Genomewide Transcriptome Profiles Reveal How *Bacillus subtilis* Lipopeptides Inhibit Microsclerotia Formation in *Verticillium dahliae*

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Verticillium dahliae is a soilborne fungus and the primary causal agent of vascular wilt diseases worldwide. The fungus produces melanized microsclerotia that are crucially important for the survival and spread of *V. dahliae*. There are no fungicides available that are both effective and environmentally friendly to suppress the fungus. Previously, *Bacillus subtilis* C232 was isolated from soil and was demonstrated to suppress microsclerotia formation in *V. dahliae*. In this study, liquid chromatography coupled with mass spectrometry revealed that the antifungal substance is actually a mixture of lipopeptides. Exposure of *V. dahliae* to these lipopeptides resulted in hyphal swelling, cell lysis, and downregulation of melanin-related genes. RNA sequencing analyses of the lipopeptide-suppressed transcriptome during microsclerotial development revealed that 5,974 genes (2,131 upregulated and 3,843 downregulated) were differentially expressed versus nonsuppressive conditions. Furthermore, gene ontology enrichment analyses revealed that genes involved in response to stress, cellular metabolic processes, and translation were significantly enriched. Additionally, the lipopeptides inhibited expression of genes associated with secondary metabolism, protein catabolism, and the high-osmolarity glycerol response signaling pathway. Together, these findings provide evidence for the mechanism by which *B. subtilis* lipopeptides suppress microsclerotia formation. The transcriptomic insight garnered here may facilitate the development of biological agents to combat *Verticillium* wilt.

The phytopathogenic fungus *Verticillium dahliae* causes *Verticillium* wilt disease on over 200 plant species and results in billions of dollars in losses around the world each year (Klosterman et al. 2009). The melanized microsclerotia produced by this fungus play critical roles in its lifecycle, aiding in its long-term survival and spread. Microsclerotia of this organism can survive in the soil without a host for many years

(Wilhelm 1955). Therefore, an effective management strategy for *Verticillium* wilts may entail control of the quantity and viability of microsclerotia in the soil (Bilodeau et al. 2012).

Light and electron microscopy studies have previously shed light on microsclerotia formation in *V. dahliae*. Microsclerotia are composed of thick-walled melanized cells and thin-walled transparent cells (Gordee and Porter 1961; Nadakavukaren 1963). In the initial stages of microsclerotia formation, the hyphae swell and form numerous septa. The septate cells subsequently enlarge, become spherical, and produce lateral buds (Griffiths 1970). At the final stage of microsclerotial maturation, melanin granules are deposited within the cell walls and between the cells. Microsclerotial development is always coupled with melanin biosynthesis in *V. dahliae*, although these two processes can be uncoupled, as is evident in genetic mutants that produce microsclerotia without pigmentation (Bell et al. 1976; Wang et al. 2018).

Because formation of microsclerotia represents a critical stage in the disease cycle of *V. dahliae*, allowing the fungus to survive for years in soil, it remains important to fully elucidate the genetics and signaling pathways that mediate microsclerotia formation. The availability of *V. dahliae* genome sequences has accelerated genetic analyses of this organism, including explorations of genes controlling microsclerotia formation and pathogenicity (Klimes et al. 2015). Dozens of genes associated with microsclerotia formation have been characterized. For example, genes encoding mitogen-activated protein kinase (MAPK) cascades were found to be required for microsclerotia formation, especially genes in the high-osmolarity glycerol response (HOG)-MAPK pathway (Qi et al. 2016; Tian et al. 2014, 2016, 2017; Wang et al. 2016).

Currently, there are no effective fungicides to cure *Verticillium*-infected plants (Klosterman et al. 2009). Fumigation of soil with methyl bromide has been an effective measure to control *Verticillium* wilt for decades; however, this method of pathogen control is no longer acceptable because of the environmental and safety problems associated with methyl bromide usage (Klosterman et al. 2009). A promising, more environmentally friendly strategy to control *Verticillium* wilt is the use of biological control agents (BCAs). Numerous studies have shown that various BCAs can suppress *Verticillium* wilt in different hosts (Berg et al. 2001; Bubici et al. 2013). Members of the *Bacillus* genus produce a variety of antagonistic compounds that suppress microsclerotia formation in *V. dahliae* (Maget-Dana and Peypoux 1994; Moyne et al. 2001; Vanittanakom et al. 1986).

Previously, *Bacillus subtilis* C232 was isolated from soil. The sterile filtrates of its culture suppressed melanin formation of

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the colony on potato dextrose agar (PDA) plates and its volatile affected the mycelium growth, showing strong antifungal activity against *V. dahliae* (Sun et al. 2015). This strain, which shows great potential as a biocontrol agent against *V. dahliae*, has been deposited in the China General Microbiological Culture Collection Center (accession number 9369) (Sun et al. 2015). We also found that this strain strongly suppressed microsclerotia formation. However, whether this suppression was due to BCAs or other chemicals has not been elucidated. To investigate the antifungal compounds produced by C232 in greater detail, we analyzed metabolites isolated from sterile C232 culture filtrates for the ability to suppress microsclerotia formation and its antifungal activity. Liquid chromatography (LC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was used to identify the composition of the antifungal metabolites. The composition was confirmed as lipopeptide mixtures. Next-generation RNA sequencing (RNA-seq) analyses were conducted to explore the mechanisms by which the antifungal lipopeptides suppressed microsclerotia formation. We demonstrated that the antifungal lipopeptides suppress microsclerotia formation by affecting cellular metabolism, translation, and peptide metabolism. Furthermore, data analyses revealed that lipopeptides of C232 caused downregulation of gene expression

affecting these processes and components of MAPK signaling pathways in *V. dahliae*.

RESULTS

B. subtilis C232 inhibits microsclerotia formation.

The sterile culture filtrate and purified lipopeptides of C232 were collected to test their potential as a biocontrol agent. The filtrate inhibited microsclerotia formation (Fig. 1A). To further explore the potential mechanism by which the C232 extract inhibits microsclerotia formation, *V. dahliae* conidia were mixed with the filtrate or water (control) and spread on a cellulose membrane on basal medium (BM) plates, as described by Xiong et al. (2014). Conidia mixed with water could form melanized microsclerotia after spread and incubation on cellulose membrane; however, treatment groups treated with the sterile culture filtrate of strain C232 did not form melanized microsclerotia (Fig. 1B). The purified lipopeptides obtained from C232 inhibited microsclerotia formation but did not affect spore germination (Supplementary Fig. S1). Observations of hyphal morphology and microsclerotia formation suggested that the lipopeptide mixture induced hyphal swelling and lysis. At 4 days post-inoculation (dpi) with the lipopeptide mixture, the hyphae became swollen and did not gather into masses. In contrast, those

Fig. 1. Effect of metabolites produced by *Bacillus subtilis* C232 on colony morphology and microsclerotia formation of *Verticillium dahliae*. **A**, Colony morphology of *V. dahliae* XS11 grown on potato dextrose agar plates with sterile water (CK) and with metabolites produced by *B. subtilis* C232 for 10 days at 25°C. **B**, Suppression of microsclerotia formation by *B. subtilis* C232 metabolites. Microsclerotia formation on basal medium (BM) plates with 1×10^5 conidia mixed with sterile water or the lipopeptide mixture (2 mg/ml) from *B. subtilis* sprayed onto the cellulose membrane for 8, 10, 12, and 14 days. **C**, Microscopy of microsclerotia development in liquid BM incubated with the lipopeptide mixture or mock treatment (sterile water) for 3, 4, 10, and 14 days. Scale bars represent 20 μ m. **D**, Bar chart showing mean microsclerotia (per square millimeter) produced by *V. dahliae* in the sterile water control- or lipopeptide mixture-treated groups at 14 days postinoculation. Data were by counts using ImageJ software. Error bars represent the standard deviations based on three independent replicates. The asterisks indicate a significant difference at $P < 0.01$.

hyphae of the control sample formed masses of microsclerotial initials and developed microsclerotia. At 10 dpi, very few melanized microsclerotia had formed across the entire membrane in the metabolite-treated group, whereas a large mass of melanized microsclerotia had been produced in the water-treated control group (Fig. 1C). At 14 dpi, nearly the entire culture was melanized in the water-treated group, whereas no visible melanization appeared in the lipopeptide-treated group. Further quantification showed that the lipopeptide mixture-treated group produced melanized microsclerotia in significantly reduced numbers (six times less than the water-treated group) (Fig. 1D). These findings demonstrated that the lipopeptide mixture inhibited microsclerotia formation by inducing hyphal lysis and by interfering with cell clustering.

Observations of cultivation status of *V. dahliae* in liquid BM medium at 3, 4, and 8 dpi revealed suppression of melanin deposition in the lipopeptide mixture-treated culture (Fig. 2A). Previously, multiple genes were reported as upregulated in their expression during melanin biosynthesis and microsclerotia formation, including *VDAG_00183* (encoding versicolorin reductase), *VDAG_00190* (encoding polyketide synthase), *VDAG_03393* (encoding scytalone dehydratase), and *VDAG_02273* (encoding a hydrophobin) (Duressa et al. 2013; Xiong et al. 2014). The transcript levels of this gene set were significantly lower in the lipopeptide-treated group than in the control group, as determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Next, we focused on *VDAG_06414*, which encodes a component of a pyruvate dehydrogenase involved in glycolysis. The transcript level of *VDAG_06414* was markedly increased by the treatment with the C232 lipopeptide mixture (Fig. 2B).

Identification of antifungal metabolites.

To identify the components of the antifungal metabolites, we performed LC-MS/MS combined with gene-specific PCR.

The antifungal metabolites were separated and were detected by LC-MS/MS. There were 16 major peaks in the high-performance liquid chromatography chromatogram (Supplementary Fig. S2), and each peak was fragmented by MS/MS. A series of ions from m/z 280 to 2,000 was detected in the MS spectrum. The molecular mass ions from m/z 900 to 1,150 and m/z 1,400 to 1,550 corresponded to antifungal lipopeptides belonging to the iturin, surfactin, and fengycin families. The protonated molecular mass ions $[M+H]^+$ at m/z 1,477.8 were assigned as a fengycin homolog with a C_{17} fatty acid side chain, according to the typical fragments at m/z 1,080 and 966 (Chen et al. 2008). The protonated molecular mass ions $[M+H]^+$ at m/z 1,021.4, 1,169.5, and 1,170.5 were indicative of iturin lipopeptides. Within the retention time of 30 to 49.9 min, various protonated $[M+H]^+$ ions of surfactins with a mass range of m/z 993 to 1,058 were observed in the ESI-MS spectrum (Pandya and Saraf 2015). Three main genes in the genus *Bacillus* contribute to the production of lipopeptides such as iturin, fengycin, and surfactin (Yao et al. 2003). The three genes (*ituC*, *fenB*, and *sfp*) associated with lipopeptide biosynthesis were successfully detected by PCR amplification (data not shown). These results suggested that the antifungal metabolite mixture produced by C232 included multiple lipopeptides, including iturin, surfactin, and fengycin.

Changes in gene transcription associated with suppression of microsclerotia formation by a lipopeptide mixture.

We determined which genes in *V. dahliae* were differentially expressed between the water-treated control and lipopeptide mixture-treated groups via microarray analysis. Eighteen libraries covering three different key time points of microsclerotial development in samples treated with the lipopeptide mix or sterile water (control) were produced by RNA-seq. After quality filtering, clean reads from the control and treated *V. dahliae* libraries were mapped to the *V. dahliae* VdLs.17

Fig. 2. Melanin production of *Verticillium dahliae* affected by a lipopeptide mixture derived from *Bacillus subtilis*. **A**, Cultures of *V. dahliae* XS11 were grown in liquid basal medium (BM) with and without the lipopeptide mixture at 3, 4, and 8 days postinoculation (dpi). **B**, Quantitative analysis of five genes (*VDAG_00183*, *VDAG_00190*, *VDAG_02273*, *VDAG_03393*, and *VDAG_06414*) associated with melanin biosynthesis and metabolism during microsclerotia formation from lipopeptide-treated or mock-treated (sterile water) at 5 dpi. RNAs were extracted from the mycelia treated by lipopeptide in liquid BM for 7 days at 25°C. Error bars represent standard deviation of three replicates. There were three biological experiments performed and three technical replicates per biological experiment.

genome, and the fragments per kilobase per transcript per million mapped reads (FPKM value) was calculated for each gene. We obtained a total of 1,116,305,696 reads from the 18 libraries, with an average of 62,016,983 reads per sample, ranging from 60,996,412 to 62,699,098 reads in each sample (Table 1). In total, 92.24% of the total reads from all 18 samples were aligned to the *V. dahliae* genome with <2-bp mismatches. Mapping analyses revealed that 9,852 predicted genes were expressed.

An average of nearly 12.27 and 11.32% of the expressed genes showed low expression values ($0 < \text{FPKM} \leq 1$) in control samples at 3 and 4 dpi, respectively, while nearly 13.84 and 13.73% of the expressed genes showed low expression values in lipopeptide mixture-treated samples at 3 and 4 dpi, respectively. There were more genes with relatively low expression values ($1 < \text{FPKM} \leq 10$) in the lipopeptide-treated samples at 3 and 4 dpi than in control samples. In addition, there were more expressed genes with high expression values ($\text{FPKM} > 10$) in control samples than in lipopeptide-treated samples, suggesting that the lipopeptide mixture downregulated gene expression (all primers used to amplify genes associated with lipopeptide biosynthesis are listed in Supplementary Table S1). To examine the correlations between gene expression patterns among the 18 libraries, Pearson's correlation coefficients of paired samples were calculated using the FPKM values of all expressed genes (Supplementary Fig. S3). All of the Pearson's correlation coefficients were >0.8 . These results illustrated the consistency of the transcriptional changes within each library.

The hierarchical clustering heatmap of 18 arrays (Fig. 3A) and principal component analysis (PCA) of 18 arrays were used (Fig. 3B) to compare samples and to explore the dynamic changes in the *V. dahliae* transcriptome after treatment with the lipopeptide mixture. In addition, the clustering affinity search technique (CAST) and screening of differentially expressed genes were used to generate gene clusters (Wang et al. 2010). The CAST analysis of 2,780 significantly expressed genes revealed 20 clusters containing 36 to 785 genes. Distinct gene patterns of several clusters of genes involving signaling pathway, central metabolism, transcription factor, and biosynthesis of secondary metabolites were produced, showing a decreased expression trend after treatment with the lipopeptide mixture (Fig. 3C to F). We randomly selected dozens of genes and

analyzed their transcript levels by RT-qPCR to verify their expression patterns. Comparisons of the FPKM and RT-qPCR data revealed the expected correlations and consistency between the two datasets.

Detailed analyses of gene expression patterns in response to multiple lipopeptides.

To elucidate the dynamic changes in gene expression during the suppression of microsclerotia formation by the lipopeptide mixture, we identified the genes that were differentially expressed among libraries (false discovery rate [FDR] < 0.05). Comparisons of gene expression between control and lipopeptide mixture-treated samples showed that 2,450 (963 upregulated and 1,487 downregulated), 2,112 (464 upregulated and 1,648 downregulated), and 1,412 (704 upregulated and 708 downregulated) genes were significantly differentially expressed at 3, 4, and 8 dpi, respectively (FDR < 0.05) (Fig. 4A and B). Venn diagrams illustrating 291 genes that were differentially expressed at 3, 4, and 8 dpi and profiles of these genes are shown in Figure 4C. Pairwise comparisons showed that the largest number of genes where change expression was evident had occurred at 3 dpi, and included a statistically significant reduction in expression. The number of differentially expressed genes varied during microsclerotia formation, affected by the lipopeptide mixture compared with water. These analyses revealed a large set of novel genes that may play roles in the suppression of microsclerotia formation by lipopeptides.

Gene ontology (GO) analysis of the genes differentially expressed between the control and lipopeptide mixture-treated samples at 3, 4, and 8 dpi revealed significant overrepresentation of genes of certain categories. Differentially expressed genes in CK_3d versus ZT_3d mainly included those in biological processes (ribosome biogenesis, ribonucleoprotein complex biogenesis, ribosomal RNA [rRNA] processing, rRNA metabolic process, translation, peptide biosynthetic process, and amide biosynthetic process) and molecular function (structural constituent of ribosome, structural molecule activity, and RNA binding). In CK_4d versus ZT_4d and CK_8d versus ZT_8d groups, differentially expressed genes mainly included those involved in biological processes (peptide metabolic process and carbohydrate metabolic process) and molecular function

Table 1. RNA sequencing statistics

Sample	Total raw reads ^a	Total clean reads (%) ^b	Clean reads Q30 (%) ^c	Total mapping ratio (%) ^d	Uniquely mapped ratio (%) ^e
Vd_3d_1	67,580,260	62,302,242 (93.62)	92.79	92.19	84.22
Vd_3d_2	67,178,027	61,965,012 (93.59)	92.92	92.24	83.93
Vd_3d_3	66,782,271	61,513,150 (93.12)	92.96	92.11	83.64
Vd_4d_1	67,317,967	61,919,066 (93.31)	92.93	91.98	84.14
Vd_4d_2	68,077,197	62,699,098 (94.38)	92.99	92.10	84.84
Vd_4d_3	67,745,143	62,359,404 (93.8)	93.11	92.05	84.62
Vd_8d_1	66,859,020	61,664,074 (92.36)	92.62	92.23	84.01
Vd_8d_2	67,612,546	62,332,006 (92.85)	92.75	92.19	84.22
Vd_8d_3	66,099,276	60,996,412 (94.27)	92.91	92.28	84.65
ZT_3d_1	67,330,337	62,294,028 (93.73)	93.01	92.52	83.99
ZT_3d_2	67,325,645	62,471,466 (93.79)	92.93	92.79	84.23
ZT_3d_3	67,458,335	62,628,318 (94.08)	92.85	92.84	84.29
ZT_4d_1	67,339,520	62,006,230 (93.43)	92.83	92.08	84.02
ZT_4d_2	66,798,556	61,421,272 (92.43)	92.8	91.95	83.87
ZT_4d_3	67,113,831	61,878,952 (93.28)	92.81	92.20	84.1
ZT_8d_1	66,748,228	61,635,314 (92.84)	92.88	92.34	84.13
ZT_8d_2	67,434,819	62,060,264 (93.23)	92.66	92.03	84.06
ZT_8d_3	67,396,062	62,159,388 (93.69)	93.06	92.23	84.26

^a Number of reads generated from the BGISEQ-500.

^b Raw data with low-quality reads, reads containing 3' adaptors, and reads containing two or more N bases removed.

^c Percentage of bases with a mass value > 30 .

^d Number of reads mapped to the reference genome within 2-bp mismatch.

^e Uniquely mapped = the number of reads mapped to the reference genome with unique sequence location.

(oxidoreductase activity, structural constituent of ribosome, and catalytic activity) (Table 2; Supplementary Fig. S4).

Enrichment pathways analyzed by gene coexpression network.

A gene coexpression network with Weighted Correlation Network Analysis (WGCNA) software was employed to determine which gene or genes may play a pivotal role in microsclerotia formation in *V. dahliae* following treatment with the lipopeptide mixture and the water control. According to the WGCNA results, the heatmap of whole genome-wide gene expression profiles revealed that 19 modules were clustered (Supplementary Fig. S5). The top 100 major genes from each module were analyzed with GO enrichment and KEGG pathway enrichment for identification of putative crucial signaling pathways influencing *V. dahliae* microsclerotia formation. Gene networks are constructed from functional gene associations. Finally, eight specially enriched pathways that may be crucial for microsclerotia formation were obtained from five modules, including those of ATP-binding cassette (ABC) transporters, MAPK signaling, citric acid cycle (TCA cycle), glycolysis/gluconeogenesis, nitrogen metabolism, endocytosis, fatty acid degradation, and RNA degradation pathways (Table 3; Supplementary Fig. S6).

Suppression of microsclerotia formation by the lipopeptide mixture is dependent on MAPK signaling.

At least two MAPK signaling pathways function in pathogenicity and microsclerotia formation, and in the regulation of cell-wall-degrading enzymes and response to stress, respectively (Inderbitzin et al. 2014). In the current study, the expression of several receptor-encoding genes was affected at 3, 4, and 8 dpi by the lipopeptide mixture. In the Slr2 signaling pathway, pivotal upstream genes *VDAG_02011*, *VDAG_09823*, *VDAG_05969* encoding Rho1, Mkk1, and an MAPK were significantly downregulated in ZT_3d compared with Vd_3d. In addition, *VDAG_08800* (carbohydrate metabolism), *VDAG_04069* (serine/threonine-protein kinase), and *VDAG_08213* (hypothetical protein) were downregulated. In the *V. dahliae* HOG signaling pathway (Wang et al. 2016), significantly downregulated upstream genes included those encoding VdPbs2 (MAPK kinase [MAPKK]; *VDAG_02783*), and an Ssk2 ortholog (*VDAG_08787*). In addition, those genes encoding components of the HOG signaling pathway orthologs such as *VdMsb2* (encoding a transmembrane protein, *VDAG_00424*) were significantly downregulated in the lipopeptide mix-treated sample, whereas *VdMsn2* (encoding a transcription factor protein *VDAG_04227*), which previously was demonstrated to be a negative regulator of microsclerotia formation (Tian et al. 2017), was significantly upregulated (Fig. 5).

Fig. 3. Analyses of the global gene expression pattern in *Verticillium dahliae* during microsclerotial development following treatment with a lipopeptide mixture from *Bacillus subtilis* C232 or mock treated with sterile water. There were three biological replicates of each stage that clustered together. Shades correspond to the $\log_2$ of fragments per kilobase per transcript per million mapped reads (FPKM) values. **B**, Principal component analysis (PCA) of the 18 samples included in this study. For the principal components 1 and 2, eigenvalues are 81.71% and 7.24%, respectively. **C** to **F**, Expression patterns of genes in four functional categories. The central metabolic pathways include **C**, nitrogen metabolism, fatty acid degradation, and glycolysis/gluconeogenesis; **D**, signal transduction and components such as mitogen-activated protein kinase (MAPK) cascades, regulators of G protein signaling (RGSs), G protein coupled receptors (GPCR), and calcium signaling; **E**, four main classes of transcription factors (i.e., C₂H₂, bHLH, bZIP, and Zn₂Cys₆); and **F**, secondary metabolism and ATP-binding cassette (ABC) transporter genes. Y-axes are $\log_2$ -transformed total intensities. Total numbers within a gene family are shown in numerals and the numbers of expressed genes within a family are shown in parentheses.

Involvement of genes regulating ABC transporter compounds, secondary metabolism, and protein catabolism in the response to the *B. subtilis* lipopeptide mixture.

Recent searches of the Joint Genome Institute (JGI) database revealed 701 genes encoding putative transporters in *V. dahliae*,

and 666 of these were detected in this study. In summary, 158 genes upregulated at three time points and 210 genes were downregulated (Fig. 6A and B). Among 3 upregulated and 13 downregulated genes differentially expressed at 3, 4, and 8 dpi, the genes *VDAG_07681* and *VDAG_01167* were significantly up- and downregulated, respectively. The genes *VDAG_08026*

Fig. 4. Differential gene expression analyses in *Verticillium dahliae* in response to a lipopeptide mixture from *Bacillus subtilis*. **A**, Venn diagram of differentially expressed genes in strain XS11 of *V. dahliae* in response to treatment with a lipopeptide mixture from *B. subtilis* or mock water control. Circles with different colors represent each pairwise comparison. The sum of numbers in each circle represents the total number of differentially expressed genes between the two samples; overlapping parts of the circle represent commonly expressed genes in response to the test treatment or control. The ratio of genes in each pairwise comparison was in parentheses. **B**, Number of significantly upregulated genes (red) or downregulated genes (green) ($P < 0.05$, false discover rate < 0.05). **C**, Hierarchical clustering of expression levels of differentially expressed genes among comparisons (CK_3d versus ZT_3d, CK_4d versus ZT_4d, and CK_8d versus ZT_8d). CK_3d, CK_4d, CK_8d, ZT_3d, ZT_4d, and ZT_8d correspond to control samples and lipopeptide mix-treated samples at 3, 4, and 8 days postinoculation, respectively.

(permease) and *VDAG_02093* (ABC transporter) were down-regulated in the lipopeptide-treated sample compared with the water control treatment. In response to the lipopeptide mixture, 23 genes of 45 ABC transporter-encoding genes mapping to the

Verticillium genome were differentially expressed. (Fig. 6C). In this study, secondary metabolism gene clusters were selected according to previous studies (Duressa et al. 2013; Wang et al. 2018; Xiong et al. 2016). The expression patterns of the genes

Table 2. Enrichment terms of significantly regulated genes treated by antifungal lipopeptide versus wild type (corrected *P* value < 0.05)

Functional categories	Terms	Differentially expressed gene number	Corrected <i>P</i> value
Molecular function			
Vd_3d vs ZT_3d			
GO:0003735	Structural constituent of ribosome	66	7.74E-13
GO:0005198	Structural molecule activity	74	1.37E-11
GO:0003723	RNA binding	94	0.02101
Vd_4d vs ZT_4d			
GO:0016491	Oxidoreductase activity	209	1.91E-05
GO:0003735	Structural constituent of ribosome	46	0.00016
GO:0005198	Structural molecule activity	50	0.00398
GO:0003824	Catalytic activity	715	0.01224
Vd_8d vs ZT_8d			
GO:0016491	Oxidoreductase activity	168	6.98E-14
GO:0003824	Catalytic activity	470	6.11E-06
GO:0020037	Heme binding	34	4.19E-05
GO:0046906	Tetrapyrrole binding	34	4.19E-05
GO:0005506	Iron ion binding	34	5.34E-05
GO:0004497	Monooxygenase activity	31	0.00028
GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	30	0.01924
Biological process			
Vd_3d vs ZT_3d			
GO:0042254	Ribosome biogenesis	63	1.82E-24
GO:0022613	Ribonucleoprotein complex biogenesis	74	1.22E-22
GO:0006364	rRNA processing	36	9.53E-14
GO:0016072	rRNA metabolic process	36	1.57E-12
GO:0006412	Translation	89	3.93E-11
GO:0043043	Peptide biosynthetic process	89	8.93E-11
GO:0043604	Amide biosynthetic process	92	4.53E-10
GO:0006518	Peptide metabolic process	92	9.49E-10
GO:0044085	Cellular component biogenesis	91	1.95E-09
GO:0043603	Cellular amide metabolic process	97	2.54E-08
GO:0034470	ncRNA processing	44	3.51E-07
GO:0044267	Cellular protein metabolic process	137	6.66E-05
GO:0019538	Protein metabolic process	140	0.00017
GO:0006417	Regulation of translation	20	0.00025
GO:0010608	Posttranscriptional regulation of gene expression	20	0.00025
GO:0034248	Regulation of cellular amide metabolic process	20	0.00025
GO:0042273	Ribosomal large subunit biogenesis	14	0.00047
GO:0002181	Cytoplasmic translation	16	0.00348
GO:0010467	Gene expression	196	0.00461
GO:0006413	Translational initiation	14	0.00495
GO:0034660	ncRNA metabolic process	52	0.00837
GO:0002183	Cytoplasmic translational initiation	13	0.0156
GO:0042274	Ribosomal small subunit biogenesis	10	0.01931
GO:0071826	Ribonucleoprotein complex subunit organization	22	0.02747
GO:0044260	Cellular macromolecule metabolic process	291	0.03031
Vd_4d vs ZT_4d			
GO:0006518	Peptide metabolic process	60	0.03201
Vd_8d vs ZT_8d			
GO:0005975	Carbohydrate metabolic process	60	5.92E-05

Table 3. Part of the enrichment KEGG pathways of five coexpression modules

Pathway ^a	Module genes	All genes	<i>P</i> value	Q value
ABC transporters	4	43	0.0007	0.03606
MAPK signaling pathway; yeast	20	1,331	0.0217	0.55359
Citrate cycle (TCA cycle)	3	37	0.0029	0.04268
Glycolysis/gluconeogenesis	6	99	0.0025	0.04392
Nitrogen metabolism	3	33	0.0136	0.36341
Endocytosis	16	617	0.0252	0.36341
Fatty acid degradation	4	81	0.0035	0.04268
RNA degradation	3	130	0.0344	0.53365

^a MAPK = mitogen-activated protein kinase.

involved in secondary metabolism showed a decreased expression in response to the lipopeptide mixture (Fig. 7A). The expression of nonribosomal protein synthase (NRPS)- and polyketide synthase (PKS)-encoding genes were decreased at 3 and 4 dpi, while the hybrid NRPS-PKS-encoding gene expression was decreased at 4 dpi but increased at 8 dpi. Melanin is major secondary metabolite of *V. dahliae* that continuously accumulates during microsclerotia development. Therefore, the expression profile of genes encoding proteins involved in the dihydroxynaphthalene (DHN)-melanin biosynthetic pathway were analyzed (Fig. 7B). *VDAG_00184* (encoding a PKS), *VDAG_00189* (laccase, *VdLac1*), *VDAG_00190* (polyketide synthase, PKS), *VDAG_00192* (putative transcription factor), *VDAG_00195* (transcription factor, *Cmr1*), *VDAG_03393*, and *VDAG_03665* were downregulated (Supplementary Fig. S7). In addition, the cluster of 12 genes spanning *VDAG_07920* to *VDAG_07931*, including lovastatin nonaketide synthase-encoding gene *VDAG_07928*, were significantly downregulated in response to the lipopeptide mixture at 4 dpi (Supplementary Fig. S8). These results showed that the lipopeptide mixture suppressed the expression of genes involved in melanogenesis and other secondary metabolic processes.

Previous studies have suggested that ubiquitin-dependent protein catabolism is involved in microsclerotia formation in *V. dahliae* (Xiong et al. 2014). To determine whether genes involved in ubiquitination and protein degradation were affected by the lipopeptide mixture, we analyzed the expression levels of genes associated with protein catabolism, including *VDAG_02479*, *VDAG_03748*, *VDAG_06944*, and *VDAG_08391*. These four genes were downregulated by the lipopeptide mixture. Genes involved in protein degradation were also significantly downregulated by the lipopeptide mixture. These findings revealed that the antifungal lipopeptides may function through suppressing protein degradation in *V. dahliae*.

DISCUSSION

Bacillus spp. have long been studied and applied for biological control of plant pathogens, due to their increased ability to produce antimicrobial secondary metabolites such as iturins and fengycins (Ongena and Jacques 2008). Several

studies have demonstrated the inhibitory activity of these metabolites against various pathogens (Ongena and Jacques 2008). Previous research showed that antimicrobial lipopeptides can damage biological membranes, causing malformation and cell leakage (Raaijmakers et al. 2010). In this study, we determined that the lipopeptide mixture produced by *B. subtilis* C232 contains more than four kinds of lipopeptides, including iturin C, iturin A, fengycins, and surfactins, and suppressed microsclerotia formation and melanin deposition of *V. dahliae*.

At present, chemical fumigation of the soil and crop rotation are key measures to manage Verticillium wilt. However, the environmental problems associated with methyl bromide and the broad host range of the pathogen have prompted researchers to explore alternative, environmentally friendly approaches to control the disease (Klosterman et al. 2009). Biological control of *V. dahliae* has developed as a promising field. Microsclerotia are desirable targets for *Verticillium* spp. control because they represent primary inoculum and are persistent for years in fields (Tjamos 2000).

Herein, we showed that the multiple lipopeptides produced by C232 inhibited microsclerotia formation of *V. dahliae*, and explored the mechanisms of this inhibition by conducting RNA-seq. In biological processes, macromolecular networks can be constructed by algorithmic predictions based on gene function correlation and expression profiles. Because of the flexibility of network models based on algorithmic predictions that derive from high-throughput gene expression tests, snapshots of protein-protein interactions may be examined, and gene expression regulatory networks and metabolic networks among different groups examined.

The MAPK signaling pathways are involved in integrating multiple extracellular and intracellular signals to regulate transcription of specific genes that help the cell adapt to the conditions in eukaryotic cells (Gustin et al. 1998). Previous works have revealed that HOG kinase signaling is required for hyphal swelling, microsclerotia formation, and the response to fungicides (Tian et al. 2016; Wang et al. 2016). In response to the lipopeptide mixture in this study, the transcript levels of *VdPbs2* (encoding MAPKK; *VDAG_02783*), *Ste7* (*VDAG_08626*), and *Mkk1* (*VDAG_09823*) were significantly decreased in *V. dahliae*. In previous studies, the *VdPbs2* deletion

Fig. 5. Analysis of genes encoding mitogen-activated protein kinase (MAPK) signaling pathway components in *Verticillium dahliae* following treatment with lipopeptide mixture. Schematic diagram of three MAPK signaling pathways conserved in both *V. dahliae* and *Saccharomyces cerevisiae*. Rectangular objects with gene IDs of the *V. dahliae*-annotated genome (Klosterman et al. 2011) were identified by BLASTp with *S. cerevisiae* proteins. The heat map from left to right shows gene expression profiles of each gene at 3, 4, and 8 days postinoculation of control and lipopeptide-treated groups.

mutant exhibited delayed microsclerotia formation and reduced virulence on smoke tree and tobacco seedlings. Furthermore, deletion of *VdPbs2* also increased sensitivity to osmotic agents while increasing resistance to some fungicides and compounds that interfered with cell wall synthesis (Tian et al. 2016). Additionally, we observed that *VDHI* (a class II hydrophobin) was significantly differentially expressed during microsclerotia formation and was clearly downregulated in the $\Delta VdHog1$ strain (Klimes and Dobinson 2006). Consistent with our previous findings, the expression of *VDHI* was significantly downregulated by the lipopeptides of C232. *VdMsn2* was discovered as a negative regulator of microsclerotia formation and its loss leads to reduced hyphal growth, increased septation, and increased microsclerotia numbers relative to the wild-type strain (Tian et al. 2017). *VdMcm1* acts as a positive regulator in microsclerotia formation (Xiong et al. 2016). Analyses of genes transcription factor-encoding genes in this study indicated decreased expression of *VdMcm1* and increased expression of *VdMsn2* relative to the wild type strain. Considering these lines of evidence, in response to lipopeptide treatment, *V. dahliae* upregulates MAPK signaling pathway gene expression, resulting in suppressed microsclerotia formation and abnormal mycelia.

Secondary metabolites play important roles in virulence in fungi. Fungal secondary metabolites include polyketides, terpenes, and indole alkaloids, and the genes responsible for their

biosynthesis are often arranged in clusters (Fox and Howlett 2008). A previous transcriptomic analysis showed that the secondary metabolism gene cluster spanning *VDAG_07920* to *VDAG_07928* was markedly upregulated during microsclerotia formation in *V. dahliae* (Xiong et al. 2016). In response to the lipopeptide mixture, most of the genes in this cluster were downregulated. Among the secondary metabolites, DHN-melanin accumulates during microsclerotia development (Bell et al. 1976; Xiong et al. 2014) and is critically important for the survival of *V. dahliae* (Wang et al. 2018). The increased expression level of a pyruvate dehydrogenase component-encoding gene (*VDAG_06414*) also indicated that perturbed metabolism suppressed microsclerotia formation through complex metabolic changes involving this dehydrogenase. Herein, after treatment with lipopeptides, the fungi showed an obvious defect in melanin accumulation. Similarly, other putative secondary metabolism gene clusters in *V. dahliae*, including those that encode PKS and NRPS genes and one PKS-NRPS hybrid gene cluster, were overall downregulated in response to the lipopeptide mixture. Protein ubiquitination is known to play an important role in microsclerotia formation (Xiong et al. 2014). Recent studies have shown that leucine has potential antiproteolytic effects through the downstream ubiquitin-proteasome system (Zanchi et al. 2008). We also noted a downregulation of genes involved in ubiquitination and, thus, may influence protein metabolism through this route (Supplementary Fig. S9).

Fig. 6. Differential expression of genes encoding transporters in *Verticillium dahliae* in response to a lipopeptide mixture obtained from *Bacillus subtilis*. Venn diagram of significantly (false discovery rate [FDR] < 0.05) **A**, upregulated or **B**, downregulated genes associated with microsclerotial development in *V. dahliae* in response to a lipopeptide mixture from *B. subtilis*, as compared with the mock-treated control (sterile water) group at 3, 4, and 8 days post-inoculation. **C**, Expression profiles of differentially expressed genes encoding components of ATP-binding cassette (ABC) transporters. Genes marked with one asterisk represent upregulated genes in all three time points, and genes marked with two asterisks represents downregulated genes in all the three time points (FDR < 0.05).

In this study, we determined the composition of the anti-fungal metabolites of C232 and identified differentially expressed genes and clusters of genes in *V. dahliae* treated with antifungal metabolites. Among lipopeptides, surfactins from *Bacillus* spp. not only have an antibacterial activity but also can inhibit fungal growth and result in mycelial malformation; fengycin shows specific antifungal activity against filamentous fungi and inhibits phospholipase A₂ (Umezawa et al. 1986). Han et al. (2015) reported that iturins produced by *Bacillus* spp. can affect spore germination, induce *V. dahliae* chitinase and glucanase secretion, and affect cellular morphology. In

previous studies reported by DeFilippi et al. (2018), the findings demonstrated that spore germination and mycelial growth of *V. dahliae* were significantly suppressed after cocultured with a *B. subtilis* strain whereas, in our study, a mixture of lipopeptides secreted by *B. subtilis* C232 showed no efficient effect on spore germination. The main role of the lipopeptide mixture was on the development process of fungal mycelial. A lipopeptide mixture was used to explore their inhibitory effects on the microsclerotia formation of *V. dahliae*. As can be expected, the lipopeptide mixture used here would have multiple action sites and complex antagonistic mechanisms of

Fig. 7. Expression patterns of gene associated with secondary metabolism in *Verticillium dahliae* in response to a lipopeptide mixture obtained from *Bacillus subtilis*. **A**, Distinct expression patterns of three gene families involved in fungal secondary metabolism: polyketide synthase (PKS), nonribosomal protein synthase (NRPS), and hybrid PKS. **B**, Heatmap showing transcript abundance of a secondary metabolism gene cluster and other genes that contribute to melanin biosynthesis in *V. dahliae*. Values were calculated by log₂ ratio of transcript abundance. CK_3d, CK_4d, CK_8d, ZT_3d, ZT_4d, and ZT_8d correspond to control samples and lipopeptide mixture-treated samples at 3, 4, and 8 days postinoculation, respectively. **C**, Schematic diagram depicting key proteins involving the dihydroxynaphthalene (DHN)-melanogenesis pathway in *V. dahliae*. DHN-melanin biosynthesis begins with a PKS-catalyzed step that catalyzes the conversion of acetyl-CoA or malonyl-CoA precursors generated from the glycolysis/gluconeogenesis pathway through a series of reduction and oxidation reactions to culminate in the product 1,8-DHN.

action that affect fungal growth and still need to be explored in more detail.

Importantly, the results presented herein increase our knowledge of how lipopeptides from *B. subtilis* C232 suppress microsclerotia formation in *V. dahliae*. It will be of interest to determine the specific effects of each antifungal metabolite on microsclerotia formation and on the malformation of fungal mycelia, and potential practical applications as biocontrol agents or target-specific pesticides.

MATERIALS AND METHODS

Fungal strain and growth conditions.

V. dahliae XS11 (nondefoliating pathotype, race 2) was isolated from a smoke tree in Fragrant Hills, Beijing, China (Xiong et al. 2014). A conidial suspension of strain XS11 was stored long term in glycerol (30% [vol/vol]) at -80°C . The strain was cultured on PDA plates at 25°C in continuous darkness. XS11 was grown on PDA plates to observe mycelial growth. To harvest conidia, XS11 was inoculated into liquid complete medium and incubated at 25°C for 3 days. The conidia were collected and washed with distilled sterile water. Conidia of XS11 were spread and grown on BM (1 liter; 10 g of glucose, 0.2 g of sodium nitrate, 0.52 g of KCl, 0.52 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.52 g of KH_2PO_4 , 3 μM thiamine HCl, 0.1 M biotin, and 15 g of agar) covered with cellulose membrane for observations of microsclerotia (Xiong et al. 2014). Conidia were cultured in liquid BM medium for sampling to observe microsclerotial development process under an optical microscope. Experiments were performed in triplicate.

Isolation and identification of antifungal metabolites.

The antifungal metabolites were isolated as follows. *Bacillus* sp. strain C232 was cultured in liquid modified number 3 medium at 30°C for 4 days (Huang et al. 2015). The culture was centrifuged at $10,000 \times g$ for 30 min at 4°C and the supernatant was collected, the pH adjusted to 2, and kept at 4°C overnight. The crude extract was obtained from the culture broth by centrifugation ($10,000 \times g$ for 30 min at 4°C), rinsed twice in sterile water, and resuspended in sterile water at a pH of approximately 7. The extract was vacuum dried and the residue was collected. The collected residues were extracted in methanol with stirring in a shaking incubator for 1 h. Methanol extraction was volatilized in a fume hood, and solid residue was resuspended in sterile water. This suspension was transferred to an ultrafiltration tube (3 kDa) and centrifuged, and the filtrate containing the lipopeptide mixture was collected.

The antifungal metabolites were identified by LC-MS/MS. An UltiMate 3000 (Dionex) system was used to separate the antifungal metabolites on an Acclaim PePmap 100- C_{18} column (Thermo Scientific) (75 μm by 2 cm by 3 cm; 100 $\text{\AA}$). Briefly, the methanol-soluble antifungal extract was eluted in acetonitrile-water-methanoic acid (1:1:0.001 [vol/vol]) with a 65-min linear gradient at 0.4 $\mu\text{L}/\text{min}$. The separated metabolites were subjected to MS analysis using a Q Exactive instrument (Thermo Scientific). The mass spectrum of the eluent was recorded over a mass range of 280 to 2,000 m/z in the positive ion mode. Tandem experiments of primary MS and secondary MS were performed in auto-ms/ms mode.

Nucleic acid extraction and quantitative real-time PCR.

DNA was extracted from C232 using a TIANamp Bacteria DNA Kit (Tiangen Biotech) to PCR amplify genes associated with lipopeptide biosynthesis. Six genes related to antimicrobial lipopeptide biosynthesis included *ituC* encoding iturin A synthetase C, *ituD* encoding an enzyme involved in iturinA production, *bamC* encoding the bacillomycin D synthetase

biosynthetic operon, *fenB* encoding an enzyme involved in fengycin production, *urfA* encoding surfactin synthetase subunit 1, and *sfp* encoding a protein related to surfactin production (Ramarathnam et al. 2007). PCR amplification and detection were performed in three biological replicates.

Total RNA was extracted from strain XS11 of *V. dahliae* that was cultured in liquid BM using Trizol reagent (Invitrogen). RNA purification was performed with the PureLink RNA mini kit (Ambion) according to the manufacturer's instructions. All samples were ground into a fine powder in liquid nitrogen with a mortar and pestle. Total RNA was eluted in RNase-free water and stored at -80°C until further use.

The RT-qPCR analyses were performed on a 7500 real-time PCR system (Applied Biosystems) using SuperReal PreMix Plus SYBR Green (Tiangen). Each PCR mixture (30 μL) contained 15 μL of 2 $\times$ SuperReal PreMix Plus, 0.25 μL of each primer, and appropriate cDNA. The *V. dahliae* β -*tubulin* gene was used as the internal reference for all RT-qPCR analyses. Three biological replicates were used to analyze the expression of each gene. Relative gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

RNA-seq and read mapping.

Fungal samples used for transcriptome profile analyses were collected from cultures grown in liquid BM or liquid BM containing multiple lipopeptides at 2 mg/ml for 3, 4, and 8 dpi at 25°C . The purity, concentration, and integrity of RNA were checked using a NanoPhotometer spectrophotometer (Implen) and an RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies). A BGISEQ-500 platform (Beijing Genomics Institute) was used to sequence three biological replicates under each condition, yielding over 60 million reads per sample.

Sequence data filtering was performed using SOAPnuke software (Beijing Genomics Institute). Raw reads were first processed using in-house Perl scripts. In this step, clean reads were obtained by removing reads containing adapter and poly-N sequences and low-quality reads from the raw data. At the same time, the Q20, Q30, and GC contents of the data were calculated. All further analyses were based on high-quality data. To estimate the abundance of each transcript, the sequenced genome was compared with the reference genome from the Broad Institute (Klosterman et al. 2011) using Hierarchical Indexing for Spliced Alignment of Transcripts software (Beijing Genomics Institute). The filtered paired-end reads were aligned to the reference genome (Klosterman et al. 2011) using Burrows-Wheeler transform (Li and Durbin 2009) to count the read numbers mapped to each gene. The FPKM values were calculated and used to estimate the effects of sequencing depth and gene length on the mapped read counts.

Differential gene expression analysis and GO enrichment.

For construction and topological analysis of the gene coexpression network, gene expression similarities and clustering modules of sequenced libraries were analyzed using WGCNA software (Langfelder and Horvath 2008). The assembled transcripts in the final transcriptome were annotated by mapping them to several public databases. The differentially expressed genes from the antifungal metabolite-treated sample and control sample were categorized in terms of biological process, molecular function, and cellular component categories in a GO enrichment analysis, using a Goseq R package (Young et al. 2010), which corrected for gene length bias. GO terms with corrected P values < 0.05 were considered to be significantly enriched within the differentially expressed gene sets. Differential expression between two conditions was detected using the DEGSeq R package (1.20.0) (Wang et al. 2010). The CAST

and screening of differentially expressed genes were used to generate gene clusters. The DEGseq package provides statistical routines for determining differential expression in digital gene expression data using a model based on negative binomial distribution (Wang et al. 2010). Six libraries (CK_3d, CK_4d, CK_8d, ZT_3d, ZT_4d, and ZT_8d) corresponding to control samples (treated with sterile water) and antifungal metabolite-treated samples at 3, 4, and 8 dpi, respectively, were used to detect differentially expressed genes. We calculated the number of upregulated and downregulated genes in each GO category. The *P* values were adjusted using the Benjamini and Hochberg method (FDR < 0.05) as the threshold for significant differential expression (Benjamini and Yekutieli 2001). MAPK pathway-related genes were identified during homology search against the *V. dahliae* genome database (JGI) using BLASTp program.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

VdLs.17 genome: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA28529>