

The role of *Aspergillus flavus* *veA* in the production of extracellular proteins during growth on starch substrates

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Abstract The aflatoxin-producer and opportunistic plant pathogenic, filamentous fungus *Aspergillus flavus* is responsible for the contamination of corn and other important agricultural commodities. In order to obtain nutrients from the host *A. flavus* produces a variety of extracellular hydrolytic enzymes. Interestingly, *A. flavus* amylase and protease activity are dependent on the global regulator *veA*, a gene known to regulate morphogenesis and secondary metabolism in numerous fungi. Analysis of starch degradation by fungal enzymes secreted into broths of starch- or corn kernel-based media showed a notable accumulation of glucose in samples of the *A. flavus* control strain while the deletion *veA* sample accumulated high levels of maltose and maltotriose and only a small amount of glucose. Furthermore, SDS-PAGE and proteomics analysis of culture broths from starch- or corn kernel-based media demonstrated differential production of a number of proteins that included a reduction in the amount of a glucoamylase protein in the *veA* mutant compared to the control strain, while an alpha-amylase was produced in greater quantities in the *veA* mutant. Quantitative real-time PCR and western blot analyses using anti-glucoamylase or alpha-

amylase antisera supported the proteomics results. Additionally, an overall reduction in protease activity was observed in the *veA* mutant including production of the alkaline protease, oryzin, compared to the control strain. These findings contribute to our knowledge of mechanisms controlling production of hydrolases and other extracellular proteins during growth of *A. flavus* on natural starch-based substrates.

Keywords *Aspergillus flavus* · *veA* · Hydrolases · Amylases · Proteases · Proteomics

Introduction

Aspergillus flavus is a filamentous, saprophytic fungus distributed worldwide that has the ability to colonize a broad range of organic substrates. Additionally, this fungus can become an opportunistic plant pathogen infecting crops of agricultural importance, including corn (maize), cotton, peanuts, tree nuts, rice, peppers, spices, and figs. *A. flavus* colonizes these agricultural commodities and can subsequently produce mycotoxins such as cyclopiazonic acid, aflatrem (Tokuoka et al. 2008; Zhang et al. 2004), and the well-known aflatoxins (AFs). AFB1 is the most mutagenic and carcinogenic natural compound known (Payne and Brown 1998; Probst et al. 2010; Sweeney and Dobson 1999; Trail et al. 1995; Turner et al. 2003). Ingestion of AF-contaminated food has been associated with hepatotoxicity, teratogenicity, and death in animals (Dvorackova and Kusak 1990; Trail et al. 1995). Consumption of AF is directly correlated with human liver cancer (Ross et al. 1992). These deleterious impacts on health are most prominent in developing countries lacking technologies to monitor and reduce AF levels in crops, and where poor storage conditions often result in greater accumulation of these mycotoxins in the food supply. In developed countries, crops

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exceeding the permitted AF levels are destroyed or reduced in value, leading to significant economic losses to producers. Losses from aflatoxin in the USA, in the hundreds of millions USD annually, are associated with market loss rather than health effects (Wu 2004). For example, losses due to aflatoxin contamination in peanut at the EU standard of 4 µg/kg would be estimated to result in US export losses of \$120 million, Argentina \$75 million, Africa \$40 million, and China \$215 million for a combined peanut export loss in these four regions of about \$450 million annually.

A. flavus is capable of producing a number of hydrolytic enzymes required for successful colonization and toxin production in crops (Dolezal et al. 2013; Mellon et al. 2007). Previous work has demonstrated that induction of AF biosynthesis in *A. flavus* during colonization of corn is the result of starch degradation by fungal amylase activities (Woloshuk et al. 1997). We have shown that the global regulatory gene *veA* is necessary for normal colonization of corn, peanuts, and cotton (Duran et al. 2009) and production of AF in seeds (Duran et al. 2009), as well as in laboratory media (Duran et al. 2007). Numerous studies have shown that *veA* controls both morphological development and secondary metabolism in many fungi (i.e., Calvo 2008 and references therein; Myung et al. 2012). In *A. flavus*, *veA* not only is required for AF biosynthesis, it also negatively controls conidiation while it is necessary for the formation of sclerotia. In the model fungus *Aspergillus nidulans* it has been shown that VeA is transported to the nucleus (Araujo-Bazan et al. 2009; Stinnett et al. 2007) where it forms a complex with other proteins, among them a putative methyltransferase, LaeA, believed to be involved in chromatin remodeling (Bayram et al. 2008). Much like VeA, LaeA is a global regulator involved in *A. flavus* development and AF biosynthesis (Kale et al. 2008) that has also been associated with seed colonization (Amaiike and Keller 2009). Whether the differences observed in the capacity to colonize plant tissues depending on the presence or absence of VeA or other associated proteins are related to a possible alteration in the production of hydrolytic enzymes is unknown.

In this study, we investigated the role of *A. flavus veA* on production of extracellular proteins during growth on starch-based substrates. Our results indicate that the *veA* mutant presents different amylase and protease hydrolytic enzyme activities compared to the control strain. Proteomic analyses also identified a number of other proteins that are differentially produced in the two strains that in addition to amylases and proteases could be critical for successful colonization of corn kernels and other susceptible crops. Similar changes in amylase activity were observed in the deletion *laeA* strain. These findings add to the significance of the *velvet* complex as a broad regulatory mechanism in fungi.

Materials and methods

A. flavus strains used in this study

A. flavus 70 pSL82, a small (S)-sclerotia morphotype derived from transformation of an *A. flavus* 70 *niaD*– strain with the plasmid pSL82 (harboring the *A. parasiticus niaD* gene) and *A. flavus* 70 deletion *veA* (ΔveA) have been previously described (Cary et al. 2007). *A. flavus* CA14 (pSL82; L morphotype), CA14 deletion *veA* (ΔveA), *A. flavus* 3357 strain (L morphotype control), 3357 deletion *laeA* ($\Delta laeA$) (Kale et al. 2008).

Amylase activity analysis

Starch-induction

Conidia (10^6 spores/mL) from Af70 pSL82 and the isogenic ΔveA mutant were inoculated in 500 mL of PMS broth (per liter: 50 g peptone; 3 g (NH₄)₂ SO₄; 10 g KH₂PO₄; 2 g MgSO₄ 7H₂O; 1 mL micronutrient solution; pH to 5.2) in a 1-L flask, and cultured in a rotary shaker incubator at 250 rpm, at 37 °C, for 24 h. Fungal mycelia were collected on sterile miracloth, washed twice with sterile deionized water. Then, approximately 1 g of wet weight of mycelium was transferred to each 50-mL flask containing 25 mL of minimal medium (GMM) (Kafer 1977) with 1 % starch as carbon source. Cultures were incubated at 30 °C on a rotary shaker (250 rpm) and the supernatants were collected at 24, 48, and 72 h. Supernatants corresponding to the respective cultures were filter-sterilized through 0.2-µm low-protein-binding filters. The experiment was carried out with three replicates. A starch-induction experiment identical to the one described was also carried out with the Af 3357 control strain and $\Delta laeA$.

Corn-induction

The experiment was performed in a similar manner as that described above with the following modifications: Aliquots of 0.2 g of initial PMS-grown mycelia from Af70 pSL82 and the ΔveA mutant were transferred to 25-mL flasks containing 9 mL suspension of ground of corn seed, line Va35, in sterile water (24 g/400 mL). Cultures were incubated as described above for starch induction. Supernatants corresponding to the respective cultures were filter-sterilized through 0.2-µm low-protein-binding filters. The experiment was also carried out with three replicates.

Enzymatic reactions

Supernatants were added to 1 % starch solution (pH 5.2) 1:1 ratio, and incubated at 40 °C for 24 h. Samples were then frozen. Three different controls were used: starch solution

with water, boiled supernatant plus starch, and supernatant alone. High-performance liquid chromatography (HPLC) analysis was performed by injecting 20 μ L of each sample into a Waters 1525 HPLC system equipped with a binary pump, a refractive index detector, and a Waters 717 Autosampler. HPLC separation was performed at ambient temperature on a Rezex ROA-Organic acid column. Compounds were eluted using an isocratic mobile phase of 5 mN sulfuric acid at a flow rate 0.60 mL/min. Quantification of glucose, maltose, and maltotriose was carried out based on standard curves generated using HPLC-grade standards (Sigma-Aldrich).

Additionally, amylase activity was examined using maltoheptaose as substrate as previously described (Fakhoury and Woloshuk 1999) with some modifications. Briefly, starch-grown culture supernatants were mixed in a 1:1 ratio with a 1 % maltoheptaose solution. The mixture was incubated at 40 °C for 18 h. The reaction was stopped by heat. Degradation products in each sample were then separated by thin layer chromatography (TLC) using silica gel plates and isopropanol/water/ammonium hydroxide (70:30:1) as solvent system for the mobile phase. Degradation products of maltoheptaose were visualized by spraying the TLC plate with 30 % sulfuric acid and charring at 100 °C for 5 min.

Protease activity assay

A. flavus 70 pSL82 and its isogenic ΔveA mutant were grown in PSM broth at 37 °C on a rotary shaker (250 rpm) for 16 h. Then approximately 1 g of mycelia was transferred into 50-mL flasks containing 25 mL of minimal medium with 0.01 % glucose and 8 mg/mL bovine serum albumin (BSA). Triplicate cultures were incubated at 250 rpm and 30 °C. Supernatants were collected at 24, 48, and 72 h after transfer by filtration through low-protein-binding filters (0.2 μ m). Protease activity was evaluated by an azocasein assay carried out as described with some modifications (Reichard et al. 1997). Azocasein (Sigma MO USA) at a concentration of 5 mg/mL was dissolved in 50 mM Tris buffer (pH 7.5), 0.2 M NaCl, 5 mM CaCl₂, 0.05 % Brij 35 and 0.01 % sodium azide. Supernatants were added to azocasein solution (1:4) and incubated for 90 min in a 37 °C stationary water bath. Twenty percent trichloroacetic acid (TCA) was then added to stop the reactions. Samples were kept at room temperatures for additional 30 min, followed by centrifugation at 8,000 $\times$ g for 3 min at 4 °C. Three hundred microliters of supernatant was then mixed with 300 μ L of 1 M NaOH. The absorbance obtained by the release of the azo group was measured in 96-well flat-bottom plates (BD Falcon) using a plate reader (Biotek) at 436 nm in duplicates. Distilled water mixed with azocasein was used as blank control.

Proteomics analysis

Isolation of secreted proteins

Fungal spores (10⁶ spores/mL) of *A. flavus* 70 pSL82 and corresponding ΔveA strain were inoculated into 250 mL PMS broth. Cultures were incubated on a rotary shaker for 16 h at 30 °C and 250 rpm in the dark. Fungal mycelia were collected on sterile miracloth, washed twice with sterile deionized water. Two grams of wet weight of mycelia was transferred into 125 mL of starch minimal medium or corn kernel medium. Corn kernels (Va35) were ground in a Wiley mill (no. 20 mesh screen) and the corn kernel medium prepared by mixing 25 g ground corn with 1 L deionized water prior to autoclaving. Cultures were incubated on a rotary shaker for 48 h at 30 °C and 250 rpm in the dark. Culture supernatants were then harvested by filtration through miracloth followed by centrifugation (2,500 $\times$ g for 20 min, 4 °C) to remove insoluble material. Collected mycelia were frozen with liquid N₂ and stored at –80 °C for subsequent RNA isolation. Supernatants (approximately 100 mL) were concentrated by lyophilization for 48 h followed by resuspension in 3.0 mL sterile deionized water. Samples were aliquoted (100 μ L) and stored at –80 °C. Sample protein concentration was determined using a Nanodrop (Thermo Scientific) and normalized to BSA as a standard. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; NuPAGE® Novex 4–12 % Bis-Tris or 7 % Tris-acetate; Life Technologies) was performed according to the manufacturer's instructions. Gels were stained with GelCode™ Blue Safe protein stain (Thermo Scientific) and imaged on a Licor Odyssey imaging system (Li-Cor Biosciences). Proteins of interest were manually excised from gels with a scalpel and transferred to microfuge tubes for in-gel digestion with trypsin. When a protein of interest from either strain was excised from a polyacrylamide gel, the corresponding gel region for the other strain was also excised for comparative purposes. Samples of proteins secreted into culture broths were prepared using methods similar to those described (Paper et al. 2007). Briefly, equivalent amounts of protein from each sample were filtered through 7-kDa molecular weight cutoff Zeba Spin Desalting Columns (Fisher Scientific), and then precipitated by the addition of 0.25 volume of 100 % TCA. Following the addition of TCA, samples were incubated at 4 °C overnight and then centrifuged for 30 min. The resulting pellets were washed with acetone and then thoroughly dried to remove acetone.

Protein sample digestion and analysis

Protein samples excised from gels were cut into small cubes, washed twice with deionized water, shrunk by incubation with acetonitrile, and then re-swelled in 100 mM ammonium carbonate solution. Samples were again shrunk with acetonitrile

and followed by drying under vacuum. The gel samples were re-swelled in 100 mM ammonium bicarbonate with 10 mM DTT and incubated at 37 °C for 30 min. After removing the supernatant, 100 mM ammonium bicarbonate with 50 mM iodoacetamide was added and the samples were incubated at room temperature in the dark for 30 min. The solution was removed and the samples were again shrunk with acetonitrile and dried under vacuum. Samples were digested in 50 mM ammonium bicarbonate containing CaCl_2 (1 mM) and 1 μl of 0.2 $\mu\text{g}/\mu\text{l}$ Sequencing Grade Modified Porcine Trypsin (Promega). Following incubation at 37 °C overnight with gentle agitation, the supernatant was collected by washes with 50 mM ammonium bicarbonate. The supernatants for each sample were pooled, dried under vacuum, and resuspended in 5 % formic acid prior to mass-spectrometric analysis.

TCA-precipitated samples were resuspended in 0.1 volume of 50 mM ammonium bicarbonate and incubated at 37 °C for 30 min following the addition of dithiothreitol (DTT, 4 mM final). Samples were then cooled to room temperature, iodoacetamide was added to 15 mM final, and samples were incubated in the dark for 30 min. Samples were digested and acidified as above prior to analysis via LC/MS/MS using an Agilent 1200 LC system, an Agilent Chip-cube interface, and an Agilent 6520 Q-TOF tandem mass spectrometer (Agilent Technologies, Santa Clara, CA). Chromatographic peptide separation was accomplished using a Chip consisting of a 40 nL enrichment column and a 43 mm analytical column packed with C18, 5 μm beads with 300A pores. One microliter of aliquots of the sample were transferred to the enrichment column via the capillary pump operating at a flow of 4 $\mu\text{l}/\text{min}$. The nano pump was operated at a flow rate of 600 nL/min. An initial gradient (Solvent A—100 % H_2O , 0.1 % Formic Acid; Solvent B—90 % Acetonitrile (ACN), 10 % H_2O and 0.1 % Formic Acid) of 97 % A was dropped to 90 % at 2 min and then 70 % at 10 min, 20 % at 13 min, 0 % at 17 min, and back to 97 % at 20 min. A post run time of 4 min was employed for column equilibration. The MS source was operated at 350 °C with 6 L/min N_2 flow and a fragmentor voltage of 175 V. N_2 was used as the collision gas with collision energy varied as a function of mass and charge using a slope of 3.7 V/100 Da and an offset of 2.5 V. Both quad and TOF were operated in positive ion mode. Reference compounds of 322.048121 and 1,221.990637 Da were continually leaked into the source for mass calibration. An initial MS scan was performed from m/z 300 to 1,600 and up to three multiply charged ions were selected for MS/MS analysis. Data files were transferred to an Agilent workstation equipped with Spectrum Mill software (Agilent Technologies, Santa Clara, CA). The raw MS/MS data files were extracted, sequenced, and searched using an *A. flavus* protein database generated with sequence from the AspGD website (<http://www.aspgd.org>). Proteins having at least two matching peptides with scores of 11 or greater

were considered valid and included in the results of the analysis.

Western blotting

Protein samples were electrophoresed on 4–12 % Bis-Tris gels. Following electrophoresis, one of the gels was stained with GelCode™ Blue Safe protein stain and imaged while the other gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond™-P; GE Healthcare) and blotted using the Amersham ECL Advance™ Western blotting detection kit (GE Healthcare). Membranes were blocked in 3 % blocking buffer (TBST with 3 % ECL Advance blocking agent (GE Healthcare) added), and following incubation with rabbit anti-*A. oryzae* alpha amylase (1:25,000 dilution; a kind gift from Dr. Jan Lehmbeck, Novozymes A/S) or rabbit anti-*A. niger* glucoamylase (1:1,000 dilution; a kind gift from Dr. Peter Punt, TNO), membranes were washed extensively and incubated with a 1:30,000 dilution of secondary antibody (ECL™ peroxidase labeled anti-rabbit antibody). Membranes were washed, detection reagent added for 5 min then a signal was visualized using the Fujifilm LAS-3000 luminescent image analyzer (Fujifilm Life Science, Stanford, CT).

qRT-PCR analysis

For real-time quantitative PCR (qRT-PCR) experiments, total RNA and cDNA were prepared from Af70 pSL82 control and ΔveA mycelia collected from the 48 h starch and corn kernel cultures as previously described (Cary et al. 2012). Quantitative PCR was performed using SYBR Green I chemistry and the iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) as previously described (Cary et al. 2012). The sequences of the oligonucleotide primers used for qRT-PCR amplification are listed in Supplementary Table S1. Gene expression levels at each time point were normalized [$2^{-\Delta\Delta CT}$ analysis; (Livak and Schmittgen 2001)] to *A. flavus* 18S rRNA gene expression levels utilizing the gene expression analysis software package for the Bio-Rad iQ5.

Results

Deletion of *veA* alters the starch-degradation profile

To determine the role of *veA* in *A. flavus* amylase activity, cell-free supernatants collected from ΔveA and control strains grown on starch or corn-based broths were added to a 1 % starch solution and the concentration of starch degradation products (glucose, maltose, and maltotriose) was measured by HPLC after 24 h. Since both L and S morphotypes produced similar results in an initial starch plate bioassay (data not

Starch-induction

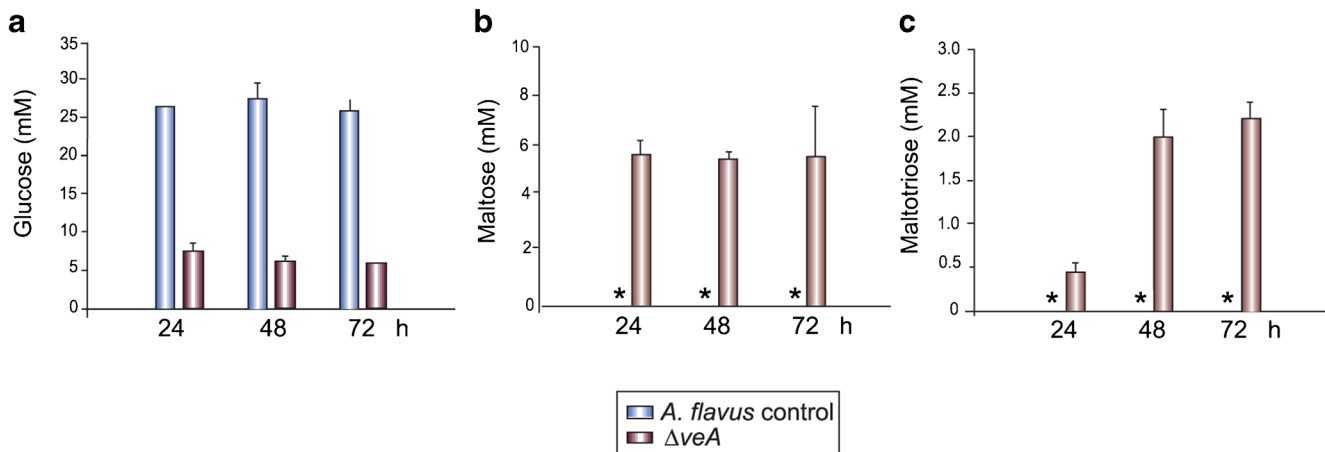


Fig. 1 Presence or absence of *A. flavus* *veA* results in different starch degradation profiles by starch-grown cultures. Starch-grown, secreted amylase activity measured by HPLC quantification of glucose (a), maltose (b), and maltotriose (c) produced by hydrolysis of starch

shown), only the Af70 (S morphotype) ΔveA and pSL82 control strains were used in this experiment. The profile of starch degradation products in the ΔveA strain differed from that obtained for the control supernatants from both starch- and corn-based culture media (Figs. 1 and 2). Supernatants from the starch-grown control cultures accumulated mainly glucose (Fig. 1a), while the ΔveA supernatants accumulated maltose and maltotriose at all time points analyzed (Fig. 1b, c). Glucose, though present in starch-grown ΔveA samples, was approximately 4.5-fold reduced with respect to that generated with the control supernatant (Fig. 1a). No maltose or maltotriose were observed in the reaction with the supernatant of the control strain.

When cultures were grown in corn-based medium, the supernatant from the control strain accumulated glucose at levels greater than observed for starch-grown cultures, while

ΔveA cultures generated approximately 7-fold less glucose compared to the control (Fig. 2a). Both corn-grown control and ΔveA culture supernatants converted starch to maltose at greater levels than observed for supernatants from starch-grown cultures (Fig. 2b). Corn-grown control supernatants produced more maltose than the ΔveA supernatants (Fig. 2b); however, levels of maltotriose were greater from ΔveA culture supernatants compared with that of the control (Fig. 2c). No degradation products were obtained with medium-only control or boiled supernatant samples (data not shown).

Amylase activity present in supernatants from control and ΔveA cultures was also assessed by analyzing the degradation products generated from a reaction with a 1 % maltoheptaose substrate. Our TLC results indicated that at all time points evaluated the control supernatants yielded glucose (Fig. 3),

Corn-induction

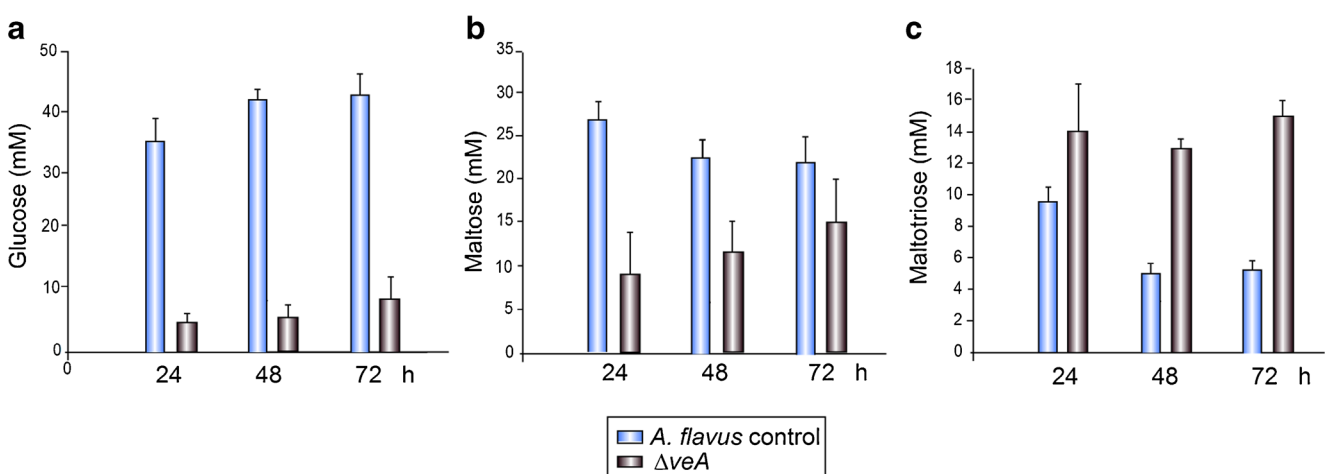
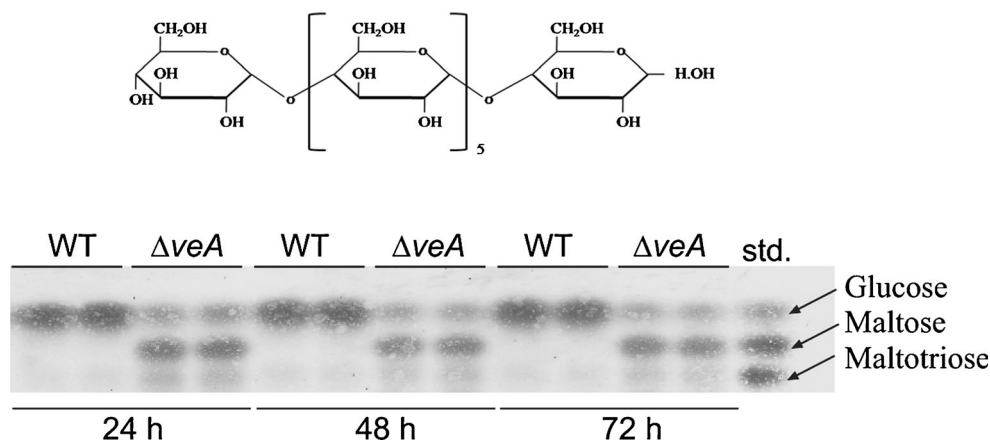


Fig. 2 Presence or absence of *A. flavus* *veA* results in different starch degradation patterns by corn-grown cultures. Corn-grown secreted amylase activity measured by HPLC quantification of glucose (a), maltose (b), and maltotriose (c) produced by hydrolysis of starch

Fig. 3 Thin-layer chromatographic (TLC) analysis of maltoheptaose degradation products generated by supernatants from starch-grown *A. flavus* control (pSL82) and ΔveA cultures. Culture supernatants were incubated with maltoheptaose for 18 h at 40 °C and separated on silica plates as described in the “Materials and method” section



while the degradation of maltoheptaose by the ΔveA samples resulted mainly in accumulation of maltose and maltotriose. As before, no degradation products were obtained with medium-only control or boiled supernatant samples (data not shown).

Deletion of *laeA* results in a change in starch-degradation profile

In the model fungus *A. nidulans*, it was first shown that the VeA protein interacts with LaeA, forming a nuclear complex with other protein components (Bayram et al. 2008). For this reason, we examined whether the presence or absence of the *laeA* gene could also regulate amylase activity as observed for *A. flavus veA*. Similar to the starch-growth experiment described above, we analyzed the effect of the *laeA* deletion ($\Delta laeA$) on amylase activity (Fig. 4a–c). Our results indicated that as in the case of ΔveA , supernatants collected from a $\Delta laeA$ strain preferentially converted starch to maltose and maltotriose, accompanied by a small amount of glucose. Supernatants from the starch-grown control cultures produced glucose as the main degradation product while maltose and maltotriose were absent at all time points analyzed.

veA is also necessary for normal protease activity

Fungal proteases are able to degrade a variety of natural protein substrates, for example those of plant origin such as corn zein or cotton seed storage protein (Mellon and Cotty 1995). Previous studies revealed that *veA* controls protease activity in *A. fumigatus* (Dhingra et al. 2012). Therefore, we also examined whether *veA* has a role in regulating protease activity in *A. flavus*. Our study revealed that this hydrolytic activity is also dependent of *veA* (Fig. 5). Azocasein analysis showed a decrease in protease activity in the ΔveA cultures compared to the control strain under the experimental conditions assayed (Fig. 5), indicating that *veA* is required for wild-type levels of protease activity in *A. flavus*.

Comparative proteomic analysis

Proteins secreted into culture broths after 48 h growth of Af70 pSL82 and ΔveA were separated by SDS-PAGE (Fig. 6). A total of 18 proteins (11 from starch media and 7 from corn-kernel) that appeared to be produced at different levels between the two strains were excised from the 1-D gels, digested with trypsin, and the resulting peptides were subjected to analysis by LC/MS/MS. Separation of proteins of approximately 30 kDa or greater was performed with 7 % Tris-acetate SDS-PAGE gels while proteins less than 30 kDa were resolved and excised from 4–12 % Bis-Tris gels. Additionally, total secreted proteins were TCA precipitated, trypsinized, and the peptides analyzed as described in “Materials and methods” section.

The identity of all protein bands excised from SDS-PAGE gels are shown in Table 1. Higher levels of alpha-amylase (C3/C17, S9, AFL2G_01841), putative glutaminase (S5, AFL2G_11013), putative endo-1,3-beta-glucanase (S6, AFL2G_02099), 1,3-beta-glucanosyltransferase (C4, AFL2G_07643), and 2 chitinases (S10, AFL2G_02328; C18, AFL2G_11085) were observed in the ΔveA mutant compared to the pSL82 control strain grown on both corn and starch media (Fig. 6).

Western blot analysis using antibody to *Aspergillus oryzae* alpha-amylase confirmed higher level production of this protein in the ΔveA mutant compared to the control (Fig. 7a, b). A number of proteins were found in higher quantities in the pSL82 control compared to the ΔveA mutant. These included an alkaline protease (oryzin, S11/S12/S15, AFL2G_01995), glucoamylase (C1/C2, S7/S8, AFL2G_11885), a putative FG-GAP repeat protein (S13, AFL2G_02090), putative amine oxidase (C2, AFL2G_07234) and a conserved hypothetical protein of unknown function (S14, AFL2G_06161). Western blot analysis using antibody to *Aspergillus niger* glucoamylase demonstrated that an immunoreactive protein of about 63 kDa was produced at slightly higher levels in the pSL82 control than the ΔveA mutant on both media (Fig. 7c, d). Additionally, a

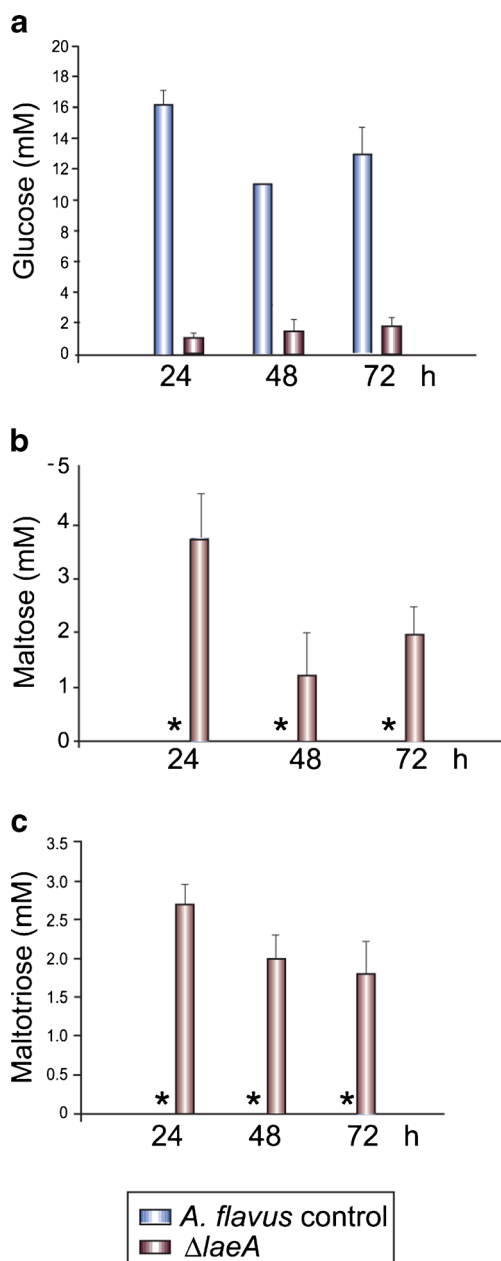


Fig. 4 Deletion of *A. flavus laeA* results in different starch degradation patterns compared to the control in starch-grown cultures. Starch-grown secreted amylase activity measured by HPLC quantification of glucose (a), maltose (b), and maltotriose (c) produced by hydrolysis of starch

second, more diffuse immunoreactive band at about 57 kDa was only observed in the pSL82 control samples. Two weakly immunoreactive bands of about 120 and 47 kDa were only observed in starch samples. The 63-kDa band corresponds to excised protein S7 and C1 while the 57 kDa corresponds to S8 and C2. LC/MS/MS data identified the 57-kDa protein as glucoamylase in starch (S8, AFL2G_11885) and a mixture of glucoamylase and amine oxidase in corn (C2).

Total secreted proteins were also analyzed by LC/MS/MS after filtration and precipitation (Table 2). A total of 33

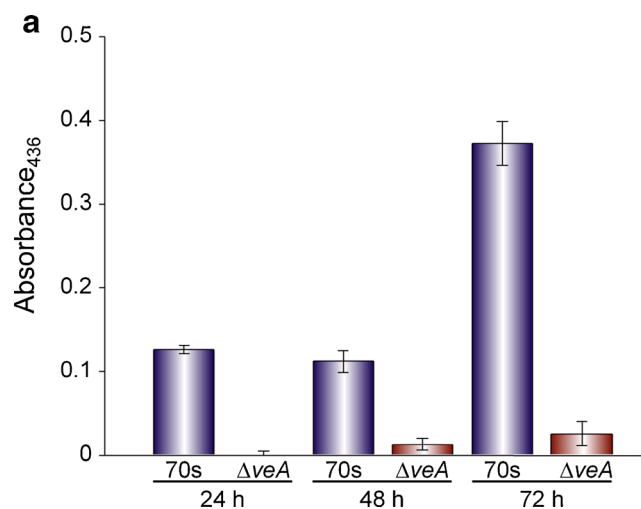
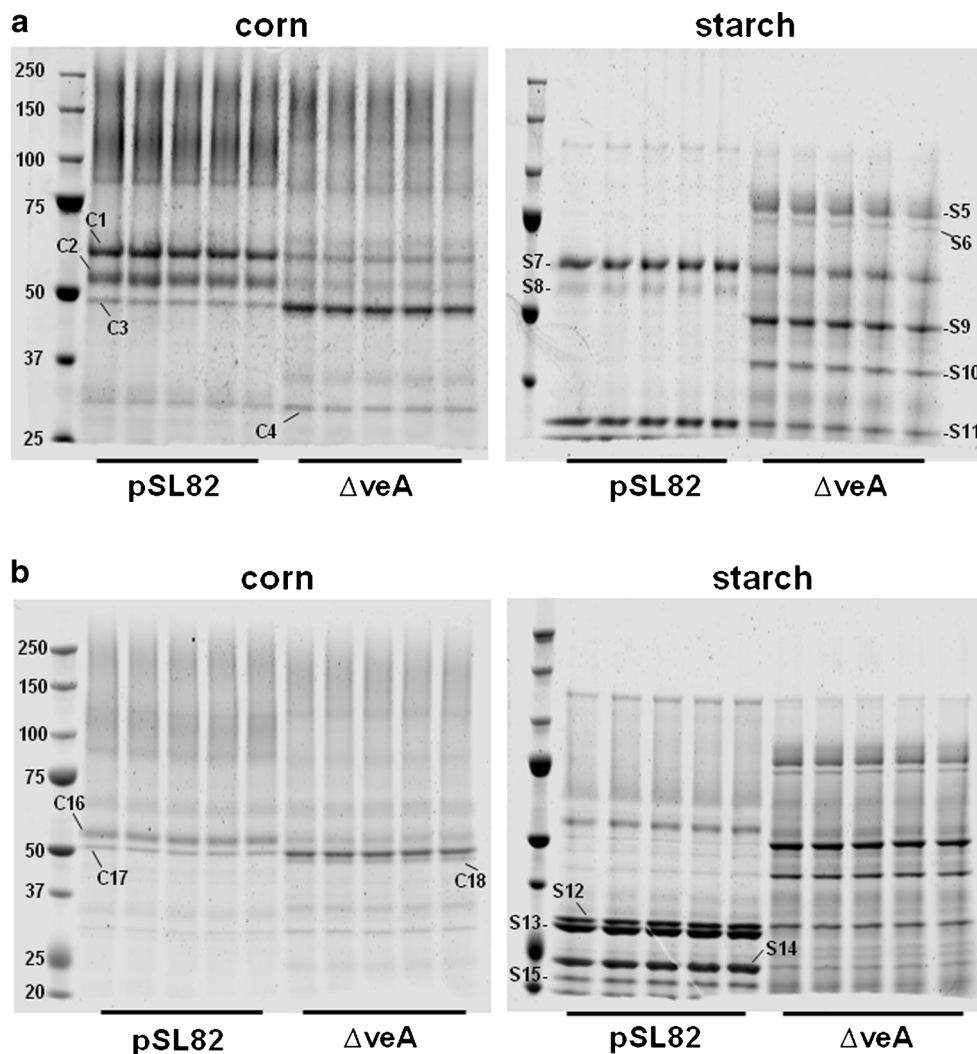


Fig. 5 *veA* is necessary for normal protease activity in *A. flavus*. (a) Af70 pSL82 and isogenic ΔveA strain were cultured in GMM with 8 mg/mL of BSA. Quantification of proteolytic activity was measured by an azocasein assay. The experiment included three replicates. Error bars represent standard error

proteins were identified in the *A. flavus* pSL82 strain grown on corn media and 25 from the starch media. Proteins were categorized into five functional classifications as presented in Fig. 8. Hypothetical proteins represented the largest class for both the secretome of the corn-induced and starch-induced cultures, 56 and 67 %, respectively. Proteins classified in proteolysis represented the second largest class for both secretomes followed by carbohydrate metabolism, metabolism, and lastly electron transport. Only 14 of the 44 identified proteins were found in both media, including a few involved in carbohydrate metabolism such as alpha-amylase (AFL2G_01841) and glucoamylase (AFL2G_11885). Maltase was not found in these supernatants. In both samples, nearly a quarter of the identified proteins were involved in proteolysis, and these included tripeptidyl aminopeptidase (AFL2G_05009), oryzin (AFL2G_01995), aspergillopepsin A (AFL2G_08462), and leucine aminopeptidase (AFL2G_04856). Greater than half of the proteins identified from both media were designated as hypothetical, and included a predicted 1,3-beta-glucanosyltransferase (AFL2G_07643) protein found in both samples. There were a number of differences in the proteins from the pSL82 strain grown on the corn and starch media, and many of these were hypothetical proteins. Within this group there were proteins predicted to be involved in cell wall functions and carbohydrate metabolism such as beta-fructofuranosidase (AFL2G_10707) and a chitosanase (AFL2G_08550).

The *A. flavus* pSL82 and ΔveA strains had variations in their secreted protein profiles when grown in either corn or starch media. There were 14 proteins found only in the pSL82 strain, 11 unique to the ΔveA strain, and 19 proteins (43 %) identified in both strains from the corn media samples. Carbohydrate metabolism proteins glucoamylase (AFL2G_11885), alpha-

Fig. 6 Preparative SDS-PAGE of concentrated culture broths. Extracellular proteins from concentrated starch or corn kernel-media-grown pSL82 and ΔveA culture supernatants were separated by SDS-PAGE using both (a) 7 % Tris-acetate or (b) 4–12 % Bis-Tris gels. Gels were stained with GelCode™ Blue Safe protein stain and imaged. Proteins displaying differential production were manually excised from gels with a scalpel, in-gel digestion with trypsin and analyzed by LC/MS/MS. When a protein of interest from either strain was excised from a polyacrylamide gel, the corresponding gel region for the other strain was also excised for comparative purposes. Proteins designated with a C prefix were from corn kernel supernatants while those with an S were from starch. A total of 18 protein bands were excised for LC/MS/MS analysis (see Table 1). Marker: Precision Plus Protein Dual Color Standard (Bio-Rad)



amylase (AFL2G_01841), chitinase (AFL2G_11085), and a predicted homolog of the *A. fumigatus* Asp f 9 allergen (AFL2G_01043) belonging to the glycosyl hydrolase family were detected in both strains grown on corn. Similarly, proteolytic proteins tripeptidyl aminopeptidase (AFL2G_05009), oryzin (AFL2G_01995), aspergillopepsin (AFL2G_08462), carboxypeptidase (AFL2G_03582), leucine aminopeptidase (AFL2G_04856), and neutral protease (AFL2G_11655) peptides were detected in both strains grown on corn media. Among those proteins missing in our analysis of the ΔveA strain grown in corn media was a predicted mannosidase (AFL2G_02514), a chitosanase (AFL2G_08550), and a carboxypeptidase (AFL2G_10957).

There was slightly less overlap among the identified proteins (31 %) when the strains were grown on starch media. There were 11 proteins found from both strains grown on starch, 10 found only in the ΔveA strain, and 14 that were only observed in the pSL82 strain. Among these, a putative muramidase (AFL2G_11586) and predicted 1,3-beta-glucanosyltransferase (AFL2G_07643) were found only in

the pSL82 strain while peptides matching a chitin binding domain protein (AFL2G_05887) were only observed in the ΔveA strain. Several of the carbohydrate metabolism and proteolysis proteins found in both strains from the corn samples were also found in both strains grown on starch media. Notable exceptions were chitinase (AFL2G_11085), predicted beta-fructofuranosidase (AFL2G_10707), carboxypeptidase (AFL2G_03582), and neutral protease (AFL2G_11655).

VeA dependence of secreted proteins

Expression of the selected genes encoding secreted proteins that displayed differential production by SDS-PAGE of starch- or corn kernel-grown Af70 ΔveA and pSL82 were analyzed by qRT-PCR. In all cases, the gene expression data correlated with the observed differences in levels of protein secreted into the culture broths. Higher level of expression was observed in the pSL82 control compared to the ΔveA mutant in both starch and corn-based medium for genes encoding oryzin (AFL2G_01995), catalase B (*catB*,

Table 1 LC/MS/MS identification of select starch and corn kernel media-grown *A. flavus* secreted proteins excised from SDS-PAGE gels

Protein	ORF	Protein (predicted)
Starch grown		
S5	AFL2G_11013	Glutaminase A
S6	AFL2G_02099	1,3-Beta glucanase
S7	AFL2G_11885	Glucoamylase
S8	AFL2G_11885	Glucoamylase
S9	AFL2G_01841	Alpha-amylase
S10	AFL2G_02328	Chitinase
S11	AFL2G_01995	Oryzin
S12	AFL2G_01995	Oryzin
S13	AFL2G_02090	FG-GAP repeat protein
S14	AFL2G_06161	Hypothetical
S15	AFL2G_01995	Oryzin
Corn-kernel grown		
C1	AFL2G_11885	Glucoamylase
C2	AFL2G_07234/11885	Amine oxidase/glucoamylase
C3	AFL2G_01841	Alpha-amylase
C4	AFL2G_07643	1,3-Beta-glucanosyltransferase
C16	AFL2G_07234/11885	Amine oxidase/glucoamylase
C17	AFL2G_01841	Alpha-amylase
C18	AFL2G_11085	Chitinase

AFL2G_08106), putative amine oxidase (AFL2G_07234), glucoamylase (AFL2G_11885), putative FG-GAP repeat protein (AFL2G_02090), and a conserved hypothetical protein (AFL2G_06161) (Fig. 9). In contrast, the ΔveA mutant demonstrated higher levels of expression of the alpha-amylase (AFL2G_01841) during growth on starch or corn-kernel media.

Discussion

Starch is an abundant polymer in corn kernels and other seeds of agriculturally important crops. Its degradation in filamentous fungi is accomplished by the concerted action of α -amylase, glucoamylase, and α -glucosidase enzymes. Degradation of this polymer by amylases has been associated with AFB1 production during *A. flavus* infection of corn (Woloshuk et al. 1997). Previous work from our laboratory demonstrated that deletion of the *A. flavus* global regulatory gene *veA* results in strains unable to produce AFs and other toxins such as aflatoxin and cyclopiazonic acid (Duran et al. 2007). Furthermore, absence of *veA* reduces pathogenicity of *A. flavus* on corn, peanut, and cottonseed (Duran et al. 2009). In this study we revealed that *veA* also influences *A. flavus* hydrolytic activity.

Fig. 7 Western blot analysis of alpha-amylase and glucoamylase production. Concentrations of proteins from broths of 48-h starch or corn kernel-grown Af70 pSL82 or ΔveA were determined and approximately equal amounts of protein loaded per lane. Proteins were separated by SDS-PAGE using 4–12 % Bis-Tris gels. Following electrophoresis, (panels a and c) one gel was stained with GelCode™ Blue Safe protein stain and imaged while the other (panels b and d) was used for Western transfer. Membranes were incubated with rabbit anti-*A. oryzae* alpha amylase (panel b) or anti-*A. niger* glucoamylase (panel d), as described in the “Materials and methods” section. Markers: M1, Precision Plus Protein Dual Color Standard (Bio-Rad); M2, Magic Mark XP Western Blot Protein Standard (Life Technologies); α -amylase, *A. oryzae* alpha-amylase. Panel d bands of immunoreactivity: a, 120 kDa; b, 63 kDa; c, 57 kDa; d, 47 kDa

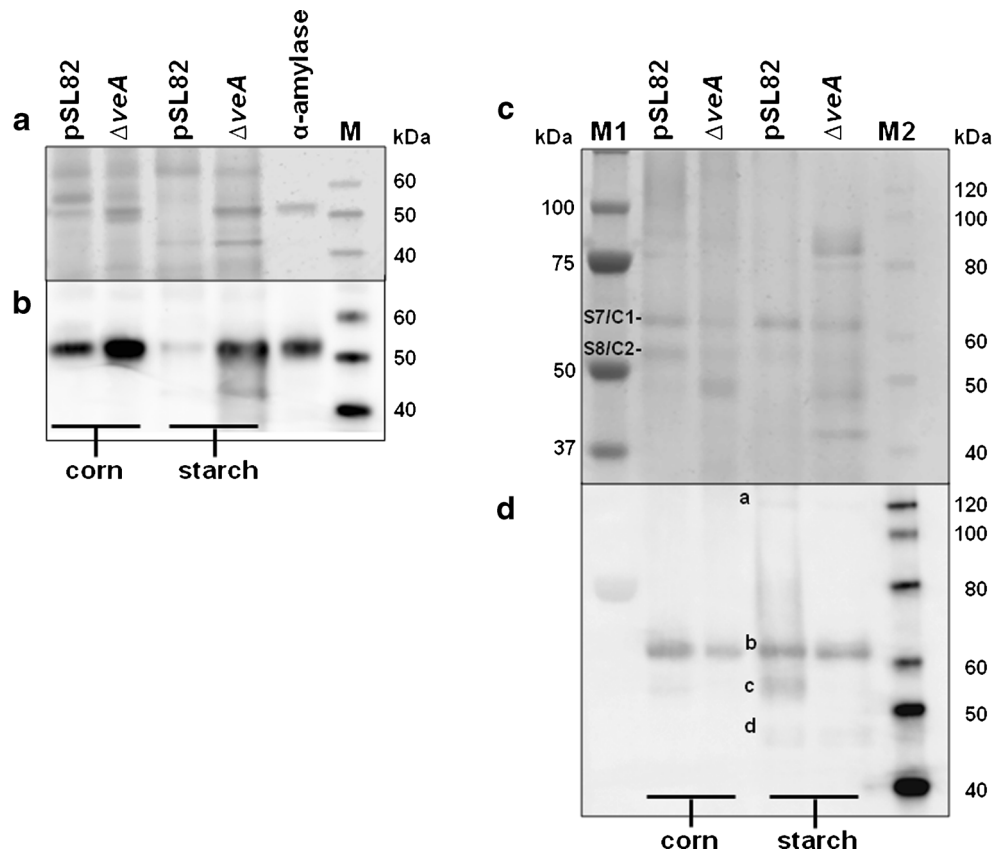


Table 2 Proteins identified from LC/MS/MS analysis of total secretome of starch and corn kernel media-grown *A. flavus*

ORF	Functional group	Function (predicted)	Protein (predicted)	Af70pSL82	Corn		Starch
					Af70pSL82	ΔveA	ΔveA
AFL2G_11885	Carbohydrate metabolism	Carbohydrate metabolism	Glucoamylase	X	X	X	X
AFL2G_01841	Carbohydrate metabolism	Carbohydrate metabolism	Alpha-amylase	X	X	X	X
AFL2G_06015	Carbohydrate metabolism	Carbohydrate metabolism	Glycolipid-anchored protein 5	X			X
AFL2G_07437	Carbohydrate metabolism	Carbohydrate metabolism	Endo 1 4-beta-xylanase				X
AFL2G_08106	Electron transport	Reduction/oxidation	Catalase B	X			
AFL2G_07643	Hypothetical	Carbohydrate metabolism	1,3-Beta-Glucanosyltransferase	X	X		X
AFL2G_07234	Hypothetical	Electron transport	Amine oxidase	X			
AFL2G_04718	Hypothetical	Cell wall	Hypothetical	X	X		X
AFL2G_04616	Hypothetical	Cell wall	Surface binding protein A	X	X	X	X
AFL2G_02631	Hypothetical	Membrane	Similar to LAP1	X			X
AFL2G_10707	Hypothetical	Carbohydrate metabolism	Beta-fructofuranosidase	X			X
AFL2G_00874	Hypothetical	Unknown	Similar to IgE-binding protein	X	X	X	X
AFL2G_07840	Hypothetical	Unknown	hypothetical	X			
AFL2G_01606	Hypothetical	Cell wall	PhiA	X			
AFL2G_04466	Hypothetical	Cell wall	GPI-anchored protein	X			
AFL2G_02090	Hypothetical	Unknown	Hypothetical	X	X	X	
AFL2G_06196	Hypothetical	Unknown	Hypothetical	X			X
AFL2G_01043	Hypothetical	Carbohydrate metabolism	Glucanase/rAsp f 9	X	X	X	X
AFL2G_10536	Hypothetical	Unknown	Predicted		X		X
AFL2G_08550	Hypothetical	Carbohydrate metabolism	Chitosanase	X			
AFL2G_01747	hypothetical	unknown	hypothetical	X			
AFL2G_10165	Hypothetical	Unknown	IgE binding protein				X
AFL2G_01569	Hypothetical	Unknown	Hypothetical	X		X	
AFL2G_05131	Hypothetical	Unknown	Hypothetical			X	X
AFL2G_03583	Hypothetical	Unknown	Hypothetical			X	
AFL2G_06133	Hypothetical	Carbohydrate metabolism	Glucoronan lyase A			X	
AFL2G_07760	Hypothetical	Unknown	Hypothetical			X	
AFL2G_11734	Hypothetical	Reduction/oxidation	FAD-dependent oxygenase			X	
AFL2G_08549	Hypothetical	Carbohydrate metabolism	Polysaccharide lyase			X	
AFL2G_01001	Metabolism	Ribonuclease	Ribonuclease T2	X	X		X
AFL2G_10143	Metabolism	Isoamyl alcohol oxidase	Isoamyl alcohol oxidase				X
AFL2G_05009	Proteolysis	Proteolysis	Tripeptidyl aminopeptidase	X	X	X	X
AFL2G_01995	Proteolysis	Proteolysis	Oryzin	X	X	X	
AFL2G_08462	Proteolysis	Proteolysis	Aspergillopepsin A	X	X		X
AFL2G_03582	Proteolysis	Proteolysis	Carboxypeptidase S1	X			X
AFL2G_04856	Proteolysis	Proteolysis	Leucine aminopeptidase	X			X
AFL2G_11655	Proteolysis	Proteolysis	Neutral protease 2	X			X
AFL2G_09418	Proteolysis	Proteolysis	FuSed4 protease		X	X	X
AFL2G_07153	Proteolysis	Proteolysis	Aorsin	X			
AFL2G_10957	Proteolysis	Proteolysis	Carboxypeptidase 4	X			

The genome of *A. flavus* contains a robust system for starch degradation, with 11 genes demonstrating homology to known amylases. Our current study revealed that starch degrading activity of *A. flavus* was reduced in the absence of *veA*. Deletion of *veA* resulted in a different profile of starch

catabolism end products than observed in the control strain. While the control strain produced glucose as the main degradation product, ΔveA accumulated maltose and maltotriose. Accumulation of glucose in the control strain as measured by HPLC analysis is consistent with the activity of gamma

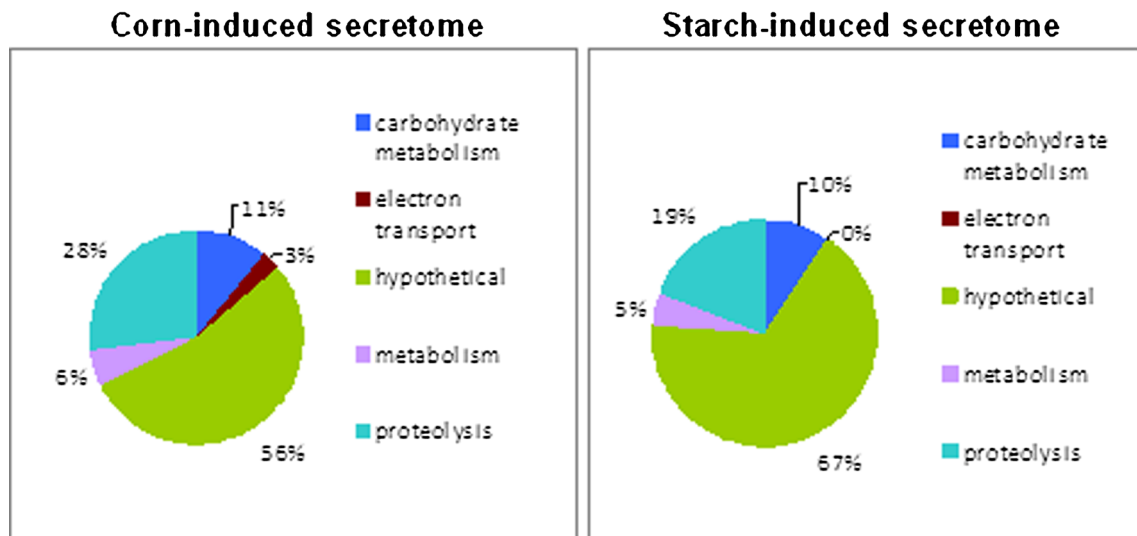
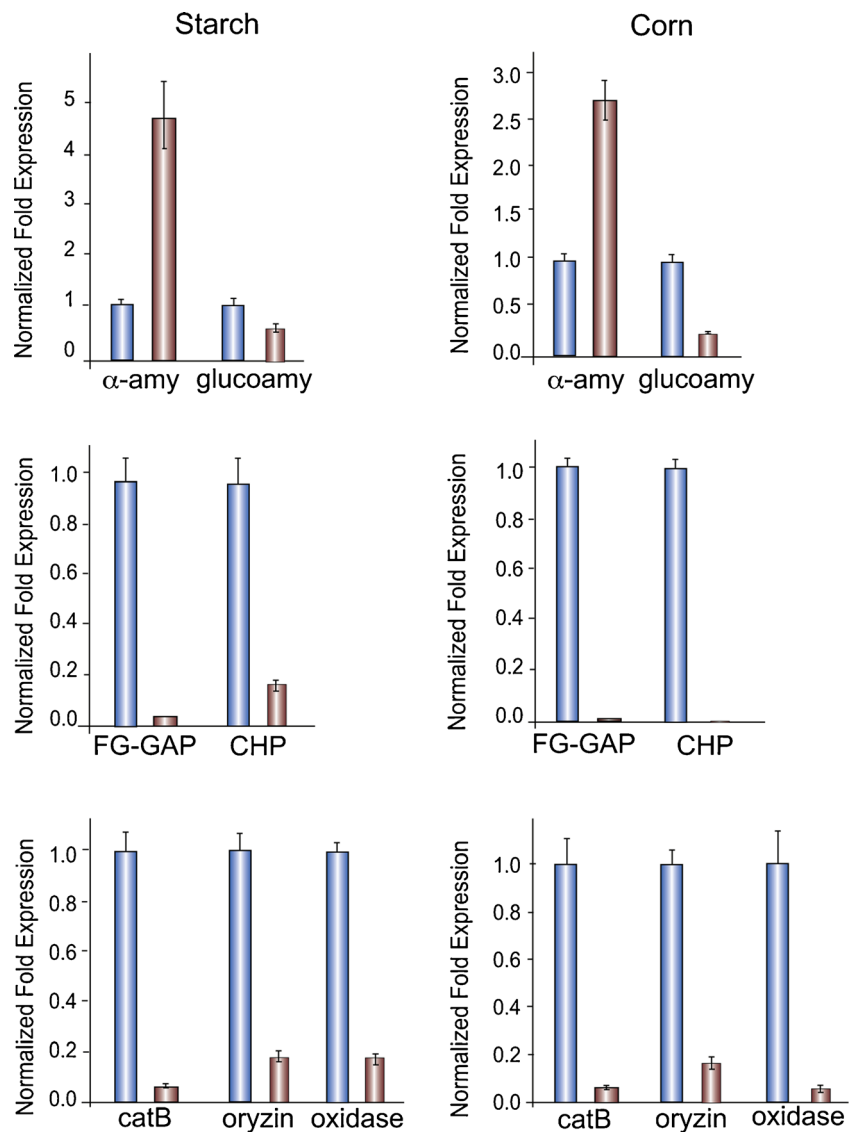


Fig. 8 Functional classification of proteins secreted by *A. flavus*. Each functional category represents the percentage of the total number of unique proteins identified in either starch or corn kernel-grown culture supernatants as determined by LC/MS/MS

Fig. 9 qRT-PCR analysis of *A. flavus* genes encoding differentially produced secreted proteins. Total RNA was extracted from Af70 pSL82 and ΔveA mycelia following 48-h growth on starch or corn kernel-based media. Gene expression levels were normalized ($2^{-\Delta\Delta CT}$ analysis) to *A. flavus* 18S rRNA gene expression levels utilizing the gene expression analysis software package for the Bio-Rad iQ5. Bars: black, Af70 pSL82 control; gray, Af70 ΔveA mutant. Samples: α -amy, alpha-amylase (AFL2G_01841); glucoamy, glucoamylase (AFL2G_11885); FG-GAP, putative FG-GAP repeat protein (AFL2G_02090); CHP, conserved hypothetical protein (AFL2G_06161); catB, catalase B (AFL2G_08106); oryzin (AFL2G_01995); oxidase, putative amine oxidase (AFL2G_07234)



amylases, also called glucoamylases, which cleave α -(1-6) glycosidic linkages and the last α -(1,4) glycosidic bonds at the non-reducing end of amylose and amylopectin, resulting in the release of glucose monomers. A similar pattern was observed when maltoheptaose was used as substrate, further supporting the presence of glucoamylase activity in the *A. flavus* wild-type strain.

Proteomic analyses of proteins secreted by *A. flavus* during growth on starch or corn kernel-based medium detected the presence of glucoamylase in both the control strain and ΔveA mutant and this was subsequently confirmed by western blot analysis. Proteins reacting with the glucoamylase antisera had estimated molecular masses of 120, 63, 57, and 47 kDa. A previous study of glucoamylase produced by *A. flavus* using SDS-PAGE zymography revealed three bands of 125, 70, and 64 kDa (Koc and Metin 2010). Further, the 57-kDa band was most prevalent in the Af70 pSL82 control samples and was especially prominent in the starch sample. The immunoreactive bands of 120 and 47 kDa were present only in the control and ΔveA starch samples. The increased levels of the 57-kDa band in the pSL82 control samples (and the 47-kDa protein in the starch samples) may explain the greater amounts of glucose being produced compared to the ΔveA mutant. The presence of observable levels of the 57 and 47-kDa immunoreactive bands in the control samples may be due to secretion of proteases into the broth that could lead to the breakdown of the 63-kDa protein. This type of breakdown of intact proteins by secreted proteases has been reported in a study of *A. flavus* proteins secreted during growth on various substrates (Medina et al. 2005). LC/MS/MS analysis of total secreted proteins identified the presence of a number of secreted proteases in both media, including the alkaline protease, oryzin (see below).

The absence of *veA* did not result in loss of alpha-amylase activity as enzymes secreted by the mutant during growth on starch or corn kernel-based media were capable of producing mainly maltose and maltotriose when either starch or maltoheptaose were used as substrate. In fact, qRT-PCR showed an increase in alpha-amylase (AFL2G_01841) expression in the ΔveA mutant compared to the control. This was also supported by western blot results showing increased levels of alpha-amylase in the mutant. It is possible that the reduction in glucoamylase activity in ΔveA strain is compensated by an increase in alpha-amylase activity generating additional maltose that could be cleaved into glucose by alpha-glucosidases. Interestingly, our enzyme assays showed that, different from the starch-grown cultures, the corn-kernel medium grown pSL82 strain also produced substantial amounts of maltose (but not maltotriose) during starch degradation. This could be possibly attributed to other additional unknown hydrolytic activity in these cultures.

Our data indicates that similar to *veA*, *laeA* also plays a role in the regulation of amylase activity. The effect of the *laeA*

deletion was comparable to that of the ΔveA mutant, displaying a reduction in starch degrading activity leading to the accumulation of maltose and maltotriose, with a small amount of glucose. Studies in the model fungus *A. nidulans* revealed that once VeA is transported to the nucleus by the α -importin KapA (Araujo-Bazan et al. 2009; Stinnett et al. 2007) this protein interacts with LaeA, forming the velvet complex, together with VelB, another protein of the velvet family (Bayram et al. 2008). The LaeA component of the velvet complex is a chromatin modifying protein also required for the synthesis of ST and other secondary metabolites (Bok and Keller 2004). In our study, we demonstrated that at least two components of the velvet complex are necessary for normal amylase activity, revealing the importance of this complex in critical hydrolytic activities that could affect plant tissue colonization.

In addition to alpha-amylase and glucoamylase, proteomic analysis of the Af70 pSL82 and ΔveA secretome following growth on starch and corn-kernel-based media also revealed the presence of additional, differentially produced proteins that could play a role in fungal infection. In most cases the control produced higher levels of the protein compared to the ΔveA strain, for instance oryzin, the Alp alkaline protease ortholog (Chen et al. 2009). Interestingly, the *A. flavus* Alp was shown have a potential role in infection of corn kernels and production of aflatoxin (Chen et al. 2009). In our study, protease activity was reduced in the absence of *veA*; however, due to the numerous protease genes present in the *A. flavus* genome it is difficult to predict whether the remarkable reduction in oryzin gene expression and concomitant reduction in the synthesis of oryzin protein could account for the observed decrease in protease activity. A similar reduction in protease activity was also observed in *A. fumigatus veA* mutants (Dhingra et al. 2012). Reduction of *A. flavus* protease activity could impair degradation of substrates such as corn zein or cotton seed storage protein thus reducing fungal virulence (Mellon and Cotty 1995).

Other proteins found in higher abundance in the control with respect to the ΔveA cultures were catalase B, a putative amine oxidase, a putative FG-GAP repeat protein, and a conserved hypothetical protein of unknown function. Catalase B has been shown to play a role in the protection of fungal hyphae from the toxic effects of H_2O_2 , for instance, *Magnaporthe grisea catB* mutants are less pathogenic on barley and rice (Kawasaki et al. 1997; Skamnioti et al. 2007). Catalase B, as well as oryzin, has been identified from analyses of extracellular proteins produced during growth of *A. flavus* and *A. oryzae* on natural and artificial substrates (Medina et al. 2005; Oda et al. 2006). The putative amine oxidase (AFL2G_07234) demonstrated 45 % amino acid identity with an amine oxidase of *A. niger* shown to be localized to the cell surface and proposed to serve as a detoxifying agent that prevents amines from entering and damaging

cells (Frebort et al. 2000). Notably, a putative FG-GAP repeat protein (AFL2G_02090) shares 57 % identity with a secreted FG-GAP repeat protein from the necrotrophic fungus *Sclerotinia sclerotiorum* (Zhu et al. 2013). This protein is proposed to be involved in suppressing host resistance at an early stage of fungal infection, suggesting that changes in the presence of this protein might also contribute to the decrease in virulence observed in the absence of *veA* (Duran et al. 2009). Glutaminase A, 1,3- β -glucanase, and chitinase were more abundant in the supernatant of ΔveA cultures compared to the control. Secretion of all three of these proteins has been reported during growth of *A. flavus* and *A. oryzae* (Medina et al. 2005; Oda et al. 2006). Glutaminase A plays key role in nitrogen metabolism (Harayama 1992) while 1,3- β -glucanase and chitinases are more than likely targeting glucans and chitins of fungal cell wall origin as these enzymes were being produced during growth on pure soluble starch that does not contain 1,3- β -glucan linkages. Genes encoding for glutaminase, glucanase, and chitinase were also found differentially expressed during corn infection (Dolezal et al. 2013).

A. flavus is a metabolically versatile fungus with the capability of producing a variety of hydrolytic enzymes that allow this organism to grow as a saprophyte as well as an opportunistic plant and animal pathogen. In this study, we have demonstrated that VeA regulates the production of numerous secreted proteins, including hydrolytic enzymes such as amylases and proteases, known to be important for seed colonization. This work provides further insight into the regulation of *A. flavus* hydrolytic enzymes that might contribute to the adaptation of this fungal species to its niche. Additionally these findings could contribute to the development of industrial applications to increase production of enzymes of commercial value. The conserved nature of VeA (Myung et al. 2012) and VeA-interacting proteins suggests that VeA orthologs could also play a role in hydrolytic activity in other fungal species.

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