Distinct Roles for VeA and LaeA in Development and Pathogenesis of *Aspergillus flavus*

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*Aspergillus flavus*, a mycotoxigenic filamentous fungus, colonizes several important agricultural crops, such as maize and peanuts. Two proteins, VeA and LaeA, known to form a nuclear complex in *Aspergillus nidulans* have been found to positively regulate developmental processes in several *Aspergillus* species. Here, an examination of near-isogenic *A. flavus* mutants differing in copy number of veA and laeA alleles (0, 1, or at least 2 each) revealed critical roles for VeA and LaeA in *A. flavus* development and seed colonization. In contrast to the wild type, both null mutants were unable to metabolize host cell lipid reserves and were inhibited by oleic acid in growth assays. The copy number of LaeA but not VeA appeared critical for a density-dependent sclerotial-to-conidial shift, since the multicopy *laeA* (MC*laeA*) strain produced relatively constant sclerotial numbers with increasing population size rather than showing the decrease in sclerotia seen in both the wild-type and MCveA*laeA* strains. The MCveA*laeA* strain yielded an intermediate phenotype. This study revealed unique roles of VeA and LaeA in seed pathogenesis and fungal biology, distinct from their cooperative regulatory functions in aflatoxin and sclerotial development.

*Aspergillus flavus*, an opportunistic pathogen of oil seeds, occurs as a saprophyte in soils worldwide and colonizes several important agricultural crops, such as maize, peanut, and cottonseed, before and after harvest (25, 29, 44). The pathogen generates asexual spores, conidia, as the source of inoculum and overwintering sclerotia which germinate to produce conidia in the subsequent season (12). *A. flavus* and other aspergilli, such as *Aspergillus parasiticus*, can produce the polyketide-derived carinogenic secondary metabolite aflatoxin (25, 33). In the United States, annual yield losses in the million-dollar range from aflatoxin contamination on peanut and maize crops are frequently reported (34). Aflatoxin-contaminated food and feed is also a major problem in developing countries, especially in Asia and Africa (45). Recently, an outbreak of aflatoxin poisoning from maize was reported to have killed a hundred people in Kenya (31). Therefore, measures to control Aspergillus infections and aflatoxin production are urgently needed to protect human and animal health.

The identification and characterization of molecules necessary for *A. flavus* conidial, sclerotial, and aflatoxin production are critical to develop rational control strategies. Recently, a heterotrimeric nuclear complex composed of three proteins, LaeA, VeA, and VeIB, has been found to regulate sporulation and secondary metabolism in the related species *Aspergillus nidulans* (1). Although the role of VeIB is not well defined, several studies have described the function of VeA and LaeA in the aspergilli. VeA is required for cleistothecial production in *A. nidulans* (24) and sclerotial production in both *A. parasiticus* (6) and *A. flavus* (13). In addition, the VeA gene regulates the expression of sterigmatocystin (a precursor of aflatoxin) and penicillin genes in *A. nidulans* (22) and aflatoxin genes in *A. parasiticus* (6) and *A. flavus* (13). The finding that VeA and LaeA are partnered in a transcriptional complex in *A. nidulans* (1) helps explain similarities in VeA and LaeA function. LaeA is a global regulator of secondary metabolite production in aspergilli, regulating the same set of metabolites as VeA in *A. nidulans* and *A. flavus* (21), as well as dozens of putative toxins in the human pathogen *Aspergillus fumigatus* (32). LaeA is also necessary for sclerotial formation in *A. flavus* (21) and affects cleistothecial development in *A. nidulans* (J. W. Bok and N. P. Keller, unpublished data). Northern analysis shows that VeA and LaeA negatively regulate each other at the transcript level in *A. nidulans* (1) and LaeA negatively regulates veA in *A. flavus* (21), leading to the concept of a feedback mechanism maintaining morphological and secondary metabolite differentiation in the aspergilli.

Other factors have been reported which link morphological development with secondary metabolism. Of particular interest are a family of oxylipin-producing oxygenases (encoded by *ppo* and *lox* genes) which have been shown to balance ascospore and conidial production in *A. nidulans* (40, 41) and sclerotial and conidial production in *A. flavus* (19), as well as secondary metabolite production in both species (19, 38). Most recently, a density-dependent switch from sclerotial-to-conidial development in *A. flavus* was found to be affected by oxylipin production (18, 19). Both oxylipin production and the response to oxylipin signaling are dependent on an intact VeA protein (5, 7). VeA is also required for *ppoA* expression, and VeA-PpoA interactions affect both sexual and asexual development in *A. nidulans* (41).

The impact of the loss of these proteins on pathogenesis has been explored to some degree for LaeA and Ppo mutants but not yet reported for VeA. LaeA is a key determinant in aspergillosis caused by *A. fumigatus* and seed rot by *A. flavus* (3,
21), and Ppo loss impacts virulence attributes of A. fumigatus (11, 40), A. nidulans (39), and A. flavus (19). Considering the interdependence of oxypillin function with VeA coupled with the VeA-LaeA interaction, we postulated that VeA mutants would also be impaired in seed pathogenesis in a manner similar to that of LaeA mutants and, furthermore, that both mutants could be affected in density-dependent development. To explore these hypotheses, we created several A. flavus isogenic mutants differing only in copy number of veA and laeA genes, including ΔveA, ΔlaeA, multiplicity laeA (MClaeA), and MCveA strains and a double MC strain (MCveA-laeA). The respective VeA and LaeA mutants exhibited critical differences in cell density responses and invasion of host tissues, despite gross similarities between sclerotial and aflatoxin production.

**MATERIALS AND METHODS**

Fungal strains and growth conditions. The Aspergillus flavus strains used and generated in this study are listed in Table 1. All strains were maintained as stocks in glycerol and grown at 29°C on glucose minimal medium (GMM) amended with appropriate supplements for spore production.

Fusion PCR and vector construction. All primers used in this study are listed in Table 2. The veA replacement PCR products were constructed using fusion PCR following Szewczyk et al. (38). The 1.3-kb fragments upstream and downstream of the veA coding region were amplified by PCR with primers 5′-veA For and Rev for the upstream fragment and primers 3′-veA For and Rev for the downstream fragment, using NRRL 3357 (prototroph) genomic DNA as a template. Next, a 1.9-kb fragment of the pyrG auxotrophy marker gene was amplified from A. fumigatus AF293 genomic DNA using primers A. fumigatus pyrG For and Rev. These three amplified PCR products were cleaned with a QIAquick gel extraction kit (Qiagen), quantified, and fused using published procedures (38). The PCR product was amplified with primers Nested For and Rev (38). All PCR steps were performed using an Expand long template PCR system (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The final construct was confirmed with endonuclease digestion and PCR using primers Int veA For and Rev for internal veA and primers A. fumigatus pyrG For and Rev for pyrG. The veA complementation vector was constructed in two steps. First, the 1.9-kb A. fumigatus pyrG PCR fragment was amplified and ligated into the pCR2.1-TOPO vector (Invitrogen) to create pSA2.4. Next, a 4.4-kb SpeI fragment containing the A. flavus veA gene was amplified from A. flavus NRRL 3357 genomic DNA with primers MC veA For and Rev and ligated into the SpeI site of pCR2.1-TOPO vector (Invitrogen) to create pSA2.4. The final fusion PCR product (53357.5 bp) was used for replacement of veA with ppyG after gel purification using a QIAquick gel extraction kit (Qiagen) to create strain TSA 1.54. The veA pyrG vector, pSA2.13, was used alone or else co-transformed with pRLM11.1, a vector containing both laeA and niaD (21), to create MC strains with multiple copies of veA alone and MC strains with multiple copies of both veA and laeA (TSA 2.46 and TSA 2.8, respectively, were used for these studies).

| Strain          | Genotype* | Source or reference |
|-----------------|-----------|---------------------|
| NRRL 3357      | Wild type | 18                  |
| NRRL 3357.5    | ppyG      | 18                  |
| TSA 1.54       | ΔveA      | This study          |
| TSA 2.46       | ΔveA:ΔpyrG| This study          |
| TJW 71.1       | ΔveA      | This study          |
| TJW 79.13      | ΔlaeA     | This study          |
| TSA 2.8        | ΔveA:ΔlaeA| This study          |

* ΔA. flavus.

**Correct transformatiens were identified by analyzing genomic DNA using PCR screens followed by Southern analyses. Primers Int veA For and Rev, Nested For and Rev for fatty acids, following the method of Maggio-Hall and Keller (27). Growth diameter was measured following a point inoculation of 5 × 10^5 conidial suspension (ORF) and 10^6 conidial suspension (ORF). The exposed sclerotia were then collected, lyophilized, and weighed (dry weight per plate). Growth diameter was measured following a point inoculation of 5 × 10^5 conidial suspension (ORF) and 10^6 conidial suspension (ORF).**

**Northern analysis. To examine the expression of veA and laeA transcripts, Northern analysis was performed. Fifty-milliliter amounts of liquid GMM were inoculated with 10^6 conidial suspension (ORF).**

**Physiological experiments.** Conidial production, sclerotial formation, and colony diameter were examined for fungal strains following the methods of Horowitz et al. Brown et al. (18, 19), Briefly, 8-mL amounts of 1.6% GMM plus 2% sorbitol agar were overlaid with 3-mL amounts of 0.7% agar GMM plus 2% sorbitol agar containing 10^6, 10^5, and 10^6 spores/plate of each A. flavus strain for culture. For conidial counts, three 1.5-cm plugs from each plate were homogenized in 5 mL of 0.01% Tween 80 (vol/vol) water, diluted to 1 ×, and counted with a hemacytometer. To visualize sclerotium formation, plates were sprayed with 70% ethanol to kill and wash away conidia. The exposed sclerotia were then collected, lyophilized, and weighed (dry weight per plate). Growth diameter was measured following a point inoculation of 5 μL of 10^6 spores/mL for each strain on 30 mL of 1.6% GMM. Cultures were grown at 29°C under continuous light or dark conditions for 3 days (conidia production), 7 days (sclerotia formation), and 3 and 6 days (colony diameter). Each treatment was replicated four times.

To assay for growth on different fatty acids, the wild-type, ΔveA, and ΔlaeA strains were examined for growth on (1) 20 mM oleic acid (6 C), 6 mM oleic acid (18 C), and 4.9 mM erucic acid (22 C) as the sole carbon source, with the fatty acids substituting for the glucose in GMM, or (2) GMM supplemented with these same molarities of fatty acids, following the method of Magni-Hall and Keller (27). Growth diameter was measured following a point inoculation of 5 μL

**TABLE 1. Aspergillus flavus strains in this study**

| Strain          | Genotype* | Source or reference |
|-----------------|-----------|---------------------|
| NRRL 3357      | Wild type | 18                  |
| NRRL 3357.5    | ppyG      | 18                  |
| TSA 1.54       | ΔveA      | This study          |
| TSA 2.46       | ΔveA:ΔpyrG| This study          |
| TJW 71.1       | ΔveA      | This study          |
| TJW 79.13      | ΔlaeA     | This study          |
| TSA 2.8        | ΔveA:ΔlaeA| This study          |

* ΔA. flavus.

**TABLE 2. Primer sequences in this study**

| Primer | Sequence (5′-3′) |
|--------|-----------------|
| 5′F veA For | AAAAAACCTTTACGGATGGATGG |
| 5′F veA Rev | GATGAGGTTGTAATGCTCTCG |
| 5′F pyrG Rev | CTGGTTAGTCTCTTGTTACCT |
| 5′F pyrG Rev | TCGTCTTCCGCTTACTCC |
| A. flavus pyrG For | GACCTCGGAGATGGAGAGG |
| A. flavus pyrG Rev | GAGAACGTGCTGATCGTCT |
| NlaeA Northern For | GATCCGTCATTCATATTTG |
| NlaeA Northern Rev | GAGGCCGAGAGGAGGCACATC |
| afluR Northern For | GACAGTCGCCGAGGAGGAGG |
| afluR Northern Rev | GTTGGACACCATCCCTT |

* Bold characters flag restriction enzyme (SpeI) site.
of $10^6$ spores/ml for each strain on 30 ml of medium. Each treatment was replicated four times. The experiment was repeated twice.

**Seed infections.** For seed/fungal studies, two cultivars (SunRunner and FloRunner) of peanut (*Arachis hypogaea*) and one (Northup King N33-P3) of non-fungicide treatment maize (*Zea mays* L.) were used. All the steps were aseptically performed as described by Kale et al. (21). Briefly, mature peanuts (20 peanut cotyledons) and maize (10 seeds) were surface sterilized and inoculated with suspensions of $10^5$ spores/ml of each respective strain, as well as with a water control (mock inoculation). Seeds were placed in 50-ml Falcon tubes containing either sterile water or the spore suspensions and shaken for 30 min in a rotary shaker at 50 rpm, after which they were placed in a high-humidity chamber. Peanut cotyledons were incubated for 3 days for peanut cultivar SunRunner or 5 days for cultivar FloRunner at 29°C under dark conditions, and maize kernels for 3 days. All seed experiments were repeated three times.

**Histological study.** Infected and control peanut cotyledons of cultivar SunRunner were collected after 3 days of inoculation and sliced with a razor blade into 2-cm pieces which were immersed in ice-cold fixative FAA (3.7% formaldehyde, 5% acetic acid, 57% ethanol in water) in vials with vacuum pressure for 30 min. Tissues were then removed, incubated with fresh FAA overnight, dehydrated through a tert-butanol series following the method of Cseke et al. (10), and embedded in paraffin (Paraplast Plus). Paraffin blocks were sectioned in 10-μm slices, and serial sections were placed on glass slides and incubated at 37°C at least overnight, until tissues adhered to the slides. Dewaxing of tissues and staining with Gomori methenamine-silver (26) were performed in the University of Wisconsin—Madison School of Veterinary Medicine histology services laboratory. For lipid staining in peanut tissues, Nile red (16) was applied to tissues following the method of Tsitsigiannis et al. (41). A tetramethyl rhodamine 5-isothiocyanate filter in a fluorescent microscope (Olympus BX-60 with 546-nm excitation and 585-nm emission filters) was used to observe Nile red-stained tissues.

**Aflatoxin analysis. (i) Extraction from medium.** Eight-milliliter amounts of 1.6% GMM–2% sorbitol agar were overlaid with 3-ml amounts of 0.7% GMM agar plus 2% sorbitol agar containing $10^2$, $10^4$, and $10^6$ spores/plate of each fungal strain. Cultures were grown for 3 days at 29°C under dark or light conditions. Three 1.5-cm plugs from each plate were homogenized in 3 ml of 0.01% Tween 80 (vol/vol) water and vortexed vigorously for 1 min. One milliliter of chloroform was added, and the sample vortexed and incubated at room temperature for 30 min. The mixture was vortexed again and then centrifuged for 15 min. The lower layer was collected, allowed to dry for 3 days, and then resuspended in 100 μl of chloroform, and 40 μl of the suspension was spotted onto TLC plates (Whatman, Maidstone, England) using a chloroform/acetone (95:5, vol/vol) solvent system. Each treatment was repeated three times.

**Aflatoxin analysis. (ii) Extraction from seed.** Peanut cotyledons and maize kernels inoculated as described above were collected in 50-ml Falcon tubes with the addition of 5 ml
of 0.01% Tween 80 and vortexed vigorously for 1 min. One milliliter was removed from each sample for conidium counting prior to aflatoxin extraction. Five milliliters of acetone was then added to the samples, followed by shaking for 10 min in a rotary shaker at 150 rpm. Samples were allowed to stand for 5 min at room temperature, and then 5 ml of chloroform was added to each sample, followed by shaking for 10 min at 150 rpm. Samples were allowed to stand for an additional 10 min at room temperature, vortexed briefly, and centrifuged for 15 min at 2,000 rpm to collect the organic lower phase. This phase was placed in a new tube and then dried completely for 3 days. Five milliliters of 0.1 M NaCl methanol/water (55:45) and 2.5 ml of hexane were added to each tube, and the mixture vortexed vigorously at high speed for 1 min. Samples were centrifuged at 2,000 rpm for 5 min. The hexane layer was collected, the remaining aqueous phase was washed with 2.5 ml of hexane, and then the collection process repeated as described above. The hexane extracts were combined, allowed to dry, and then resuspended in 500 µl of chloroform, and 10 µl of each extract was separated on a silica gel TLC plate using the chloroform/acetone (95:5 vol/vol) solvent system. Each treatment was repeated three times.

Statistical analysis. Statistical differences were analyzed using the JMP software package, version 3.2.6 (SAS Institute, Inc., Cary, NC). Multiple comparisons of results for all strains were calculated for growth diameter, lipase activity, and sporulation on seed. To assess the density-dependent development of each strain, we first obtained the MC laeA and MC veA mutants of A. flavus. A 5-µl amount of a suspension of 10⁶ spores/ml of each strain was point inoculated on 30 ml of 1.6% GMM. Cultures were grown at 29°C under continuous dark or light conditions, and growth diameters measured at 3 and 6 days after inoculation. Letters indicate differences between strains that were statistically significant (P < 0.05) according to the Tukey-Kramer multiple comparison test. Error bars show the standard deviations of the results of four replications. Strains were grown in both light and dark conditions. WT, wild type.

RESULTS

Creation of veA and laeA mutant strains in A. flavus. This study required creating near-isogenic strains varying in the number of laeA and veA alleles in the same A. flavus isolate. As ΔlaeA and MC strains of the genome-sequenced strain A. flavus 3357 already existed (21), the first goal was to obtain near-isogenic strains of A. flavus 3357 with loss of or overexpression of veA.

The sequence of the A. flavus 3357 veA ortholog was obtained by designing primers from the A. flavus ATCC MYA384 veA gene (GenBank DQ296645) (13). The sequences of the two genes were found to be 99% identical. All primers and probes in this study were designed from this sequence (Table 2). Figure 1A shows the strategy of replacement of veA with A. fumigatus pyrG. Transformants were first screened for loss of production of sclerotia on GMM plus 2% sorbitol, medium, a phenotype associated with the A. flavus ATCC MYA384 ΔveA mutant (13). Several asclerotial A. flavus 3357 transformants were identified and their DNA extracted and analyzed by PCR and Southern analysis. Seventeen out of 100 transformants were found to contain the 4.6-kb and 4.3-kb fragments expected of KpnI (Fig. 1B) and SapI (data not shown) digests, respectively, as expected for a veA replacement with A. fumigatus pyrG. One of these strains, TSA 1.54, was chosen for further studies (Fig. 1B). A strain with at least two copies of veA was obtained by transforming NRRL 3357.5 with plasmid pSA3.13. Several strains were obtained, as determined by Southern analysis, and one, the MCveA strain TSA 2.46, was chosen for further studies (Fig. 1B). A strain with at least two copies of laeA was obtained by transforming NRRL 3357.5 with plasmids pSA3.13 and pLRM11.1. One of these transformants, the MCveA-laeA strain TSA2.8, was chosen for further studies (Fig. 1B).

The strains with the six genotypes (the wild type and five mutants) exhibited clear differences in development and morphology, as described below, and additionally, the ΔlaeA strain showed a statistically significant inhibition in growth diameter compared to the growth of most other strains under both light and dark conditions. Conversely, the MClaeA strain’s growth diameter was greater than the growth diameters of most other strains in both light and dark regimes (Fig. 2).

veA and laeA affect each other’s transcription. Kale et al. (21) recently found that laeA expression negatively affects transcription of veA in A. flavus; this result was replicated in our work (Fig. 3). We also found evidence for veA regulation of laeA expression. Although Northern analysis revealed that the ΔveA strain did not show an increase of laeA expression, the MCveA strain had decreased laeA expression compared to that of the wild type. The MCveA-laeA strain showed relatively high levels of expression of both veA and laeA but not as high as the individual MC strains.

We also examined the expression of the aflatoxin-specific transcription factor afIR in all strains. As expected and as previously described (13, 15, 21), there was no afIR expression in ΔveA and ΔlaeA strains. Similarly to the MClaeA strain, both
FIG. 4. Effects of \( veA \) and \( laeA \) allele numbers on density-dependent conidial and sclerotial production in \( A. \ flavus \). Each strain was grown from \( 10^2 \), \( 10^4 \), and \( 10^6 \) spores/plate as described in Materials and Methods. (A) Conidial counts. (B) Sclerotial weight. Letters indicate statistically significant differences (\( P < 0.05 \)) for each strain at different population levels according to the Tukey-Kramer multiple comparison test. Error bars show standard deviations of the results of four replications. WT, wild type.
the MCveA and MCveA-laeA strain showed higher levels of aflR expression than the wild type with this treatment. **Conidial and sclerotial density-dependent production is affected by VeA and LaeA.** A recent study has shown that conidial and sclerotial production is density dependent in *A. flavus*, for which low cell densities resulted in high sclerotial formation and high cell densities in low sclerotial formation, with an inverse effect on conidial production (18). This quorum-like signaling system regulating the sclerotial-to-conidial shift was impaired in oxylipin-generating oxygenase mutants (18,19). Because VeA has been shown to be important in oxylipin signaling responses (40,41) and forms a complex with LaeA in the nucleus (1), we predicted that changes in veA and laeA expression could affect the density-dependent sclerotial-to-conidial shift.

The relative abilities of the wild type and the veA and laeA mutants to form sclerotia and conidia were determined by inoculating 10^2, 10^4, and 10^6 conidia onto GMM plus 2% sorbitol plates which were placed in constant dark at 29°C for 3 (conidia) and 7 (sclerotia) days. Similar to prior results (18), sclerotial production diminished and conidial production increased in the wild type with increasing cell population levels (Fig. 4A and B). The veA and laeA null mutants were incapable of producing sclerotia at any population level and yielded relatively constant levels of conidial production regardless of population levels (Fig. 4A and B).

However, clear differences between effects of loss of or overexpression (MC) of veA compared to the results for cognate laeA mutants emerged in both conidial and sclerotial development. Previous studies have suggested a “balance” in sclerotial and conidial production, i.e., when sclerotial production is low, conidial is high and vice versa (18,41). This appeared to hold true for the ΔlaeA strain (no sclerotial production at any cell density and high conidial counts at all densities) but not the ΔveA strain, for which conidial counts were very low at all population levels (Fig. 4A) despite the lack of sclerotial production (Fig. 4B). The MC mutants also showed clear differences in their density-dependent responses. The MCveA strain still exhibited a density-dependent response in sclerotial production with declining numbers in both light and dark regimes at high population levels (Fig. 4B). This was in contrast to the MClaeA strain, which maintained constant sclerotial numbers at all population levels (Fig. 4B). The MCveA-laeA double mutant exhibited an intermediate response. The trend to increased conidial numbers at high population levels was maintained in the MCveA and MCveA-laeA strains but not in the MClaeA strain (Fig. 4A). These results are summarized in Table 3.

**Density-dependent production of aflatoxin is controlled by LaeA.** We also examined the strains for possible effects of laeA and veA expression on aflatoxin production at all cell densities, as aflatoxin production in the wild type is highest at low population levels (19). Regardless of cell densities, the ΔveA and ΔlaeA strains never produced observable aflatoxin under the growth conditions used here, whereas all the MC strains produced aflatoxin in all treatments (Fig. 5). The MCveA strain also showed a density-dependent decrease of aflatoxin with increasing cell population, similar to the wild type, whereas the MClaeA strain did not, and the double mutant showed an intermediate result. Aflatoxin production correlated with sclerotial production.

**VeA and LaeA are important factors for seed colonization.** Recently, Kale et al. (21) reported that laeA mutants were aberrant in host colonization and aflatoxin production on both peanut and maize seed, but there are no reports for the role of VeA in *A. flavus* pathogenicity. Here, we examined and contrasted colonization attributes of the different veA and laeA mutants on two peanut cultivars and one maize hybrid. Each fungal strain maintained similar growth patterns regardless of the host seed. Figure 6A shows that both null mutants produced fewer conidia than the wild type during growth on seeds, with the ΔveA strain developing significantly fewer conidia than the ΔlaeA strain. Visually, the ΔveA strain was most crippled in its ability to grow on any seed (data not shown). The MCveA and MCveA-laeA strains also produced fewer conidia than the wild type; however, the MClaeA strain was similar to the wild type in conidial production, depending on the host seed, as reported earlier (21). The MC strains also formed sclerotia on the seeds (data not shown).

The colonized seeds were next examined for aflatoxin contamination. All MC strains and the wild type produced aflatoxin in all hosts, in contrast to the lack of aflatoxin production by both the ΔveA and ΔlaeA strain (Fig. 6B). The considerably higher aflatoxin production by some MC mutants in vitro (Fig. 5), however, was not replicated in growth on seed under the conditions in this study.

To further investigate the ability of the strains to colonize seed, histological studies were performed. We were specifically interested in assaying for maceration effects and reasoned that this could be partially measured by host cell lipid utilization. The staining techniques did not show any obvious difference in host penetration by MC strains compared to that of the wild

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**TABLE 3. Summary of density-dependent phenomena in A. flavus mutants**

| Mutation | Conidia | Sclerotia | Conidia | Sclerotia |
|----------|---------|----------|---------|----------|
| None (WT) | +       | +        | +       | +        |
| ΔveA     | ±       | ±        | ±       | ±        |
| ΔlaeA    | –       | –        | –       | –        |
| MCveA    | ±       | ±        | ±       | ±        |
| MClaeA   | –       | –        | –       | –        |
| MCveA-laeA | ±       | ±        | ±       | ±        |

*+, presence of density-dependent development; –, absence of density-dependent development; ±, intermediate result.

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**FIG. 5.** Aflatoxin production of veA and laeA mutants. Aflatoxin from each strain was assessed at three different spore inoculation levels. The experiment was replicated three times, as shown. C, aflatoxin B1 control; WT, wild type.
However, the two null mutants exhibited different host invasion patterns. The results in Fig. 7A and B show that wild-type hyphae penetrated several layers of host epidermal and mesophyll cells, with accompanying dissolution of host lipid reserves. Although the ΔlaeA strain also penetrated the host cells intracellularly, host lipid reserves were largely intact and the cell integrity appeared less damaged (Fig. 7A and B). In contrast, hyphae of the ΔveA strain grew intercellularly in epidermal cells and did not appear to penetrate peanut cells as well as hyphae of other strains (Fig. 7A). This mutant, like the ΔlaeA mutant, was also less able to degrade host cell lipid reserves than the wild type (Fig. 7B). However, an in vitro assay for general lipase activity revealed no significant difference between these strains (data not shown).

The wild type and the ΔlaeA and ΔveA strains were then grown on media amended with different fatty acids either as sole carbon source or supplemented with glucose to determine if there might be any gross difference in the ability to utilize or be inhibited by short-, medium-, or long-chain fatty acids. The results did not support any critical difference between the wild type and the two mutants when grown on a fatty acid as the sole carbon source, but the two mutants showed significant inhibition of growth compared to that of the wild type when cultured on GMM amended with oleic acid (Fig. 8). This experiment was repeated twice with similar results (data not shown).

**DISCUSSION**

In this study, we characterized the function and cross-regulation of VeA and LaeA in *A. flavus* development and pathogenesis. The results, while confirming that VeA and LaeA share functions in regulating aflatoxin and sclerotial production, also demonstrate distinct roles of VeA and LaeA in terms of vegetative growth, conidiation, density-
dependent responses, and pattern of colonization of host tissues.

A requirement for LaeA in density-dependent sensing. Quorum-sensing systems in bacteria contribute to the production of virulence factors and biofilm formation in interactions between bacteria and host (14, 42). In fungi, a quorum-sensing system governing morphological shifts and virulence has been uncovered in the human pathogen *Candida albicans* (17, 35). Recently, oxylipin-deficient lipoxygenase and dioxygenase mutants have been found to affect a newly discovered quorum-sensing-like, density-dependent sclerotial-to-conidial morphology shift in *A. flavus* (18, 19). Because oxylipin signaling is dependent on VeA function (5, 7, 40) and VeA is part of a nuclear complex with LaeA (1), we asked if VeA or LaeA mutants could be affected in this quorum-like morphology shift in *A. flavus*. Both null mutants were blocked in sclerotial formation regardless of cell population, and perhaps due to an inability to produce sclerotia, conidial production was relatively stable for each mutant at all three population levels, although it was much higher in the ΔlaeA strain. The MC strains showed clear differences in density-dependent development in that an extra copy of LaeA but not VeA abolished this quorum-like phenomenon (Fig. 4A and B). To date, there are no chemical data identifying molecules regulating the sclerotial-to-conidial switch in *A. flavus*, although oxylipins are hypothesized to fulfill this function at least in part (18, 19). Quorum-sensing molecules for *Candida albicans* (farnesol and tyrosol) and *Saccharomyces cerevisiae* (phenylethanol and tryptophan) are aromatic alcohols and control the morphological switch from the yeast to filamentous growth in these fungi (8, 9). Interestingly, the yeast-to-filamentous growth switch in the fungus *Ceratocystis ulmi* is attenuated by lipoxygenase inhibitors and may implicate oxylipins in quorum sensing in this tree pathogen (20). We speculate that *A. flavus* MCΔlaeA mutants are aberrant in oxylipin production and/or sensing but that this can be remediated to some degree when VeA levels also increase, as demonstrated by the intermediate density-dependent phenotype of the MCveA-ΔlaeA strain. The effects of gene loss and gain on density-dependent development are summarized in Table 3.

**VeA and LaeA feedback regulation.** Both veA and laeA have been reported to be global regulators of secondary metabolites in *A. flavus* (13, 21), as well as in other aspergilli (2, 6). Here, the results indicate that the MCveA and MClaeA strains—particularly the MClaeA strain—produce more aflatoxin and sclerotia than the wild type. The MCveA-ΔlaeA double mutant did not show increased toxin production compared to that of the single mutants or an additive effect on sclerotial production. Prior work indicated that LaeA negatively regulated veA expression (21), and here, we show evidence for VeA regulation of LaeA (Fig. 3), as was described for *A. nidulans* (1). These results support a mechanism of mutual repression of veA and laeA expression and may explain, in part, a dampening of the expression of both genes in the MCveA-ΔlaeA strain.
compared to the expression of the single genes in the MCreA and MClaeA strains which, in turn, may affect aflatoxin and sclerotial output in the double mutant.

Requirement for VeA and LæA in host cell penetration and degradation. Host lipid reserves are depleted during seed colonization by *Aspergillus* (23, 37), with lipase and esterase activities implicated in seed pathogenesis (37, 39). Both null mutants were impaired in seed colonization, where neither strain could degrade lipid reserves despite hyphal penetration of at least some layers of the host seeds (Fig. 7A and B).

The crippled ability of both null mutants to utilize lipid reserves brings to mind several lipid biosynthesis mutants also impaired in *Aspergillus* colonization of seed, including β-oxidation mutants (28), odead mutants [(delta)12-desaturase] (43), and the oxylipin oxygenase mutants in *A. nidulans* (4, 39) and *A. flavus* (19). The inhibition of both null mutants by oleic acid (not seen in the wild type) (Fig. 8) suggests a possible toxic effect of this fatty acid on these strains which may relate to their impairment in growth on seed. It is less likely that the inhibition is associated with defects in β-oxidation, since the mutants grew equally as well as the wild type on oleic acid as a sole carbon source (data not shown), although we cannot rule out this possibility. Regardless of mechanism, the results of all of these studies together may support lipid utilization and/or signaling as an important factor in *Aspergillus* seed pathogenesis.

Interestingly, the hyphal penetration patterns of the two null mutants as revealed by Gomori staining were quite diverse, indicating loss of degradative enzymes in this strain and may explain its poor production of conidia on host seed. However, we note that the strain is crippled in conidial production on medium also. The relative decrease of conidial production by the ΔlaeA strain on seed (compared to its vigorous conidial production in medium) might be attributable to a loss in lipid degradation or the possible toxicity effects mentioned above. Histology of the MC strains presented an invasion and lipid degradation pattern similar to that of the wild type. The relatively decreased conidial production from these strains is possibly a function of their skewed sclerotial development rather than an inability to obtain nutrients from the seed.

In conclusion, this study provides evidence for distinct roles of LæA and VeA in the development and pathogenesis of *A. flavus* despite the considerable overlapping of functions previously reported (13, 21). The loss of both genes blocks the production of sclerotia and aflatoxin, but under our conditions, only læA overexpression abolishes density-dependent phenomena, including a sclerotial-to-conidial shift and decreased aflatoxin production with cell population increase. The null mutants, while both were reduced in host lipid utilization, displayed distinct cell ingress abilities as reflected in patterns of hyphal penetration of host cells.

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ADDENDUM

A study with some similar conclusions has been published by Duran and colleagues (13a).

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VOL. 8, 2009  
*ASPERGILLUS FLAVUS* LæA AND VeA  
1059
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