

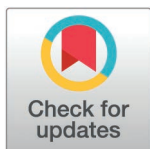
RESEARCH ARTICLE

The *adapt-to-nutrient NRPS-like* secondary metabolite gene cluster facilitates *Verticillium dahliae* adaptation to different nutrient environments

Ying-Yu Chen¹, Lea S. Steglich¹, Nadja Spasovski¹, Marcel H. W. Franzius¹, Merle Aden^{1,2}, Isabel Maurus¹, Rebekka Harting¹, Gerhard H. Braus^{1*}

1 Department of Molecular Microbiology, Institute of Microbiology and Genetics, Georg-August-Universität Göttingen, Göttingen, Germany, **2** Service Unit LCMS Protein Analytics, Göttingen Center for Molecular Biosciences (GZMB), Georg-August-Universität Göttingen, Göttingen, Germany

* gbraus@gwdg.de



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Data availability statement: The raw RNA sequencing data have been deposited in online repositories (NCBI database, BioProject ID PRJNA1346639 and PRJNA1364727). Other data presented in this study are available in the supplementary material (S4 Table).

Abstract

Filamentous fungi produce a wide range of secondary metabolites to adapt to changing environments. RNA sequencing revealed that nine biosynthetic gene clusters (BGCs) of the phytopathogenic *Verticillium dahliae* react to different nutrient environments. The *adapt-to-nutrient NRPS-like* (ANN) cluster contributes to antibacterial activity and developmental processes important for the early biotrophic life cycle, but is dispensable for virulence on tomato (*Solanum lycopersicum*). Transcription of the core biosynthetic enzyme-encoding ANN3 is highly induced in nutrient-poor environment. ANN3 is transcriptionally controlled by global and in-cluster transcription factors. ANN3 is activated by early colonisation transcription factors Som1 and Vta2, but repressed by Mtf1, which governs late stages of disease progression. The in-cluster transcription factor Ann1, which represses ANN3, is less abundant in nutrient-poor environment or when *V. dahliae* encounters antagonists. Ann1 promotes resting structure formation but suppresses conidiation and antibacterial activity. Possible products of the ANN cluster were revealed by comparing metabolites extracted from ANN3 regulator mutants and from the bacterial-fungal interaction zone. Our findings revealed that *V. dahliae* perceives different nutrient environments and changes its survival strategy by differential expression of the ANN secondary metabolite gene cluster.

Author summary

Verticillium dahliae is an economically significant phytopathogen that is widely distributed. The fungus adjusts and adapts its survival strategy according to the surrounding environment. Transcriptome data revealed that the core biosynthetic gene ANN3 of the *adapt-to-nutrient NRPS-like* (ANN) cluster is most expressed in nutrient-poor environments. The expression of ANN3 is governed by

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in-cluster repressor Ann1 and global transcriptional regulators that regulate other metabolic processes. Transcription factors Som1 and Vta2 are involved in the early plant-root infection process, whereas Mtf1 regulates late stage of disease development. *ANN3* is activated by Som1 and Vta2, but repressed by Mtf1. The repressor Ann1 is less present in nutrient-poor environments or when bacterial competitors are present. Ann1 promotes dormancy and represses spreading by conidiation. Vegetative growth is reduced but antibacterial activity is promoted when *ANN1* is deleted. Possible chemical products of the *ANN* cluster were identified by comparing the metabolites extracted from the regulator mutant strains and the bacterial-fungal interaction zone. In summary, our findings show how *V. dahliae* reacts to environmental signals to balance growth, survival, and competition through the *ANN* cluster.

Introduction

Verticillium dahliae is a haploid soil-born plant pathogen that infects a wide range of plant hosts [1]. Verticillium wilt, the monocyclic plant disease caused by *V. dahliae* infection, results in significant losses in agriculture worldwide [1]. The control of the fungus is difficult due to its resistant resting structures known as microsclerotia, and the rapid spread within the plant vasculature system by asexual conidiospores [1]. Heavily melanised microsclerotia are typically released into the environment from decaying plant material, and the dormancy of *Verticillium* can persist for years [2]. Environmental conditions such as favourable temperature, moisture levels, and chemical signals produced by soil bacteria as well as plant root exudates induce germination from microsclerotia [3–5]. The *ex planta* life cycle following dormancy is relatively short. Upon arrival at its plant host, the fungus adheres, colonises, and penetrates the root surface [6,7]. As *V. dahliae* crosses through the cortex and enters the xylem vessels, it produces conidiospores to cause systemic infection [1]. Along with the senescence of the plant host, *V. dahliae* transitions to saprophytic growth, and forms microsclerotia, which are subsequently released into the environment along with decaying plant materials [1].

V. dahliae encounters antagonists including different soil bacteria in the rhizosphere during the short saprophytic *ex planta* phase [5,8]. Filamentous fungi protect themselves and outcompete other microbes mainly by chemical defence strategies [9]. Secondary metabolites (SMs) are molecules that are derived from primary metabolic pathways. These natural products do not directly contribute to survival, but are rather produced under specific conditions to increase fitness. For example, DHN-melanin is produced during *V. dahliae* microsclerotia formation, and lagopodin B is synthesised by *Coprinopsis cinerea* when it's confronted with bacteria [10,11]. For the ease of regulation, genes involved in the synthesis of a specific SM are often located in close proximity within the genome, thus forming biosynthetic gene clusters (BGCs) [12]. The increased availability of whole genome sequences and

bioinformatic algorithms made studies on cryptic or uncharacterised BGCs possible, leading to the discovery of previously unknown natural products [13]. Transcriptional regulation of BGCs takes place at several levels. Whereas global transcriptional regulators or protein complexes may act on multiple metabolic processes, some BGCs also contain cluster-specific transcription factors [14].

Previous observations indicate that *V. dahliae* can physically escape antagonists by changing growth directions and reducing metabolic activities in lab experiments when confronted with bacterial isolates [8]. Whereas more studies focused on the inhibition of *Verticillium* spp. growth by soil bacteria, recent studies suggest that the fungus can also chemically defend itself from competitors [8,15–17]. To date, understanding the metabolite producing potential of *V. dahliae* remains incomplete. Only three BGCs in the *V. dahliae* genome have been studied. The melanin biosynthesis *PKS2* (also published as *PKS1*) cluster is regulated by in-cluster transcription factors *Vta1* and *Cmr1* [11,18–20], *Nag1* in the *HYB1* cluster is involved in key developmental processes and melanin biosynthesis [19,21], and the nonribosomal peptide synthetases in the *NRPS1* cluster contributes to the regulation of developmental processes and pathogenicity towards plants [22].

Transition from the fungal *ex planta* to the *in planta* life cycle is controlled by a series of sequentially acting transcription factors [23]. The *V. dahliae* *Som1* regulator is involved in the control of adherence and penetration of root surfaces and corresponds to *S. cerevisiae* *FLO8* that activates adherence and flocculation. The *Verticillium* transcription activator of adhesion 3 (*Vta3*) controls the colonisation of root surfaces, and subsequently *Vta2* is involved in the fungal proliferation within roots [6,7]. The *Som1*-*Vta3*-*Vta2* regulatory network coordinates physiological processes to adapt to different phases of the *V. dahliae* life cycle and its corresponding nutrient environment [24,25]. Transcription factors active during early phases of infection can promote regulatory subnetworks that also drive the onset of plant infection, such as the activation of *VTA3* by *Som1*, and the increased expression of *VTA2* by *Som1* and *Vta3* [6,7]. On the contrary, *Vta3* and *Vta2* decrease the expression of the *Mtf1*-regulated subnetwork that is involved in later phases of disease development [24].

In this study, we found that the presence of a nutrient-poor environment activates the *adapt-to-nutrient NRPS-like* cluster (*ANN* cluster, formerly termed *OTHER3* cluster [19]) in *V. dahliae*. The in-cluster transcription factor *Ann1* is a transcriptional repressor of the core biosynthetic enzyme-encoding gene *ANN3*. The abundance of *Ann1* is affected by the surrounding nutrient environment. *ANN3* expression can be controlled by global transcription factors that are involved in different phases of disease development. Our analysis disclosed the importance of *ANN1* in the *ex planta* life cycle of *V. dahliae*, such as the promotion of microsclerotia formation, the control of antibacterial activities, and the resistance towards osmotic stress.

Results

V. dahliae reacts to nutrient poor environments by increasing the transcript level of *ANN3* in the *ANN* secondary metabolite gene cluster

25 secondary metabolite gene clusters were identified by *in silico* prediction to be present in the *V. dahliae* JR2 genome [19]. Transcriptional profiles of *V. dahliae* in nutrient-poor and nutrient-rich growth conditions were compared. RNA-seq experiments were carried out on RNA samples harvested from cultures grown only in the minimal medium CDM or in cultures initially grown in CDM and subsequently shifted to pectin-rich simulated xylem medium (SXM). Transcriptome analyses revealed that expression of the core biosynthetic enzyme-encoding genes in eight BGCs were decreased in pectin-rich SXM in comparison to the nutrient-poor CDM, whereas the biosynthetic gene in one BGC was more expressed in SXM than in CDM (Table 1).

ANN3 in the *ANN* cluster (formerly termed *OTHER3* and the *OTHER3* cluster [19]) along with the *PKS2* (also known as *PKS1* in publications [11,18,20]) in the melanin biosynthesis cluster were two of the most differentially expressed core biosynthetic genes in nutrient-poor environments. The *ANN* cluster is located on the 5th chromosome of the *V. dahliae* JR2 genome, and consists of 14 ORFs, including two genes for transcription factors as well as three biosynthetic and four

Table 1. Differences in transcript level of each core biosynthetic gene of *V. dahliae* BGCs in samples shifted to SXM after initial CDM cultivation compared to samples cultured only in CDM.

BGC	Core biosynthetic gene	Transcript level difference (log ₂ FC) ¹
PKS1	PKS1 (VDAG_JR2_Chr1g11000)	ns
PKS2	PKS2 (VDAG_JR2_Chr1g15880)	-7.76
PKS3	PKS3 (VDAG_JR2_Chr2g00450)	ns
PKS4	PKS4 (VDAG_JR2_Chr2g06950)	1.52
PKS5	PKS5 (VDAG_JR2_Chr2g07190)	ns
PKS6	PKS6 (VDAG_JR2_Chr3g00930)	ns
PKS7	PKS7 (VDAG_JR2_Chr4g11250)	ns
PKS8	PKS8 (VDAG_JR2_Chr5g10150)	-2.75
PKS9	PKS9 (VDAG_JR2_Chr8g10310)	-3.47
HYB1	HYB1 (VDAG_JR2_Chr1g23880)	-3.62
NRPS1	NPS (VDAG_JR2_Chr3g13240)	-2.81
NRPS2	NRPS2 (VDAG_JR2_Chr6g06000)	ns
NRPS3	NRPS3 (VDAG_JR2_Chr7g10250)	-2.29
T3PKS	T3PKS (VDAG_JR2_Chr4g09550)	ns
TERP1	TERP1 (VDAG_JR2_Chr1g12450)	ns
TERP2	TERP2 (VDAG_JR2_Chr1g17230)	-1.79
TERP3	TERP3 (VDAG_JR2_Chr2g09130)	ns
TERP4	TERP4 (VDAG_JR2_Chr7g02950)	ns
OTHER1	OTHER1 (VDAG_JR2_Chr2g03880)	ns
OTHER2	OTHER2 (VDAG_JR2_Chr4g04680)	ns
ANN (OTHER3 ²)	ANN3 (OTHER3 ² ; VDAG_JR2_Chr5g11480)	-7.41
OTHER4	OTHER4 (VDAG_JR2_Chr5g11790)	ns
OTHER5	OTHER5 (VDAG_JR2_Chr6g00660)	ns
OTHER6	OTHER6 (VDAG_JR2_Chr6g01090)	ns
OTHER7	OTHER7 (VDAG_JR2_Chr8g01170)	ns

ns = not significantly different.

¹Differences in core biosynthetic gene transcript levels are presented in log₂ fold-change. Negative values indicate significant reduced transcript level in SXM compared to samples only cultured in CDM.

²Formerly termed as the OTHER3 cluster with the core biosynthetic gene named as OTHER3 [19].

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transport-related genes, respectively. The function of the five remaining encoded proteins could not yet be predicted (Fig 1a). RNA-seq results that compared RNA samples harvested from cultures initially grown in CDM and subsequently shifted to xylem sap extracted from tomato plants are differentially regulated in a similar way as those that were shifted to the pectin-rich-SXM (S1 Fig). ANN3 encodes the core biosynthetic enzyme, which is predicted to contain an AMP-dependent synthetase/ligase domain, a phosphopantetheine binding ACP domain, and five transmembrane domains (S2 Fig). Transcript levels of ANN3 were analysed by qRT-PCR to compare the gene expression in different nutrient environments. The results confirmed that ANN3 is highly induced in nutrient-poor growth condition compared to nutrient-rich media (Fig 1b).

Transcription factor Ann1 represses expression of the biosynthetic gene ANN3 and is less present in nutrient-poor culturing conditions

Fungal secondary metabolism is usually tightly regulated on several levels, allowing the fungus to react to specific environmental cues. Such regulation can be executed by global regulators that govern several cellular processes, or by

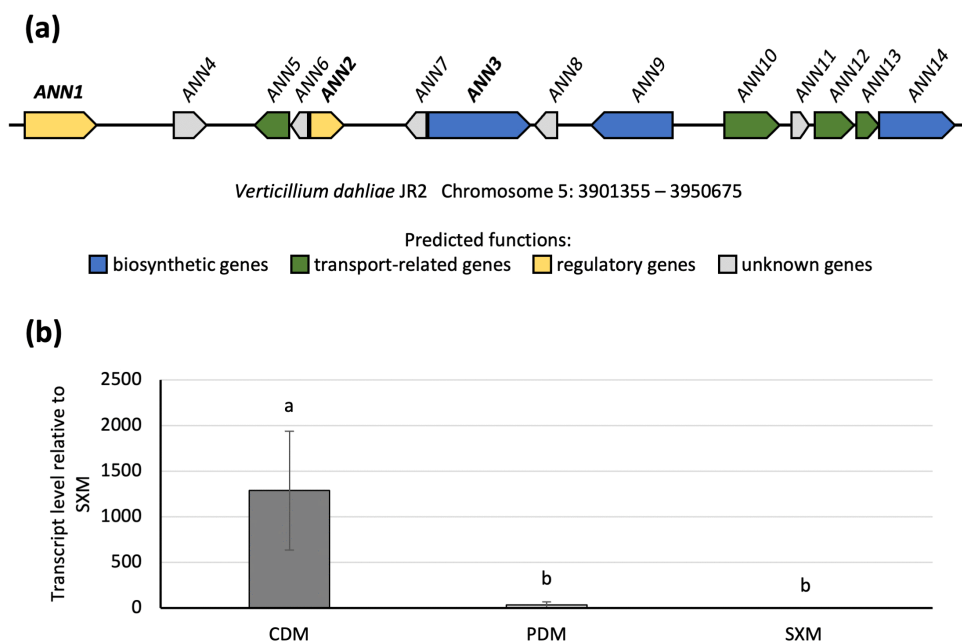


Fig 1. The *V. dahliae* ANN secondary metabolite cluster differentially expresses its core biosynthetic gene ANN3 in different nutrient environments. (a) The ANN cluster contains two predicted regulatory genes (yellow), three predicted biosynthetic genes (blue), four predicted transport-related genes (green), and five genes with no predicted functions yet. (b) Transcript level of the core biosynthetic gene ANN3 is significantly higher when cultured in nutrient-poor CDM compared to nutrient-rich PDM or pectin-rich SXM. qRT-PCR was performed with three biological replicates. One-way ANOVA with post-hoc Tukey HSD test was performed to compare transcript levels of ANN3 in different culture media. A difference of the lower-case letter on top of each bar indicates significant difference ($P < 0.05$).

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cluster-specific transcription factors that are usually pathway-specific [12]. Transcription factor-encoding genes within the ANN cluster, ANN1 (VDAG_JR2_Ch5g11420a) and ANN2 (VDAG_JR2_Ch5g11460a) were studied in detail to elucidate the regulatory mechanism of the nutrient-dependent expression of ANN3 (VDAG_JR2_Ch5g11480a). ANN1 is the first gene in the ANN cluster and encodes an ORF of 4751 base pairs (bp), which consists of three exons and two introns. The predicted Ann1 protein is 1172 amino acids (aa) in length and contains a C2H2 type zinc finger domain (PF00096) as well as a fungal specific transcription factor domain (PF04082; S3 Fig).

A BLAST search for proteins in other species revealed that Ann1 orthologs are widespread among the phylum Ascomycota, with orthologs from other *Verticillium* species sharing sequence similarities above 90%, and 167 orthologs from various species that share more than 50% similarity. Among the species that contain an Ann1 ortholog, we discovered other pathogenic and non-pathogenic filamentous fungi or yeasts with wide-spread industrial applications. Such as crop pathogen *Colletotrichum graminicola*, caterpillar fungus *Cordyceps militaris*, human pathogen *Aspergillus fumigatus*, methylophilic yeast *Komagataella phaffii* (formerly known as *Pichia pastoris*), and baker's yeast *Saccharomyces cerevisiae* (Fig 2). AmdX from *Aspergillus nidulans* was previously studied to be involved in the nitrogen utilisation regulatory network [26], and Adr1 from *S. cerevisiae* controls the utilisation of non-fermentable carbon sources [27,28].

The deletion, the GFP-fused complementation, as well as the over expression strains of ANN1 or ANN2 were generated and confirmed by Southern hybridisation to examine how the ANN cluster is being governed by transcription factors Ann1 and Ann2. All strains were cultured in PDM, SXM or CDM for 5 days before mycelia were harvested for RNA extraction. qRT-PCR experiments were performed to examine if Ann1 or Ann2 regulate the core biosynthetic enzyme-encoding gene ANN3, and whether Ann1 and Ann2 mutually influence each other's expression. Our results verified the transcript levels of the mutant strains and revealed that the absence of ANN1 resulted in a two-fold increase in ANN3 transcript level when

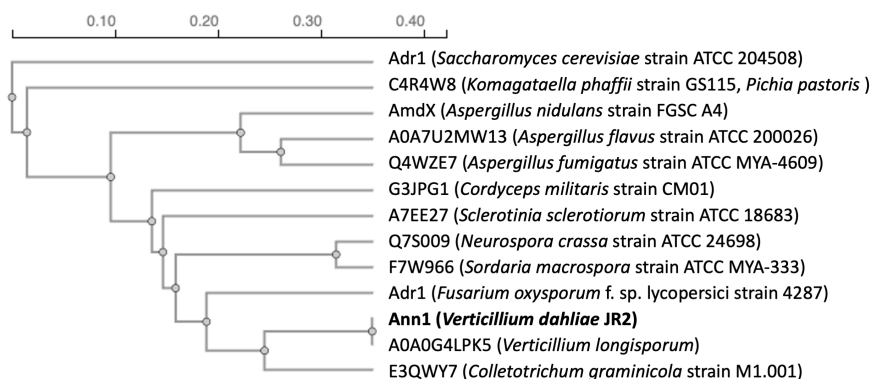


Fig 2. Phylogenetic tree generated from the protein sequences of Ann1 and its potential orthologs in other fungal species. The names of each ortholog or the UniProt identifier are listed when a name is not given. The species and strain from which each protein originates are listed in brackets. Horizontal distance of each branch represents the number of substitutions per site.

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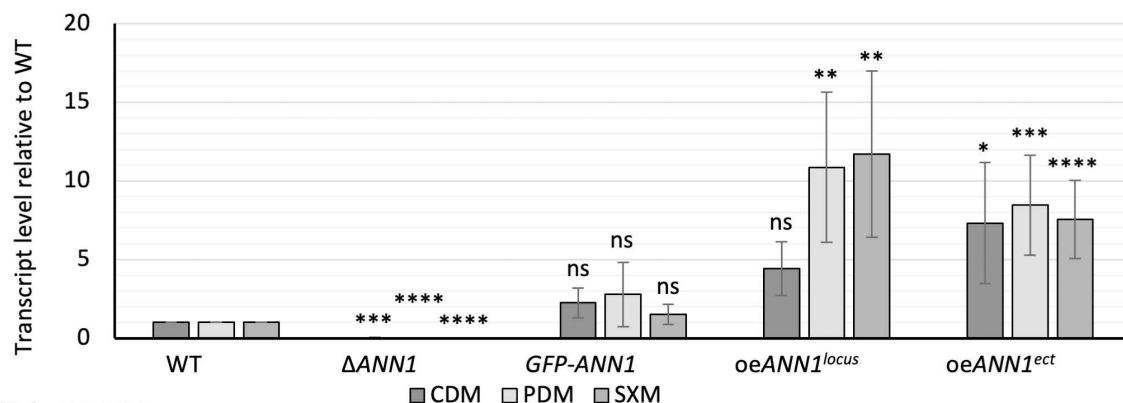
cultured in nutrient-rich PDM and SXM, but it doesn't further increase in CDM. In contrast, ectopically over expressing *ANN1* seven-fold resulted in a five-fold decrease in *ANN3* transcripts when cultured in minimal medium CDM, but the expression of *ANN3* doesn't further reduce in PDM and SXM (Fig 3a and 3b). *Ann1* therefore acts as a transcriptional repressor of the core biosynthetic enzyme-encoding gene *ANN3*. Nutrient availability in each tested media affect *ANN3* expression levels more than the transcript levels of transcription factor *ANN1*. We also discovered that deletion of *ANN1* resulted in a decrease in expression levels of the second transcription factor-encoding gene *ANN2* in all culture media (Fig 3c). On the contrary, neither *ANN1* nor *ANN3* expression levels changed more than two-fold upon the deletion or over expression of *ANN2* (S4 Fig). Our results suggest that *Ann1* acts upstream of *Ann2* as a transcriptional activator, and expression of *ANN3* is mainly controlled by the transcriptional repressor *Ann1* in the tested conditions and not by the functionally elusive *Ann2*.

Western experiments were performed to study how *Ann1* is involved in the nutrient-dependent regulation of *ANN3*. The presence of the *Ann1* protein in different nutrient environments was examined. The complementation strain that expresses GFP-fused *Ann1* under the control of its native promoter and the WT strain were cultured in either CDM, PDM, or SXM for five days before mycelia were harvested for protein extraction. The full-length GFP-*Ann1* protein is only detectable when the culture was grown in the nutrient-rich PDM. In contrast, full-length GFP-*Ann1* is undetectable in the minimal medium CDM, and the free-GFP signal was prominent (Fig 4a). Intracellular localisation of *Ann1* was investigated to verify the presence of *Ann1* as a transcriptional regulator in the nucleus. The GFP-*Ann1* complementation strain was transformed to ectopically express RFP-fused histone H2B for nuclear visualisation. The strain was cultured in PDM and observed under the fluorescence microscope. Since the GFP-*Ann1* signal co-localises with the histone-RFP signal, we confirmed the nuclear localisation of GFP-*Ann1* (Fig 4b). Our results indicate that *Ann1* is most present in nutrient-rich environments, and it locates in the nucleus. This aligns with the *Ann1* function as a transcription factor, and the elevated *ANN3* transcript levels in nutrient-poor environments, in which the *Ann1* repressor is less abundant.

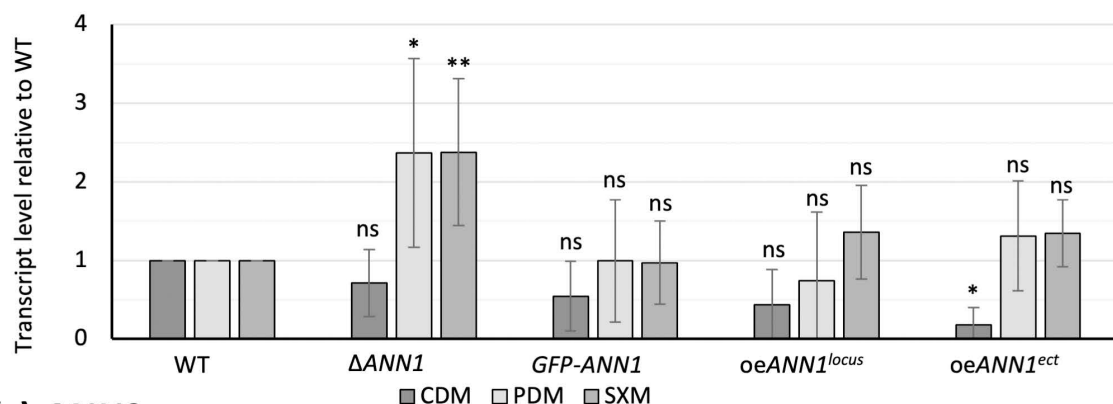
The *ANN* secondary metabolite gene cluster is regulated by the *Som1-Vta3-Vta2-Mtf1* network

Som1, *Vta3*, and *Vta2* are transcription factors that regulate sequential steps in the early plant root infection processes of *V. dahliae*, including adhesion, root surface propagation, and colonisation of roots. The transcription factor *Som1* promotes the expression of *VTA3* and *VTA2*, while *VTA2* expression is also governed by *Vta3* [6,7]. In contrast, transcription factor *Mtf1* controls the expression of genes involved in the later phase of the infection, and its expression is repressed by *Vta3* and *Vta2* [24]. In addition to the transcription factors locally present within the cluster, we further investigated whether the *ANN* cluster is regulated by these global regulatory proteins. qRT-PCR experiments were performed to compare the

(a) *ANN1*



(b) *ANN3*



(c) *ANN2*

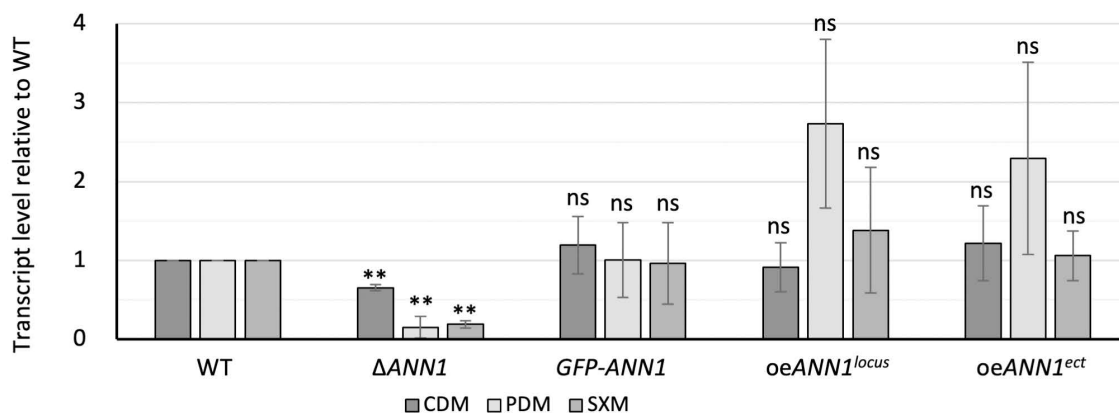


Fig 3. *V. dahliae* *Ann1* is a transcriptional repressor of the core biosynthetic enzyme-encoding gene *ANN3*, and an activator of transcription factor-encoding *ANN2*. Transcript levels of (a) *ANN1*, (b) *ANN3*, and (c) *ANN2* in the *ANN1* mutant strains were analysed by qRT-PCR. *ANN1* transcript levels were verified to be elevated in both in locus (locus) and ectopic (ect.) over expression strains. Compared to the WT gene expression levels, the transcript of *ANN3* is increased more than 2-fold in PDM and SXM when *ANN1* is deleted. Transcript level of *ANN3* is reduced more than 5-fold in the ectopic *ANN1* over expression strains when cultured in CDM. Expression of the transcription factor-encoding gene *ANN2* is reduced in all tested media when *ANN1* is deleted. qRT-PCR were performed with at least three biological replicates. One-sample t-test was performed to compare transcript levels of each tested strain to the WT expression levels under the same culturing condition (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P \leq 0.0001$; ns, not significantly different).

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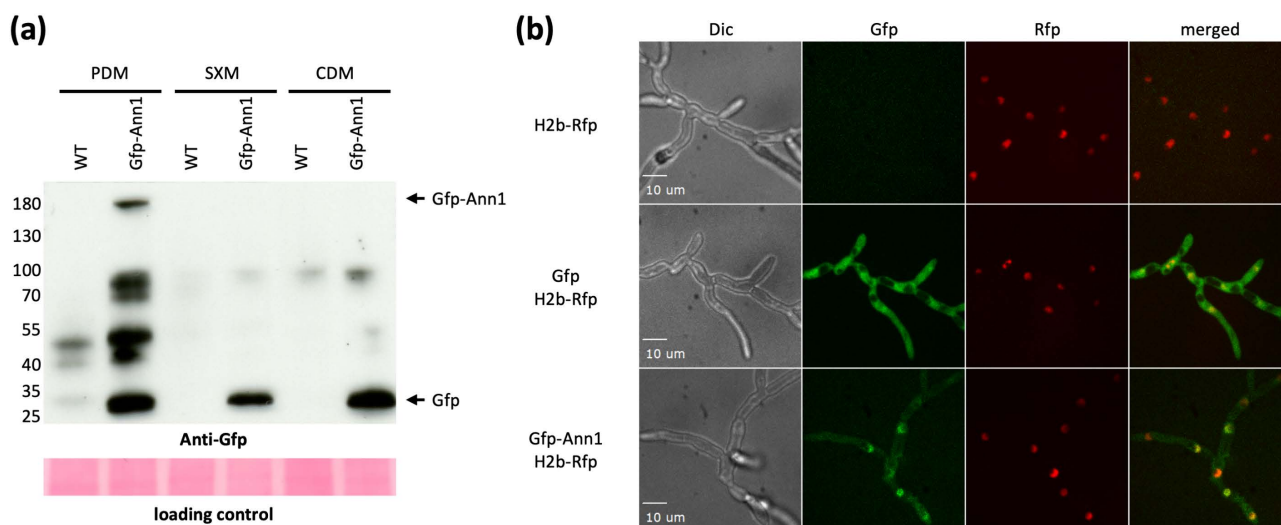


Fig 4. Less GFP-Ann1 fusion protein is present when *V. dahliae* is cultured in nutrient-poor conditions and it is mainly located in the nucleus in nutrient-rich PDM. (a) The full-length GFP-Ann1 is only present when *V. dahliae* is cultured in nutrient-rich PDM. Less GFP-Ann1 is present when *V. dahliae* is cultured in minimal medium CDM and pectin-rich medium SXM. Western experiments were performed with antibody recognising GFP, and the loading control was done by Ponceau staining or TGX Stain-Free Protein Gels (Bio-Rad) to ensure that an equal amount of protein was loaded in each lane. The expected size of free GFP is 28 kDa, and GFP-Ann1 is 155 kDa, and the expected positions are labelled on the right of the image. The expected protein sizes at each position were marked on the left according to the position of the protein ladder. (b) Fluorescence microscopy images were taken for the WT strain, the free GFP strain, and the strain expressing the GFP-Ann1 fusion protein. All strains were cultured in PDM and ectopically express the histone H2B-RFP fusion protein for nuclear visualisation. This figure presents differential interference contrast (Dic) view, green fluorescent protein filter view (GFP), red fluorescent protein filter view (RFP), and a merged view of the GFP and RFP channels.

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gene expression levels of *ANN1* and *ANN3* in the WT or in the *SOM1*, *VTA3*, *VTA2*, or *MTF1* deletion strains. The tested strains were inoculated in liquid CDM, and mycelia were harvested 5 days post inoculation (dpi) for RNA extraction. The absence of *SOM1* or *VTA2* led to a 19-fold and three-fold decrease in *ANN3* transcripts respectively (Fig 5). The changes in *ANN3* transcripts were less than two-fold in the absence of *VTA3*. In contrast, deletion of *MTF1* resulted in a 6-fold increase in *ANN3* transcript levels, indicating that the expression of *ANN3* is directly or indirectly repressed by Mtf1. By contrast, the *ANN1* transcript level remained within a two-fold range of variation in all the tested deletion strains. These results suggest that *ANN3* can be independently regulated by multiple transcriptional regulators, and its expression is favoured during the *ex planta* to *in planta* transition but suppressed in later phases of disease development.

Ann1 promotes melanisation and osmotic stress resistance

V. dahliae forms microsclerotia, which are heavily melanised resting structures that allow survival in unfavourable growth conditions [1]. The active phase of the *ex planta* life cycle of *V. dahliae* is short and transitory. However, it involves survival in competitive and potentially nutrient-limiting conditions, before the fungus reaches the plant host [1]. We examined the *ex planta* growth phenotype by point inoculating an equal number of conidiospores of each *ANN1* or *ANN2* mutant strains and the WT strain on agar plates with different media. Colonies of the $\Delta ANN1$ strain on every tested medium were smaller in diameter, implying that Ann1 is required for regular vegetative growth (Fig 6a and 6b). The diameters of $\Delta ANN1$ colonies cultured on CDM plates were 27% smaller than WT colonies. In comparison, $\Delta ANN1$ colonies cultured on CDM plates with cell wall stressors Congo red or sorbitol were 35% smaller, while the colonies on CDM plates with osmotic stressor NaCl were 70% smaller (Fig 6a and 6b). This suggests that *ANN1* promotes osmotic stress resistance but not cell wall-stress resistance. Melanisation levels of the colonies grown on pectin-rich SXM and cellulose-containing CDM plates

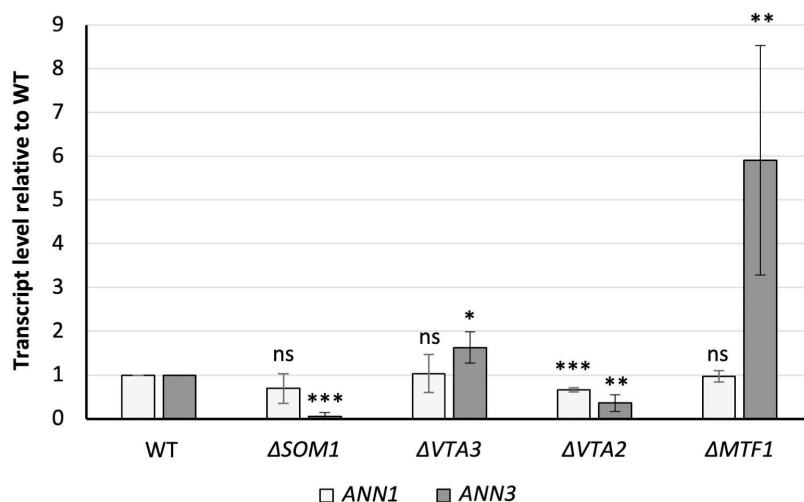


Fig 5. The *V. dahliae* Som1-Vta-Mtf1 regulatory network is directly or indirectly controlling the expression levels of *ANN1* and *ANN3*. Expression of *ANN3* is promoted by Som1 and Vta2 but repressed by Mtf1, and *ANN1* transcript level is decreased in the $\Delta VTA2$ strain. Transcript levels of *ANN1* and *ANN3* in the deletion mutants of potential upstream regulators were analysed by qRT-PCR with at least four biological replicates performed. All tested strains were cultured in liquid CDM for 5 days before the mycelia were harvested for RNA extraction. One-sample t-test was performed to compare transcript levels of each tested strain to the WT expression levels under the same culturing condition (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significantly different).

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were quantified. The $\Delta ANN1$ colonies hardly melanised in either medium, whereas the *ANN1* over expression colonies showed an increased level of melanisation (Fig 6a and 6c). Similar growth assays were also performed with the *ANN2* mutant strains. All colonies of the *ANN2* mutant strains were indistinguishable from WT colonies in size (S5 Fig). The *ANN2* over expression strain was less melanised at 3 dpi when cultured on SXM plates. However, no differences in melanisation can be observed between the different *ANN2* mutant strains and the WT strain at 10 dpi (S5 Fig). These results imply that Ann1 is required for vegetative growth and facilitates resistance to unfavourable growth conditions by resisting osmotic stress and promoting microsclerotia formation.

Ann1 regulates antibacterial activity of *V. dahliae*

The *ex planta* life cycle of *V. dahliae* consists of a potentially long period of dormancy in the form of microsclerotia, and a relatively short period of time when it germinates upon encountering suitable growth conditions [1]. Complex interactions in the soil between *Verticillium* species, its plant host, and soil bacteria shape this short *ex planta* phase in the form of competition and communications [1,5]. Here we investigated how *V. dahliae* chemically defends itself from bacterial competitors. As Ann1 is shown to be involved in the transition from the *in planta* to *ex planta* life cycle by promoting microsclerotia formation, and less Ann1 protein is present in nutrient-poor growth conditions, we further studied whether Ann1 is also involved in other phases of the *ex planta* life cycle. Bacterial-fungal interaction assays were performed by co-culturing the *ANN1* mutant strains as well as the WT strain with the Gram-positive *B. subtilis* 168, *B. thuringiensis* GOE4 isolated from tomato soil samples [15], and the Gram-negative *E. coli* DH5 α . The interaction interphase between *B. subtilis* 168 and the *V. dahliae* colonies showed that they grow close or touch each other when either the *V. dahliae* WT or the *GFP-ANN1* complementation strain was used (Fig 7a). However, a sharp edge of the *B. subtilis* 168 colony that is positioned apart from the *V. dahliae* colony can be observed when the $\Delta ANN1$ strain was grown on the same plate (Fig 7a), indicating the presence of an antibacterial compound secreted by the $\Delta ANN1$ strain. On the contrary, the *B. subtilis* 168 and the *V. dahliae* colonies touch or even grow into each other when the *ANN1* over expression strains were inoculated (Fig 7a). When the WT and the

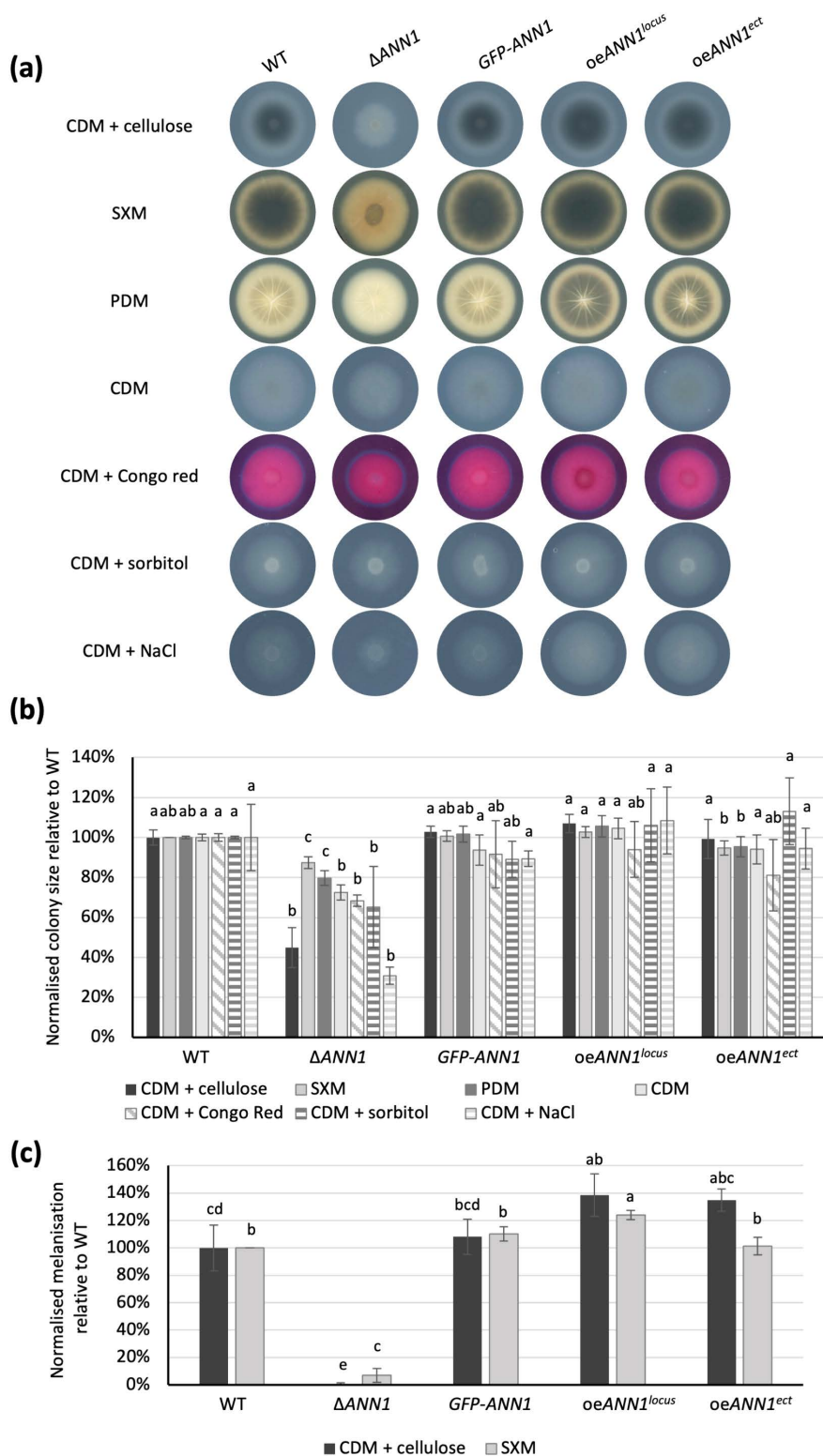


Fig 6. Ann1 is involved in *V. dahliae* filamentous growth and microsclerotia formation. (a) The *ex planta* growth phenotype was compared between WT and the *ANN1* mutant strains at 10 dpi on minimal medium supplemented with cellulose (CDM + cellulose), pectin-rich medium (SXM), potato dextrose medium (PDM), minimal medium (CDM), minimal medium supplemented with Congo red (CDM + Congo red), minimal medium

supplemented with sorbitol (CDM + sorbitol), and minimal medium supplemented with NaCl (CDM + NaCl). 50,000 spores were point-inoculated on each agar plate and the plates were scanned 10 dpi. (b) Colony sizes were measured and normalised to the size of the WT colony in the corresponding culture medium. The $\Delta ANN1$ strain had smaller colony size when cultured on CDM + NaCl, which suggests that it is less resistant to osmotic stress conditions. (c) Colony sizes and melanisation were measured by Fiji ImageJ and normalised by the colony size or degree of melanisation of WT in the respective culture medium. Melanisation is increased in *ANN1* over expression strains and reduced in the $\Delta ANN1$ strain. One-way ANOVA with post-hoc Tukey HSD test was performed to compare the colony size and the degree of melanisation of each strain on the same culture medium. A difference of the lower-case letter on top of each bar indicates significant difference ($P < 0.05$).

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ANN1 mutant strains were co-cultivated with the more aggressive *B. thuringiensis* GOE4, bacterial colonies always ended up growing into the *V. dahliae* colony. However, more contact between the bacterial and the fungal colonies was observed in the *ANN1* over expression strain that ectopically over expresses an extra copy of *ANN1* (Fig 7a). The distances between the *B. subtilis* 168 and the fungal colonies, and the contact between the *B. thuringiensis* GOE4 and the *V. dahliae* colonies was determined as a measurement of the antibacterial activity. The absence of *ANN1* significantly increases the ability of *V. dahliae* to cope with antagonistic interactions against *B. subtilis* 168, and the ectopic over expression of *ANN1* reduces its resistance towards *B. thuringiensis* GOE4 (Fig 7b, 7c). Statistical analyses were also performed on the results from the *E. coli* DH5 α interaction assays. However, no statistical significance can be observed possibly due to the limited growth of *E. coli* DH5 α in the unfavourable culturing conditions and a large variation between experimental results (S6 Fig). Since previous results indicated that less GFP-Ann1 protein is present in nutrient-poor conditions, we examined if the co-cultivation of *V. dahliae* with bacteria affects the expression of the *ANN* cluster. No significant differences in *ANN1* transcript levels were observed between the *V. dahliae* pure culture and the cultures co-cultivated with *B. subtilis* 168, *B. thuringiensis* GOE4 or *E. coli* DH5 α (S7 Fig). However, signal intensity of the full-length GFP-Ann1 protein was reduced and more free GFP was detected in the co-cultivated samples compared to the *V. dahliae* GFP-*ANN1* pure culture (Fig 7d). The *ANN3* transcript level increased six-fold during *B. subtilis* 168 co-cultivation in comparison to *V. dahliae* WT pure culture expression levels (Fig 7e). The reduction of Ann1 protein upon encountering antagonists, and the complete deletion of *ANN1* may have resulted in an increased transcription of *ANN3* and the presence of a secreted antibacterial compound.

Ann1 inhibits conidiation but is dispensable for pathogenicity

Upon entry into the plant vasculature system, *V. dahliae* spreads rapidly by forming asexual conidiospores that cause systemic infection [1]. Conidiation is a process that takes place during the *in planta* life cycle of *V. dahliae*. Equal numbers of conidiospores of WT and each *ANN1* mutant strains were inoculated in liquid SXM and the conidiospores were harvested and counted 5 dpi to study if *ANN1* is involved in the process of conidiation. The $\Delta ANN1$ and the GFP-*ANN1* strains showed no significant difference in their ability to form conidiospores compared to the WT strain, whereas the *ANN1* over expression strains showed a conidiospore reduction of approximately 50% compared to WT counts (Fig 8a). Although the *ANN1* mutant strains differ in their conidiation ability, tomato plant infection experiments revealed no significant differences in the disease scores between seedlings inoculated with different *ANN1* mutant strains at 21 dpi, and all tomato plants infected by *V. dahliae* WT and each of the *ANN1* mutant strains showed similar disease symptoms (Fig 8b). Therefore, *ANN1* is likely dispensable for pathogenicity.

Four identified metabolites represent potential ANN cluster products

We aimed to identify the products of the *ANN* cluster, because manipulation of transcript levels of the *ANN3*-controlling *ANN1* led to an altered antibacterial activity. Metabolites from the *ANN1* and the *SOM1* mutant strains were analysed as Ann1 represses, but Som1 activates the transcription of *ANN3*. CDM agar plates were inoculated with WT or the *ANN1/SOM1* mutant strains and incubated for 14 days before metabolites were extracted for LC-MS analysis. Empty CDM agar plates served as control. By analysing the total ion chromatogram of the LC-MS results, 11 metabolites were discovered to have altered abundance in either the $\Delta ANN1$ strain or the *ANN1* over expression strains. The $\Delta ANN1$ strain had an increased abundance of substances (I), (II), (IX), and (X) compared to the WT or over expression strains. (Fig 9).

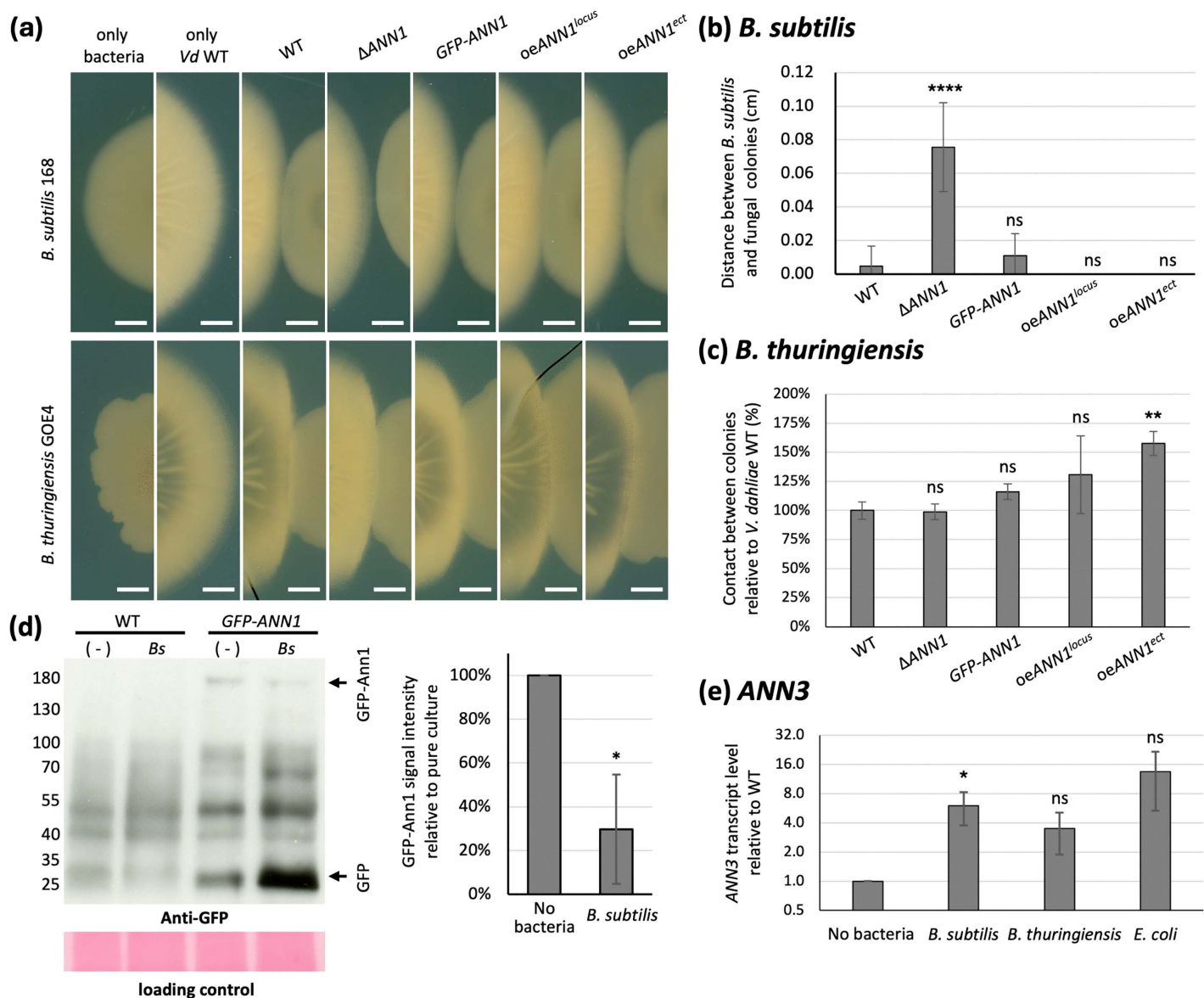


Fig 7. Ann1 negatively regulates antibacterial activity of *V. dahliae*. (a) Distance between the *V. dahliae* and *B. subtilis* 168 colonies was increased when *ANN1* is deleted, whereas contact between *V. dahliae* and *B. thuringiensis* GOE4 increased when *ANN1* is over expressed. The WT *V. dahliae* and the *ANN1* mutant strains were spotted on 100 mL PDM plates, and 10 μ L of bacterial suspension at OD₆₀₀ = 0.01 was spotted 2.5 cm apart from the centre of the *V. dahliae* colony at 3 dpi. The *V. dahliae* and bacterial colonies were co-cultured for an additional 7 days before the results were evaluated (white bar = 1 cm). (b) Antibacterial activity of *V. dahliae* against *B. subtilis* 168 was quantified by the distance between the two colonies. Unpaired t-test was performed to compare the antibacterial activity of each strain towards *B. subtilis* 168 (****, $P \leq 0.0001$; ns, not significantly different). (c) Antibacterial activity of *V. dahliae* against *B. thuringiensis* GOE4 was quantified by the contact between the two colonies. Contact between the *B. thuringiensis* GOE4 colony and each *ANN1* mutant strain colonies was normalised by the average length of contact between the bacterial and the *V. dahliae* WT colony. Unpaired t-test was performed to compare the antibacterial activity of each strain towards *B. subtilis* 168 (**, $P < 0.01$; ns, not significantly different). (d) The abundance of full-length GFP-Ann1 protein is reduced when *V. dahliae* is cultured with *B. subtilis* 168. The *GFP-ANN1* and WT strains were cultured in liquid PDM for 5 days, *B. subtilis* 168 were co-cultured with *V. dahliae* for 24 hr in designated samples. Western experiments were performed with antibody recognising GFP, and Ponceau staining or TGX Stain-Free Protein Gels (Bio-Rad) served as loading control to ensure that an equal amount of protein was loaded in each lane. The expected size of free GFP is 28 kDa, and GFP-Ann1 is 155 kDa. The expected positions are labelled on the right of the image. The expected protein sizes at each position were marked on the left according to the position of the protein ladder. The intensity of the GFP-Ann1 signals were quantified and normalised by *V. dahliae* *GFP-ANN1* pure culture signals. One-sample t-test was performed to compare signal intensity of the co-cultivated samples to the pure culture samples (*, $P < 0.05$). (e) The transcript level of *ANN3* significantly increases when *V. dahliae*

is co-cultured with *B. subtilis* 168. qRT-PCR was performed to assess the transcript levels of *ANN3* in different culturing conditions, and the transcript level of co-cultivated samples was normalised by the WT level in the same biological replicate. All samples were cultured in liquid PDM for 5 days before the mycelia were harvested for RNA extraction. Bacterial cultures were inoculated to the co-cultured samples 4 dpi at an initial OD₆₀₀ of 0.01. The y-axis is plotted on a log₂ scale. One-sample t-test was performed to compare transcript levels of the co-cultivated samples to the pure culture samples (*, $P < 0.05$; ns, not significantly different).

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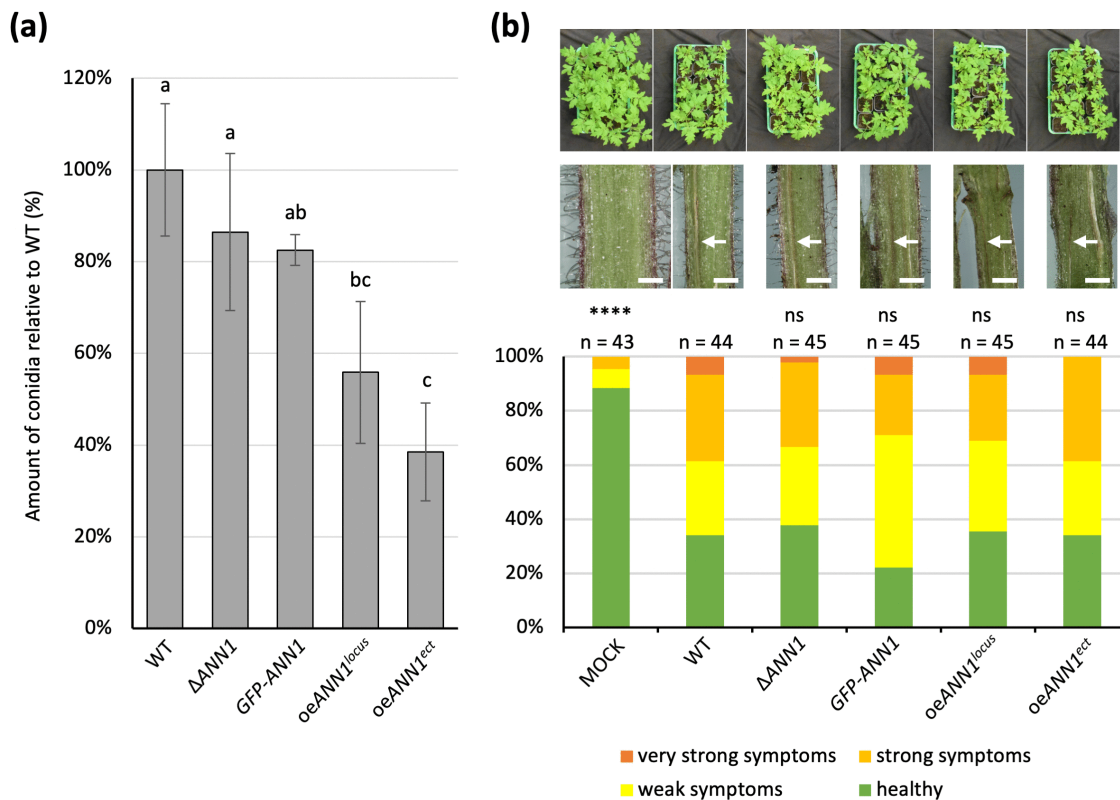


Fig 8. Ann1 inhibits conidiation but is not involved in virulence towards tomato plants. (a) The *ANN1* over expression strains produce less conidia than WT and other *ANN1* mutant strains. The *ANN1* mutant strains and the WT strain were cultured in SXM for 5 days before the conidio-spores were harvested and counted. Four biological replicates with four technical replicates each were performed, and all results of the same biological replicate were normalised by the WT conidiation level. One-way ANOVA with post-hoc Tukey HSD test was performed to compare conidiation of each strain. A difference of the lower-case letter on top of each bar indicates significant difference ($P < 0.05$). (b) WT and the *ANN1* mutant strains were tested for *in planta* phenotype by infecting 10-days old tomato seedlings, and the disease scores were assigned 21 dpi. An overview of the treated plants and representative images of hypocotyl cross-sections are shown on top. The white arrow indicates sites of hypocotyl discolouration, and the white bar represents 1 mm. The disease scores of seedlings inoculated by water (MOCK) and each *ANN1* mutant strain were compared to the disease scores of the WT-inoculated seedlings by two-tailed Mann-Whitney U test (****, $P < 0.0001$; ns, no significant difference).

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The production of 15 metabolites was affected by the deletion or the over expression of *SOM1* (S8 Fig). The deletion of *SOM1* reduced the production of 13 metabolites. Within which, substances (I) – (IV) and (VI) – (X) also had altered abundance in the *ANN1* mutant strains, and the production of substances (XII) – (XIV) is known to be controlled by the velvet proteins [29]. The velvet proteins are also global transcriptional regulators, and the expression of *VEL1* is known to be governed by *Som1* [6,29]. Since the expression of the core biosynthetic gene *ANN3* is repressed by *Ann1* and promoted by *Som1*, possible candidates for the metabolite produced by the *ANN* cluster or the derivatives of the product can be limited to compounds (I), (II), (IX), and (X).

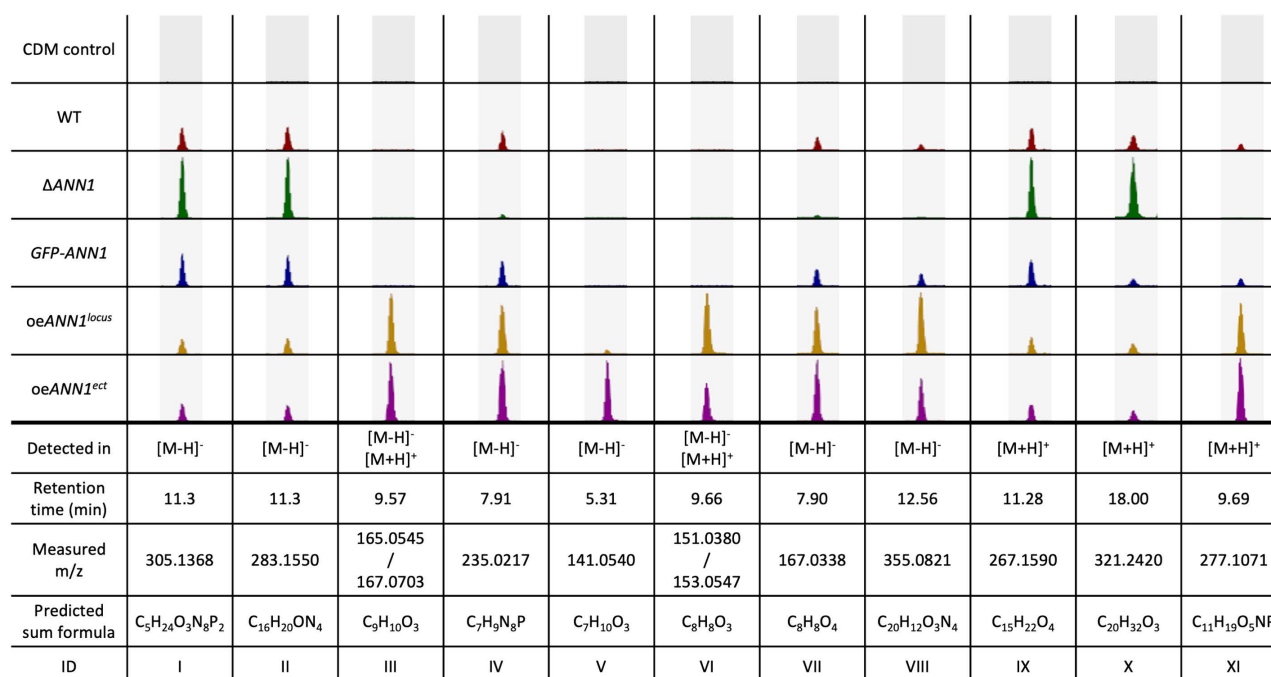


Fig 9. Eleven metabolites had altered abundance in the *V. dahliae* ANN1 mutant strains. Four of the listed metabolites are more abundant in the ΔANN1 strain (I), (II), (IX), (X), and seven are more abundant in the over expression ANN1 strains (III) – (VIII), (XI). All tested strains were grown on CDM plates for two weeks before the metabolites were extracted by 50% ethyl acetate from the homogenised agar. Extracted ion chromatograms of masses that had differed abundance in the tested strains are shown, and 5 ppm of mass deviation was tolerated. The height of each peak corresponds to the relative abundance of a certain mass within the tested strains. The predicted sum formula of each compound is calculated by the calculated exact mass.

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Metabolites were also extracted from the interaction interface between the *B. subtilis* 168 colony and the *V. dahliae* WT or ANN1 mutant strain colony on PDM plates (Fig 7a) to analyse if the metabolite produced by the ANN cluster or the derivatives are possibly contributing to the antibacterial activity. Out of the 18 fungal metabolites with altered abundance in the ANN mutant strains detected in the co-culture interface on PDM, substances (I), (II), (IV), (VII), (IX), and (X) overlapped with previously detected metabolite samples from pure cultures of ANN1 mutant strains on CDM (S9 Fig). Combined with the metabolites discovered from pure cultures of ANN1 or SOM1 mutant strain, compounds (I), (II), (IX), and (X) are the most probable candidates as the product or its derivatives of the ANN cluster, and it is possible that they are involved in antibacterial activity.

Discussion

Phytopathogenic *Verticillium* perceive different growth conditions during their life cycle and react by adjusting their transcriptome or secretome [8,25,30]. By comparing the *V. dahliae* transcriptome in nutrient-rich and nutrient-poor environments, we revealed that eight biosynthetic gene clusters (BGCs) in the *V. dahliae* genome had their core biosynthetic enzyme-encoding genes differentially expressed. Among which, ANN3 of the ANN cluster and PKS2 (also published as PKS1 [11,19]) in the melanin biosynthesis cluster were the most highly transcribed core genes in nutrient-poor environments. Melanin ensures the dormancy phase of the life cycle by protecting microsclerotia [18,20], whereas the biological role of the ANN cluster was so far unstudied. The regulation of fungal secondary metabolism often involves complex signalling pathways. Specific environmental cues activate certain pathways, and transcription factors act in the end to activate or repress genes involved in secondary metabolite biosynthesis [12]. In this study, we discovered that the expression of ANN3 is governed by the in-cluster transcriptional repressor Ann1 (Fig 3), as well as global transcriptional regulators Som1, Vta2, and Mtf1 (Fig 5). It

is estimated that 60% of fungal biosynthetic gene clusters encode in-cluster transcription factors that act as pathway-specific regulators [12]. Although most of these clusters only have one pathway-specific transcription factor, in rare cases, they can also include more regulatory genes. For example, both *VTA1* and *CMR1* are located within the melanin biosynthesis cluster in the *V. dahliae* genome [18,20], and *AflR* and *AflS* in the aflatoxin biosynthesis cluster of *A. flavus* [31]. In the case of *VTA1* and *CMR1*, the presence of two transcription factors enables the activation of melanin biosynthesis by two independent pathways, which can be triggered by different environmental cues. An additional in-cluster transcription factor also gives regulatory flexibility, as each member gene within the melanin biosynthesis cluster can also be controlled differently by *VTA1* and *CMR1* [18,20]. The transcription factor Ann1 mainly controls the nutrient-based expression of *ANN3* (Figs 3, 4), but the regulatory role of Ann2 towards *ANN3* remains yet unknown in our tested conditions (S4 Fig). The presence of *ANN2* suggests a potential of the *ANN* cluster to be activated or repressed by environmental stimuli other than nutrient availability, or the possibility of a different modification or transportation of the produced metabolite.

Our results revealed that Ann1 negatively regulates the core biosynthetic enzyme-encoding *ANN3*, and the presence of Ann1 is reduced in nutrient-poor culturing conditions (Figs 3b & 4a). To date, two orthologs of Ann1 have reported functions (Fig 2). Adr1 in *S. cerevisiae* regulates the utilisation of non-glucose carbon sources [27,28], whereas *A. nidulans* AmdX allows the fungus to utilise simple amides as the sole nitrogen source by activating the expression of acetamidase-encoding AmdS [26]. Genes regulated by ScAdr1 and AnAmdX are repressed when the preferred nutrient source is available [27,32]. In the case of ScAdr1, the presence of glucose results in the phosphorylation of ScAdr1, which leads to the subsequent inactivation of downstream target genes [33]. The similar involvement of Ann1, ScAdr1, and AnAmdX in adapting to limited or unfavourable nutrient sources suggests an evolutionarily conserved function within the Ascomycota phylum.

This study further investigated the regulatory network that governs *ANN1* and *ANN3* to understand the role of the *ANN* cluster in the *V. dahliae* life cycle. Som1 and Vta2 are known to be regulating the early root-colonisation process. In contrast, Mtf1 is involved in later phases of the infection [6,7,24]. The fact that *ANN3* expression is promoted by Som1 and Vta2 and repressed by Mtf1 (Fig 5) suggests that the *ANN* cluster is less needed in the later phases of disease development and is more important during the *ex planta* to *in planta* lifestyle transition. The fact that altered *ANN1* expression levels did not result in differences in disease symptoms further suggests that the *ANN* cluster is dispensable for later disease development and virulence (Fig 8b). As *V. dahliae* is mostly dormant in resistant microsclerotia during its *ex planta* phase, and there are no effective control methods once the fungus enters the plant vasculature system [34], the short biotrophic *ex planta* period presents a window of opportunity to prevent Verticillium wilt. Earlier studies on different *Fusarium oxysporum* strains suggested that pathogenic isolates are less well-adapted to competitions in the soil compared to non-pathogenic isolates [35]. Supporting this model, the phytopathogen *V. longisporum* decreases the expression of genes involved in metabolic processes and cell wall organisation upon encountering antagonists, and the hyphal tip also grows away from co-cultivated bacteria to avoid contact [8]. The characterisation of the *ANN* cluster supports the idea that *V. dahliae* can also act on bacterial antagonists as alternative to taking the “wait or escape” strategy. The *ANN* cluster acts when the fungus must cope with unfavourable growth conditions and has to counteract against bacterial competitors. Competition from antagonists can lead to nutrient limitation, thus resulting in the reduction of Ann1 protein (Fig 7d). The reduced Ann1 de-represses the expression of the core biosynthetic enzyme-encoding *ANN3* and possibly results in the increased production of metabolites (I), (II), (IX), or (IX) that may potentially be involved in inhibiting bacterial growth (Figs 7 & 9). Since the expression of genes involved in metabolic processes and cell wall organisation decrease during competition [8], the reduced vegetative growth and resistance of the *V. dahliae* $\Delta ANN1$ strain towards osmotic stress may be a result of the growth-defence trade-off. As *V. dahliae* spends most of its biotrophic phase in the plant host, *in planta* developmental processes such as conidiation and microsclerotia formation are subjected to complex regulations [1,6,29,36]. Upon the initial root infection, rapid spread and growth is needed to secure a successful host colonisation. The *ANN* cluster is less involved in the *in planta* life cycle presumably because it is a mechanism for defence. Instead of supporting processes needed for plant colonisation, Ann1 is shown to withhold conidiation and promote dormancy (Figs 6 & 8a).

In summary, our study reports the *ANN* cluster as a novel defence mechanism for the phytopathogenic *V. dahliae* to cope with antagonists and unfavourable nutrient environments. The biological functions and the regulatory relationship between members of the *ANN* cluster are summarised in Fig 10. The in-cluster transcription factor Ann1 influences growth, developmental processes, and interspecies competition. Involvement of the *ANN* cluster in the short yet exposed *ex planta* growth phase makes it an interesting target for the future development of effective Verticillium wilt controlling methods.

Materials and methods

Strains and growth conditions

Verticillium dahliae, *Escherichia coli*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, and *B. thuringiensis* strains used in this study are listed in S1 Table. Bacterial strains were cultured in lysogeny broth medium [37] and supplemented with kanamycin (AppliChem) with a final concentration of 100 µg/mL when required. *E. coli*, *A. tumefaciens*, *B. subtilis*, and *B. thuringiensis* were incubated at 37°C, 25°C, 30°C, and 30°C respectively when cultured alone. *E. coli*, *B. subtilis*, and *B. thuringiensis* were incubated at 25 °C when co-cultivated with *V. dahliae*. *V. dahliae* strains were grown at 25°C in

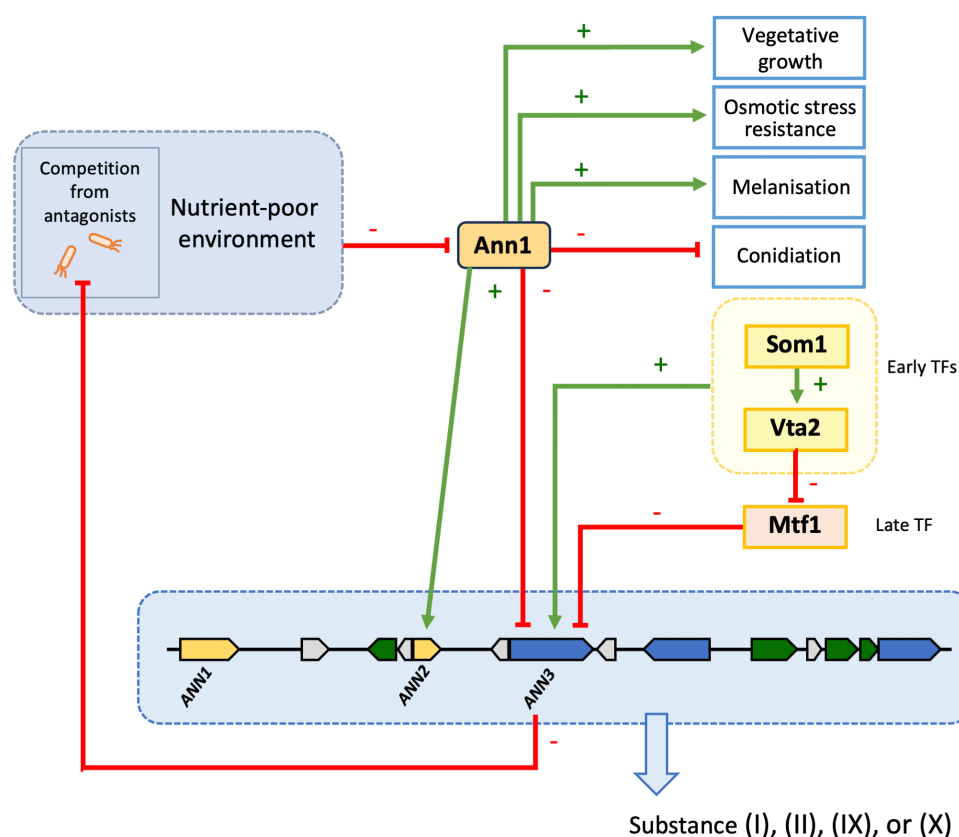


Fig 10. Regulatory relationships between the members of the *V. dahliae* *ANN* cluster and their functions in biological processes. The in-cluster transcription factor Ann1 represses the expression of the core-biosynthetic enzyme-encoding *ANN3* but activates the transcription factor-encoding *ANN2*. Nutrient-poor environments and the presence of antagonists lead to the reduction of Ann1 protein and increased transcript level of *ANN3*. Ann1 withholds antibacterial activity possibly by inhibiting the production of metabolite synthesised by Ann3. Ann1 promotes melanisation, whereas Ann2 plays a minor role in inhibiting it. Ann1 additionally promotes vegetative growth and osmotic stress resistance but inhibits conidiation. Early transcription factors (TFs) that regulate plant root colonisation processes such as Som1 and Vta2 activate *ANN3* expression, and are presented in yellow boxes. The late TF Mtf1 that controls later phases of disease development represses *ANN3* expression, and is depicted in an orange box. The *ANN* cluster genes are shown in a blue box with dashed border, and substances (I), (II), (IX), or (X) are the potential metabolite products of the *ANN* cluster or its derivatives.

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simulated xylem medium (SXM), potato dextrose medium (PDM), Czapek Dox medium (CDM), or xylem sap extracted from tomato plants as previously described [38]. Tomato xylem sap was extracted as described in an earlier study [24]. SXM was supplemented with 300 µg/mL cefotaxime (Fujifilm Wako Chemicals), and PDM was supplemented with 50 µg/mL hygromycin B (Invivogen) or 72 µg/mL nourseothricin (Werner BioAgents) when required. Conidiospores were harvested and quantified as described in earlier studies [24].

Bioinformatic tools

Information on gene annotations was obtained from the Ensembl Fungi database [39]. Protein sequence of *V. dahliae* JR2 was obtained from the Ensembl Fungi database, whereas protein sequences of all other fungi mentioned in this study were obtained from the UniProt database [39,40]. Protein sequences were compared and phylograms were generated using the Clustal Omega multiple sequence alignment website [41].

Verticillium mutant strain construction

Plasmids containing the deletion, complementation, or over expression cassette were transformed into *E. coli* DH5α by heat shock as established in prior studies [42], and the sequence-confirmed plasmids were transformed to *A. tumefaciens* AGL1 as mentioned previously [43]. Plasmids were designed and constructed according to the principles of earlier studies, and the plasmids and primers are listed in S2 and S3 Tables. *Agrobacterium*-mediated transformation was performed to manipulate the *V. dahliae* genome [6]. The *ANN1* and *ANN2* deletion strains were constructed by replacing the open reading frame with a resistance cassette via homologous recombination. The complementation strains were generated by reintroducing the *ANN1* gene with *GFP* fused to the 5' end, and the *ANN2* gene with *GFP* fused to the 3' to the genomic locus of the respective deletion strains. The in locus over expression strains of *ANN1* and *ANN2* were generated by adding a strong *A. nidulans* *gpdA* promoter in the 5' end of the respective genes in the respective genomic locus. The *ANN1* ectopic over expression strain was generated by ectopically integrating an extra copy of *ANN1* gene with *gpdA* promoter in the 5' end in the WT background, whereas the *SOM1* over expression strain was generated by ectopically reintroducing the *SOM1* gene with *gpdA* promoter in the 5' end in the *SOM1* deletion strain. Histone *H2B* with *RFP* fused to the 3' end was ectopically integrated into the genome of the WT strain, a strain over expressing free GFP, and the *ANN1* complementation strain for localisation studies. Genotypes of the mutant strains were verified by Southern hybridisation (S10 & S11 Figs)

RNA extraction and quantitative real-time PCR (qRT-PCR)

5×10^7 conidiospores of each tested *V. dahliae* strain were inoculated in 50 mL of CDM, PDM, or SXM and incubated at 25°C. Mycelia of each tested strains were harvested 5 days post inoculation (5 dpi) and ground into powder. For samples involving bacterial-fungal co-cultivations, *E. coli* DH5α, *B. subtilis* 168, and *B. thuringiensis* GOE4 cultures were inoculated into 4-days old *V. dahliae* cultures to reach a final bacterial concentration of $OD_{600} = 0.01$, and fungal mycelia were harvested a day after the inoculation of bacterial culture. RNA was extracted from each mycelial sample with TRIzol (Invitrogen) [29]. DNase treatments were performed on each crude RNA extract with TURBO DNA-free kit (Invitrogen), and cDNA was synthesised from DNase-treated RNA samples by RevertAid RT kit (Thermo Scientific) according to the manufacturer's protocol. qRT-PCRs were performed to analyse transcript levels of *ANN1*, *ANN2*, and *ANN3* according to previously described methods [24], and the primers used are listed in S2 Table. Transcript levels of each tested mutant strain were normalised by the WT levels of the corresponding culture condition in the same biological replicate. One-sample t-test was performed for statistical analysis.

Protein extraction and Western experiments

5×10^7 conidiospores of each tested *V. dahliae* strain were inoculated into 50 mL of CDM, PDM, or SXM and incubated at 25°C for 5 days. For samples involving bacterial-fungal co-cultivations, *B. subtilis* 168 cultures were inoculated into 4-days old *V. dahliae* culture to reach a final *B. subtilis* 168 concentration of $OD_{600} = 0.01$, and fungal mycelia were harvested a

day after the inoculation of bacterial culture. Mycelia of each sample were ground into powder and weighed. 500 μ L of modified lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA pH 8, 1 mM PMSF, 2 mM DTT, 0.5% Nonidet-P40, 2x cOmplete EDTA-free Proteinase Inhibitor Cocktail, 4% SDS) were added per 1 g of mycelial powder [44]. Mycelial-lysis buffer mixtures were incubated at 65°C for 5 min, and centrifuged at 10,000 rpm for 20 min to separate protein extracts from cell debris. Protein concentration was determined by Bradford assay [29], and Western experiments and signal detection were done as previously described [38]. Loading controls were either done by Ponceau staining, or by 4–20% Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad) with ChemiDoc Touch Imaging System (Bio-Rad) to ensure equal loading of proteins in each well.

Confocal microscopy

The localisation of GFP-fused Ann1 was visualised by fluorescence confocal microscopy with GFP filters [36]. Nuclei were visualised by ectopic expression of RFP-fused histone H2B, and observed under the fluorescence confocal microscopy with RFP filters [36].

Phenotypic analysis

5 $\times$ 10⁴ of freshly harvested conidiospores were point-inoculated onto 30 mL agar plates. In addition to SXM, PDM, and CDM plates, CDM plates with 3% cellulose instead of sucrose as carbon source were used as a microsclerotia-inducing condition. CDM plates supplemented with 0.5 M NaCl or 0.8 M sorbitol were used for osmotic stress tests, and CDM plates supplemented with 20 μ g/mL Congo red were used for cell wall stress tests. Plates were incubated at 25°C for 10 days and the bottom view of the plates was documented. Sizes of each documented colony were measured by the Fiji (ImageJ) software [45]. Melanisation was measured as a representation of microsclerotia formation. Melanisation levels were quantified using Fiji (ImageJ) by converting images to 8-bit greyscale, adjusting the threshold to isolate melanised regions, and measuring the area using the ROI Manager tool [45]. Colony size and melanisation level of each colony were normalised to the corresponding value of the WT colony within the same biological replicate. One-way ANOVA with post-hoc Tukey HSD test was performed for statistical analysis.

Quantification of conidiation

Experiments performed to compare conidiation between different mutant strains were done as reported in earlier studies [24,29]. The amount of conidiospores counted from each strain was normalised by the WT levels in the same biological replicate. One-way ANOVA with post-hoc Tukey HSD test was performed for statistical analysis.

Bacterial-fungal interaction assay

Bacterial-fungal interaction assays were performed by point-inoculating 5 $\times$ 10⁴ of freshly harvested conidiospores on 100 mL PDM agar plates. 10 μ L of bacterial suspension at OD₆₀₀ = 0.01 2.5 cm apart from the point of the fungal inoculation 3 days after inoculating the fungus. *E. coli* DH5 α , *B. subtilis* 168, or *B. thuringiensis* GOE4 were used to interact with the *V. dahliae* WT or *ANN1* mutant strains. Distances between the bacterial and the fungal colonies, or the contact between the two colonies were measured 10 days after fungal inoculation. Unpaired t-test was performed for statistical analysis.

Tomato plant infection

Pathogenicity of the *V. dahliae* WT and *ANN1* mutant strains was investigated by tomato plant infection experiments as described reported in earlier studies [18]. Two-tailed Mann-Whitney U test was performed to compare the disease scores of seedlings inoculated WT with those inoculated with deionised water or with other *V. dahliae* strains.

Secondary metabolite extraction and analysis

Two 30 mL CDM agar plates were each plated with 1×10^6 of freshly harvested conidiospores and incubated for two weeks at 25°C. A total of 30 mL of agar were homogenised per tested strain, and metabolites were extracted from homogenised agar [29]. Metabolite samples were measured and analysed as previously described [29,46], and extracted ion chromatograms were generated by the FreeStyle 1.6 software (ThermoFisher Scientific). Each tested condition was at least performed in three biological replicates. MS2 spectra of the metabolites were compared between experiments to confirm the reproducibility of a certain compound in different experiments. MS2 spectra of all metabolites described in this study are listed in [S4 Table](#).

Supporting information

S1 Fig. *ANN* cluster genes express similarly in tomato xylem sap as in the pectin-rich SXM.

(DOCX)

S2 Fig. The genomic structure of the core biosynthetic enzyme-encoding gene *ANN3*.

(DOCX)

S3 Fig. The genomic structure of the transcription factor-encoding gene *ANN1*.

(DOCX)

S4 Fig. *V. dahliae* Ann2 is not regulating *ANN3* in the tested conditions.

(DOCX)

S5 Fig. *V. dahliae* Ann2 plays a minor role in microsclerotia formation.

(DOCX)

S6 Fig. The *V. dahliae* Ann1-regulated antibacterial activity has no significant impact on *E. coli* DH5α.

(DOCX)

S7 Fig. Co-cultivation of *V. dahliae* with bacteria does not affect *ANN1* transcript levels.

(DOCX)

S8 Fig. 15 metabolites had altered abundance in the *V. dahliae* *SOM1* mutant strains.

(DOCX)

S9 Fig. 18 fungal metabolites had altered abundance in the *V. dahliae* *ANN1* mutant strains during the co-cultivation with *B. subtilis* 168.

(DOCX)

S10 Fig. Verification of the *V. dahliae* *ANN1* mutant strains.

(DOCX)

S11 Fig. Verification of the *V. dahliae* *ANN2* mutant strains.

(DOCX)

S1 Table. Bacterial and fungal strains used in this study.

(DOCX)

S2 Table. Plasmids used in this study.

(DOCX)

S3 Table. Primers used in this study.

(DOCX)

S4 Table. MS2 spectra of all metabolites described in this study.

(DOCX)

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Author contributions

Conceptualization: Ying-Yu Chen, Rebekka Harting, Gerhard H. Braus.

Data curation: Ying-Yu Chen, Merle Aden, Isabel Maurus.

Formal analysis: Ying-Yu Chen, Lea S. Steglich, Nadja Spasovski, Merle Aden, Isabel Maurus, Rebekka Harting.

Funding acquisition: Gerhard H. Braus.

Investigation: Ying-Yu Chen, Lea S. Steglich, Nadja Spasovski, Marcel H. W. Franzius, Merle Aden, Isabel Maurus, Rebekka Harting.

Methodology: Ying-Yu Chen, Isabel Maurus, Rebekka Harting.

Project administration: Ying-Yu Chen, Rebekka Harting, Gerhard H. Braus.

Resources: Gerhard H. Braus.

Supervision: Rebekka Harting, Gerhard H. Braus.

Validation: Ying-Yu Chen, Lea S. Steglich, Nadja Spasovski, Marcel H. W. Franzius, Merle Aden, Isabel Maurus, Rebekka Harting, Gerhard H. Braus.

Writing – original draft: Ying-Yu Chen.

Writing – review & editing: Ying-Yu Chen.

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