Microbes typically secrete a plethora of molecules to promote niche colonization. Soil-dwelling microbes are well-known producers of antimicrobials that are exploited to outcompete microbial coinhabitants. Also, plant pathogenic microbes secrete a diversity of molecules into their environment for niche establishment. Upon plant colonization, microbial pathogens secrete so-called effector proteins that promote disease development. While such effectors are typically considered to exclusively act through direct host manipulation, we recently reported that the soil-borne, fungal, xylem-colonizing vascular wilt pathogen *Verticillium dahliae* exploits effector proteins with antibacterial properties to promote host colonization through the manipulation of beneficial host microbiota. Since fungal evolution preceded land plant evolution, we now speculate that a subset of the pathogen effectors involved in host microbiota manipulation evolved from ancient antimicrobial proteins of terrestrial fungal ancestors that served in microbial competition prior to the evolution of plant pathogenicity. Here, we show that *V. dahliae* has co-opted an ancient antimicrobial protein as effector, named VdAMP3, for microbiome manipulation in plants. We show that VdAMP3 is specifically expressed to ward off fungal niche competitors during resting structure formation in senescing mesophyll tissues. Our findings indicate that effector-mediated microbiome manipulation by plant pathogenic microbes extends beyond bacteria and also concerns eukaryotic members of the plant microbiome. Finally, we demonstrate that fungal pathogens can exploit plant microbiome-manipulating effectors in a life stage-specific manner and that a subset of these effectors has evolved from ancient antimicrobial proteins of fungal ancestors that likely originally functioned in manipulation of terrestrial biota.

Microbes secrete a Diversity of Molecules into Their Environment to Mediate Niche Colonization. During host ingress, plant pathogenic microbes secrete effector proteins that facilitate disease development, many of which deregulate host immune responses. We recently demonstrated that plant pathogens additionally exploit effectors with antibacterial activities to manipulate beneficial plant microbiota to promote host colonization. Here, we show that the fungal pathogen *Verticillium dahliae* has co-opted an ancient antimicrobial protein, which likely served in microbial competition in terrestrial environments before land plants existed, as effector for the manipulation of fungal competitors during host colonization. Thus, we demonstrate that pathogen effector repertoires comprise antifungal proteins and speculate that such effectors could be exploited for the development of antimycotics.

**Significance**

Microbes secrete a diversity of molecules into their environment to mediate niche colonization. During host ingress, plant pathogenic microbes secrete effector proteins that facilitate disease development, many of which deregulate host immune responses. We recently demonstrated that plant pathogens additionally exploit effectors with antibacterial activities to manipulate beneficial plant microbiota to promote host colonization. Here, we show that the fungal pathogen *Verticillium dahliae* has co-opted an ancient antimicrobial protein, which likely served in microbial competition in terrestrial environments before land plants existed, as effector for the manipulation of fungal competitors during host colonization. Thus, we demonstrate that pathogen effector repertoires comprise antifungal proteins and speculate that such effectors could be exploited for the development of antimycotics.
Results

To identify effectors potentially acting in microbiome manipulation, we recently queried the *V. dahliae* secreteme for structural homologs of known antimicrobial proteins (AMPs), which led to the identification of 10 candidates including the functionally characterized VdAMP2 (18). Among the remaining nine candidates, we now identified a small cysteine-rich protein of $\sim$4.9 kDa, which we name VdAMP3 (Ensembl: VDAG_JR2-Chr3g05620a). As a first step in the characterization of VdAMP3, we assessed its predicted structure. Interestingly, VdAMP3 is predicted to adopt a cysteine-stabilized $\alpha\beta$ (CS$\alpha\beta$) fold that is also found in defense-like proteins (Fig. L4) (24–26). CS$\alpha\beta$ defensins represent a widespread and well-characterized family of antimicrobial proteins that are presumed to share a single ancient origin in the last common ancestor of animals, plants, and fungi that produce these proteins today (24–27). It is important to note, however, that many typical small cysteine-rich pathogen effectors adopt AMP-like conformations and that tertiary structures of several AMP families strongly resemble each other (27, 28). Hence, structure prediction can easily lead to false-positive classifications as AMP or allocation to the wrong AMP family.

CS$\alpha\beta$ defensins, or so-called cis-defensins, owe their structure to highly conserved cis-orientated disulfide bonds that establish an interaction between a double- or triple-stranded antiparallel $\beta$-sheet with an $\alpha$-helix (25, 27). To validate the prediction of VdAMP3 as a member of this ancient antimicrobial protein family, we aligned its amino acid sequence with the antibacterial CS$\alpha\beta$ defensins plectasin and eurocin, from the saprophytic Ascomycete species *Pseudoplectania nigrella* and *Eurotium amstelodami* (formerly *Aspergillus amstelodami*), respectively (29–31). Although the biological relevance of these defensins for the respective fungi remains unclear, their antibacterial activity and protein structure have been well characterized, which led to their recognition as genuine CS$\alpha\beta$ defensins (29–31). Although the overall identity between the three proteins was rather low (25 to 40%), protein sequence alignment revealed that VdAMP3 contains the six highly conserved CS$\alpha\beta$ defensin-like fold. The emerging picture that VdAMP3 belongs to this particular protein family and that the detected similarities with plectasin and eurocin are not the result of convergent protein evolution, we
queried the predicted proteomes of the fungi from the Joint Genome Institute (JGI) 1000 Fungal Genomes Project (32) for homologs of VdAMP3 with higher sequence identity and included a subset of those in the protein alignment (Fig. 1C). Interestingly, besides homologs in Ascomycota and Basidiomycota, our sequence similarity search also revealed homologs in early-diverging fungi from the subphyla Macromycotina and Zoopagomycota [both formerly classified as Zygomycota (33)] (Fig. 1C). Importantly, this divergence is estimated to have taken place >900 million years ago (34), indicating it preceded the evolution of the first land plants ~450 million years later (34–37). Consequently, this analysis indicates that VdAMP3 evolved from an ancestral fungal gene hundreds of millions of years ago before land plants existed.

As a first step to determine a potential role of VdAMP3 in *V. dahliae* infection biology, we assessed whether we could find evidence for VdAMP3 expression during host colonization. Analysis of previously generated transcriptome datasets of diverse *V. dahliae* strains during colonization of a diversity of hosts did not reveal in planta expression of VdAMP3 (17, 38–40). However, strong induction of this effector gene was reported during microsclerotia formation in a transcriptome analysis of *V. dahliae* strain XS11 grown in vitro (24). To validate this finding, we analyzed in vitro expression of VdAMP3 in *V. dahliae* strain JR2. To this end, *V. dahliae* conidiospores were spread on nitrocellulose membranes placed on top of solid minimal medium and fungal material was harvested prior to microsclerotia formation after 48 h of incubation and after the onset of microsclerotia formation after 96 h of incubation. Expression of VdAMP3 was determined at both time points with real-time PCR alongside expression of the Chrg002430 gene that encodes a putative cytochrome P450 enzyme that acts as a marker for microsclerotia formation (24, 41). Consistent with the observations for *V. dahliae* strain XS11 (24), no VdAMP3 expression was detected at 48 h, when Chrg002430 was also not expressed and no visual microsclerotia formation could be observed on the growth medium (Fig. 2A). However, induction of VdAMP3, as well as Chrg002430, was observed after 96 h of incubation, at which time point the formation of microsclerotia on the growth medium also became apparent (Fig. 2A). Collectively, these data demonstrate that expression of VdAMP3 coincides with microsclerotia formation in vitro also for *V. dahliae* strain JR2.

Although previous transcriptome analyses failed to detect in planta expression of VdAMP3, we realized that these analyses were predominantly performed for infection stages when the fungus was still confined to the xylem vessels and microsclerotia formation had not yet been initiated. Accordingly, in planta expression of VdAMP3 may have been missed. Thus, we inoculated *Nicotiana benthamiana* with *V. dahliae* and determined expression of VdAMP3 in leaves and petioles sampled at different time points and displaying different disease phenotypes, ranging from asymptomatic at 7 d postinoculation (dpi) to complete necrosis at 22 dpi. As expected, a strong induction of the previously characterized VdAVF1 effector gene was detected at 7 and 14 dpi (Fig. 2B) (17, 18). In contrast, however, no expression of VdAMP3 was recorded, even at the latest time point, when the leaf tissue had become completely necrotic (Fig. 2B). Importantly, no Chrg002430 expression was detected at any of these time points either (Fig. 2B), suggesting that microsclerotia formation had not yet started in these tissues. Indeed, visual inspection of the necrotic plant tissue collected at 22 dpi did not reveal microsclerotia presence. To induce microsclerotia formation, *V. dahliae*-inoculated *N. benthamiana* plants harbored at 22 dpi were sealed in plastic bags and incubated in the dark to increase the relative humidity and mimic conditions that occur during tissue decomposition in the soil. Interestingly, after 8 d of incubation, the first microsclerotia could be observed and induction of VdAMP3, as well as Chrg002430, was detected (Fig. 2C). Notably, the induction of both genes in planta is markedly weaker when compared with their expression in vitro (Fig. 2D). However, this is likely explained by a much smaller proportion of the total population of *V. dahliae* cells undergoing synchronized development into microsclerotia, also because the time window from conidial germination through hyphal growth to microsclerotia formation is much smaller in vitro than in planta. Collectively, our findings suggest that in planta expression of VdAMP3 coincides with microsclerotia formation, similar to our observations in vitro. Moreover, our data suggest that VdAMP3 expression primarily depends on a developmental stage of *V. dahliae* rather than on host factors such as tissue necrosis.

To determine more precisely where VdAMP3 is expressed and to improve our understanding of how *V. dahliae* may benefit from effector expression during microsclerotia formation, we generated a *V. dahliae* reporter strain expressing eGFP under control of the VdAMP3 promoter. Intriguingly, microscopic analysis of the reporter strain during microsclerotia formation stages in vitro (Fig. 2D) revealed that VdAMP3 is expressed by swollen hyphal cells that act as primordia that subsequently develop into microsclerotia but not by the adjacent hyphal cells or recently developed microsclerotia cells (Fig. 2E–G). This highly specific expression of VdAMP3 suggests that the effector may facilitate the formation of microsclerotia in decaying host tissue. Given its presumed antimicrobial activity, VdAMP3 may be involved in antagonistic activity against opportunistic decay organisms in this microbiobially competitive niche.

To determine if VdAMP3 indeed exerts antimicrobial activity, we tried to produce VdAMP3 heterologously in the yeast *Pichia pastoris* and in the bacterium *Escherichia coli*, but these attempts failed, indicative of potential antimicrobial activity of the effector protein. Therefore, chemical synthesis of VdAMP3 was pursued. Next, we incubated a randomly selected panel of bacterial isolates with the effector protein and monitored their growth in vitro. VdAMP3 concentrations as high as 20 μM resulted in no or only marginal bacterial growth inhibition (*SI Appendix, Fig. 1A*). A similar assay with fungal isolates showed that incubation with 5 μM VdAMP3 already markedly affected growth of the filamentous fungi *Alternaria brassicicola* and *Cladosporium cucumerinum* and the yeasts *P. pastoris* and *Saccharomyces cerevisiae* (Fig. 3 A and B). This finding suggests that VdAMP3 displays more potent activity against fungi than against bacteria. Importantly, a thorough heat treatment involving boiling of VdAMP3 abolished its antifungal activity (*SI Appendix, Fig. 2*), indicating that the specificity of this activity depends on its correct three-dimensional confirmation.

Considering its antifungal activity, but also the highly controlled timely and topical expression of VdAMP3, we tested if exogenous VdAMP3 application negatively impacts hyphal growth of *V. dahliae*. Interestingly, incubation of *V. dahliae* with 5 μM VdAMP3 markedly affected its growth (*SI Appendix, Fig. 3 A and B*). However, it needs to be realized that this effector protein is produced by the time when most hyphae of the fungus have lost their function, as the host tissue has become senescent and will soon decompose, and the fungus produces microsclerotia for long-term survival. Next, to verify if growth or development of *V. dahliae* is affected by VdAMP3, we generated a VdAMP3 deletion mutant (*SI Appendix, Fig. 4*), which we cultivated in vitro alongside wild-type (WT) *V. dahliae*. As anticipated, deletion of VdAMP3 did not accelerate growth of the fungus (*SI Appendix, Fig. 3C*), confirming that the effector gene does not compromise the development of the fungus during the life stages prior to microsclerotia formation. Moreover, deletion of VdAMP3 also did not impair the ability of *V. dahliae* to form resting structures, nor their ability to infect new plants and cause disease (*SI Appendix, Fig. 3 C–E*). Next, we aimed to
determine if the antifungal activity of VdAMP3 contributes to Verticillium wilt disease development. To this end, N. benthamiana plants were inoculated with V. dahliae WT as well as with VdAMP3 complementation and deletion mutants (SI Appendix, Fig. 4). In line with our inability to detect expression during early infection stages, disease phenotypes and V. dahliae biomass quantification using real-time PCR did not reveal a contribution of VdAMP3 to host colonization up to 2 wk after inoculation (Fig. 3C and D). Considering the cell type–specific expression of VdAMP3 in developing microsclerotia, we speculated that the effector protein contributes to V. dahliae niche establishment during host plant senescence when the fungus has emerged from the xylem and has colonized the mesophyll. To test this hypothesis, we performed additional disease assays using V. dahliae WT and the VdAMP3 deletion mutant and sealed the N. benthamiana plants in plastic bags after harvesting to stimulate the onset of tissue decomposition and microsclerotia formation. Intriguingly, when we visually inspected the plants after 4 wk of incubation, we detected dispersed patches of dark mycelium, typical for V. dahliae microsclerotia, on the surface of plants colonized by V. dahliae WT (SI Appendix, Fig. 5). Strikingly, we did not identify such patches on plants colonized by the VdAMP3 deletion mutant, suggesting that V. dahliae depends on the antifungal activity of VdAMP3 to form microsclerotia in decaying host phyllospheres. It needs to be noted that an experimental setup that depends on a largely unpredictable occurrence of visibly detectable patches of microsclerotia on the surface of decaying plant parts that are colonized by diverse assemblages of opportunistic microbes that seize their opportunity to prosper while plant defenses fade is hardly feasible for standardized, robust quantification of microsclerotia formation. Also, this setup does not permit assessment of microsclerotia formation deeper in the decaying tissues. Instead, we quantified V. dahliae biomass using real-time PCR. As anticipated, we detected a
significant reduction in biomass of the VdAMP3 deletion mutant when compared with V. dahliae WT and the complementation mutants (Fig. 3 E and F), confirming that VdAMP3 indeed is essential during microsclerotia formation in planta presumably by acting in self-protection against other microbes.

To investigate if the effects of VdAMP3 are limited to N. benthamiana or whether those also extend to other hosts, we inoculated Arabidopsis thaliana plants with V. dahliae WT and the VdAMP3 deletion mutant. Consistent with our observations for N. benthamiana, deletion of VdAMP3 did not affect establishment of Verticillium wilt in A. thaliana (SI Appendix, Fig. 6 A and B). However, V. dahliae biomass quantification in aboveground A. thaliana tissues at 3 wk postinoculation revealed reduced accumulation of V. dahliae in the absence of VdAMP3 (SI Appendix, Fig. 6C). Thus, the effects of VdAMP3 are not restricted to a single host.

As in vitro antimicrobial activity assays pointed toward fungi as the primary targets of VdAMP3, we speculated that V. dahliae exploits VdAMP3 to suppress fungal competitors in decomposing host tissues to safeguard the formation of its resisting structures. To characterize the microbiota associated with N. benthamiana decomposition and to determine the impact of VdAMP3 on these microbial communities, we characterized the phyllosphere microbiota of fresh mock-inoculated N. benthamiana plants, and decaying plants diseased by V. dahliae WT or the VdAMP3 deletion mutant incubated in plastic

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Fig. 3. VdAMP3 is an antifungal protein that contributes to V. dahliae biomass accumulation in the decaying host phyllosphere. (A) Microscopic pictures of fungal isolates grown in 5% PDB supplemented with 5 μM VdAMP3 or ultrapure water (Milli-Q). VdAMP3 impair growth of A. brassicicola, C. cucumerinum, P. pastoris, and S. cerevisiae. Pictures were taken after 24 (A. brassicicola, C. cucumerinum, and S. cerevisiae) or 64 (P. pastoris) h of incubation. (B) Fungal growth as displayed in A was quantified using imagej (unpaired two-sided Student's t test; n = 4). (C) VdAMP3 does not contribute to establishment of Verticillium wilt disease in N. benthamiana. Photos display representative phenotypes of N. benthamiana plants infected by wild-type V. dahliae (WT), the VdAMP3 deletion (ΔVdAMP3), and two complementation (Comp) mutants 14 dpi. (D) Relative V. dahliae biomass in aboveground N. benthamiana tissues determined with real-time PCR. Different letter labels represent significant differences (one-way ANOVA and Tukey's post hoc test; P < 0.05; n ≥ 27). (E) Representative phenotypes of N. benthamiana plants as shown in C after 28 d of incubation in plastic bags. (F) Relative V. dahliae biomass in N. benthamiana tissues as displayed in E. Letters represent significant differences (one-way ANOVA and Tukey's post hoc test; P < 0.05; n ≥ 27).
Fig. 4. VdAMP3 manipulates the mycobiome of the decaying N. benthamiana phyllosphere. (A and B) V. dahliae–induced decay of the N. benthamiana phyllosphere is associated with a decreased bacterial and increased fungal abundance. Relative abundance of bacteria (A) and fungi (B), excluding V. dahliae, in the phyllosphere of decaying N. benthamiana plants colonized by WT V. dahliae (WT) or the VdAMP3 deletion mutant (14 dpi and after 28 d of incubation in plastic bags) and in the phyllosphere of fresh N. benthamiana plants (mock). Letters represent significant differences in total bacterial/fungal abundance between the three treatments (one-way ANOVA and Tukey’s post hoc test; P < 0.05; n = 3). (C) V. dahliae–induced decay of N. benthamiana plants impacts alpha diversity of the phyllosphere. The plot displays the average Shannon index ± SD; letters represent significant differences (one-way ANOVA and Tukey’s post hoc test; P < 0.05; n = 3). (D) PCoA based on Bray–Curtis dissimilarities (beta diversity) reveals separation of the microbiomes based on the three different treatments. (E) Differential abundance analysis of microbial genera between the microbiomes colonized by V. dahliae WT and the VdAMP3 deletion mutant indicates that secretion of VdAMP3 significantly impacts a larger proportion of the fungi than of the bacteria (two-tailed Fisher’s exact test). (F) Of the differentially abundant microbial genera, significantly more fungi display a decreased abundance in the presence of VdAMP3 when compared with the bacteria (two-tailed Fisher’s exact test). (G and H) Overview of the differentially abundant bacterial (G) and fungal (H) genera. The plots display increased (positive log2 fold change) or decreased (negative log2 fold change) abundance in the presence of VdAMP3 when compared with the microbiome of fresh N. benthamiana plants (mock). (i.e., by V. dahliae WT).
An ancient antimicrobial protein co-opted by a fungal plant pathogen for in planta microbiome manipulation

PNAS | 7 of 11
https://doi.org/10.1073/pnas.2110968118

V. dahliae

while VdAMP3 deletion mutant revealed a significant reduction of microsclerotia formed by the VdAMP3 deletion mutant when compared with V. dahliae WT and the complementation mutants in the presence of both fungal species. We cocultivated T. viride and V. dahliae to test if VdAMP3 indeed is essential for microsclerotia formation in the presence of fungal communities, presence of a single microbial interactor but also facilitates microsclerotia formation in the presence of fungal communities, microsclerotia formation in the presence of other fungi, we cocultivated T. viride and V. dahliae to test if VdAMP3 indeed is essential for microsclerotia formation in the presence of fungal communities, strongly suggests that secretion of VdAMP3 manipulates microbiome compositions (Fig. 4D). Intriguingly, when we compared the abundances of the identified microbial genera between the microbiomes colonized by V. dahliae WT and the VdAMP3 deletion mutant, we detected significantly more differentially abundant fungi (10.1%) than bacteria (3.8%) (Fig. 4F, SI Appendix, Tables 1 and 2). Interestingly, whereas the number of bacterial genera that display an increased or a decreased abundance in the presence of VdAMP3 is more or less equal, the vast majority of the differentially abundant fungal genera (82.1%) are repressed in the presence of VdAMP3 (Fig. 4F). Moreover, while no consistent suppression of bacterial genera from the same class could be detected, we exclusively identified suppression of the differentially abundant fungal genera from the Saccharomyces or Sordariomycetes in the presence of VdAMP3 (Fig. 4G and H). Thus, these observations indicate that V. dahliae VdAMP3 mainly acts as an antifungal effector protein that displays selective activity that predominantly impacts the mycobiome in the decaying host phyllosphere.

To further substantiate that the suppression of the Saccharomyces and Sordariomycetes is a direct consequence of the VdAMP3 activity, we incubated fungal species belonging to the suppressed genera with the effector to determine their sensitivity. In line with the previously observed sensitivity of the Saccharomyces P. pastoris and S. cerevisiae, the Saccharomyceae species Cyberlindnera jadinii, Debaryomyces vanrijiae, Rhodotorula bogoriensis, and Meyerozyma amylolytica also displayed markedly reduced growth in the presence of VdAMP3 (Fig. 5A and B). Similarly, growth of the Sordariomycetes Cordyceps militaris and Trichoderma viride was inhibited by the effector (Fig. 5A and B). Hence, these findings support the observed suppression of the Saccharomyces and Sordariomycetes in the N. benthamiana phyllosphere microbiome as a direct consequence of VdAMP3 activity.

The cell type–specific expression of VdAMP3, combined with its role in microbiome manipulation, strongly suggests that VdAMP3 is exploited to ward off fungal niche competitors in planta to safeguard the formation of V. dahliae microsclerotia. To test if VdAMP3 indeed is essential for V. dahliae microscler- otia formation in the presence of other fungi, we cocultivated V. dahliae WT and the VdAMP3 deletion and complementation mutants with D. vanrijiae and M. amylolytica. Once microsclerotia formation by V. dahliae WT became apparent (Fig. 6A), we quantified the number of resting structures that were formed by the different V. dahliae genotypes. As anticipated, we detected a significant reduction of microsclerotia formed by the VdAMP3 deletion mutant when compared with V. dahliae WT and the complementation mutants in the presence of both fungal species, confirming that V. dahliae relies on the antifungal activity of VdAMP3 to form microsclerotia in the presence of particular fungal niche competitors (Fig. 6B and C). Additionally, to confirm that this activity is not only relevant in the presence of a single microbial interactor but also facilitates microsclerotia formation in the presence of fungal communities,

Consistent with a primary role for fungi in the decomposition of dead plant material (44–48), we detected a significant increase of fungi and decrease of bacteria in the phyllosphere of the N. benthamiana plants diseased by the V. dahliae strains when compared with healthy mock-treated plants (Fig. 4A and B). These changes are accompanied by a reduced alpha diversity in the decaying phyllospheres (Fig. 4C). Additionally, principal coordinate analysis (PCoA) based on Bray–Curtsis dissimilar- ities (beta diversity) uncovered clear separation of the micro- biota of the healthy plants from those in decay (Fig. 4D). The PCoA also revealed a weaker, yet potentially relevant, separation of the microbiota colonized by V. dahliae WT and the VdAMP3 deletion mutant, which suggests that secretion of VdAMP3 manipulates microbiome compositions (Fig. 4D). Notably, we also detected a significant increase of resting structures that were formed by V. dahliae genotypes. As anticipated, we detected a significant increase of resting structures that were formed by V. dahliae genotypes. As anticipated, we detected a significant increase of resting structures that were formed by V. dahliae genotypes. As anticipated, we detected a significant increase of resting structures that were formed by V. dahliae genotypes.

we performed similar experiments using two synthetic communi- ties that, besides D. vanrijiae and M. amylolytica, also comprised the filamentous fungus C. militaris or the yeast C. jadinii plus the filamentous mycoparasite T. viride. Also in these experiments, we detected a significant reduction of microsclerotia formed by the VdAMP3 deletion mutant when compared with V. dahliae WT and the complementation mutants (Fig. 6B and C). Collectively,

Fig. 5. VdAMP3 negatively affects Saccharomyces and Sordariomycetes. (A) Microscopic pictures of fungal isolates grown in 5% PDB supplemented with 5 μM VdAMP3 or ultrapure water (Milli-Q). VdAMP3 impairs growth of D. vanrijiae, M. amylolytica, C. jadinii, R. bogoriensis, C. militaris, and T. viride. Pictures were taken after 10 (D. vanrijiae and C. jadinii), 24 (M. amylolytica and R. bogoriensis), or 30 (C. militaris and T. viride) h of cultivation. (B) Fungal growth as displayed in A was quantified using ImageJ (unpaired two-sided Student’s t test; n = 4).
these findings underpin the idea that *V. dahliae* exploits the anti-fungal activity of VdAMP3 to safeguard the formation of its resting structures by warding off fungal niche competitors in senescing host mesophyll tissues.

**Discussion**

Microbes secrete a plethora of molecules to promote niche colonization (4). Free-living microbes are well-known producers of antimicrobials that are secreted to outcompete microbial coinhabitants to establish themselves in a microbial community. Microbial plant pathogens secrete a diversity of so-called effector molecules during host ingress, many of which are small cysteine-rich proteins that deregulate host immune responses to promote colonization (4, 6, 7). While investigating the vascular wilt fungus *V. dahliae*, we recently demonstrated that plant pathogens not only exploit effector proteins to promote disease establishment through direct host manipulation but also through the manipulation of plant microbiota by means of antibacterial activities (18). Considering that the advent of fungi on earth preceded land plant evolution, we speculated that a subset of the pathogen effectors involved in host microbiota manipulation may have evolved from antimicrobial proteins that originally functioned in microbial competition in terrestrial niches before the first land plants appeared and plant pathogenicity evolved. Here, we demonstrated that the soil-borne fungal plant pathogen *V. dahliae* has co-opted an ancient antimicrobial protein as effector for mycobiome manipulation in planta to safeguard the formation of its resting structures. Thus, our findings indicate that plant pathogenicity in fungi is
not exclusively associated with the evolution of novel effectors that manipulate plants or their associated microbial communities but also with the co-option of previously evolved secreted proteins that initially served alternative lifestyles, such as saprophytism, as effectors to promote host colonization. Moreover, our findings indicate that effector-mediated manipulation of plant microbiota by microbial plant pathogens is not confined to bacterial targets but extends to eukaryotic microbes.

Functional characterization of VdAMP3 unveiled that the effector evolved to play a life stage–specific role in microbiome manipulation during microsclerotia formation by V. dahliae. Recently, we described the characterization of the first microbiome-manipulating effectors secreted by V. dahliae, VdAve1 and VdAMP2 (18). VdAve1 is a ubiquitously expressed bactericidal effector that promotes V. dahliae host colonization through the selective manipulation of host microbiota in the roots as well as in the xylem by suppressing microbial antagonists. Moreover, VdAve1 is also expressed in the soil biome, where it similarly contributes to niche colonization. Intriguingly, VdAMP2 is exclusively expressed in soil and, like VdAve1, exerts antibacterial activity that contributes to niche establishment. Interestingly, VdAMP2 and VdAve1 display divergent activity spectra and, therefore, likely complement each other for optimal soil colonization. In decaying host tissues, neither VdAve1 nor VdAMP2 are expressed, yet VdAMP3 expression occurs. Collectively, our findings for VdAve1, VdAMP2, and VdAMP3 demonstrate that V. dahliae dedicates a substantial part of its catalog of effector proteins toward microbiome manipulation and that each of these effectors act in a life stage–specific manner.

The life stage–specific exploitation of the in planta secreted antimicrobial effectors VdAve1 and VdAMP3 is well reflected by their antimicrobial activities and by the microbiota of the niches in which they act. Contrary to previous V. dahliae transcriptome analyses that repeatedly identified VdAve1 as one of the most highly expressed effector genes in planta (17, 38–40), we detected a repression of the effector gene in decomposing N. benthamiana tissues (Fig. 1 B and C). Characterization of the antimicrobial activity exerted by VdAve1 previously uncovered that the protein exclusively affects bacteria and does not impact fungi (18). Thanks to their ability to produce a wide diversity of enzymes, fungal pathogens are the primary decomposers of plant debris on earth (44). The phyllosphere of plants comprises a diversity of fungi (49–51). Importantly, upon plant senescence, these fungi are provided the first access to decaying plant tissues (44). The phyllosphere of plants diversifies with the growth and development of the plant, contributing to niche colonization. Contrary to previous studies, the phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44). The phyllosphere of plants is not a sterile environment but a diverse ecosystem that manipulates the development of the plant (44).

In Vitro Microbial Growth Assays. Bacterial isolates were grown on l-lysogeny broth agar at 28 °C. Single colonies were selected and grown overnight in low-salt lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, and 0.5 g/L sodium chloride) at 28 °C. Overnight cultures were resuspended to optical density (OD)_{600} = 0.025 in fresh low-salt LB supplemented with 20 μM VdAMP3 or ultrapure water (Milli-Q). In vitro growth was quantified using a CLARIOstar plate reader (BMG Labtech) as described previously (18).

Fungal isolates were grown on potato dextrose agar (PDA) at 22 °C. For yeasts, single colonies were selected and grown overnight in 0.05x potato dextrose broth (PDB) at 28 °C. Overnight cultures were resuspended to OD_{600} = 0.01 in fresh 5% PDB supplemented with ultrapure water (Milli-Q), 0.5 μM VdAMP3, or 0.5 μM VdAMP3 that was incubated in a PCR thermocycler at 95 °C for 16 h. Alternatively, for filamentous fungi, spores were harvested from PDA and suspended in 5% PDB supplemented with 5 μM VdAMP3 or ultrapure water (Milli-Q) to a final concentration of 10^6 spores/mL. Next, 200 μL of the fungal suspensions was aliquoted in clear 96-well flat-bottom polystyrene tissue-culture plates. Plates were incubated at 28 °C, and fungal growth was imaged using an SXK10 stereo microscope (Olympus) with an EP50 camera (Olympus).

Microbiome Analysis. Inoculation and incubation of N. benthamiana plants were performed as described above. Subsequent genomic DNA isolation and V. dahliae biomass quantification were performed as described previously using the primers listed in Table S3 (PNAS, 2018). After 4 wk of incubation in plastic bags at room temperature in the dark, the decaying N. benthamiana phyllosphere was collected and the VdAve1 expression mutant were collected. The phyllospheres of fresh 3-wk-old N. benthamiana plants were included as controls. All samples were flash-frozen in liquid nitrogen and ground using mortar and pestle, and genomic DNA was isolated generally thrive in soil or decaying organic matter but can opportunistically cause disease in immunocompromised patients (52–54). Azoles are an important class of antifungal agents that are used to treat fungal infections in humans. Unfortunately, agricultural practices involving massive spraying of azoles to control fungal plant pathogens, but also the extensive use of azoles in personal care products, ultraviolet stabilizers, and antioxidant aircrafts, for instance, gives rise to an enhanced evolution ofazole resistance in opportunistic pathogens of mammals in the environment (52, 55). For instance,azole resistant Aspergillus fumigatus strains are ubiquitous in agricultural soils and in decomposing crop waste material, where they thrive as saprophytes (56, 57). Thus, fungal pathogens of mammals, like A. fumigatus, comprise niche competitors of fungal plant pathogens. Hence, we speculate that, like V. dahliae, other plant pathogenic fungi may also carry potent antifungal proteins in their effector catalogs that aid in niche competition with these fungi. Possibly, the identification of such effectors could contribute to the development of novel antimycotics.

Materials and Methods
Gene Expression Analyses. In vitro cultivation of V. dahliae strain JR2 for analysis of VdAMP3 and Chrg02430 expression was performed as described previously (24). Additionally, for in planta expression analyses, total RNA was isolated from individual leaves or complete N. benthamiana plants harvested at different time points after V. dahliae root dip inoculation. To induce microsclerotia formation, N. benthamiana plants were harvested at 22 dpi and incubated in sealed plastic bags (volume = 500 mL) for 8 d prior to RNA isolation. RNA isolations were performed using the the Maxwell 16 LEV Plant RNA Kit (Promega). Real-time PCR was performed as described previously using the primers listed in SI Appendix, Table 3 (17).

Generation of V. dahliae Mutants. The VdAMP3 deletion and complementation mutants, as well as the eGFP expression mutant, were generated as described previously using the primers listed in SI Appendix, Table 3 (18). To generate the VdAMP3 complementation construct, the VdAMP3 coding sequence was amplified with flanking sequences (~0.9 kb upstream and ~0.8 kb downstream) and cloned into pCG (58). Finally, the construct was used for Agrobacterium tumefaciens–mediated transformation of V. dahliae as described previously (59). In vitro growth and microsclerotia production of the VdAMP3 deletion mutant were tested and quantified as described previously (18).

Antimicrobial resistance in bacteria and fungi is posing an increasing threat to human health. Possibly, microbiome-manipulating effectors represent a valuable source for the identification and development of novel antimicrobials that can be deployed to treat microbial infections. Arguably, our findings that microbiome-manipulating effectors secreted by plant pathogens also comprise antifungal proteins open up opportunities for the identification and development of antimycotics.

Most fungal pathogens of mammals are saprophytes that

Snelders et al.
An ancient antimicrobial protein co-opted by a fungal plant pathogen for in planta microbiome manipulation
using the DNeasy PowerSoil Kit (Qiagen). Sequencing libraries were prepared using the TruSeq DNA Nano kit (Illumina), and paired-end 150-bp sequencing was performed on the Illumina NextSeq500 platform at the Utrecht Sequencing Facility.

The sequencing data were processed as follows. Quality control of the reads, adapter trimming, and removal of N. benthamiana reads were performed with the ATLAS metagenomic workflow using the default parameters of the configuration file (61). Reads of the different samples were combined and assembled using metaSPAdes (used k-mer sizes: 21, 33, and 55) to obtain a single metagenome cross-assemble (62). Subsequently, the cross-assembled contigs were taxonomically classified using Contig Annotation Tool and binned per genus (63). The reads of the individual samples were mapped to the binned contigs using Burrows-Wheeler Aligner Maximal Exact Match (64). Next, the mapping files were converted to bam format using SAMTools (version 1.10), and the number of reads mapped to the contigs of a single genus were used to estimate the microbial mass for the individual samples. The generated taxonomy table and abundance table were subsequently transformed into a phyloseq (66) object (version 1.30.0) in R (version 3.6.1) to facilitate analysis of the microbiomes. The alpha diversity (Shannon index) and beta diversity (Bray-Curtis dissimilarity) were determined as described previously (66, 67). The DESeq2 extension of phyloseq was used to identify differentially abundant microbial genera (68). To this end, a parametric model was applied to the data and a negative binomial Wald test was used to test for significant differential abundance.

**Fungal Cocultivation Assays.** Fungal isolates were grown on PDA at room temperature. For D. vanrijiae, M. amylolytica, and C. jadinii, single colonies were selected and grown overnight in 5% PDB at 28 °C while shaking at 50 rpm. The overnight cultures of D. vanrijiae and M. amylolytica were resuspended to OD500 = 0.001 in fresh 5% PDB. For the synthetic communities, D. vanrijiae, M. amylolytica, and C. jadinii were resuspended to OD500 = 0.001 and spores of C. militaris and T. viride were harvested from PDA and resuspended to 10⁶ spores/mL. Next, equal volumes of the various fungal suspensions were mixed to obtain two syncoms: (A) D. vanrijiae, M. amylolytica, and C. militaris and (B) D. vanrijiae, M. amylolytica, C. jadinii, and T. viride, which were stored at −20 °C in 5% PDB supplemented with 10% glycerol (w/v). Upon use, the syncoms were thawed at room temperature and diluted 10× (A) or 25× (B) in fresh 5% PDB, after which they were mixed with V. dahliae. To this end, conidia of V. dahliae strain JR2 and the VgAMP3 deletion and complementation mutants were harvested from PDA plates and diluted in ultrapure water (Milli-Q) to a final concentration of 10⁵ or 10⁶ conidia/mL. Next, 150 μL of the fungal suspensions was mixed with 150 μL of the V. dahliae conidospore suspensions (10⁹ conidia/mL). For cultivation with syncom A or M. amylolytica and 10⁶ conidia/mL for the individual syncoms for the microbial samples. The generated taxonomy table and abundance table were subsequently transformed into a phyloseq object (version 1.30.0) in R (version 3.6.1) to facilitate analysis of the microbiomes. The alpha diversity (Shannon index) and beta diversity (Bray-Curtis dissimilarity) were determined as described previously (66, 67). The DESeq2 extension of phyloseq was used to identify differentially abundant microbial genera (68). To this end, a parametric model was applied to the data and a negative binomial Wald test was used to test for significant differential abundance.

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**ACKNOWLEDGMENTS.** B.P.H.J.T. is supported by the Research Council Earth and Life Sciences of the Netherlands Organization of Scientific Research (NWO). B.P.H.J.T. acknowledges funding by the Alexander von Humboldt Foundation in the framework of an Alexander von Humboldt Professorship endowed by the German Federal Ministry of Education, and research is further supported by the Deutsche Forschungsgemeinschaft (German Research Foundation) under Germany’s Excellence Strategy—EXC 2048/1-Project: Identification (391349590). We thank the Utrecht Sequencing Facility, subsidized by the University Medical Center Utrecht, Hubrecht Institute, Utrecht University, and The Netherlands X-omics Initiative (NWO Project 184.034.019) for providing sequencing services.
