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Population genomics demystifies the defoliation phenotype in the plant pathogen *Verticillium dahliae*

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Summary

- *Verticillium dahliae* is a broad host-range pathogen that causes vascular wilts in plants. Interactions between three hosts and specific *V. dahliae* genotypes result in severe defoliation. The underlying mechanisms of defoliation are unresolved.
- Genome resequencing, gene deletion and complementation, gene expression analysis, sequence divergence, defoliating phenotype identification, virulence analysis, and quantification of *V. dahliae* secondary metabolites were performed.
- Population genomics previously revealed that G-LSR2 was horizontally transferred from the fungus *Fusarium oxysporum* f. sp. *vasinfectum* to *V. dahliae* and is exclusively found in the genomes of defoliating (D) strains. Deletion of seven genes within G-LSR2, designated as VdDf7 genes, produced the nondefoliation phenotype on cotton, olive, and okra but complementation of two genes restored the defoliation phenotype. Genes VdDf5 and VdDf6 associated with defoliation shared homology with polyketide synthases involved in secondary metabolism, whereas VdDf7 shared homology with proteins involved in the biosynthesis of N-lauroylethanolamine (N-acylethanolamine (NAE) 12:0), a compound that induces defoliation. NAE overbiosynthesis by D strains also appears to disrupt NAE metabolism in cotton by inducing overexpression of fatty acid amidase hydroxylase.
- The VdDfs modulate the synthesis and overproduction of secondary metabolites, such as NAE 12:0, that cause defoliation either by altering abscisic acid sensitivity, hormone disruption, or sensitivity to the pathogen.

Introduction

*Verticillium dahliae* is a widely distributed soilborne pathogenic fungus that invades xylem vessels of susceptible plants, causing an intractable vascular wilt disease (Fradin & Thomma, 2006; Klosterman et al., 2009). Over 200 plant species, including many economically important agricultural crops, are infected by *V. dahliae* (Fradin & Thomma, 2006; Klosterman et al., 2009; Inderbitzin & Subbarao, 2014). *V. dahliae* produces numerous microsclerotia in infected plant tissues, which are released into the soil with the decomposition of crop residue and can survive in the soil for at least 15 yr (Fradin & Thomma, 2006; Inderbitzin & Subbarao, 2014). The melanized resting structures produced by the fungus germinate in the presence of root exudates, and the emerging hyphae penetrate roots through the tip or through sites on lateral roots (Bishop & Cooper, 1983). After crossing the root cortex, hyphae grow into the xylem vessels. The hyphae remain exclusively in these vessels and produce conidia, which are transported acropetally within the xylem throughout the plant (Fradin & Thomma, 2006). This leads to characteristic symptoms that include wilting, stunting, leaf chlorosis and necrosis, vein clearing, and vascular discoloration (Schnathorst & Mathre, 1966; Fradin & Thomma, 2006).

Historically, pathotypes among *V. dahliae* strains have been classified based on the types of plant symptoms observed, and population genetic analyses typically support this classification. The *V. dahliae* strains that infect some plant species (cotton (*Gossypium hirsutum*), okra, and olive) are classified as defoliating (D) or nondefoliating (ND) based on their ability to completely...
verticillium dahliae has a highly clonal population structure with little or no evidence of recombination (de Jonge et al., 2013; Milgroom et al., 2014; Short et al., 2015), and investigations of the population structure by heterokaryon incompatibility (het) loci or microsatellite loci showed that isolates of V. dahliae can be divided into several clonal lineages (Joaquim & Rowe, 1991; Milgroom et al., 2014; Short et al., 2015). In addition, isolates of V. dahliae are classified into race 1 and race 2 based on the response of differential tomato (Solanum lycopersicum) and lettuce cultivars, and the compatible or incompatible reactions are conditioned by an assortment of resistance genes (R-genes) in the host (Diwan et al., 1999). Population analyses further revealed that the D pathotype isolates belonged to the vegetative compatibility group 1A (VCG 1A), and defoliation in cotton, okra, and olive is limited to isolates within VCG 1A (Schnathorst & Mathre, 1966; Jiménez-Díaz et al., 2006; Korolev et al., 2008). This is in contrast to other VCG groups, which generally cause wilting without defoliation (Schnathorst & Mathre, 1966; Korolev et al., 2008; Jiménez-Díaz et al., 2011); ND and D strains of V. dahliae from cotton sort as races 1 and 2, respectively (Hu et al., 2015).

The physiological and biochemical processes of leaf senescence and defoliation are complex and are linked to crop yield (Lewis et al., 2006). During leaf abscission that occurs naturally as part of the plant’s developmental sequence of maturation and senescence, nutrients are recycled from source tissues to reproductive organs (Munne-Bosch & Alegre, 2004). Phytohormones (e.g. abscisic acid (ABA), ethylene) coordinate leaf senescence and defoliation, and external factors can influence these processes as well. Phytotoxic compounds (atrazine, thiadiazuron, etc.), abiotic stresses (drought, osmotic stress, low light, salinity, etc.), and biotic stresses (e.g. pathogens and pests) can also promote leaf senescence and defoliation (Lim et al., 2007; Sade et al., 2017).

Among the biotic stresses, pathogen infection commonly results in early leaf chlorosis and defoliation (Bertamini et al., 2002; Bertaccini et al., 2014). These symptoms are in part related to water blockage caused by the colonization and proliferation of pathogenic microbes in the xylem (Fradin & Thomma, 2006; Klosterman et al., 2009), but they can also be caused by secreted toxins from V. dahliae (Zhang D. D. et al., 2016; Zhang W. Q. et al., 2017). The deleterious effects of some V. dahliae secondary metabolites have been reported (Bhatnagar et al., 2003; Zhang et al., 2012). In general, fungal secondary metabolite synthesis, transport, and secretion require the activity of various oxidoreductases and transporters (Yu et al., 1995; Desjardins et al., 1996; Proctor et al., 1999; Brown et al., 2004).

Several studies have investigated molecular mechanisms that underpin the pathogenicity and virulence of V. dahliae, including the involvement of secreted proteins, a battery of cell-wall-degrading enzymes, transcription factors, and membrane receptors (Klimes et al., 2015). In addition, mechanisms of host adaptation in V. dahliae reveal that this asexual pathogen may evolve through chromosomal rearrangement or horizontal gene transfer from other pathogens that enables rapid development of novel effector genes contributing to virulence (de Jonge et al., 2012, 2013). Comparative genomic analyses suggested that genes for signaling/transcriptional regulation and iron/lipid metabolism encoded by lineage-specific (LS) regions play important roles in niche and host adaptation (Klosterman et al., 2011), and some LS genes are clearly upregulated in planta (de Jonge et al., 2012, 2013). Despite these advances, the mechanistic bases for the defoliation phenotype in V. dahliae are unresolved.

The D and ND pathotypes are associated with specific genomic DNA sequences (Pérez-Artéz et al., 2000). Seven virulence factors are encoded in an LS region in the D V. dahliae strain Vd991 from cotton (G. hirsutum), known as G-LSR2. Some of the gene products encoded within the G-LSR2 region share homology with proteins involved in redox reactions that contribute to this isolate’s virulence and adaptation to cotton (Chen et al., 2018).

The objectives of this study were to: (1) confirm the association of the LS region of G-LSR2 exclusively to D pathotypes by whole-genome resequencing; (2) investigate the function of seven cotton-specific virulence factors in G-LSR2 in defoliation; (3) investigate the evolution of defoliation-associated genes between V. dahliae and the putative origin of G-LSR2; and (4) examine the role of secondary metabolites encoded by G-LSR2 genes in defoliation.

Materials and Methods

Fungal strains and culture conditions

V. dahliae Vd991 and an additional 75 isolates were collected from infected cotton plants in nine provinces in China (Supporting Information Table S1). The V. dahliae reference strains JR2 and VdLs.17 are from tomato and lettuce (Lactuca sativa) (Bhat & Subbarao, 1999; de Jonge et al., 2013), respectively. Five strains of Fusarium oxysporum f. sp. vasinfectum (FOV05, FOV07, FOV16, FOV17, and FOV18) were isolated from cotton plants. Unless specified otherwise, cultures of these fungi were maintained on potato dextrose agar (PDA) or potato dextrose broth (PDB) at 25°C before use.

Molecular validation of D and ND phenotypes

Genomic DNA was extracted from fresh mycelia using a cetyl trimethylammonium bromide method (Springer, 2010). The primers described by Pérez-Artéz et al. (2000) were employed to identify D and ND strains of V. dahliae. All PCRs were performed separately in 25 μl reaction volumes containing 12.5 μl of 2 × FastPfu Fly PCR SuperMix (TransStart, Beijing, China), 1 μl of genomic DNA (c. 30 ng μl⁻¹), and 1 μl of each primer at a concentration of 10 μM. The annealing temperature was 58°C for both D and ND primers. The D and ND primers and other primers used in this study are listed in Table S1.

The D/ND phenotypes were validated on cotton seedlings using root-dip inoculation (Hu et al., 2015). Briefly, susceptible
cotton plants (G. hirsutum cv Junmian No. 1) were maintained in a glasshouse at 28°C under a 14 h : 10 h light : dark photoperiod for 4 wk. Seedlings were gently uprooted, washed, and dipped into 1 × 10⁷ conidia ml⁻¹ suspension (5 ml per seedling) for 5 min. Three independent replicates consisting of 12 plants each were inoculated for each V. dahliae isolate. Seedlings treated with sterile distilled water were used as controls. Seedlings were maintained at 25°C under a 14 h : 10 h light : dark photoperiod. Defoliation was assessed 4 wk after inoculation. Fungal biomass in cotton roots was determined as described by Santhanam et al. (2013). Quantitative PCR (qPCR) was performed using the qPCR SYBR premix Ex Taq II kit (TaKaRa, Tokyo, Japan) with primers from the cotton, okra and olive 18S gene (Table S1). Differences between inoculated and noninoculated treatment groups were considered significant if paired Student’s t-test probability was ≤0.05. Presence or absence of vascular discoloration in shoots was assessed visually at 4 wk after inoculation.

To identify the D/ND phenotypes on olive and okra, 3-wk-old susceptible okra plants (Qiukui 101) and 1-yr old olive seedlings (Olea europaea ‘Leccino’) were also inoculated with 1 × 10⁷ conidia ml⁻³. Roots were dipped in V. dahliae isolate suspensions (5 ml per seedling) for 5 min and then maintained at 25°C under a 14 h : 10 h light : dark photoperiod for 3 wk and defoliation was assessed.

Genome resequencing and mapping

For V. dahliae genome resequencing, c. 5 μg of genomic DNA was used for each isolate to construct paired-end sequencing libraries with insert sizes of 500 bp according to the manufacturer’s instructions (Illumina, San Diego, CA, USA). Cluster generation, template hybridization, isothermal amplification, linearization, blocking, denaturation, and hybridization of the sequencing primers were performed according to the manufacturer’s instructions (Illumina). The SolexaPipeline-0.3 was used to call bases for 90 bp reads from raw fluorescent images. The insert size distribution of each library was determined by ELAND in SolexaPipeline. Reads were aligned to the Vd991 reference genome (Chen et al., 2018) using the SOAPaligner (Li et al., 2009) with the following parameters: soap2.20 -p 4 –a 1.fq -b 2.fq -D IRGSP_chromosomes_build04.fa.index –o sample.soap -2 sample.single –u unmapped.fa –m 435*1 –x 501*1 –s 35 -l 24 –v 7 (*1: the insert size was estimated by ELAND and served as input for each library). To identify the gene absences in the G-LSR2 region within the genomes of V. dahliae isolates, the coverage depth and breadth (mapping length/gene length) of each gene (including 20 flanking genes) were calculated. Reference genome G-LSR2 regions with corresponding coverage depth <2× and breadth <25% in resequenced genomes were considered absent.

Sequence divergence of VdDf homologues from F. oxysporum f. sp. vasinfectum

Putative homologs of the seven genes within G-LSR2 (VEDA_05193–VEDA_05199; Chen et al. (2018)) hereinafter referred to as Verticillium dahliae Defoliation-associated genes 1–7 (VdDf1–VdDf7) were amplified from the genomic DNA of five isolates of F. oxysporum f. sp. vasinfectum (FOVA_5, FOVA_7, FOVA_16, FOVA_17, and FOVA_18). The PCR was conducted by using the primers for cloning VdDf1–VdDf7, with an initial 94°C denaturation step for 10 min, followed by 36 cycles of 94°C for 30 s, 55°C for 1–2 min, and 72°C for 3 min. The protein sequences were deduced on the basis of VdDf1–VdDf7 sequences. The sequence polymorphisms were identified by comparison with reference sequences.

Fungal transformation

Gene deletion strains were produced using a homologous recombination following Mullins & Kang (2001). VdDf1–VdDf7 were independently deleted, and VdDf5 and VdDf6 were deleted together (VdDf5_6). To generate the deletion constructs, the flanking sequences of corresponding genes/sequences were amplified from the Vd991 genomic DNA and integrated with the hygromycin cassette using fusion PCR (Liu et al., 2013). The amplified products were then cloned into the pGKO2-gateway vector (Khang et al., 2005). For the complementation transformants, the sequence of VdDf5 and VdDf6 together accompanied by two flanking sequences (1765 bp upstream and 1186 bp downstream) was amplified from V. dahliae strain Vd991 genomic DNA and cloned into the binary vector pCOM that carries genticin resistance (Zhou et al., 2013) and reintroduced to the ΔDfs-1 (constructed by the homologous recombination following Chen et al., 2018), VdLs.17 and VDG78 strains. For ectopic expression of VdDH1, the full-length sequence was amplified from the Vd991 genomic DNA, cloned into the binary vector pCT-HN with the TrpC, constitutive promoter, and integrated into the ΔDfs-1, ΔDf5_6-1, VdLs.17 and VDG78 strains. For ectopic expression of F. oxysporum f. sp. vasinfectum genes in VdDf5 and VdDf6 (ΔDf5_6�), the homologous genes identified in F. oxysporum f. sp. vasinfectum FOV05, and two flanking sequences (1773 bp upstream and 1186 bp downstream) were amplified from genomic DNA and cloned into the binary vector pCOM that carries genticin resistance (Zhou et al., 2013). These were reintroduced into VDG78 and VdLs.17 strains. Agrobacterium tumefaciens-mediated transformation of V. dahliae was conducted as described previously (Liu et al., 2013), and the transformants were selected on PDA (potato, 200 g l⁻¹ agar, 15 g l⁻¹) supplemented with antibiotics (60 μg ml⁻¹ hygromycin or 50 μg ml⁻¹ genticin). Homologous recombination of the deletion mutants and ectopic transformants were verified by PCR with the corresponding primers (Table S1).

Gene expression analysis

To analyze gene expression in V. dahliae in response to cotton, 4-wk-old cotton seedlings were root dip-inoculated with 5 ml of 1 × 10⁷ conidia ml⁻¹ from the wild-type strains Vd991 and VDG78, and the gene deletion mutants ΔDfs, ΔDf5-1, ΔDf6-1 and ΔDf5_6. The roots were harvested at 12, 24, 48, 120, and 144 h post-inoculation and flash frozen in liquid nitrogen (N₂) for RNA extraction. V. dahliae strains cultured on PDA were
used as controls. After grinding, 100 mg of ground material was used for total RNA extraction using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen, New York, NY, USA) and first-strand complementary DNA was synthesized using a ReverTra Aid First cDNA Synthesis Kit (Thermo, Vilnius, EU Lithuania). The transcript levels of the different genes examined during different infection stages were determined using the qPCR SYBR premix Taq II kit (TaKaRa) by reverse transcription (RT)-qPCR with the corresponding primers (Table S1), using the 2–ΔΔCt method (Livak & Schmittgen, 2001). *V. dahliae* elongation factor 1-α (EF-1α) was used as an endogenous control, and reactions were performed in triplicate. PCR conditions consisted of an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 30 s.

Expression analysis of cotton genes encoding fatty acid amide hydrolases (FAAHs) was carried out using 4-wk old seedlings of *G. hirsutum* cv Junnian No. 1. Plants were inoculated with a 1 × 10⁷ conidia ml⁻¹ suspension of *V. dahliae* or fed with 20 mM N-acylethanolamine (NAE 12:0). The seedling roots were harvested in three independent replicates at specific times after inoculation and then flash-frozen in liquid N₂ and stored at −80°C until use. The expression of eight cotton *FAAH* gene family members (*GhFAAH1–GhFAAH8*) were quantified by RT-qPCR. The cotton 18S gene was used as an internal control to normalize the variance among samples.

D phenotype and virulence assays of *V. dahliae* secondary metabolites on cotton

Secondary metabolites were extracted from culture suspensions of strains as follows. Three independent transformants of each strain were cultured in 200 ml Czapek-Dox medium without antibiotics at 25°C in a shaking incubator at 180 rpm for 5 d. The liquid cultures were centrifuged at 21 000 g at 4°C for 20 min and the supernatants were filtered through a cellulose acetate filter (0.45 μm) and then cleaned by liquid–liquid extraction using 250 ml of chloroform : acetonitrile (1 : 1 v/v) twice. Next, the lower layer (organic solvent) was collected in a round-bottom flask and concentrated almost to dryness using a rotary evaporator (Yarong Machiners, Shanghai, China) at 35°C. The residues were reconstituted in 2 ml of acetonitrile and filtered through 0.22 μm syringe filters for ultrahigh performance liquid chromatography–tandem mass spectrometry analysis.

Chromatographic separation was carried out using an Agilent 1290 LC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a four-channel online degasser, a standard binary pump, and an Agilent Poroshell120 EC-C18 column (2.1 mm × 50 mm, 2.7 μm particle size). The mobile phase consisted of ultrapure water containing 0.1% formic acid (eluents A) and methanol (eluents B). The gradient elution program was 30% B at injection time, with linear increase to 90% B in 5.0 min, where it was maintained for 2.0 min before returning to the initial conditions of 30% B (70% A) in 4.0 min. The flow rate was 0.4 ml min⁻¹, and all compounds were eluted within 11.0 min. The temperature of the sample vial holder was set at 5°C, and the column temperature was maintained at 40°C to decrease viscosity. The injected volume was 1 μl.

An Agilent 6495 triple quadrupole mass spectrometer equipped with a conventional electrospray ionization source was used to quantify the three compounds of interest. N₂ (99.95%) and argon (99.99%) were used as the nebulizer gas and the collision gas, respectively, and the pressure in the T-wave cell was 3.2 × 10⁻⁵ MPa. The positive electrospray ionization mode and multiple reaction monitoring were used for the detection of the three compounds, and the tandem mass spectrometry conditions were optimized for the target compounds. Typical conditions were: source temperature, 200°C; capillary voltage, 3.0 kV; and desolvation temperature, 370°C. A cone gas flow of 50 l h⁻¹ and a desolvation gas flow of 600 l h⁻¹ were used. All other mass spectrometry parameters were optimized individually for each target compound, and the optimized parameters are listed later. MassHunter software (Agilent) was used to collect and analyze the data.

Defoliation function of NAE 12:0 on cotton plants

Four-week-old cotton plants were inoculated with a 1 × 10⁷ conidia ml⁻¹ suspension of strain VDG78. One week later, 5 ml of 20 mM NAE 12:0 was fed as nutrition to the cotton seedlings. Plants fed with sterile distilled water or NAEs and plants inoculated only with Vd991 strain served as negative and positive controls, respectively. The D or ND phenotype of cotton plants was determined 10 d later.
Data availability

The raw data of 75 *V. dahliae* strains have been deposited in GenBank under PRJNA171348 and the CNGB Nucleotide Sequence Archive (CNSA: https://db.cngb.org/cnsa; accession number CNP0000156).

The homologous gene IDs of *GhFAAH1–GhFAAH8* in *Gossypium raimondii* was Gorai.004G133000.1, Gorai.005 G091000.1, Gorai.007G067000.1, Gorai.008G018500.1, Gorai.010G111500.1, Gorai.011G117700.1, Gorai.012G03720 0.1, and Gorai.013G053300.1, respectively.

Results

Population analyses reveal the presence of LS region G-LSR2 only in the D pathotype strains of *V. dahliae*

Through comparative genomics of *V. dahliae* strains JR2 and VdLs.17, we previously showed that the lineage-specific region G-LSR2 encodes host-specific adaptation factors, explaining the high virulence of Vd991 on cotton (Chen et al., 2018). Highly virulent strains generally cause defoliation in cotton, suggesting that G-LSR2 itself may be associated with the defoliation function. Characterization of isolates from cotton with D and ND genetic markers indicated that the Vd991 genome contains the D marker but lacks the ND marker. The inverse is true for the genomes of JR2 and VdLs.17, which contain only the ND marker (Fig. S1a). Cotton plants (*G. hirsutum* cv Junmian No. 1) displayed the D phenotype following inoculation with Vd991, but not with JR2 or VdLs.17 (Fig. S1b). Fungal biomass in planta and vascular discoloration were significantly higher with Vd991, in contrast to either JR2 or VdLs.17 (Fig. S1c,d).

To investigate the association between G-LSR2 and the D pathotype, we performed whole-genome resequencing of 75 isolates of *V. dahliae*. PCR data using D- and ND-specific primer sets revealed 59 D isolates and 16 ND isolates (Table S2). PCR genotypes were validated by assessing the phenotypes of a subset of arbitrarily chosen three D and ND phenotypes (Fig. S2a,b). Additionally, c. 4 million paired-end Illumina reads (PE = 90 bp) were generated for resequencing isolates for comparing G-LSR2 between strains. Reads were mapped to the reference genes from G-LSR2 and two flanking genomic regions that both harbor an additional 20 genes. As expected, with the exception of one gene (*VEDA_0518l*) in G-LSR2, sequencing reads from all 59 D isolates were mapped to 22 genes encoded by G-LSR2, whereas sequence reads from the 16 ND isolates did not map with high significance (Fig. 1). The coverage depth and breadth of coverage of each gene in G-LSR2 from the D isolates suggested the sequence data in this region were reliable (Table S3). Subsequent mapping of the total reads to G-LSR2 flanking sequences revealed their presence in all the D and ND isolates (Fig. 1). These data suggest that G-LSR2 is an LS region present only in the D isolates from cotton.

Seven genes in G-LSR2 confer the D function in *V. dahliae*

Seven genes within G-LSR2 (*VdDf1–VdDf7*) contribute to the adaptation of *V. dahliae* Vd991 to cotton (Chen et al., 2018). Whether VdDf6 are linked to the D phenotype in cotton was evaluated. Almost complete defoliation was observed 3–4 wk after inoculation with the wild-type strain Vd991 (Figs 2a, S3). However, the D phenotype was not observed after inoculation with the VdDf deletion mutant ΔDf6 (as in Chen et al. (2018)) (Figs 2a, S3). The ΔDf6 strains caused less vascular discoloration than the wild-type strain Vd991 (Fig. 2b), and contained significantly less fungal biomass in cotton roots (<40%) compared with the biomass in plants inoculated with the wild-type strain Vd991 (Fig. 2c). These results suggested that the VdDf6 contribute to the D phenotype.

**VdDf5** and **VdDf6** confer the D phenotype in the LS region G-LSR2

Single-gene knockout mutants of genes in VdDf6 were generated by homologous recombination (Fig. S4a–g). The virulence assays showed that deletion of VdDf5 or VdDf6 in the Vd991 background resulted in loss of the D phenotype in cotton at 4 wk after inoculation (Fig. 3a). Cotton plants inoculated with ΔDf5 and ΔDf6 strains displayed less vascular discoloration and fungal biomass, compared with other gene deletion strains and wild-type (Figs 3b, S5a). Transcript abundances of the VdDf6 genes were highly upregulated at 24 h post-inoculation in cotton (Chen et al., 2018). Expression analysis of VdDf1–VdDf7 revealed that the transcript levels, except for VdDf3 and VdDf4, were significantly upregulated at 0.5–7 d after inoculation of cotton, especially with VdDf5 and VdDf6, which were strongly upregulated (up to 20-fold change) 2 d after inoculation (Fig. S6). These results suggested that VdDf5 and VdDf6 from VdDf6 are the central genes involved in conferring the D phenotype. To confirm this, targeted replacement of VdDf5 and VdDf6 together was performed (Fig. S4h). Remarkably, the two independent VdDf5 and VdDf6 double deletion mutants (ΔDf5_6-1 and ΔDf5_6-2) lost the ability to defoliate cotton (Figs 3c, S5b). Simultaneous complementation of VdDf5 and VdDf6 (VdDf5_6) in the ΔDf6 strains resulted in the restoration of the defoliation phenotype (Figs 3c, S7a). Degrees of vascular discoloration and fungal biomass supported that VdDf5 and VdDf6 contribute to virulence on cotton (Figs 3d, S5b).

The D phenotype was evaluated on olive and okra, two other hosts that also exhibit defoliation of infected plants (Jiménez-Díaz et al., 2006; Korolev et al., 2008). Both ΔVdDf6 and ΔVdDf5_6 lost the D phenotype on olive and okra, but this was recovered following the restoration of only VdDf5 and VdDf6 in the VdDf6 deletion strains (Figs 3e, S8a). Fungal biomass levels also supported these observations (Figs 3f, S8b). Thus, only VdDf5 and VdDf6 confer the D phenotype in the three plant hosts.
Sequence divergence of defoliation genes VdDf5 and VdDf6 following transfer from F. oxysporum f. sp. vasinfectum

To investigate whether VdDf5 and VdDf6 confer the D phenotype in different genetic backgrounds, ectopic mutants expressing VdDf5 and VdDf6 together were generated in the ND strains VDG78 (from cotton) and VdLs.17 (from lettuce) (Fig. S7b,c). Remarkably, the ND strain VDG78 gained the ability to cause the D phenotype following the simultaneous introduction of VdDf5 and VdDf6 (Fig. 4a), as well as to cause severe vascular discoloration in inoculated plants that also harbored higher fungal biomass than those inoculated with the wild-type ND strain (Fig. 4a,b). The introduction of VdDf5 and VdDf6 together into VdLs.17 resulted in similar disease phenotypes (Fig. S9a,b). However, the defoliation symptoms caused by these ND transformants following the introduction of VdDf5 and VdDf6 were weaker than symptoms caused by the D strain Vd991 (Figs 4a,b, S9a,b).

Previous studies (Chen et al., 2018) indicated that G-LSR2 was likely horizontally transferred from F. oxysporum f. sp. vasinfectum and encodes genes that may explain the dominant adaptation to cotton. To explore the molecular evolution of VdDf5 following transfer from F. oxysporum f. sp. vasinfectum, the homologues of VdDf5 in F. oxysporum f. sp. vasinfectum were aligned with V. dahliae sequences. This revealed several polymorphisms, including start codon changes, nonsynonymous single nucleotide polymorphisms, and truncation variations (Fig. S10). The VdDf5 and its homologous gene in F. oxysporum Df5FO alignments revealed three nonsynonymous mutations (Fig. 4c), and eight single nucleotide polymorphisms resulting in four nonsynonymous changes in VdDf6 and its homologous gene in F. oxysporum Df6FO, and an 18 bp truncation resulting in a different start codon (Fig. 4d).

Next, we assessed the function of the VdDf5 and VdDf6 orthologues from F. oxysporum f. sp. vasinfectum (Df5FO and Df6FO, Df5_6FO) by ectopic expression in a cotton ND isolate. RT-qPCR analysis showed that the Df5FO and Df6FO were
Seven genes encoded by lineage-specific region of VdDfs in *Verticillium dahliae* are required for the defoliating phenotype on cotton.

(a) Pathogenicity assay for investigating the role of the genomic region VdDfs in defoliation by *V. dahliae*. Four-week-old susceptible cotton plants (*Gossypium hirsutum* cv Junmian No. 1) were inoculated with a $1 \times 10^7$ conidia ml$^{-1}$ conidial suspension of wild-type *V. dahliae* Vd991, knockout mutants of the entire VdDfs region (ΔDfs-1 and ΔDfs-2, constructed by the homologous recombination that has already been described in Chen et al., 2018), or sterile water using a standard root-dip method. There were three independent replicates consisting of 12 plants. Plants were photographed 3 wk post-inoculation. (b) Vascular discoloration in cotton after inoculation with gene deletion mutants at 4 wk post-inoculation. Uninoculated plants were used as controls. (c) In planta fungal biomass development of the VdDfs knockout mutants (ΔDfs-1 and ΔDfs-2) in inoculated cotton roots, and quantified by quantitative PCR. Error bars represent SE. Statistical significance has been represented (according to unpaired Student’s t-tests): **, *P* < 0.01.

successfully expressed in the ectopic transformants and the expression levels were similar to VdDf5 and VdDf6 from strain Vd991 during infection of cotton (Fig. S11). Unlike VdDf5 and VdDf6, which conferred the D phenotype to *V. dahliae* (Figs 4a, b, S9a,b), Df5_6+F0 expression did not confer either the D phenotype to the cotton ND isolate (VDG78) (Fig. 4e) or higher virulence (Fig. 4f). These results suggested that amino acid substitutions in VdDf5 and VdDf6 resulting in higher virulence/defoliation were selected for in *V. dahliae* following the horizontal transfer from *F. oxysporum f. sp. vasinfectum.*

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Fig. 2 Identification of functional genes in VdDfs from *Verticillium dahliae* involved in the defoliating phenotype during infection on cotton. (a) Discovery of the genes involved in the defoliating phenotype using single-gene deletion mutants. Plants of four-week-old cotton (*G. hirsutum* cv Junmian No. 1) were root-dip-inoculated with single gene knockout mutants of each of the seven genes (ΔDfs-1 to ΔDfs-7). A conidial suspension of $1 \times 10^7$ conidia ml$^{-1}$ for each mutant strain was prepared and inoculated. Three replicates consisting of 12 cotton plants each were included for each experiment. The wild-type *V. dahliae* Vd991 and sterile water (Mock) treatments were used as positive and negative controls, respectively. Plants were maintained at 25°C in a glasshouse under a 14 h : 10 h, light : dark cycle. The defoliation phenotypes were investigated 4 wk after inoculation. (b) Quantitative PCR of in planta fungal biomass development of single gene knockout mutants. Error bars represent SE. Statistical significance has been represented (according to unpaired Student’s t-tests): **, *P* < 0.01.

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Fig. 3 Identification of functional genes in VdDfs from *Verticillium dahliae* involved in the defoliating phenotype during infection on cotton. (a) Discovery of the genes involved in the defoliating phenotype using single-gene deletion mutants. Plants of four-week-old cotton (*G. hirsutum* cv Junmian No. 1) were root-dip-inoculated with single gene knockout mutants of each of the seven genes (ΔDfs-1 to ΔDfs-7). A conidial suspension of $1 \times 10^7$ conidia ml$^{-1}$ for each mutant strain was prepared and inoculated. Three replicates consisting of 12 cotton plants each were included for each experiment. The wild-type *V. dahliae* Vd991 and sterile water (Mock) treatments were used as positive and negative controls, respectively. Plants were maintained at 25°C in a glasshouse under a 14 h : 10 h, light : dark cycle. The defoliation phenotypes were investigated 4 wk after inoculation. (b) Quantitative PCR of in planta fungal biomass development of single gene knockout mutants. Error bars represent SE. (c) Assessment of the defoliating role of genes VdDfs and VdDf6 together in *V. dahliae*. Four-week-old cotton plants were inoculated with a $1 \times 10^7$ conidia ml$^{-1}$ conidial suspension using a root-dip method. Plants were inoculated with two independent targeted gene deletions of VdDf5 and VdDf6 genes into the VdDfs mutant background, or the wild-type. Plants treated with sterile water were used as control (Mock). Three independent replicates were performed, each consisting of 12 plants. The defoliation phenotype was investigated 4 wk after inoculation. (d) Quantitative PCR analyses of fungal biomass development of VdDfs and VdDf6 knockout mutants and corresponding ectopic transformants in cotton. Error bars represent SE. (e) The defoliating phenotype of strains (Vd991, ΔDfs-1, ΔDfs-5_6-1, EC-Dfs_5_6 (ΔDfs-1)) on olive plants. The defoliation/nondefoliating phenotype of different strains was assessed using 4-wk-old olive plants 3 wk after root-dip inoculation with a conidial suspension ($1 \times 10^7$ conidia ml$^{-1}$). There were three independent replicates, each consisting of 12 plants. (f) Quantitative PCR analyses of in planta fungal biomass of different strains in olive. Error bars represent SE. Statistical significance has been represented (according to unpaired Student’s t-tests): **, *P* < 0.05; ***, *P* < 0.01.
G-LSR2 is a secondary metabolite gene cluster controlling biosynthesis of a compound with D activity

The annotation of 22 genes encoded by G-LSR2 predicted a variety of functions. Twelve genes were homologous to F. oxysporum f. sp. vasinfectum stress response genes (Table S4). VdDF5 and VdDF6 share homology with polyketide synthases, whereas VdDF1 and VdDF3 are oxidoreductase homologues, VdDF2 encodes an NmrA transcriptional regulator, and VdDF4 and VdDF7 share homology with a major facilitator superfamy gene and an N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD), respectively (Fig. 5a). Therefore, G-LSR2 is likely a secondary metabolism gene cluster, which characteristically encodes polyketide synthases, oxidoreductases, transporters, transcriptional regulators, and hydrolases (Yu & Keller, 2005; Brakhage, 2013). Also, at least six protein-coding genes in this cluster share a conserved NACHT and TPR domain or a P-loop nucleoside triphosphate hydrolase (P-loop NTPase) domain, such as the genes VDEA_05186, VDEA_05185, and VDEA_05183 (Fig. 5a), which are often involved in host–pathogen interactions by transcriptional regulation (Leipe et al., 2004).

To investigate whether genes in G-LSR2 function in secondary metabolism production and secretion that leads to defoliation, the toxicity of putative secondary metabolites from G-LSR2 wild-type and mutant V. dahliae strains were assayed on plants. Cotton seedlings were treated with secondary metabolites extracted from culture supernatants from the wild-type and the ΔDfs strain, and the ΔDfs strain simultaneously complemented with VdDF5 and VdDF6. As expected, cotton leaves displayed wilting, chlorosis, and began to defoliate 2 wk after treatment with the culture supernatant extracted from wild-type strains in vitro, but showed no D phenotype following treatment with the ΔDfs culture supernatant (Fig. 5b). Treatment with supernatant collected from the VdDF5-6 complemented-ΔDfs strain also resulted in defoliation (Fig. 5b).

Interestingly, VdDF7 shares homology to a gene encoding NAPE-PLD, which hydrolyzes N-acylphosphatidylethanolamines (NAPEs) to produce NAEs. Intriguingly, NAEs are known to affect plants through ABA signaling (Blancaflor et al., 2014), impacting leaf senescence and stomatal closure (Murata et al., 2015). Quantification of three NAEs (NAE 12:0, NAE 14:0, and NAE 16:0) showed that the concentration of NAE 12:0 was significantly lower (c. 100-fold less) in culture suspensions of the deletion strains (ΔDfs-1, ΔDfs-5-1, ΔDfs-6-1, and ΔDfs-6-1, and ΔDfs-6-1) relative to the wild-type, and NAE content was restored after the simultaneous re-introduction of VdDF5 and VdDF6 into the ΔDfs mutant and ND strain VDG78 (Fig. 5c). Furthermore, the concentrations of NAE 12:0 were significantly lower in ND strains than in D strains in culture (Fig. 5d). However, the concentration of NAE 16:0 between D and ND strains was not statistically significant (Fig. S12). In contrast, the concentration of NAE 14:0 was significantly (P < 0.05) higher in the ND strains than in D strains (Fig. S12). Correspondingly, cotton plants inoculated with the ND isolate VDG78 also displayed the D phenotype when combined with the NAE 12:0 treatment (Fig. 5c). Therefore, VdDF5 and VdDF6 within the G-LSR2 cluster are crucial to the NAE 12:0 biosynthesis.

NAEs are involved in defoliation by interfering with normal NAE metabolism in cotton plants

NAEs are a class of bioactive lipids, and FAAH is one of the enzymes responsible for degrading NAEs to fatty acid amide and ethanolamine in plants (Chapman, 2004). The annotation of conserved domains by InterProScan previously revealed that no FAAH (InterPro ID: IPR030560) enzymes were present in the set of protein coding genes in V. dahliae Vd991 (Chen et al., 2018). Since V. dahliae lacks FAAH, potentially resulting in the inability to catabolise NAEs, the function of NAEs in V. dahliae may be related strictly to pathogenicity. Interestingly, RT-qPCR analysis of the eight cotton FAAH gene family members (GhFAAH1–GhFAAH8) revealed that the transcript levels of GhFAAH3, GhFAAH6, GhFAAH7, and GhFAAH8 were significantly upregulated (c. 8- to 23-fold) in susceptible cotton at 5 d after inoculation with the D strain Vd991 compared with inoculation with ND strain VDG78 (Figs 6a, S13), which supported the idea that plant expression of FAAH occurs in response to the high concentration of NAE 12:0 transported from V. dahliae. In vitro assays demonstrated that the expression of GhFAAH6, GhFAAH7, and
\textit{GhFAAH8} was significantly higher at 48 h in NAE-12:0-fed cotton roots (Fig. 6b). Furthermore, the expression levels of cotton \textit{FAAH} genes were relatively higher in plants treated with the culture filtrate from D strain (Vd991) than that from ND strain (VDG78) (Fig. 6c). Therefore, the secondary metabolite gene cluster (\textit{VdDf}s) from \textit{F. oxysporum} enables efficient NAE biosynthesis.
and perhaps also the transport into the host plant cell; cotton plant FAAHs were significantly upregulated in response to the high level of NAEs transported from the V. dahliae D strains (Fig. 6).

**Discussion**

Even though V. dahliae is a well-known wilt pathogen with a very broad host range, it is able to cause defoliation in only three hosts; namely, cotton, olive, and okra (Schnathorst & Mathre, 1966; Jiménez-Díaz et al., 2006; Korolev et al., 2008). The reasons for this symptom dichotomy among the more than 200 hosts on which V. dahliae causes wilt has largely remained unresolved. In this study, we examined the molecular and genetic underpinnings of defoliation based on insights gleaned from our previous work (Chen et al., 2018). Earlier, we showed that a seven-gene sequence within the flexible genomic DNA region G-LSR2 of V. dahliae Vd991, acquired through horizontal transfer from F. oxysporum f. sp. vasinfectum, conferred higher virulence and adaptation towards cotton (Chen et al., 2018). Of the seven genes (VdDF7–VdDF5) within G-LSR2, we have demonstrated that VdDF5 and VdDF6 are critical for the D phenotype. Interestingly, these two genes appear to have undergone sequence evolution following the transfer from F. oxysporum f. sp. vasinfectum. G-LSR2 has the characteristics of a secondary metabolism gene cluster. VdDF5 and VdDF6 encode polyketide synthase homologues that appear to function in conjunction with oxireductases, transporters, and a transcriptional regulator in the biosynthesis, metabolism, and transport of NAE 12:0, a compound that can induce the D phenotype.

Although molecular markers for identifying the D and ND pathotypes have been available for some time (Pérez-Artés et al., 2000), the functional genes associated with or linked to the D marker have not been understood. Interestingly, BLAST searches indicated that the sequence of the D marker displayed high identity to the G-LSR2 border sequence (Scaffold 4: 354531–355476), specifically in the coding sequence of VEDA_05203 (Fig. S14). In addition, population genomics of V. dahliae from cotton showed that the presence/absence of G-LSR2 correlated precisely with the D and ND PCR markers (Pérez-Artés et al., 2000). Deletion of G-LSR2 in D strains results in the ND phenotype on cotton, olive, and okra, providing strong genetic evidence that the LS region G-LSR2 underlies the D phenotype in V. dahliae.

Acquisition of genes through horizontal gene transfer allows microbes to rapidly gain new capabilities and adapt to new and changing environments (Bonham et al., 2017). Various mechanisms have been described that result in the domestication of exogenous genetic material, including compensatory evolution, positive selection, and changes in gene expression (Wiedenbeck & Cohan, 2011). Comparative analyses of VdDF1–VdDF7 nucleotide and encoded amino acid sequences relative to the donor sequences from F. oxysporum f. sp. vasinfectum (also a cotton wilt pathogen) revealed divergence, including nonsynonymous/synonymous mutations, and alterations in start or stop codon usage (Figs 4c,d, S10). Expression analysis showed that Df8Fo was significantly upregulated, but Df8Go was not expressed (or at lower levels) during F. oxysporum f. sp. vasinfectum infection of cotton plants (Fig. S15). This suggested that F. oxysporum f. sp. vasinfectum was unable to cause defoliation owing to the ineffective expression of both Df8Fo and Df8Go genes. Since the key genes VdDF5 and VdDF6 were transcribed and expressed stably and are critical to the defoliation phenotype, the F. oxysporum f. sp. vasinfectum homologues were insufficient to induce defoliation, even when Df8Fo and Df8Go were successfully expressed in V. dahliae (Fig. S11). Furthermore, even though NAE 12:0 was also detected in V. dahliae, its concentration was representative of that displayed by ND strains and significantly lower than in D strains (Fig. 5d). These results suggested that Df8Fo and Df8Go genes are functionally defective in F. oxysporum f. sp. vasinfectum. It therefore appears that genes in VdDF8 underwent positive...
selection for virulence in *V. dahliae* following the horizontal transfer from *F. oxysporum* f. sp. *vasinfectum*.

Fungal secondary metabolites are extremely diverse and perform a range of functions, including iron acquisition, stress defense, and toxic assaults on living hosts, and so on (Macheleidt et al., 2016). Secondary metabolism genes are usually organized in clusters and typically encode polyketide and nonribosomal peptide synthases involved in the synthesis of a diverse array of...
Fig. 6 Detection of the cotton fatty acid amide hydrolase (FAAH) gene family members' (GhFAAHs') responses to defoliating (D). (a) Expression analysis of GhFAAHs during Verticillium dahliae infection on cotton roots. Four-week-old cotton plants (Gossypium hirsutum cv Junmian No. 1) were root-dip inoculated with V. dahliae D strain Vd991 or nondefoliating (ND) strain VDG78 and harvested at 5 d post-inoculation. Reverse transcription quantitative PCR was performed to determine the relative expression levels of GhFAAHs using the cotton 18S gene as a reference (set as 1.0) and compared with expression levels of GhFAAHs observed in uninoculated cotton treated with distilled water. (b) Expression analysis of GhFAAHs by feeding N-acylethanolamine (NAE) 12:0 on cotton roots. Four-week-old cotton plants were root-dip fed with NAE 12:0 and the roots were harvested at 48 h post-treatment. The relative expression levels of GhFAAHs' responses to NAE 12:0 were compared with the treatment with distilled water (set as 1.0). (c) Expression analysis of GhFAAHs by treating with culture filtrate on cotton roots. Four-week-old cotton plants were treating with culture filtrate of ND strain VDG78 and D strain Vd991, the roots were harvested at 48 h post-treatment. The relative expression levels of GhFAAHs' responses to the culture filter of D strain Vd991 were compared with the treatment with the culture filter of ND strain VDG78 (set as 1.0). Error bars represent SE; statistical significance has been represented (according to unpaired Student's t-tests): **, P < 0.01.
compounds, such as terpenes and indole alkaloids (Brown et al., 1996; Kennedy et al., 1999; Yu & Keller, 2005; Brakhage, 2013) and melanin, as in V. dahliae (Wang et al., 2018). During infection, V. dahliae secretes a diverse range of effectors (de Jonge et al., 2012; Gui et al., 2017; Zhang et al., 2017) and secondary metabolites (Xiong et al., 2016; Zhang et al., 2016). Interestingly, secondary metabolite production mediated by genes in VdDs can induce defoliation in the absence of the pathogen (Fig. 5b). The arrangement and functional annotation of genes in G-LSR2 are characteristic of a fungal secondary metabolism cluster, which typically contains polyketide synthases, oxidoredutases, transporters, and transcriptional regulators (Brown et al., 1996; Kennedy et al., 1999). The proteins encoded by secondary metabolism gene clusters often contain the conserved NACHT, TPR, or P-loop NTPase domains (Weijn et al., 2013) and share homology with protein domains encoded in G-LSR2 (Fig. 5a). Furthermore, VdDf1–VdDf7 were co-expressed during infection of cotton (Fig. S6), as predicted for coordinately regulated genes that are located in the secondary metabolism clusters (Collemare et al., 2008) known to be vital for virulence.

Multiple secreted secondary metabolites are known to function in pathogenesis (Macheleidt et al., 2016). NAEs are bioactive lipids derived from the hydrolysis of the membrane phospholipid NAPE and are known to regulate a variety of physiological processes in animals and plants (Chapman et al., 1999; Chapman, 2004; Kilaru et al., 2012). In plants, the NAEs occur in low concentrations (nanograms per gram FW range in vegetative tissues of plants) and are involved in cytoskeleton modification, chloroplast degradation, root development, defense response, and interaction with ABA (Chapman, 2004). NAEs may also participate in signal transduction pathways that induce ABA-responsive genes, block stomatal closure, and affect seed germination and seedling growth (Austin-Brown & Chapman, 2002; Teaster et al., 2007; Kang et al., 2008; Keerettaweep et al., 2013; Blancaflor et al., 2014). Many of the physiological processes regulated by NAEs are consistent with the disease phenotypes induced by V. dahliae, including the inhibition of seedling growth (Veronese et al., 2003; Pradin & Thomma, 2006), alterations to the cytoskeleton in plants treated with Vd-toxin extracted from the culture filtrate of strain Vd991 (Hu et al., 2014). Moreover, ABA is a key plant hormone mediating plant responses to environmental stresses, and promotes leaf abscission and senescence (Lim et al., 2007). Therefore, interactions between NAEs and ABA signaling could also contribute to leaf abscission and the defoliation phenotype. In our study, VdDf6 played a critical role in NAE 12:0 production associated with V. dahliae D pathotype. NAE 12:0 also caused defoliation in plants treated a week after inoculation with the ND strain. Although NAE 14:0 had little role in defoliation, the metabolic equilibrium of NAEs (Chapman, 2004) resulted in NAE 14:0 being higher in ND strains than in D strains (Fig. S12a). This further confirmed that the production of NAE 12:0 by V. dahliae contributes to leaf abscission and defoliation phenotype.

This study also demonstrated that VdDf5 and VdDf6 encode putative polyketide synthases likely involved in NAE 12:0 biosynthesis. Polyketide synthases play a critical role in the lipid metabolism, and NAEs and NAEs are N-containing lipids (Mohanthy et al., 2011; Quadri, 2014; Miyazawa et al., 2015). Furthermore, VdDf7 was predicted to encode NAPE-PLD that hydrolyzes NAPEs to produce NAEs. High levels of NAE 12:0 observed in D strains caused leaf abscission, and cotton inoculated with the ND strain also displayed leaf abscission following treatment with the exogenous NAE 12:0, indicating that the high level of NAE 12:0 in cotton secreted by D V. dahliae strain causes leaf abscission. Furthermore, NAE 12:0, known to inhibit phospholipase Dα activity and block ABA-induced stomatal closure (Austin-Brown & Chapman, 2002), could also significantly facilitate pathogen infection of cotton. Functional annotation of the V. dahliae genome showed that a duplicate orthologue encoding NAPE-PLD exists in the V. dahliae strain Vd991 (VEDA_04083), which was highly homologous to VdLs17 (VDAG_10085) and JR2 (evm.model.contig44686.491). The apparent redundant function of two NAPE-PLD genes in Vd991 may explain why the defoliation phenotype was not abolished after deletion of VdDf7 (Fig. 3). Furthermore, the functional homologues of VdDf6 (NmrA transcriptional regulator, major facilitator superfamily, etc.) are ubiquitous in V. dahliae, which likely provide the complementary functionality for NAE 12:0 biosynthesis by VdDf5 and VdDf6 in the ND strain (Fig. 4a). The production of NAE was restored following the simultaneous reintroduction of VdDf5 and VdDf6 into the ΔDf6 mutant EC-Df5_6(ADfs) or ND strains, suggesting that VdDf5 and VdDf6 are vital for NAE biosynthesis under the premise that NAPE-PLD is involved in the hydrolysis of NAPE to NAE; but the exact function needs to be elucidated further.

NAEs, comprising a family of functionally diverse signaling lipids in plants and animals, are transformed into free fatty acids and ethanolamine by FAAHs (Blancaflor et al., 2014). In plants, overexpression of FAAHs also disturbs the hormone balance, enhances ABA sensitivity, and compromises innate immunity (Blancaflor et al., 2014). Although the NAEs could be synthesized in microbes (Ellingson, 1980; Merkel et al., 2005), the transformation of NAEs by FAAH has not been reported in microbes. Also, in V. dahliae no conserved domains of FAAH were found among any of the encoded proteins in the genome (Chen et al., 2018). In Arabidopsis, the enzymes encoded by At5g44440 (AtFAAH) and At1g08980 (AMT1) have been shown to exhibit NAE hydrolytic activity (Shrestha et al., 2003). Interestingly, direct inoculation of cotton plants with the D strain or treatment with NAE 12:0 or treatment of the plants with the culture filtrate from the D strain uniformly resulted in the upregulation of FAAH genes (Fig. 6), suggesting that cotton increases the expression level of FAAH genes in response to high-level NAEs that were putatively released into the plant from the D V. dahliae strains (Fig. 5c,d). The D strains displayed a high efficiency of NAE biosynthesis (Fig. 5d), which appeared to induce the over-expression of FAAH gene family members (Fig. 6) and likely disrupt the host plant native NAE metabolism. Previous research also found that the overexpression of AtFAAH in Arabidopsis affected salicylic acid and jasmonic acid production, rendering the host more sensitive to ABA and pathogen infection (Kang et al., 2008). Therefore, upregulation of FAAH genes may also
increase cotton plant sensitivity to the ABA, disrupt hormone sensitivity, and predispose plants to pathogen infection accompanied by defoliation (Fig. 7).

In conclusion, our study provides strong evidence that the defoliation and high virulence of D pathotype is the result of secondary metabolite NAE 12:0, the biosynthesis of which is controlled by genes in the lineage-specific region G-LSR2 in *V. dahliae*.

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**Author contributions**

XFD, JYC and KVS conceived the study and designed all experiments, while JYC, DDZ, ZQK, LZ, DW, JQH and CL performed the data analysis and secondary metabolites identification, and RXL performed the additional secondary metabolites data of *F. oxysporum* in the revision stage. JW, DDZ, YJG, JJL,
BLW, CMY and TGL performed the targeted gene deletion, ectopic expression analysis, and pathogenicity and D/ND phenotype identification. JYC, DPGS, RMB, SJK, DDZ, J LW and GYZ contributed to the writing of the manuscript. KVS conceptualized the study, reviewed the data, and edited the manuscript. All authors have read, commented, and approved the manuscript. D-DZ, JW, DW, Z-QK and LZ contributed equally to this work.

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References

**Fig. S1** Cotton defoliation and non-defoliation phenotypes of *Verticillium dahliae* strains Vd991, JR2 and VdLs.17.

**Fig. S2** Defoliation/non-defoliation phenotypes and PCR genotypes of different *Verticillium dahliae* isolates.

**Fig. S3** Cotton defoliation phenotype dynamics from one to four weeks following inoculation with genomic region G-LSR2 deletion mutant **ADf**.

**Fig. S4** Screening of gene deletion mutants.

**Fig. S5** Identification of functional genes encoded by **VdDf** involved in vascular discoloration during infection of cotton by *Verticillium dahliae*.

**Fig. S6** Expression analysis of **VdDf1–VdDf7**, the seven G-LSR2 genes during infection of cotton by *Verticillium dahliae*.

**Fig. S7** PCR verification of the transfer of two genes **Df5-6** from the wild-type *Verticillium dahliae* strain Vd991 to strains VdLs.17 and VDG78, and mutant background **ADf**-1, respectively.

**Fig. S8** Defoliation phenotypes on okra caused by different *Verticillium dahliae* strains.

**Fig. S9** The collaborative role of genes **Df5** and **Df6** in conferring the defoliation phenotype in *Verticillium dahliae* strain VdLs.17.

**Fig. S10** Comparison of the sequence divergence of **VdDf** between *Verticillium dahliae* Vd991 and homologous genes from six different *Fusarium oxysporum* f. sp. *vasinfectum* strains.

**Fig. S11** Expression analysis of the **VdDf5** and **VdDf6** homologs, **Df5FO** and **Df6FO**, during infection of cotton by the ectopic transformant.

**Fig. S12** Quantification of NAE 14:0 and NAE 16:0 extracted from different strains by UHPLC-MS/MS.

**Fig. S13** Relative expression level analysis of the cotton *GhFAAH* genes in response to D strain Vd991.

**Fig. S14** Nucleotide alignment of the published defoliating marker sequence to the genomic region G-LSR2 of *Verticillium dahliae* Vd991.

**Fig. S15** Expression analysis of the **VdDf5** and **VdDf6** homologs genes (**Df5FO** and **Df6FO**) during infection of cotton by *F. oxysporum* f. sp. *vasinfectum*.

**Table S1** Information on isolates for which re-sequenced genomes were obtained for this study.

**Table S2** Primers used in this study.

**Table S3** The coverage breadth and depth of resequenced isolates mapped to encoding genes in G-LSR2.

**Table S4** Information of lineage-specific genes in Vd991.

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