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Regular Articles

Vayg1 is required for microsclerotium formation and melanin production in *Verticillium dahliae*

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ABSTRACT

The fungus *Verticillium dahliae* causes vascular wilt disease on many plant species, including economically important crop and ornamental plants worldwide. It produces darkly pigmented resting structures known as microsclerotia, which are able to survive for up to 14 years in soil, and represent one of the defining characteristics of this species. The pigment produced in *V. dahliae* is dihydroxynaphthalene (DHN)-melanin, a form of melanin common among fungi and named so for the intermediary of this melanin biosynthetic pathway. In this study, we characterized the function of the *V. dahliae Vayg1* gene, whose homologs were involved in melanin biosynthesis in *Exophiala dermatitidis* (*Wayg1*) and *Aspergillus fumigatus* (*Aayg1*), by deletion and complementation of the gene and co-incubating deletion mutant with wild-type strain. Results showed that melanin production and microsclerotial formation in deletion mutants are inhibited. The *Vayg1* deletion mutant also exhibited reduced pathogenicity. These results showed that *Vayg1* is necessary for melanin and microsclerotium production, and we may thus hypothesize that the *Vayg1* product may catalyze two different precursors, one of which is essential for DHN melanin production and the other one is involved in a signal network for microsclerotial formation in *V. dahliae*.

1. Introduction

Verticillium dahliae Kleb. is a soilborne plant pathogenic fungus classified in the Sordariomycetes of the Ascomycota, although a sexual reproductive phase has not been identified yet (Inderbitzin et al., 2011). It causes Verticillium wilt on more than 200 plant species, including many agriculturally important crops (Pegg and Brady, 2002). The list of plant hosts affected by *V. dahliae* is continually expanding as new hosts are identified (Atallah et al., 2011; Bhat and Subbarao, 1999; Lu et al., 2013).

The disease cycle of *V. dahliae* consists of a parasitic stage in which the plant is colonized, and a brief saprophytic stage where the pathogen can decompose plant tissues, followed by a dormant stage in which the pathogen can survive for years in the soil (Klosterman et al., 2009). The dormant survival structures that enable *V. dahliae* to survive up to 14 years, even in the absence of a host (Wilhelm, 1955), are known as microsclerotia that are resistant to extreme temperatures, desiccation and other environmental stresses (Bell and Wheeler, 1986; Butler and Day, 1998). Primary infections are typically initiated in the root by germinating microsclerotia, which produce infectious hyphae that directly penetrate the root (Klosterman et al., 2009). Following a period of colonization and growth in the xylem, large numbers of microsclerotia are produced on senescent infected plants (Powelson, 1970), and released into soil with decomposed plant debris. Thus, microsclerotia play a key role in the disease cycle of *V. dahliae*.

Microsclerotia in *V. dahliae* are composed of compact masses of thick-walled, darkly pigmented (melanized) cells, which originate from swollen, septate hyphae by a process of budding (Brandt, 1964; Fradin and Thomma, 2006). The cell walls of the microsclerotia are impregnated with melanin as the propagules mature (Brandt, 1964). Most fungal melanin has been identified as dihydroxynaphthalene (DHN)-melanin, named for the pathway intermediary, 1,8-DHN. DHN-melanin biosynthesis starts with a polyketide synthase (PKS) to produce 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), which is reduced to scytalone via a reductase

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(T4HR). Then with a series of downstream reactions, 1,8-DHN is produced.

Melanin is essential in *V. dahliae* for the development of fully functional microsclerotia (Bell and Wheeler, 1986). Production of melanin may be critical for long-term survival, as melanin enables *V. dahliae* and other fungi to ward off damage from UV irradiation, extreme temperatures and enzymatic degradation from β-1,3 glucanases and chitinases present in soil microorganisms (Bell et al., 1976; Butler and Day, 1998). In wild-type *V. dahliae*, the presence of melanin implies the existence of microsclerotia and its absence implies lack of microsclerotia (Brandt, 1964; Duressa et al., 2013). Nevertheless, melanin biosynthesis and microsclerotial development are not inextricably linked and melanin or products from later stages of the biosynthetic pathway are not necessary for formation of microsclerotia in *V. dahliae* (Bell et al., 1976).

In view of the importance of microsclerotia in the disease cycle of *V. dahliae* (Klosterman et al., 2009), an increased understanding of the genetics and molecular mechanisms that regulate their formation, along with DHN-melanin biosynthesis, may lead to novel disease control targets or approaches. Previously, a *V. dahliae* homolog of *Aspergillus fumigatus Aayg1* (Fujii et al., 2004) was expressed at 165-fold higher levels in melanized microsclerotium-producing cultures than in non-pigmented amicrosclerotial cultures by RNA-Seq analyses (Duressa et al., 2013). The *A. fumigatus Aayg1* gene is responsible for polyketide chain-length shortening, and catalyzing the conversion of the heptaketide naphthopyrone (YWA1) to 1,3,6,8-THN (Fujii et al., 2004), a key initial step during melanin biosynthesis in some fungi. However, homologs of *Aayg1* are not present in all fungal species, including several species in the *Fusarium* genus (Klosterman et al., 2011).

There is limited amount of published information on the function of *Aayg1* and its homologs. Only its homologs in *Exophiala dermatitidis* (*Wayg1*) and *Botrytis cinerea* (*Bcygh1*) have been shown to be required for catalyzing 2-acetyl-1,3,6,8-tetrahydroxynaphtha lene (AT4HN) derived from PKS1 to melanin in *E. dermatitidis* or catalyzing one PKS product in conidia pigment biosynthesis pathway in *B. cinerea*, respectively (Schumacher, 2016; Wheeler et al., 2008). Though functions of *Aayg1* homologs in melanin biosynthesis pathway are quite conserved, a couple of unique features have been identified. For example, in contrast to the wild-type conidia that are covered with rodlet structure, some patches of organized rodlet layers were observed on the conidial surface of *Aayg1* deletion mutants in *A. fumigatus*, indicating that the cell surface defects resulted in defective virulence (Bayry et al., 2014). The roles of melanin production related genes in pathogenicity have been well studied for many plant fungal pathogens, including *Magnaporthe oryzae* and *Colletotrichum* species (Chumley and Valent, 1990; Howard and Ferrari, 1989). However, the role of *Aayg1* homolog has not been elucidated in *V. dahliae*. Although several genes have been identified to be involved in microsclerotial formation or infection process in *V. dahliae* (Gao et al., 2010; Rauyaree et al., 2005; Tzima et al., 2011), mechanism of microsclerotial formation still remains unclear.

In the present study, we demonstrated the involvement of *Vayg1* in melanin production and microsclerotial formation by characterization of the phenotypes in deletion mutants of the single *Aayg1* gene homolog in *V. dahliae* (*Vayg1*) and its complementation and co-incubation the deletion mutant with the wild-type strain. Results showed that deletion of *Vayg1* prevented not only the melanin production but also microsclerotial formation. Given the dual requirement of *Vayg1* for both microsclerotium production and melanin biosynthesis, we hypothesize that *Vayg1* may catalyze two precursors, one of which is like YWA1 or At4HN that is essential for melanin production and the other is involved in the signal network for microsclerotial formation in *V. dahliae*. In addition we have obtained data which suggested that the *Vayg1* deletion mutant also had reduced virulence.

2. Materials and methods

2.1. Fungal isolates and culture conditions

The virulent, defoliating *V. dahliae* strain JY, isolated from cotton in Shaanxi province, China (Hu et al., 2013), was used as a wild-type (WT) strain. The transformants produced in the present study were derived from strain JY, and stored as suspensions of conidia in 25% glycerol at −80 °C. The WT strain was inoculated onto potato dextrose agar (PDA) medium or into potato dextrose broth (PDB) unless otherwise specified. Hygromycin B-resistant ($HygB^r$) strains and geneticin (G418, Invitrogen, USA)-resistant strains were grown on Czapek Dox agar or in liquid Czapek Dox medium amended with 50 μg/ml hygromycin B or 200 μg/ml G418, respectively. For microsclerotium formation assays, conidial suspensions were spread evenly on basal agar modified medium (BMM) (Hu et al., 2013). Conidia harvested from liquid Czapek Dox medium were re-suspended to approximately 2×10^6 spores/ml in sterile distilled water for pathogenicity assays.

2.2. Generation of constructs and fungal transformations

The OSCAR method (Paz et al., 2011) was used to construct *Vayg1* deletion vector with the primers used to amplify the flanks (Supplementary Table S1). Then the deletion vector was cloned into *Agrobacterium tumefaciens* strain EHA105. After *A. tumefaciens*-mediated transformation (ATMT) of *V. dahliae*, hygromycin B-resistant transformants were obtained and went through the single-spore purification process as described previously (Dobinson, 1995). To verify the mutation in the *Vayg1* deletion strains, PCR amplification with the primer pairs Hyg-F/R and RT-F/R (Supplementary Table S1) and DNA blot hybridizations with the probe generated from primers Ph-F/R and PV-F/R (Supplementary Table S1) were carried out.

For *Vayg1* mutant complementation, a 2.6 kb fragment spanning the promoter region and open reading frame of the *Vayg1* homologous gene was amplified via PCR with the primer pair Vayg1C-F/R (Supplementary Table S1). The PCR product was purified, sequenced, and then cloned into the linearized plasmid pFL2 which contains *neo* (Zhou et al., 2011) by means of yeast *in vivo* homologous recombination as previously described (Bruno et al., 2004). The resulting construct, p*Vayg1*, was transformed into the *Vayg1* deletion mutant strain using the polyethylene glycol-mediated protoplast transformation approach (Wei et al., 2004). Transformants resistant to G418 were screened by PCR analyses using the primer pair neo-F/R (Supplementary Table S1) and further verified by Southern blot analysis with the probe PV-F/R (Supplementary Table S1). Finally, the transcript levels of *Vayg1* in the deletion mutant and complemented mutant were quantified by RT-PCR with the *Vayg1*-specific primer pair q-F/R (Supplementary Table S1), and the *β-tubulin* gene was used for normalization (Duressa et al., 2013; Hu et al., 2014).

2.3. Southern blot analysis

Genomic DNA was extracted from *V. dahliae* cultures that were incubated in liquid Czapek Dox medium for 1 week using a DNeasy® Plant Mini Kit (Qiagen, Germany). Ten μg of the total DNA extracted from each culture was digested with PstI; the digested genomic DNA was separated on a 0.7% agarose gel and then transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech, USA). The appropriate deletion or complementation

sequence was used for probing the *hph* gene (hygromycin B phosphotransferase, conferring resistance to hygromycin B) or *Vayg1*, respectively. Hybridization was performed using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). A DIG-labeled DNA molecular marker (Roche) was used to estimate hybridization fragment sizes.

2.4. Evaluation of colony morphology and microsclerotium formation

Morphological evaluations of the WT, two *Vayg1* deletions (Δ*Vayg1-1* and Δ*Vayg1-2*) and the *Vayg1* complementation strains were conducted on Czapek Dox agar medium. For the analysis of microsclerotium production, BMM plates were prepared as described previously (Hu et al., 2013). The cultures of the WT, two *Vayg1* deletions (Δ*Vayg1-1* and Δ*Vayg1-2*) and complementary mutants in BMM plates were used for observations under an Olympus BX51 microscope (Olympus, Japan) at 1, 2, and 4 weeks post incubation at 20 °C in dark.

For localization of *Vayg1*'s expression in *V. dahliae*, the complementary mutant containing *Vayg1*-GFP construct was used to perform green fluorescence observation with an Olympus BX51 microscope (Olympus, Japan) after 2 weeks of incubation on Czapek Dox agar medium at 25 °C.

2.5. Co-incubation assays

In order to determine whether microsclerotial formation and melanin production in the deletion mutant could be restored in the presence of chemical precursors, the *Vayg1* deletion mutant Δ*Vayg1-1* was co-incubated with WT strain on the same PDA plate for two weeks assuming that chemical compounds (precursors) needed for microsclerotial and melanin can be dispersed in the media. Then microsclerotial formation in Δ*Vayg1-1* was monitored under a microscope; melanin production was visually assessed. Both melanised and non-menalised microsclerotia were observed in Δ*Vayg1-1*. To assess whether microsclerotial or melanin production was permanently restored in Δ*Vayg1-1*, four mycelium plugs from the melanized colony edge or non-melanized center of Δ*Vayg1-1* were incubated separately on PDA for two weeks to check. Experiments were triple replicated.

2.6. Analysis of gene expression

For analyses of gene expression of *Vayg1* at different stages of microsclerotium development in the WT, cultures of the WT were prepared in BMM plates (Hu et al., 2013). Samples were collected at different time points: 60, 72, 96 and 336 hpi (hours post inoculation), representing four stages of microsclerotium development in the WT strain, similar to those described for another *V. dahliae* strain (Xiong et al., 2014). In order to assess the effects of *Vayg1* deletion on melanin biosynthesis, expression levels of six melanin biosynthesis related genes (*T4HR*, *SCD*, *T3HR*, *laccase*, *Cmr1* and *Pig1*) were quantified in samples from BMM plates after inoculation with the WT, three *Vayg1* deletion mutants (Δ*Vayg1-1*, Δ*Vayg1-2*, and Δ*Vayg1-7*) and the Δ*Vayg1*-C strain for 60, 72, 96 and 336 hpi. All samples were stored at −80 °C until use. Approximately 100 mg of fungal materials was used for each sample and was ground into fine powder in liquid nitrogen.

Total RNA was isolated with TRIzol Reagent (Invitrogen, USA) and cDNA was synthesized with the primerScript RT reagent Kit (TaKaRa, Japan) following the manufacturer's procedure. The qRT-PCR was performed to analyze *Vayg1* expression with the primer pair q-F/R (Supplementary Table S1) with the *V. dahliae* β-*tubulin* gene (Supplementary Table S1) used for normalization (Duressa et al., 2013; Hu et al., 2014). The amplification reaction was carried out using 2 × UltraSYBR green Mixture (ComWin Biotech, Beijing, China) and the ICycler instrument iQ5 (Bio-Rad Laboratories, CA, USA). Amplification of each sample was conducted in quadruplicate. The results were analyzed by iQ5 software 2.1. Expression ratios were calculated from CT values using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mean and standard error of gene expressions were estimated from three biological replicates.

2.7. Virulence assays

Virulence assays were performed on the susceptible cv. JM11 cotton seedlings that were inoculated at the two true leaf stages using a root dip-inoculation method (Zhang et al., 2013). Cultures of the WT, the *Vayg1* deletion mutant Δ*Vayg1-1* and Δ*Vayg1*-C strains were incubated in liquid Czapek Dox medium for 7 days. Conidia were harvested from liquid Czapek Dox medium and re-suspended to approximately 2×10^6 spores/ml in sterile distilled water for the pathogenicity assays. Seedlings were removed from pots and washed under tap water; approximately 2 cm was trimmed from the root tips. Inoculations were performed by dipping the seedling roots in conidial suspensions of each *V. dahliae* strain for 15 min and then replanting each in pots of 15-cm-diameter. The control was mock-inoculated with sterile distilled water for 15 min. Disease severity of each seedling was recorded at 6 wpi (weeks post inoculation) on the following five categories: 0 = healthy, no symptoms on leaves; 1 = one or two cotyledon leaves showing symptoms; 2 = a single true leaf showing symptoms; 3 = more than two leaves showing symptoms; and 4 = plant dead. The assay was repeated three times for each strain. At each time for each strain, there were three pots, each with five seedlings. Plant height was recorded at 6 wpi. At 6 wpi, seedlings were removed from soil and washed under running water. The degree of discoloration of vascular tissue was recorded visually on the root and stem that were longitudinally cut. A scale of 0–5 was followed to assess the disease severity: 0 = no vascular discoloration, 1 = less than 25% discoloration of vascular area, 2 = between 26% and 50%, 3 = between 51 and 75%, 4 = between 75 and 100% (but <100%), and 5 = complete discoloration. An overall disease index was calculated as $\frac{\sum_i^N \times i}{M\sum_i^N} \times 100$, where N_i is number of plants with a disease score of i and M (=4) is the maximum scale level value.

2.8. Assays for root adhesion, penetration and colonization

Roots of JM11 cotton seedlings (c.a. two true leaves stage) were placed in the Erlenmeyer flasks with the conidial suspension of the Δ*Vayg1-1*, Δ*Vayg1*-C, and the WT strains at a concentration of 10^6 - spores/ml, and incubated at 25 °C, 120 rpm for 72 h. Adhesion of germinating conidia to the root surface was observed macroscopically after inoculation (Di Pietro et al., 2001). To observe fungal penetration of plant tissues, onion epidermis sections were inoculated with conidial suspensions (1×10^4 spores/ml) of each genotype. After incubation for 48 h in a humid chamber at 25 °C, the epidermis was stained with Coomassie Brilliant blue followed by microscopic examination (Yang et al., 2013). Cotton tissue samples from root, stem (between soil line and the cotyledon point), and leaves of 11 inoculated plants were collected at 4 wpi for *in planta* quantification of fungal biomass. DNA was extracted and quantified by Nanodrop 2000, and for each sample 100 ng of DNA was employed for quantitative real time PCR reaction with the primer pair Vd-F/R (Tzima et al., 2011). The cotton *ubiquitin* gene (CK738219) was used for normalization (Dowd et al., 2004; Mcfadden et al., 2006) using primers ubiquitin-F/R (Supplementary Table S1). All of the assays for root adhesion, penetration and colonization were repeated three times.

2.9. Statistical analysis

Statistical analyses were performed using R software (v 3.0.2). One-way ANOVA was applied and followed by Student–Newman–Keuls (SNK) test to determine significant differences between treatments at P = 0.05.

3. Results

3.1. Identification of V. dahliae Vayg1

The *Vayg1* sequence, corresponding to locus *VDAG_04954* (Broad Institute designation), was used as a query to search the public *V. dahliae* genome database. The result revealed that *Vayg1* is a single copy gene in the genome of *V. dahliae* strain VdLs.17, encoding a protein with a predicted length of 418 amino acids, similar in length to Aayg1 from *A. fumigatus* and Wayg1 from *E. dermatitidis* (Fig. 1A). BLAST analysis of *Vayg1* complement sequence from the *V. dahliae* strain JY in National Center for Biotechnology Information databases showed an 100% identity with the VdLs.17. Analyses of the Vayg1 amino acid sequence revealed the presence of an esterase/lipase (Aes) (COG0657.1) domain, and alignments of Vayg1 with Aes family proteins revealed the presence of conserved amino acid residues T201, E228, S273, R289 and H396 (Fig. 1A), which are important for enzyme activity (Fujii et al., 2004; Wheeler et al., 2008). BlastP analysis showed that the amino acid sequence of Vayg1 shares a close relationship with the deduced sequence of Aayg1 from *A. fumigatus* (55% identity) and Wayg1 from *E. dermatitidis* (50% identity), which had been investigated for their functions in converting the PKS product of YWA1 or AT4HN into 1,3,6,8-THN respectively, a further modified step in the DHN pathway to produce melanin (Fujii et al., 2004; Heinekamp et al., 2012; Tsai et al., 2001; Wheeler et al., 2008) (Fig. 1B). No similar homolog was found in *Saccharomyces* spp., although phylogenetic analysis revealed close relationships with uncharacterized Vayg1 homologs in some other filamentous fungi (Fig. 1C).

3.2. Disruption and complementation of the Vayg1 gene

After construction of recombinant plasmid and ATMT, the *V. dahliae Vayg1* gene (*VDAG_04954*) was deleted by homologous recombination (Fig. 2A). Deletion mutants were obtained after screening hygromycin-resistant transformants by PCR analysis, using the primer pairs of Hyg-F/R and RT-F/R (Supplementary Table S1). In total we obtained three *Vayg1* deletion mutants which had the identical morphology. Southern blot analysis using a 520 bp PCR product of *hph* as the probe (*hph*-probe; Fig. 2A), applied to only one of the three mutants (Δ*Vayg1-1*), indicated a

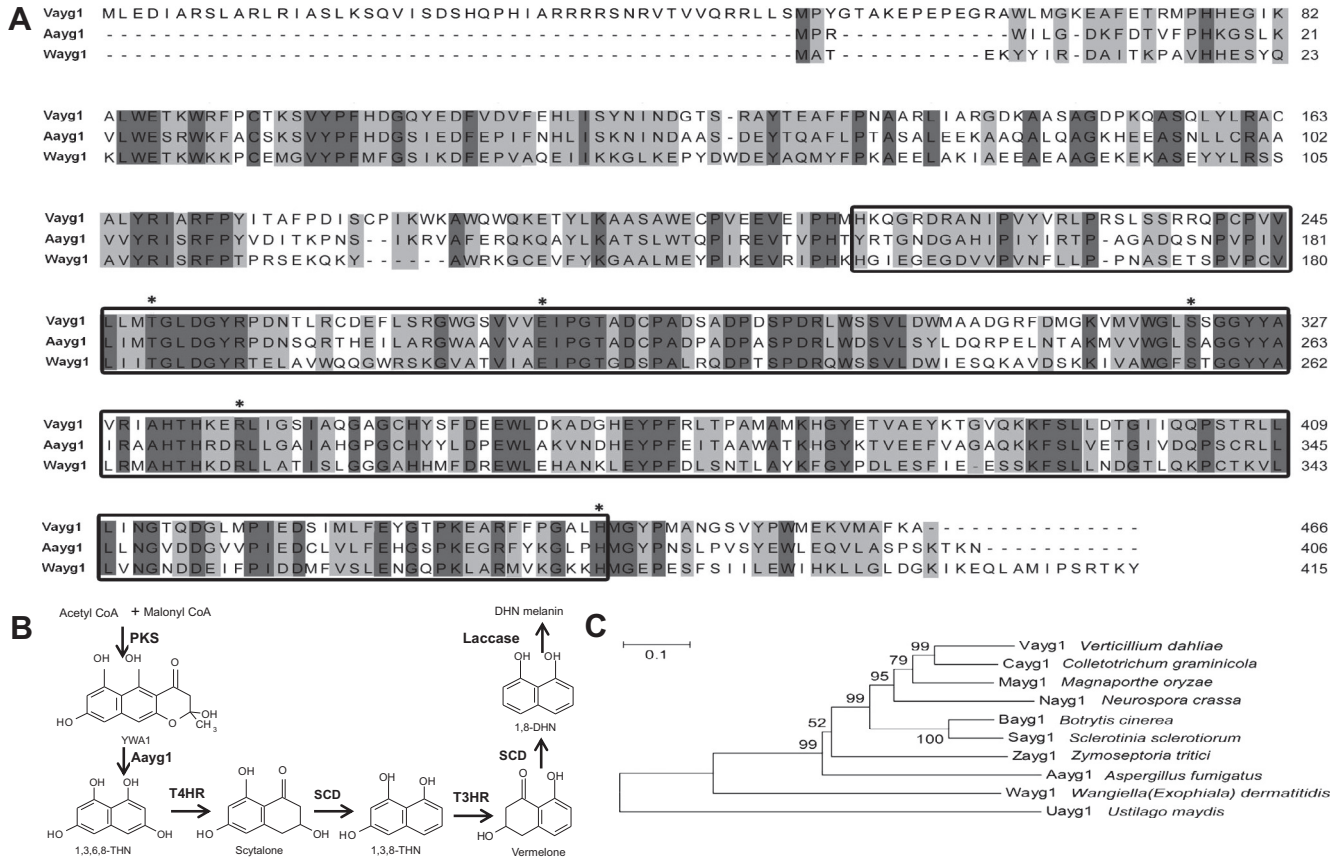

Fig. 1. Sequence alignment and phylogenetic analysis of the *Verticillium dahliae* Vayg1 protein with related fungal homologs. A. Amino acid alignment of Vayg1 with Aayg1 from *Aspergillus fumigatus* (AHA42449.1) and Wayg1 from *Exophiala dermatitidis* (AAT81166.2). Conserved amino acid residues are shaded. The boxed areas indicate the esterase/lipase (Aes) enzymatic domain, where the conserved amino acid residues for the enzyme activity are indicated by the asterisks. B. The DHN melanin biosynthesis pathway in *A. fumigatus*. The figure was based on the original published in The Journal of Biological Chemistry. Tsai et al. Pentaketide melanin biosynthesis in *Aspergillus fumigatus* requires chain-length shortening of a heptaketide precursor. J Biol Chem. 2001; 276: 29292–29298. © the American Society for Biochemistry and Molecular Biology. C. Phylogenetic tree of Vayg1 using a neighbor-joining method with homologs from other fungal species. In addition to the homologs described above, accession numbers of homologs and fungal sources are as follows: *Colletotrichum graminicola* (Cayg1), EFQ29456.1; *Magnaporthe oryzae* (Mayg1), ELQ40198.1; *Neurospora crassa* (Nayg1), EAA36298.3; *Botrytis cinerea* (Bayg1), EMR86570.1; *Sclerotinia sclerotiorum* (Sayg1), EDN96456.1; *Zymoseptoria tritici* (Zayg1), EGP88990.1; *Ustilago maydis* (Uayg1), XP_011388424.1. Bootstrap percentages over 50% are indicated at the nodes.

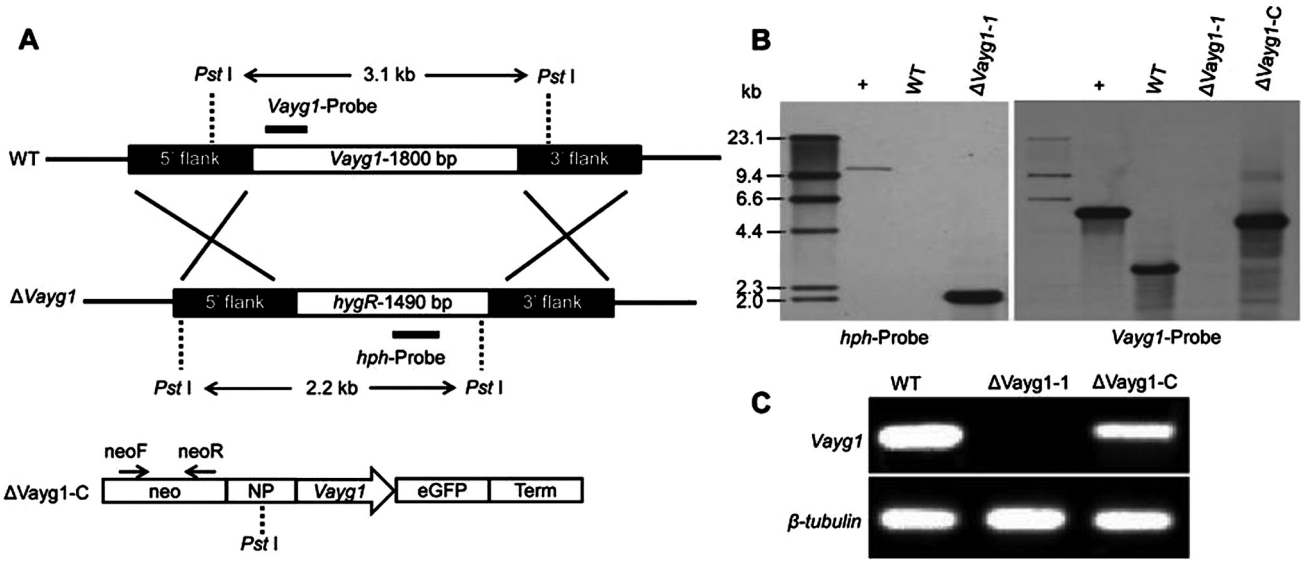

Fig. 2. Disruption and complementation of the *Verticillium dahliae Vayg1* gene. A. Schematic diagram of the strategy for deleting the *Vayg1* gene (*VDAG_04954*) in *Verticillium dahliae* strain JY by homologous recombination. The *Vayg1*-Probe and *hph*-Probe were used in Southern blot analyses. Positions of the restriction site (PstI), the construct components of the hygromycin B-resistance gene (*hygR*), native promoter of the *Vayg1* gene (NP), enhanced green fluorescent protein (eGFP), terminator (Term) sequence from the *M. oryzae β-tubulin* gene, neomycin-resistance sequence (neo), and the PCR primer pair to detect the *neo* gene (neoF/neoR) are shown. B. Confirmation of the gene replacement event by Southern blot analysis. The *hph* gene fragment as probe (*hph*-Probe) was employed to verify insertion and copy number of *hygR* in the *Vayg1* deletion mutant strain Δ*Vayg1-1*. A region of *Vayg1* was employed as the probe (*Vayg1*-Probe) to confirm the presence or absence of *Vayg1* in PstI-digested genomic DNA of the wild-type (WT) strain JY and *Vayg1* mutant strains. The deletion vector and complemented vector were used as positive control (+), respectively. C. Confirmation of the gene replacement event by reverse transcription PCR (RT-PCR) analysis using the primer pair of q-F/R for *Vayg1*; the *V. dahliae β-tubulin* gene was used for normalization.

lack of hybridization signal when probed against the PstI-digested DNA from wild-type strain of *V. dahliae*. In addition, the *hph*-probed blot revealed integration of the hygromycin B phosphotransferase coding region, and its presence as a single copy integrant in the *Vayg1* mutant strain (Fig. 2B). The complementation mutants were confirmed by PCR analysis with primer pairs of Vayg1C-F/R and neo-F/R (Supplementary Table S1) and Southern blot analysis. The probe corresponding to a region of *Vayg1* (*Vayg1*-probe; Fig. 2A) used in Southern blot analyses included a 447 bp PCR fragment. When probed with the *Vayg1*-probe, a 3.1 kb band was observed, corresponding to the anticipated *Pst*I restriction pattern of *Vayg1* in the wild-type strain (Fig. 2B). Hybridization with the *Vayg1*-probe resulted in a band at approximately 5.0 kb in the DNA digest from the *Vayg1* complementation mutant Δ*Vayg1*-C, but not in that of the Δ*Vayg1-1* strain (Fig. 2B). The transcription level of *Vayg1* in Δ*Vayg1-1* and Δ*Vayg1*-C mutants was also confirmed by reverse transcription PCR (RT-PCR) analyses using cDNA of the Δ*Vayg1*, Δ*Vayg1*-C mutants and the wild-type strain; the results were consistent with those of the Southern blot analysis (Fig. 2C). Taken together the results above indicate deletion of *Vayg1* (strain Δ*Vayg1-1*) and mutant complementation with *Vayg1* to produce strain Δ*Vayg1*-C.

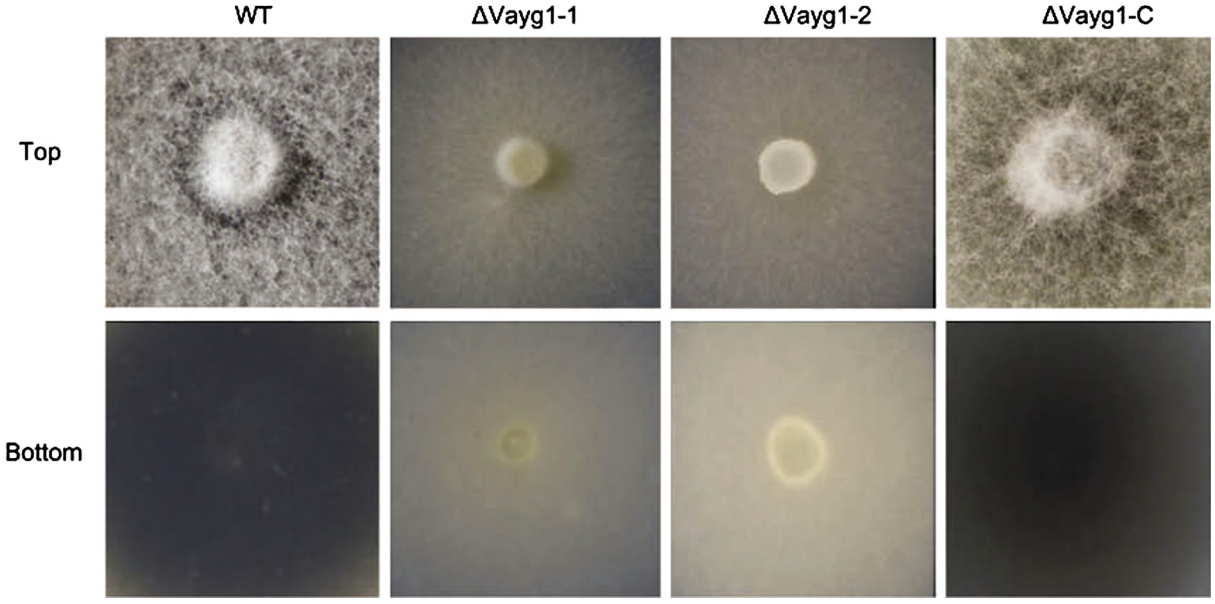

Fig. 3. Colony appearance of *Verticillium dahliae* wild-type (WT) strain JY, the two *Vayg1* deletion mutants (Δ*Vayg1-1 and* Δ*Vayg1-2*), and the complemented Δ*Vayg1* strain (Δ*Vayg1*-C) on Czapek Dox agar medium after two week incubation at 25 °C with top and bottom views.

3.3. Vayg1 is required for microsclerotial formation in V. dahliae

After incubation on Czapek Dox agar medium for two weeks, *Vayg1* deletion mutant strains displayed a significant reduction in aerial hyphae and lacked the typical dark melanization as observed in the wild-type strain under the same culture conditions (Fig. 3). Melanization and aerial hyphae production were restored to the WT levels in the Δ*Vayg1*-C strains (Fig. 3). Cultures of *Vayg1* deletion and complementary strains and the WT strain were assessed under a microscope to monitor microsclerotia development. No microsclerotia were observed for the *Vayg1* deletion mutant strains Δ*Vayg1-1* and Δ*Vayg1-2* at 1, 2 and 4 wpi. However, there were abundant, darkly pigmented microsclerotia in the WT and Δ*Vayg1*-C strains (Fig. 4A). Septate hyphae, but no obvious swollen

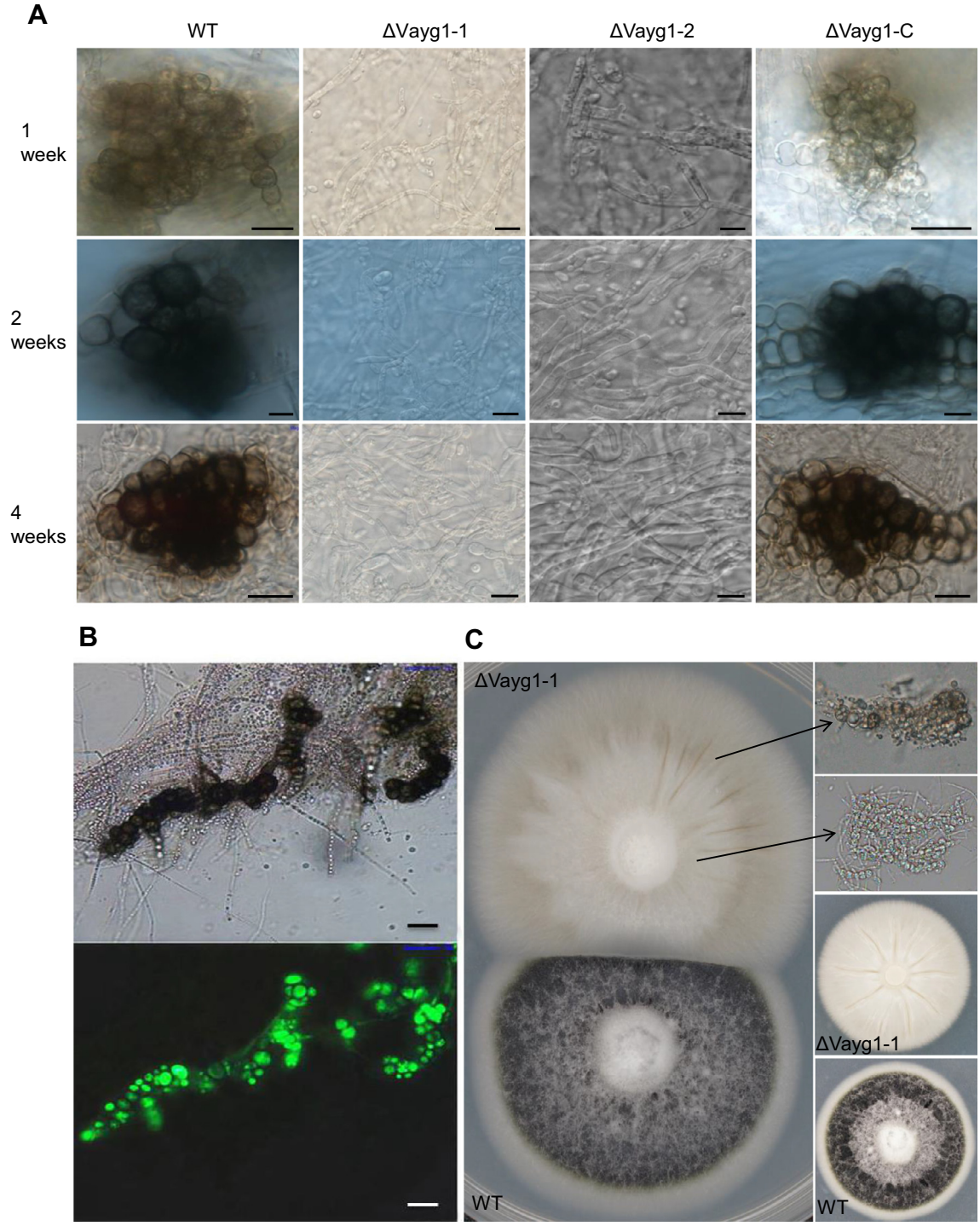

Fig. 4. Assays of the role of *Vayg1* in microsclerotium production. A. Microscopic observation for microsclerotium formation in wild-type (WT) *Verticillium dahliae* strain JY, the two *Vayg1* mutant strains (Δ*Vayg1-1 and* Δ*Vayg1-2*), and the complement *Vayg1* mutant strain (Δ*Vayg1*-C). A spore suspension of 150 μL, adjusted to the concentration of 10^6 spores/ml for each strain, was spread onto BMM and cultivated at 20 °C in dark. Microscopy images were taken of each genotype at 1 week, 2 weeks, and 4 weeks after inoculation. Bar = 20 μm. B. Analysis of the *Vayg1*-GFP fusion in *V. dahliae* by fluorescence microscopy. Bar = 20 μm. C. Morphology of co-incubated Δ*Vayg1-1* with the WT strain and the Δ*Vayg1-1* or WT strain incubated alone on PDA media for two weeks and microscopic observation of restored microsclerotia in co-incubated Δ*Vayg1-1* colony.

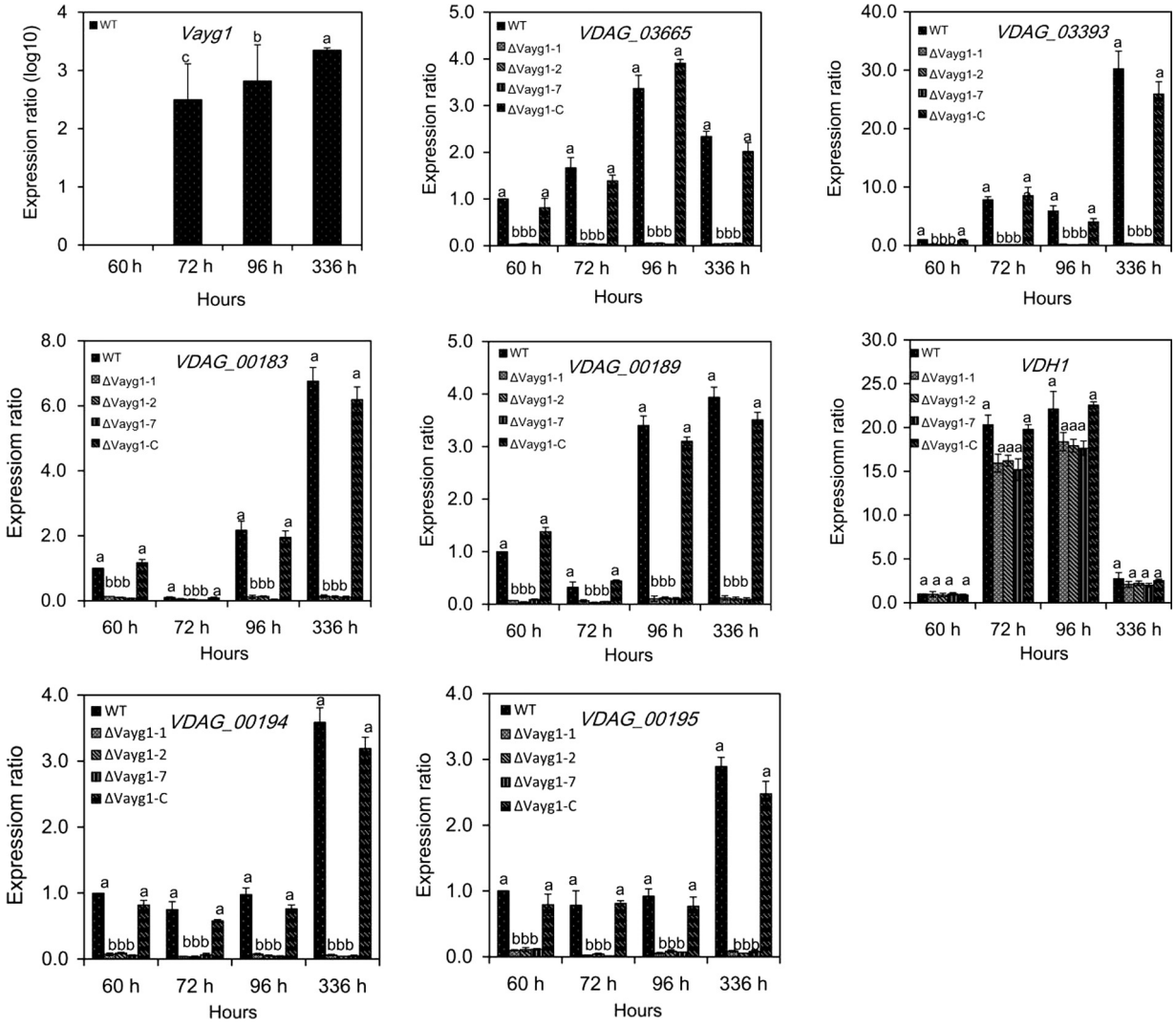

Fig. 5. Quantitative real-time reverse transcription PCR (qRT-PCR) analyses of the expression of *Verticillium dahliae Vayg1* during microsclerotia development in the wild-type (WT) strain and expression levels of six melanin biosynthesis related genes (*VDAG_03665* encoding T4HR, *VDAG_03393* encoding SCD, *VDAG_00183* encoding T3HR, *VDAG_00189* encoding Laccase and *VDAG_00194*/*VDAG_00195* encoding putative transcriptional regulator Pig1/Cmr1 for melanin production) in the WT strain, three *Vayg1* deletion mutants (Δ*Vayg1-1*, Δ*Vayg1-2* and Δ*Vayg1-7*) and the complement *Vayg1* mutant (Δ*Vayg1*-C). RNA was extracted at the time points shown following growth on BMM at 20 °C in the dark. The *V. dahliae β-tubulin* gene was used for normalization (Duressa et al., 2013; Hu et al., 2014). Amplification of each sample was conducted in quadruplicate. The expression ratio of the gene was calculated from cycle threshold (CT) values using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Expression of the genes in cultures of wild-type *V. dahliae* at 60 hpi was set to 1.0. Data are shown as the mean of three independent biological experiments. Vertical bar represents standard error of means. Comparisons of gene expression among strains at specific time point were conducted using an SNK test with R software (v 3.0.2) and $P = 0.05$.

chains of hyphal cells, were observed in Δ*Vayg1-1* and Δ*Vayg1-2* strains (Fig. 4A). Swollen chains of hyphal cells are typically formed in wild-type cultures of *V. dahliae* at the onset of microsclerotial development (Klimes and Dobinson, 2006; Xiong et al., 2014). Localization of *Vayg1* fused with *gfp* gene was observed by fluorescence microscopy. The green fluorescence associated with the GFP fusion protein was present primarily in microsclerotia (Fig. 4B), suggesting that *Vayg1* gene was principally expressed in these resting structures.

Vayg1 deletion mutant Δ*Vayg1-1* strain was also co-incubated with the WT strain in the same plate. After two weeks, microsclerotia were produced in Δ*Vayg1-1* and a melanized colony edge was visually observed (Fig. 4C). Further incubation of mycelial plugs from the melanized colony edge or non-melanized center of this co-incubated Δ*Vayg1-1* colony alone produced identical morphology with the original (not co-incubated with the WT strain) Δ*Vayg1-1* which is incubated alone, i.e. neither microsclerotial formation nor melanin production was observed (data not shown).

3.4. Vayg1 is involved in DHN melanin production

Since microscopic observations indicated a complete lack of microsclerotia in the culture of the two *Vayg1* deletion strains, and further assays were performed to understand the role of *Vayg1* in microsclerotial development. First, the expression of *Vayg1* was tested during microsclerotial development in the WT to confirm its involvement in the process. The BMM plates were inoculated with conidia of the WT strain. The cultures were harvested for quantitative real-time reverse transcription PCR (qRT-PCR) at several time points: 60 hpi when the cultures were of all hyphae, at 72 hpi when cells of hyphae were swollen and initial cells of microsclerotia were observed, at 96 hpi when there was marked increase in accumulation of brown and lightly-pigment microsclerotia, and at 336 hpi when large numbers of mature and melanin-enriched microsclerotia were present (Supplementary Fig. S1). Expression of *Vayg1* was significantly induced in cultures at the 72 hpi, 96 hpi, and 336 hpi developmental time points than at 60 hpi in the WT strain (Fig. 5). Pigment and microsclerotia accumulated

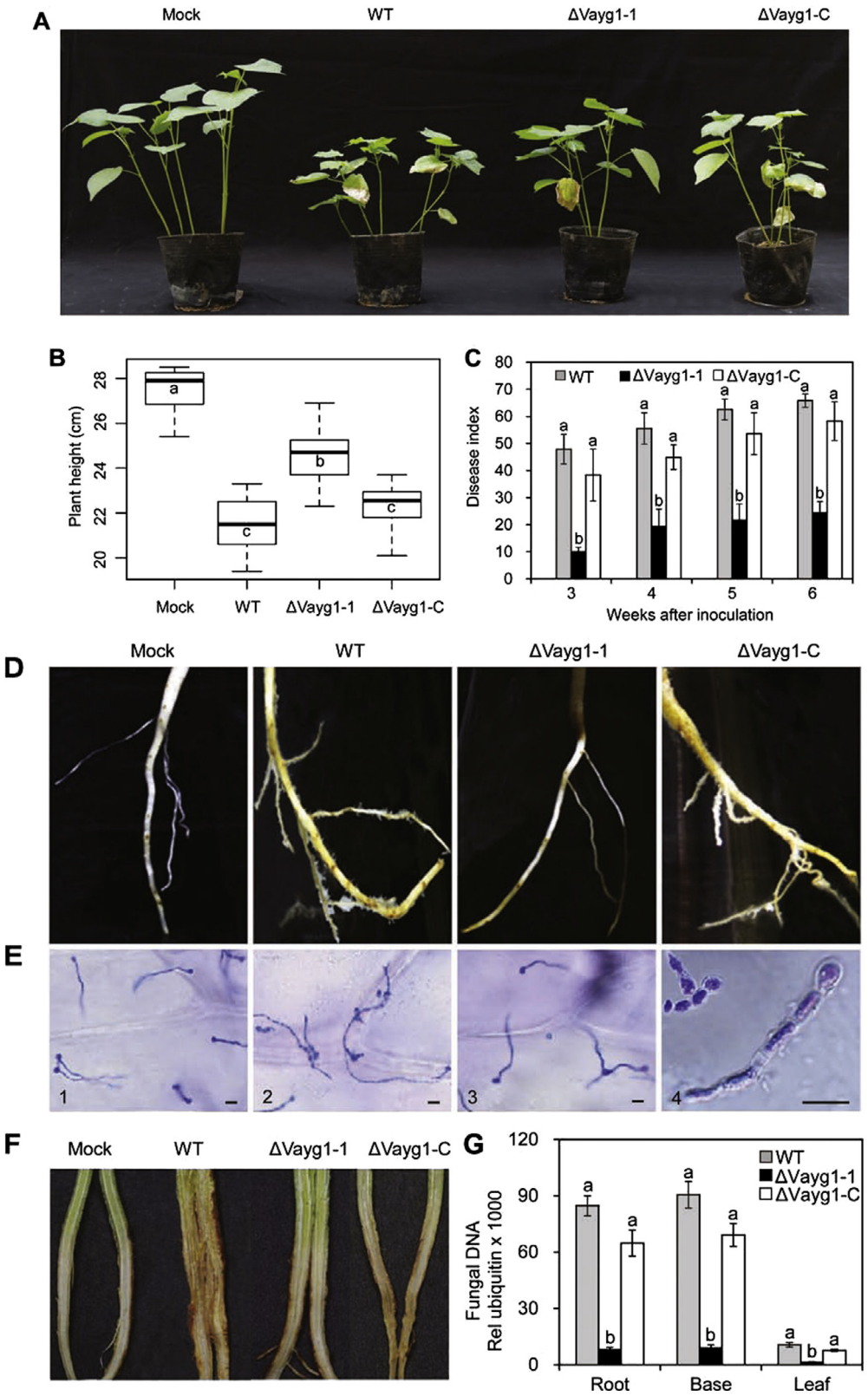

Fig. 6. *Verticillium dahliae Vayg1* is a virulence factor on cotton. A. Cotton cv. JM11 plants were mock-inoculated or inoculated with the wild-type *V. dahliae* strain JY (WT), the *Vayg1* deletion mutant (Δ*Vayg1-1*), or the Δ*Vayg1* complemented strain (Δ*Vayg1*-C) and photographed at 6 weeks post-inoculation (wpi). B. Height of cotton cv. JM 11 plants mock inoculated (Mock), or inoculated with WT *V. dahliae*, the Δ*Vayg1-1* strain, or the Δ*Vayg1*-C strain and examined at 6 wpi. C. Disease index of cotton cv. JM 11 plants with mock-inoculated, or inoculated with WT *V. dahliae*, the Δ*Vayg1-1* strain, or the Δ*Vayg1*-C strain at 6 wpi. D. Attachment of WT, Δ*Vayg1-1*, and Δ*Vayg1*-C strains to cotton roots. Roots of cotton plants with 2 true leaves were immersed for 72 h with gentle shaking in liquid Czapek Dox medium containing conidia of strains mentioned above. E. Penetration assays of WT, Δ*Vayg1-1* and Δ*Vayg1*-C strains. Penetration into onion epidermis sections by the WT (1), Δ*Vayg1-1* (2), and Δ*Vayg1*-C (3) strains 48 h after inoculation. Bar = 10 μm. F. Colonization of WT, Δ*Vayg1-1* and Δ*Vayg1*-C strains in cotton plants at 4 wpi. G. Detection of fungal biomass in infected plant tissues at 4 wpi. Data are shown as the mean of three replicated experiments. Vertical bar represents standard error of means from three biological experiments. At least 11 inoculated plants were included in each independent biological experiment for each strain when testing plant height, disease index and the quantification of fungal biomass. Comparisons of difference among strains were conducted using an SNK test with R software (v 3.0.2) and $P = 0.05$.

in accordance with the increasing incubation time on BMM (Supplementary Fig. S1). Expressions of six melanin biosynthesis related genes were down regulated in the *Vayg1* deletion mutants, while their expression levels were comparable in the WT and in the complemented strain Δ*Vayg1*-C (Fig. 5). In addition, analyses of expression of the *V. dahliae* hydrophobin gene, *VDH1*, which plays an important role in microsclerotium production, were performed. The transcript levels of *VDH1* observed in the deletion mutants were not significantly different from those in the WT and the complement strains at each time point (Fig. 5), suggesting that *Vayg1* was not required for *VDH1* expression.

The *Vayg1* deletion mutant was co-incubated with the WT strain on PDA media for two weeks. Microsclerotial formation was restored and a dark colony edge was visible in the *Vayg1* deletion mutant, differed from the *Vayg1* deletion mutant incubated alone (Fig. 4C). Even though microsclerotia were restored in the center of the *Vayg1* deletion mutant colony when co-incubated with the WT strain after microscopic observation, no visually melanization of microsclerotia was observed (Fig. 4C), suggesting the absence of melanin production in the *Vayg1* deletion mutant.

3.5. Vayg1 is involved in host attachment, penetration and colonization

The susceptible cotton plants infected with the WT and the complement strain Δ*Vayg1*-C showed severe leaf chlorosis at 6 wpi compared to the plants inoculated with the *Vayg1* deletion mutant Δ*Vayg1-1* (Fig. 6A). Cotton seedlings inoculated with Δ*Vayg1-1* were significantly higher than those inoculated with the WT and complement strains but significantly lower than the control plants (mock-inoculated) (Fig. 6B). Disease index on plants inoculated with Δ*Vayg1-1* was significantly less than plants inoculated with the WT (Fig. 6C).

After 24 h of gentle shaking of the cotton roots in conidia of each of the three strains (WT, Δ*Vayg1-1* and Δ*Vayg1*-C), nearly 95% of the conidia of all three strains had germinated which indicated a similar conidial germination efficiency in the three genotypes strains. After incubation for 72 h, germinated conidia of the WT and the Δ*Vayg1*-C strains attached to the root visibly, while a similar attachment was not observed for Δ*Vayg1-1* (Fig. 6D). Coomassie Brilliant Blue was used to stain the *V. dahliae* cells that germinated on the surface of onion epidermis. After incubation for 48 h at 25 °C in humid conditions, samples were stained and evaluated for hyphal penetration. Microscopic observations of these samples revealed that all of the conidia of the WT, Δ*Vayg1-1*, and Δ*Vayg1*-C strains had germinated. Hyphae of the WT (Fig. 6E1) and the *Vayg1* complemented mutant strains frequently penetrated into the onion epidermal cells (Fig. 6E3); however, Δ*Vayg1-1* strain rarely penetrated the epidermal cells (Fig. 6E2). In contrast, most of the Δ*Vayg1-1* hyphae grew along the surface of onion epidermis (Fig. 6E2) and the Δ*Vayg1-1* hyphae were swollen and vacuolated when compared to those of the WT (Fig. 6E4).

Colonization assays showed that disease severity scores, based on xylem discoloration, ranged from 2 to 4 in the plants inoculated with the WT and the *Vayg1* complemented mutant strains, whereas they were below 2 at 4 wpi for those plants inoculated with Δ*Vayg1-1* (Fig. 6F). Compared to the higher biomass observed for the WT and Δ*Vayg1*-C strains, the amount of fungal DNA in the Δ*Vayg1-1* strain infected tissues was significantly lower, in all host tissues examined (leaf, stem and root) (Fig. 6G).

4. Discussion

The DHN-melanin produced during microsclerotial maturation in *V. dahliae* and other fungi is important for protection against a variety of environmental stresses (Butler and Day, 1998), and hence for the long-term survival of the pathogen (Bell et al., 1976; Butler and Day, 1998). In the present study, we characterized the *V. dahliae Vayg1* gene that was shown to be upregulated during development of melanized microsclerotia (Duressa et al., 2013). The present results showed that *Vayg1* gene is required for DHN melanin production and is also involved in microsclerotial formation. In addition, reduced virulence was also observed in the *Vayg1* deletion mutant.

Use of mutation techniques, such as gene knockout and gene disruption, makes it possible to identify genes involved in microsclerotial development in *V. dahliae* (Gao et al., 2010; Rauyaree et al., 2005). Klimes and Dobinson (2006) reported a hydrophobin gene, *VDH1*, is essential for microsclerotial development. A subsequent study confirmed that *VDH1* mediates the development of microsclerotia from conidiophores and other hyphal structure (Klimes et al., 2008). The present study showed that *Vayg1* expression was high during microsclerotial development on BMM. A localization assay monitoring a *Vayg1*-GFP fusion revealed that green fluorescence was mainly present at microsclerotia, consistent with the transcript data (Duressa et al., 2013). The expression of *VDH1*, which is necessary for microsclerotium formation in *V. dahliae*, was not regulated by *Vayg1*, suggesting that the regulation of *Vayg1* and *VDH1* on microsclerotial production in *V. dahliae* is separate or that the precursor that Vayg1 catalyzed is located in the downstream of VDH1. Microsclerotial formation of *Vayg1* deletion mutant was restored when co-incubated with the WT strain in the same plate for two weeks; however, further incubation of mycelial plus from the "restored cultures" failed to produce microsclerotia. This suggests that microsclerotial formation in the *Vayg1* deletion mutant was not restored genetically. The rescue of microsclerotia production in *Vayg1* deletion mutant during co-incubation is probably due to the fact that chemical compounds diffused in the media from WT strain which were easily utilized by the mutant as signaling and/or precursors to form microsclerotia. Thus, we may conclude that *Vayg1* is required for microsclerotial formation and may catalyze a precursor that plays a crucial role in microsclerotial formation. However, further investigation is needed to elucidate the exact roles of *Vayg1* played in microsclerotial formation.

Amino acid alignment of Vayg1 and its homologs in fungi suggested that the conserved amino acid residuals important for enzyme activity in DHN melanin biosynthesis in other fungi are present in Vayg1. Co-incubation with the WT strain partially restored melanin in the colony edge of *Vayg1* deletion mutant. However, when co-incubating the WT *E. dermatitidis* with its albino PKS deletion mutant, WT cultures did not secrete appreciable amounts of soluble melanin metabolites, so the albino failed to darken and did not produce downstream metabolites (Wheeler et al., 2008). Colony edge is the most metabolically active tissue during fungal growth, which may explain why it is the colony edge of *Vayg1* deletion mutants that sufficiently utilized the limited amount of molecules that diffused in the media from the WT strain, leading to observed melanin in the *Vayg1* deletion mutants. Even though microsclerotia were restored in the colony center of *Vayg1* deletion mutants, no melanization was observed in this area, indicating that melanin production in the *Vayg1* deletion mutant is likely not restored. Gene expression data indicated, at least in part, that *Vayg1* played a positive role in regulating a set of melanin biosynthesis genes since the expression levels of these genes were significantly reduced in the *Vayg1* deletion mutants. Considering these findings together, it suggests that *Vayg1* is required for melanin production in *V. dahliae*.

Results in this study also showed a reduction of virulence in the *Vayg1* deletion strain in cotton, and this defect was complemented by the wild-type *Vayg1*. Reduced mycelia growth and sporulation

(Supplementary Fig. S2A and B), and resistance to oxidative stress (Supplementary Fig. S3) in the *Vayg1* deletion mutant might have contributed to the reduced virulence. Hyphae of *Vayg1* deletion mutant adhering cotton roots were reduced than those of the wild-type strain. Adhesive proteins mediate attachment of fungal pathogens to their hosts, and these attachments are crucial at several stages during host–parasite interactions (Braun and Howard, 1994; Hostetter, 2000). Analogous to the proposed function of *Aayg1* in *A. fumigatus* in altering cell adhesion characteristics (Bayry et al., 2014), the present result showed that the *Vayg1* deletion strain of *V. dahliae* has defects in adhesion to cotton roots, which could also be partially attributable to reduced vegetative growth (Supplementary Fig. S2A) of the *Vayg1* deletion mutant. Staining analysis revealed that reduced adhesion may have resulted primarily from a developmental defect of hyphae in the *Vayg1* deletion mutant. In contrast to the hyphae in the WT strain, germinating conidia of the *Vayg1* deletion mutant failed to penetrate host cells but differentiated into swollen and vacuolated hyphal structures and these structures may not produce sufficient turgor pressure or maintain cell-wall rigidity necessary for successful penetration (Howard and Ferrari, 1989; Ludwig et al., 2014).

In summary, phenotypic analyses of the *V. dahliae Vayg1* mutant strains in this study revealed the involvement of *Vayg1* in melanin production, microsclerotial formation, and virulence. Bell et al. (1976) reported that melanin or products in later stage of melanin biosynthesis pathway are not required for microsclerotial formation. However, homologs of Vayg1 in *A. fumigatus* and *E. dermatitidis* were confirmed to catalyze the PKS product to 1,3,6,8-THN, which is located in upstream of melanin production pathway. In addition, *Vayg1* is also involved in microsclerotial formation in *V. dahliae*, and thus we hypothesize that the Vayg1 protein may catalyze two precursors, one of which is essential for DHN melanin production and the other is involved in a signal network for microsclerotial formation in *V. dahliae*. Considering the important role of microsclerotia, further studies are underway aiming at identification of intermediary compound(s) by high performance liquid chromatography (HPLC) in *Vayg1* mutants, which is (are) necessary for microsclerotial formation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.11.003.

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