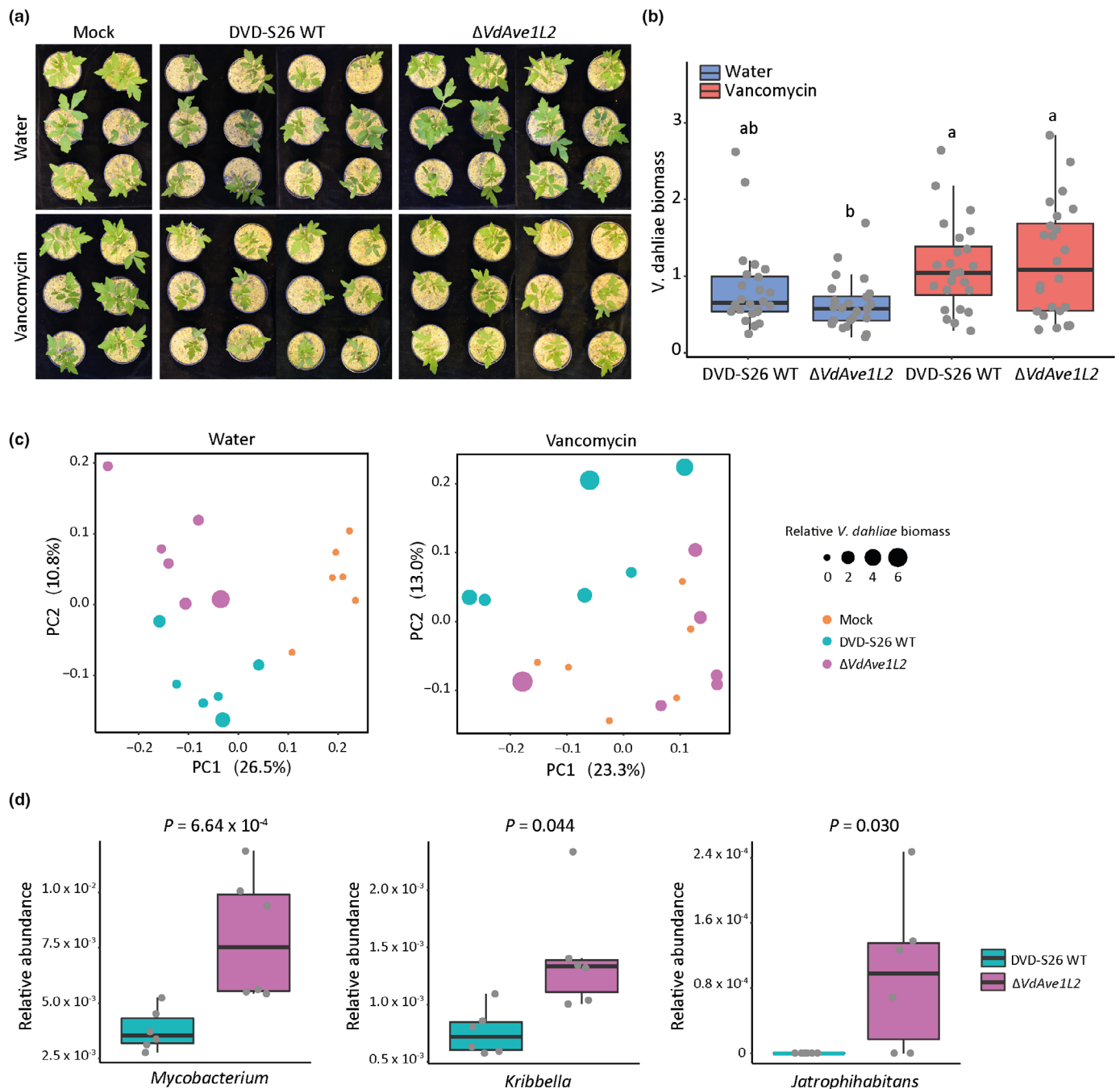
**Fig. 4** VdAve1L2 impacts Actinobacteria in the tomato root microbiota. (a) VdAve1L2 is an antibacterial effector protein. *In vitro* growth of *Bacillus subtilis* is inhibited by VdAve1L2. The previously characterized antibacterial effector VdAve1 (Snelders *et al.*, 2020) was included as a positive control for bacterial growth inhibition and displays a differential activity compared with VdAve1L2. Ultrapure water (MQ) was included as a negative control for bacterial growth inhibition and displays a differential activity compared with VdAve1L2. Graph displays the average OD<sub>600</sub> of three biological replicates ± SD. (b) Relative abundance of bacterial phyla in tomato root microbiota 10 d after inoculation with WT *Verticillium dahliae* strain DVD-S26, the corresponding DVD-S26 VdAve1L2 deletion mutant, a *V. dahliae* strain JR2 VdAve1 deletion mutant, and the corresponding VdAve1L2 expression mutant as determined by 16S ribosomal DNA profiling. (c) *Verticillium dahliae* colonization does not impact the  $\alpha$ -diversity of tomato root microbiota. The plot displays the average Shannon index ± SD (one-way ANOVA and Tukey's *post hoc* test;  $P < 0.05$ ;  $n = 3$ ). (d) Principal coordinate analysis based on Bray–Curtis dissimilarities uncovers the separation of root microbiome compositions based on the presence of VdAve1L2. (e) Differential abundance analysis of bacterial phyla identified Actinobacteria as the sole phylum that is significantly suppressed in the microbiota colonized by *V. dahliae* strains secreting VdAve1L2. The plot displays the average relative abundance ± SD of Actinobacteria in the different microbiota (Wald test,  $n = 3$ ).

Next, we aimed to determine the importance of the suppression of Actinobacteria by VdAve1L2 for tomato colonization by *V. dahliae*. Following a previously described protocol (Lee *et al.*, 2021), we extracted the root microbiota from tomato plants followed by incubation with the gram-positive bacteria-specific antibiotic vancomycin to affect Actinobacteria. Subsequently, the vancomycin-treated communities and water-treated control communities were allowed to establish on tomato seedlings grown under sterile conditions. Finally, the plants were inoculated with *V. dahliae* strain DVD-S26 and the VdAve1L2

deletion mutant to determine whether manipulation of the Actinobacteria affected *V. dahliae* host colonization and assess the virulence contribution of VdAve1L2. As determined using 16S ribosomal DNA profiling, tomato plants exposed to vancomycin-treated microbiota did not harbor a dramatically altered community of bacterial phyla when compared to plants exposed to the water-treated microbiota (Fig. S9a). Moreover, the vancomycin treatment did not affect the  $\alpha$ -diversity or total abundance of bacteria in the plant microbiota (Fig. S9b,c). However, as anticipated, we detected a severe impact of vancomycin treatment on



**Fig. 5** *Verticillium dahliae* VdAve1L2 affects antagonistic Actinobacteria. (a) Actinobacteria are inhibited by VdAve1L2. Graphs display the average  $OD_{600}$  of three biological replicates  $\pm$  SD. The names of the Actinobacteria are colored based on their corresponding family; the negative control bacterium (*Agrobacterium tumefaciens*) is indicated in black. (b) VdAve1L2 supports *V. dahliae* growth in the presence of antagonistic Actinobacteria. Representative microscopic pictures displaying the VdAve1L2 deletion mutant and two mutants expressing VdAve1L2 under the control of the VdAve1 promoter cultivated for 6 d in the presence of the VdAve1L2-insensitive proteobacterium *Agrobacterium tumefaciens* and the VdAve1L2-sensitive Actinobacteria *Frondihabians peucedani* and *Cellulomonas chitinilytica*.



**Fig. 6** Treatment of tomato root microbiota with vancomycin diminishes the virulence contribution of *VdAve1L2*. (a) Phenotypes of tomato plants harboring vancomycin-treated or water-treated microbial communities infected by WT *Verticillium dahliae* and the *VdAve1L2* deletion mutant at 19-d postinoculation. (b) Relative *V. dahliae* biomass in tomato stem tissue determined with real-time PCR (one-way ANOVA and Tukey's *post hoc* test;  $P < 0.05$ ;  $n = 24$ ). (c) Principal coordinate analysis based on Bray–Curtis dissimilarities uncovers the separation of root microbiota compositions in tomato plants harboring water-treated microbial communities, but not vancomycin-treated microbial communities colonized by both *V. dahliae* strains and upon mock treatment. (d) Relative abundance of the three Actinobacteria genera that are depleted from the tomato plants harboring the water-treated microbial community colonized by *V. dahliae* WT when compared to colonization by the *VdAve1L2* deletion mutant, as determined by 16S ribosomal DNA profiling (Wald test;  $n = 6$ ). All box plots are generated with GGPLOT2 and display all data points, with the box plot indicating the 25th percentile Q1, the median (thick line) and the 75th percentile Q3 of a distribution. The whiskers capture roughly 99% of the distribution.

the Actinobacteria community structure (Fig. S9d). Moreover, phenotypic assessment revealed markedly increased stunting of tomato plants harboring the vancomycin-treated microbiota when compared to plants containing the water-treated

community when inoculated with the *VdAve1L2* deletion mutant, showing disease suppression by Actinobacteria in the water-treated community (Fig. 6a). Quantification of *V. dahliae* biomass in the root microbiomes using real-time PCR confirmed

significantly increased colonization by the *VdAve1L2* deletion mutant in the presence of the vancomycin-treated microbiota (Fig. 6b). Importantly, while on plants that were treated with the water-treated community *VdAve1L2* markedly contributes to virulence (Fig. 6a), this virulence contribution is not observed on plants that were treated with the vancomycin-treated community, in line with the hypothesis that the Actinobacteria that are targeted by *VdAve1L2* are no longer present in the host microbiota (Fig. 6a). Accordingly, in contrast to the microbiomes with the water-treated microbial community, principal coordinate analysis based on Bray–Curtis dissimilarities ( $\beta$ -diversity) failed to reveal a clear separation of the root microbiomes with the vancomycin-treated community based on their colonization by the different *V. dahliae* strains or mock treatment (Fig. 6c). Moreover, we detected a *VdAve1L2*-mediated repression of Actinobacteria genera only in plants that received the water-treated communities (Fig. 6d). Likely, treatment with vancomycin limited the abundance of antagonistic Actinobacteria such that interference by *VdAve1L2* is no longer required for optimal *V. dahliae* colonization. In conclusion, our findings suggest that Actinobacteria in the tomato root microbiota antagonize host colonization by *V. dahliae* and that the fungus exploits *VdAve1L2* in turn to suppress these antagonists and promote disease.

## Discussion

To escape recognition by host immune receptors, microbial plant pathogens rely on the inactivation, loss, or mutation of effectors that become recognized. Unlike many microbial plant pathogens for which allelic effector gene variants escaping recognition have been reported (Armstrong *et al.*, 2005; Wu *et al.*, 2014), *V. dahliae* was known to evade effector recognition only through purging complete virulence genes, particularly of *VdAve1* and *VdAv2* (de Jonge *et al.*, 2012; Chavarro-Carrero *et al.*, 2021). Here, we report the discovery of *VdAve1L*, an effector gene with considerable sequence similarity to *VdAve1*, yet that displays extraordinary allelic variation in the *V. dahliae* population, which most likely results from selection pressure imposed by a plant immune receptor.

Functional characterization of the full-length effector variant *VdAve1L2* uncovered that this effector, like its homolog *VdAve1*, exerts antibacterial activity to directly suppress microbial competitors *in planta*. Remarkably, however, *VdAve1L2* is exclusively expressed during plant colonization and, in contrast to *VdAve1*, not expressed *in vitro* or in soil. Moreover, the two effectors display distinct antibacterial activities *in vitro*. Accordingly, we observed that the secretion of *VdAve1L2* by the race 2 strain DVD-S26, unlike *VdAve1* secreted by the race 1 strain JR2, impacts the abundance of Actinobacteria, and not of Sphingomonadales in tomato. *VdAve1L2* and *VdAve1* thus functionally diverged from each other. So far, the mode of action of *VdAve1* remains unclear and, accordingly, it is unclear how *VdAve1L2* functionally diverged from *VdAve1* to target Actinobacteria rather than Sphingomonadales.

Actinobacteria represent a core phylum that is found in virtually any plant grown in any environment. Several Actinobacterial species were shown to fulfill beneficial roles in plant holobionts,

such as suppression of plant diseases (Berendsen *et al.*, 2018; Chen *et al.*, 2020; Lee *et al.*, 2021). Additionally, Actinobacteria are keystone taxa that impact and benefit microbial community structures in plants (Carlström *et al.*, 2019; Gómez-Pérez *et al.*, 2022). Considering these beneficial traits, it is not surprising that members of this phylum are targeted by microbial plant pathogens to weaken plant holobionts. Interestingly, the oomycete *Arabidopsis* pathogen *Albugo candida* was recently reported to deposit several antibacterial effector proteins in the leaf apoplast (Gómez-Pérez *et al.*, 2022). Interestingly, some of these effectors impact the growth of Actinobacterial keystone taxa of the *Arabidopsis* phyllosphere *in vitro*, suggesting that the suppression of Actinobacteria in host microbiota might be a strategy adopted by diverse microbial plant pathogens. These findings furthermore support the hypothesis that effector-mediated manipulation of host microbiota communities may be a widely deployed strategy of plant pathogens to support host colonization (Snelders *et al.*, 2022).

*VdAve1* is recognized by the tomato immune receptor SIVe1, encoded by a gene in a locus that also encodes the highly similar orphan receptor SIVe2 (Fradin *et al.*, 2014). Considering the similarity between *VdAve1* and *VdAve1L*, we tested whether SIVe2 was able to recognize any of the current *VdAve1L* alleles or their putative progenitors, but none of those evoked a detectable HR upon overexpression in combination with SIVe2. It needs to be noted that due to the fact that we expressed the *VdAve1L* alleles without N- or C-terminal affinity tags, to prevent interference with possible recognition, we could not confirm the successful expression of the *VdAve1L* effectors. However, if recognition took place in tomato, it may equally well have been mediated by any other putative immune receptor encoded in the tomato genome. Perhaps even more likely, recognition may also have occurred in any of the hundreds of other *V. dahliae* hosts. Importantly, Actinobacteria are ubiquitously present in a wide diversity of plants, and thus, *V. dahliae* is likely to benefit from the antibacterial activity of *VdAve1L2* in plant species beyond tomato.

We previously showed that *VdAve1* was horizontally acquired from plants, where the abundantly present homologs are generally annotated as plant natriuretic peptides (de Jonge *et al.*, 2012). The fact that some of the sequenced *V. dahliae* isolates carry both *VdAve1* and *VdAve1L* raises the question whether both genes have been introduced by two separate horizontal gene transfer (HGT) events, or whether only a single HGT event took place that was followed by gene duplication and divergence. Although we have tried to resolve what scenario is most likely, our (phylogenetic) analyses rendered inconclusive results. Hence, at present, the exact relationship between *VdAve1* and *VdAve1L* remains unclear. Nevertheless, with *VdAve1L2*, we report the characterization of the fourth *V. dahliae* effector protein that acts in microbiota manipulation (Snelders *et al.*, 2020, 2021). Following *VdAve1* (de Jonge *et al.*, 2012), *VdAve1L2* is likely the second microbiota-manipulating effector secreted by *V. dahliae* that is recognized by a plant immune receptor, although it needs to be noted that the presumed recognition has not been confirmed experimentally. In light of the view that the microbiota

constitutes an extrinsic layer of the plant immune system (Dini-Andreote, 2020), microbiota-manipulating effectors target a critical immune component of the host and thus constitute a relevant target for surveillance by plants to mediate timely pathogen detection, in a similar fashion as effector proteins that interfere with intrinsic immune components are perceived. As a consequence of recognition, microbial plant pathogens need to mutate, purge, or inactivate their microbiota-manipulating effector proteins to escape host recognition, which leads to pathogen races with divergent suites of antimicrobial effectors. A possibility for the more effective use of microbial biocontrol agents could be to base their selection on the genotype of a plant pathogen, for instance by selecting antagonists that are insensitive to the activity of a specific (LS) effector. Conversely, in case a resistance gene has been described to recognize a microbiota-manipulating effector protein, the application of a strong antagonistic biocontrol agent that is sensitive toward the activity of the corresponding effector can be considered. In this manner, a strong selection pressure is exerted to retain that particular effector gene in the pathogen, which may contribute to enhanced durability of the resistance in turn. In this manner, further identification and characterization of microbiota-manipulating effectors secreted by microbial plant pathogens may aid in the development of more sophisticated, and perhaps more successful, biocontrol strategies.

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## Competing interests












None declared.

## Author contributions

NCS, JCB and BPHJT conceived the project. NCS, JCB, YS, NS, GLF, HR, GCMvdB, DET, GCP, ZP, LF, MFS and BPHJT designed and performed the experiments. NCS, JCB, YS, NS, GLF, HR, GCMvdB, DET, LF, MFS and BPHJT analyzed the data. NCS, JCB, GLF and BPHJT wrote the

manuscript. All authors read and approved the final manuscript. NCS and JCB contributed equally to this work. YS, NS and GLF contributed equally to this work.

## ORCID

Luigi Faino  <https://orcid.org/0000-0002-6807-4191>  
 Gabriel L. Fiorin  <https://orcid.org/0000-0001-9706-4964>  
 Gabriella C. Petti  <https://orcid.org/0000-0001-9250-7746>  
 Zoe Prockl  <https://orcid.org/0000-0001-6802-5800>  
 Hanna Rovenich  <https://orcid.org/0000-0001-6138-4480>  
 Michael F. Seidl  <https://orcid.org/0000-0002-5218-2083>  
 Nick C. Snelders  <https://orcid.org/0000-0002-2576-1928>  
 Yin Song  <https://orcid.org/0000-0002-6037-777X>  
 Bart P. H. J. Thomma  <https://orcid.org/0000-0003-4125-4181>  
 David E. Torres  <https://orcid.org/0000-0003-3711-0548>  
 Grady C. M. van den Berg  <https://orcid.org/0000-0002-6576-8948>

## Data availability

The 16S profiling data have been deposited in the NCBI GenBank database under BioProject [PRJNA742137](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA742137).

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## Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Presence/absence of *VdAve1L* in *Verticillium dahliae* strains as determined by PCR.

**Fig. S2** *Verticillium dahliae VdAve1L3* is disrupted by a transposable element.

**Fig. S3** *Verticillium dahliae VdAve1L* allele displays extraordinary sequence variation.

**Fig. S4** Single-nucleotide polymorphism locations in the coding sequence of the *Verticillium dahliae VdAve1L* alleles.

**Fig. S5** *VdAve1L2* is expressed by *Verticillium dahliae* during tomato colonization.

**Fig. S6** Verification of *Verticillium dahliae VdAve1L2* deletion and complementation mutants.

**Fig. S7** *Verticillium dahliae VdAve1L2* is an antibacterial effector protein.

**Fig. S8** Expression of *VdAve1L2* in *Verticillium dahliae* mutants.

**Fig. S9** Metagenomic characterization of tomato root microbiota with water-treated and vancomycin-treated microbial communities.

**Table S1** List of primers used in this study.

**Table S2** *Verticillium dahliae* isolates.

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