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SUBCELLULAR LOCALIZATION OF AFLATOXIN ENZYMES AND AFLATOXIN IN *ASPERGILLUS PARASITICUS*

presented by

ANINDYA CHANDA

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SUBCELLULAR LOCALIZATION OF AFLATOXIN ENZYMES AND AFLATOXIN IN *ASPERGILLUS PARASITICUS*

By

Anindya Chanda

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ABSTRACT

SUBCELLULAR LOCALIZATION OF AFLATOXIN ENZYMES AND AFLATOXIN IN *ASPERGILLUS PARASITICUS*

By

Anindya Chanda

Aflatoxin biosynthesis initiates as a response to environmental changes, involves a tightly regulated expression of a 75kb gene cluster resulting at least 17 enzyme activities and finally ends with the secretion of end products outside the cell. It is one of the most characterized eukaryotic secondary metabolic pathways and hence serves as a useful model to understand secondary metabolism in fungi and plants. Understanding the spatial distribution of proteins and biosynthetic products in *Aspergillus parasiticus* during aflatoxin synthesis helps us to understand fungal and plant secondary metabolism in general at a cellular level. Prior to our current work, the intracellular site of aflatoxin synthesis was unknown although our previous subcellular studies demonstrated localization of early, middle and late aflatoxin enzymes in vesicles and vacuoles during aflatoxin synthesis at peak levels.

Under aflatoxin inducing conditions in liquid yeast-extract sucrose medium, *A. parasiticus* generates vesicles and vacuoles which exhibit significant heterogeneity in size (20nm to 5μm) and density. The hypothesis tested in this study is **vesicles and vacuoles are the intracellular site(s) for aflatoxin biosynthesis.** Two independent approaches were used to test the hypothesis.

As a first step in conducting a detailed analysis of the role of these organelles in aflatoxin synthesis, a novel "high density sucrose cushion" method was developed to purify a vesicle-vacuole fraction using protoplasts prepared from cells harvested during aflatoxin synthesis. Western blot analysis demonstrated the presence of three aflatoxin enzymes (Ver-1, Vbs and OmtA) in this fraction. Vesicles and vacuoles also were observed to represent a primary site to sequester aflatoxin. This purified fraction converted sterigmatocystin, a late intermediate in aflatoxin biosynthesis, to aflatoxin B_1 showing that the last two steps of aflatoxin biosynthesis occur in or on vesicles and vacuoles.

Second, to clarify which compartments (vesicles or vacuoles) were functionally involved aflatoxin biosynthesis vesicle-vacuole fusion (a late step in vacuole biogenesis in eukaryotes) was interrupted by application of Sortin3 (a low mass bioactive compound that interferes with protein trafficking to the vacuole and produces a *vps16* phenotype in yeast) and by disruption of the Rab7/Ypt7 GTPase homologue, *vb1*. Both treatments resulted in accumulation of vesicles and significantly higher levels of aflatoxins in the medium compared to wild-type (ELISA). This increase in aflatoxin was not due to up-regulation in aflatoxin gene expression (RT-PCR) but was due to significantly higher accumulation of functional enzymes (Western blot analysis) caused by elevated accumulation of vesicles. These data provide the first evidence that manipulation of the vesicle transport machinery in Aspergillus affects secondary metabolism. We hypothesize that vesicles represent the intracellular site for the late steps of aflatoxin biosynthesis and aflatoxin export, whereas the vacuole is primarily responsible for aflatoxin storage and aflatoxin enzyme turnover.

To my parents, who worked so hard to provide me with a good education

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LIST OF ABBREVIATIONS

YES:	Yeast-extract sucrose growth medium
YEP:	Yeast-extract peptone growth medium
AFB_1:	Aflatoxin B_1
TEM:	Transmission electron microscopy
ELISA:	Enzyme-linked immunosorbent assay
CLSM:	Confocal laser scanning microscopy
SAM:	S-adenosyl methionine
ST:	Sterigmatocystin
OMST:	*o*-Methylsterigmatocystin
PCR:	Polymerase chain reaction
RT-PCR:	Reverse transcriptase polymerase chain reaction
EGFP:	Enhanced green fluorescence protein
NA:	Norsorolinic acid
PKS:	Polyketide synthase

CHAPTER 1

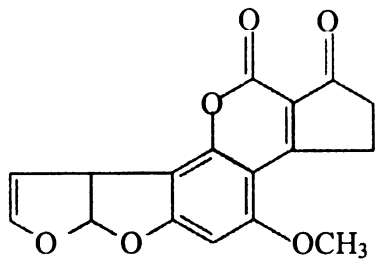
LITERATURE REVIEW

Aflatoxins: Background, toxicity and economic significance

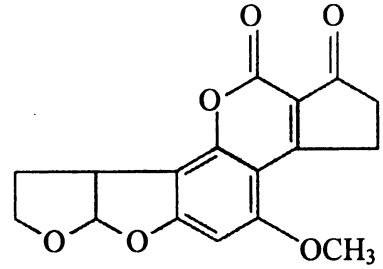
The discovery

In 1960, London witnessed a huge loss in the poultry industry. More than 100,000 turkeys died as a result of an illness named "Turkey X disease" (Nesbitt *et al.*, 1962; WP, 1961). The causative agents of this illness were contaminants in feed that were later characterized as "aflatoxins" by thin layer chromatography on the suspect feed (Diener, 1969).

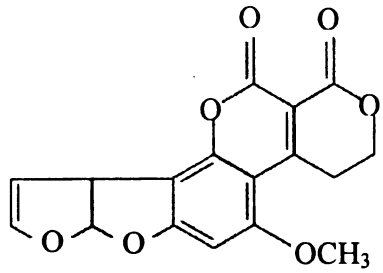
Aflatoxins are a group of biologically active secondary metabolites (mycotoxins) produced by certain strains of imperfect fungi (Bennett & Klich, 2003). They are polyketide-derived furanocoumarins and consist of four major structural groups (Figure 1.1) - B_1, B_2, G_1 and G_2. These groups are named on the basis of their R_f values and fluorescent color under ultraviolet light (Carnaghan *et al.*, 1963). *Aspergillus flavus*, *Aspergillus pseudotamarii,* and *Aspergillus ochraceoroseus* produce only the B aflatoxins, and *Aspergillus nomius*, *Aspergillus bombycis,* and *Aspergillus parasiticus* produce both B and G aflatoxins (Bennett & Klich, 2003; Yu *et al.*, 2002). Aflatoxins are the most toxic and carcinogenic compounds among the known mycotoxins (Squire, 1981) and, therefore, they have been extensively studied. Aflatoxins derived their name from *A. flavus* from which they were originally isolated. Other significant members of the aflatoxin family are M_1 and M_2, that are oxidative forms of aflatoxin B_1 modified in the



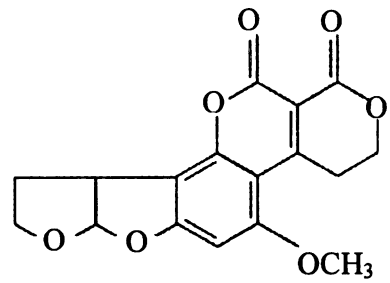
AFB_1



AFB_2



AFG_1



AFG_2

Figure 1.1. Molecular structure of the four major types of aflatoxins

digestive tract of some animals and isolated from milk, urine and feces (Allcroft *et al.*, 1966; Allcroft *et al.*, 1967; Yu *et al.*, 2002).

Toxicity

Mycotoxins (low molecular weight secondary metabolites, produced by filamentous fungi, including *Aspergillus*, *Penicillium* and *Fusarium*), are known to cause a toxic response, called mycotoxicosis, if ingested by higher vertebrates and other animals (Bennett & Klich, 2003; Yabe & Nakajima, 2004; Yu *et al.*, 2004b). Since the discovery of Turkey X disease in 1960, aflatoxins are recognized as the most harmful mycotoxins and are of greatest significance in agriculture and human health. The diseases caused by aflatoxin consumption are collectively called 'aflatoxicoses'. According to the studies done on laboratory animals and domestic animal species, the major target organ for aflatoxin toxicity is the liver (Busby, 1984). Aflatoxins are immunosuppressive, mutagenic, teratogenic and hepatocarcinogenic in experimental animals (Yu *et al.*, 2002). The effects of aflatoxin ingestion are dependent on dose, time of exposure and nutritional status of patients (CAST, 2003). Acute effects may involve lack of appetite, loss of weight, gastrointestinal infection coupled with abnormal liver function tests, liver centrilobular necrosis, bile duct proliferation, acute hepatitis, and even death (Edds, 1973; Mwanda *et al.*, 2005; Probst *et al.*, 2007; Robens & Richard, 1992). Chronic aflatoxicosis results in cancer and immune suppression (Bennett & Klich, 2003; Busby, 1984). The liver is the primary target organ for these potent naturally occurring carcinogens, which cause liver damage, liver cirrhosis, tumor induction, teratogenesis and hepato-cellular carcinoma(Bennett & Klich, 2003). Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2

(AFG_2) are the major aflatoxins (where B and G refer to the blue and green fluorescence observed upon UV exposure) produced by the filamentous fungi with AFB_1 being the most toxic and most abundant (McLean & Dutton, 1995; Yu *et al.*, 2004b) (the order of toxicity is $B_1>G_1>B_2>G_2$).

Epidemiological studies have shown that aflatoxins, together with Hepatitis B virus, cause 250,000 deaths annually in certain parts of China and sub-Saharan Africa from hepatocellular carcinoma (Yabe & Nakajima, 2004). Aflatoxins are a major concern in areas where climates are warm and humid, and grains are stored with poor drying. Although humans and animals are susceptible to the effects of acute aflatoxicosis, the likelihood of human exposure to acute levels of aflatoxin is more remote in well-developed countries. In undeveloped countries, human susceptibility can vary with age, health, and level and duration of exposure. Some notable outbreaks include the deaths of 3 Taiwanese in 1967, the deaths of more than 100 people in Northwest India in 1974 and 12 deaths in Kenya in 1982 (Agag, 2004). Aflatoxins also can be detected in meat, milk, and eggs from animals that have consumed feed ingredients containing aflatoxins. Hence, apart from direct risks to humans from consumption of aflatoxin-contaminated grains, there also are indirect health risks to those who consume animal products containing residues of the toxin.

Mechanism of carcinogenicity

AFB1 is metabolized by the liver through the cytochrome p450 enzyme system to the major carcinogenic metabolite AFB1-8,9 epoxide (AFBO), or to less mutagenic forms such as AFM1, Q1 or P1. There are several pathways that AFBO can take,

resulting in cancer, toxicity or excretion through the urine. This highly reactive molecule readily binds DNA and protein to form adducts; for example N^7-guanine adducts lead to gene mutations and cancer. One such mutation occurs in the human p53 tumour suppressor gene at codon 249. Studies have shown that AFBO induces G>T or G>C transversions in this codon, making it a mutational 'hotspot' (McLean & Dutton, 1995; Newberne, 1973; Rundle, 2006; Staib *et al.*, 2003). Inhibition of AFBO formation (through disruption of the cytochrome p450 system) and/or adduct formation are important strategies for prevention of these damaging mutations. In animal models, metabolic detoxification of AFBO is facilitated by induction of glutathione S-transferase (GST), which catalyzes the reaction that binds glutathione to AFBO and renders it non-carcinogenic.

Economic significance of aflatoxin contamination

Because of the serious health concerns that result from aflatoxin contamination of foods and feeds worldwide, regulatory guidelines have been imposed by the U.S. Food and Drug Administration for consumption and for interstate shipment of foods and feeds. A maximum of 20 parts total aflatoxin per billion parts of food or feed substrate (ppb) is the maximum allowable limit (action level) imposed according to FDA(CAST, 2003). In some European countries total aflatoxin levels are regulated below 5 ppb. The stringency in allowable limits of aflatoxin results in an enormous wastage of agricultural commodities because they must be destroyed or decontaminated. One report says that the yearly loss due to aflatoxin contamination in peanut and corn has been estimated to be about $47 million (CAST, 2003). Another report says that the average aflatoxin

management costs in United States alone is at least $100 million per year (Robens, 2001). The problem is further complicated because these toxins are resistant to milling, processing and cooking (CAST, 2003) and the presence of other mycotoxins might result in synergistic effects (Casado J.M, 2001). Aflatoxins along with other mycotoxins (fumonisins, etc.) also might have serious impacts on the recently booming biofuel industry. Their presence may affect negatively the biological conversion of lignocellulosic materials to liquid fuel (Kyung-Hwan Han, 2007); an old study suggests that aflatoxin contamination may affect biological conversion of carbohydrates to ethanol by affecting the activity of alcohol dehydrogenase (Reiss, 1973). There are also concerns with regard to occupational hazard associated with production of biofuels from biomass contaminated with aflatoxins (A. M. Madsen, 2003).

Environmental significance

The increasing concern over global warming and a constant depletion of stratospheric ozone layer of approximately 6% per decade (Björn, 2007), raise concerns regarding a possible trend to increased accumulation of aflatoxins in the environment. In a previous study, near UV light exposure enhanced accumulation of aflatoxin B_1 in *A. flavus* culture, caused a mutagenic effect and induced production of aflatoxin by non-toxigenic strains *A. flavus* P-63, *A. niger* EN-200 and *A. ochraceus* P-157 (Nagy H. Aziz, 2002; Nagy H. Aziz, 1997).

Therefore, elimination of aflatoxins is a critical economical, environmental and health concern worldwide.

Aflatoxin biosynthesis

Our previous work

1. **The discovery of the aflatoxin biosynthetic genes and aflatoxin gene cluster**

   Our laboratory first cloned the aflatoxin structural genes *nor*-1 and *ver-1* by genetic complementation of the recipient strains B-62 (Chang *et al.*, 1992) and CS-10 (Skory *et al.*, 1992). A year later genetic linkage between *nor-1* and *ver- 1* (Skory *et al.*, 1993) was demonstrated and by 1995, a physical and transcript map of the aflatoxin gene cluster was generated and the gene cluster was characterized (Trail *et al.*, 1995; Yu *et al.*, 1995). By the early 2000s through the collaborative efforts of different laboratories working on the aflatoxin biosynthetic pathway the complete nucleotide sequence and the gene function for the entire aflatoxin cluster was delineated (Yu *et al.*, 2004a; Yu *et al.*, 2004b). Briefly the pathway (Figure 1.2.) is : acetate → polyketide (decaketide) → anthraquinones → xanthones → aflatoxins (Yabe & Nakajima, 2004; Yu *et al.*, 2004b). Initially, acetate and malonyl CoA are converted to a hexanoyl starter unit by a specialized fatty acid synthase, which is then extended by a polyketide synthase to the early intermediate norsolorinic acid, the first stable precursor in the pathway. The polyketide then undergoes several enzymatic conversions, through a series of pathway intermediates. Following the formation of the middle pathway intermediate versicolorin B, the pathway branches to form AFB1, and AFG1, which contain dihydrobisfuran rings and are produced from demethylsterigmatocystin (DMST); the other branch forms AFB2, AFG2, which contain tetrabisfuran rings and are produced from dihydrodemethylsterigmatocystin

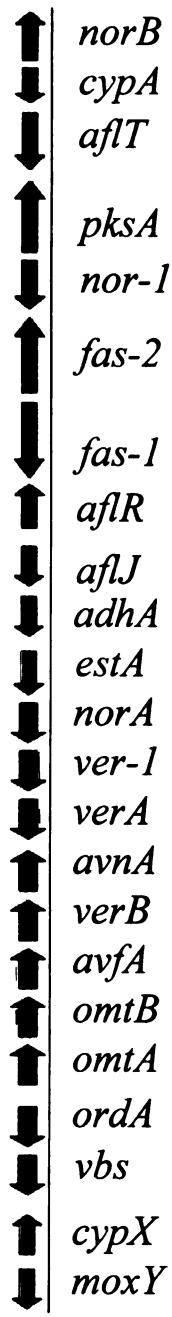


Figure 1.2. The 75kb aflatoxin gene cluster in *A. parasiticus.* Arrowheads represent direction of transcription.

(DHDMST). *A. parasiticus* generally produces all four kinds of major aflatoxins, whereas *A. flavus* produces only AFB1 and AFB2 (Yabe & Nakajima, 2004). More than 20 *Aspergillus* species produce sterigmatocystin (ST) as their final product, which is a potent mycotoxin as well. At least 17 different enzymatic steps are involved in aflatoxin biosynthetic pathway. The genes for this biosynthetic pathway are well conserved between *Aspergilli* and are located within a 75kb gene cluster in the genomes of *A. parasiticus* and *A.* flavus (Yabe & Nakajima, 2004; Yu *et al.*, 2004b).

2. Regulatory factors involved in aflatoxin gene expression

A very important event in aflatoxin research was the discovery of a key positive regulator in the pathway called AflR (Chang *et al.*, 1993; Payne *et al.*, 1993). AflR belongs to a family of fungal transcriptional activators (zinc binuclear cluster), and positively regulates the expression of most genes involved in the AF/ST biosynthesis pathway. Gene disruption of (knocking out) *aflR* completely blocks the expression of (most) genes in this biosynthetic pathway and eliminates aflatoxin or sterigmatocystin production (Chang P.K, 1999; Woloshuk C.P, 1994). Our studies show that AflR requires other regulatory factors to carry out its regulatory activities. Novel transcription factors (NorLbp and CRE1bp), TATA binding protein and their associated *cis*-acting binding sites have been identified that participate in regulation of aflatoxin biosynthesis at the level of the individual gene (*nor*-1) and the entire aflatoxin gene cluster possibly through their association with AflR regulation (Miller *et al.*, 2005; Roze *et al.*, 2004c).

3. **Association between fungal development and aflatoxin synthesis**

Studies by us and others suggested that aflatoxin biosynthesis and fungal development are associated. Strains that accumulate intermediates between norsorolinic acid and versicolorin A have reduced sclerotial (asexual survival structure) development (Skory *et al.*, 1992; Trail *et al.*, 1995). On the other hand, a gene knock-out of *fas-1A* or *pksA* that affect the pathway upstream to *nor-1* increased sclerotia production (Mahanti *et al.*, 1996). Another gene encoding a polyketide synthase (*fluP*), was studied by our laboratory; disruption of *fluP* reduced sclerotial development (Zhou *et al.*, 2000). Sclerotial development also is affected in the o-methylsterigmatocystin accumulating strain SRRC2043 (Yu *et al.*, 1998). Various conidiation mutants showed reduction in aflatoxin production (Kale *et al.*, 2003). A recent study showed that disruption of *veA* gene in *A. parasiticus* resulted in the blockage of sclerotial formation as well as a blockage in the production of aflatoxin intermediates (Calvo *et al.*, 2004). According to a recent finding, the VeA protein in *A. nidulans* participates in a complex that connects light-dependant developmental regulation and control of secondary metabolism (Bayram *et al.*, 2008). These data strongly support a regulatory link between fungal development and secondary metabolism in *A. parasiticus.*

4. **Studies on signal transduction pathways and molecular mechanisms that mediate aflatoxin gene expression**

Aflatoxin gene expression is affected by many environmental factors [carbon, nitrogen, trace elements, pH and temperature] (Miller MJ, 2006). These studies provided the rationale for our research on signal transduction mechanisms in *A. parasiticus*. One identified signaling pathway associated with aflatoxin biosynthesis is a G-protein mediated signaling pathway. *A. parasiticus* makes aflatoxin in yeast extract sucrose [YES] medium but not in yeast-extract-peptone (YEP) medium (when peptone replaces sucrose) (Cary *et al.*, 2000). In a 100mL YES liquid shake culture (standard growth conditions, batch fermentation) *A. parasiticus* starts aflatoxin production from 24h and the production increases exponentially until 48h. After 48h the production slows down (Liang, 1996). Aflatoxin proteins and transcripts are first detected in the same system between 24h to 40h (Roze *et al.*, 2007a). We think that the transition from high to low glucose/sucrose is what activates a signal transduction pathway that is finally transduced into aflatoxin gene expression. A regulatory model was first proposed in *A. nidulans* where it was shown that FadA, an α-subunit of the heterotrimeric G protein, binds GTP, the result of which promotes growth and inhibits conidiation and sterigmatocystin production (Hicks *et al.*, 1997). The involvement of G protein, cAMP and PKA in regulation of aflatoxin biosynthesis and conidiation was later established in the Keller laboratory (Shimizu & Keller, 2001) and in our laboratory in *A. parasiticus* (Roze *et al.*, 2004a). It was proposed that FadA/PKA regulation mechanisms are similar in *A. parasiticus* and *A. nidulans* and cAMP levels are involved in mediating the PKA-dependent regulatory influence on conidiation and

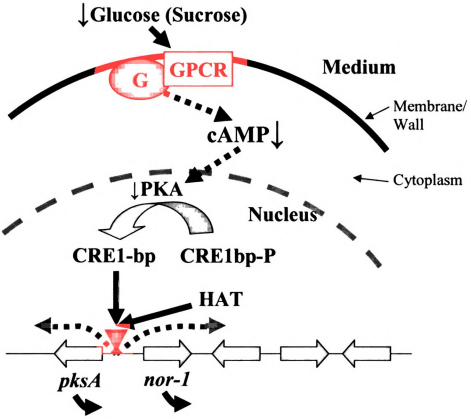


Figure 1.3. Model: GPCR mediated sensing of declining glucose/sucrose concentration in the growth medium by 24 h results in a decrease of cAMP/PKA pathway activity. CRE1bp bound to 15 CRE-like sites in the *pks*A/*nor*-1 intergenic region, is activated by dephosphorylation and forms a complex with HAT (histone acetyltransferase) ; this initiates bidirectional acetylation of histone H4 followed by activation of gene transcription (Roze *et al.*, 2007a).

aflatoxin biosynthesis in *A. parasiticus.* Recently we have also found a positive correlation between the initiation and spread of histone H4 acetylation in aflatoxin promoters and the onset of accumulation of aflatoxin proteins and aflatoxin (Roze *et al.*, 2007a). We proposed that the declining glucose/sucrose levels in the growth medium is sensed by a G-protein coupled receptor, which turns on a signaling cascade through a cAMP/PKA signaling pathway. This results in activation of a cyclic AMP response element binding protein, called CRE1bp (Roze *et al.*, 2004c), which then recruits histone acetyltransferase (HAT) to initiate a wave of histone H4 acetylation at or near the *pksA*/*nor*-1 intergenic region. The wave then spreads bidirectionally from this point. The CRE1-like sites present in the promoters throughout the cluster reinforce spreading of the wave by recruiting new CRE1bp/AflR/HAT complexes to the aflatoxin promoters as they are made accessible in the conversion of heterochromatin to euchromatin (Figure 1.3.)

5. Studies on compounds that inhibit aflatoxin production

As mentioned earlier, aflatoxins have received greater attention than other mycotoxins because of their acute health and economic impacts worldwide. The long-term goal of gaining knowledge about the aflatoxin biosynthetic pathway aims at eliminating aflatoxin contamination from foodstuffs. Extensive research has been focused on identifying compounds that inhibit aflatoxin biosynthesis. Our laboratory's contributions in this area include the findings of inhibitory effects of ethylene and carbon dioxide (Gunterus *et al.*, 2007; Roze *et al.*, 2004b), wortmannin (Lee *et al.*, 2007) and volatiles (Roze *et al.*, 2007b) on aflatoxin biosynthesis. An

excellent detailed review on this area of research has recently been published (Holmes *et al.*, 2008).

6. Studies on the subcellular localization of aflatoxin enzymes and aflatoxins

In order to design efficient ways to block aflatoxin production (our long term goal) sufficient information (apart from understanding the genes and enzymes involved in aflatoxin biosynthesis) is necessary to understand how the aflatoxin enzymes localize and interact within the fungal cell to complete the biosynthetic pathway. So apart from studying aflatoxin biosynthesis at the molecular level we also focused on studying aflatoxin biosynthesis at the cellular and colony levels. My research was aimed at contributing to this area. Our initial incentive for this study was to block aflatoxin biosynthesis by blocking the destined localization of aflatoxin enzymes. But along the way we found that secondary metabolic pathway in plants and fungi share striking similarities in their use of the endomembrane system to effectively compartmentalize proteins, substrates, intermediates and products for completion of the orderly biosynthetic process. The knowledge of orchestration and regulation of secondary metabolism at a cellular level in an Aspergillus model could therefore be useful to discover novel methods to control and manipulate secondary metabolism in nature.

Presented in the following section is a brief review on what is known about compartmentalization of secondary metabolism in plants and fungi. The review ends with a summary of our previous subcellular localization studies of aflatoxin proteins in *A. parasiticus*.

Compartmentalization in secondary metabolism

Plants

Plant secondary metabolites are broadly classified on the basis of their biosynthetic origin into alkaloids and other nitrogen compounds, phenolics and terpenoids (Harborne, 1999.). Based on a thorough search of the literature, most sub-cellular work has been done on alkaloid and flavonoid (phenolic compounds) metabolism.

Alkaloids are secondary metabolites derived from amino acids. Approximately 12000 alkaloids are produced by plants (Facchini & St-Pierre, 2005). Their source can be traced to the aliphatic amino acids ornithine and lysine and the aromatic amino acids phenylalanine, tyrosine and tryptophan (Mann, 1987). Different specialized cell types [endodermis, laticifers, idioblasts, pericycle and cortex] have been associated with alkaloid biosynthesis and accumulation in plants. Recent studies on trafficking and metabolic channeling in alkaloid metabolism suggest that alkaloid biosynthetic enzymes can effectively form complexes in sub-cellular compartments. The compartmentalization of enzyme complexes helps in sequestration of the toxic endproducts in those compartments and protects the cell from self-toxicty. Studies done on *Catharanthus roseus* have associated vacuoles with the sequestration of endogeneous alkaloids (Deus-Neumann B, 1984; Deus-Neumann B, 1986; McCaskill *et al.*, 1988; Roytrakul S, 2007). For example, berberine has been shown to accumulate in vacuoles mediated by two different transporters – an ABC transporter and a H^+/berberine antiporter (Otani *et al.*, 2005). Nicotine, that is toxic to the cells, also accumulates in vacuoles (Saunders, 1979). Sanguinarine, a toxic benzophenanthridine alkaloid made in opium poppy cells is made

in vesicles originating from the ER and which are thought to fuse with the central vacuole (Alcantara *et al.*, 2005). A similar vesicle-mediated-vacuolar accumulation has also been suggested for camptothecin, a terpenoid indole alkaloid (Sirikantaramas *et al.*, 2007). Three excellent reviews are available on alkaloid metabolism- one on biosynthesis (Facchini, 2001), another on synthesis and trafficking (Facchini & St-Pierre, 2005) and the most recent one on trafficking and metabolic channeling (Ziegler & Facchini, 2008).

Flavonoids, the most common among the phenolics, belong to the class of phenylpropanoids. These are a diverse group of secondary metabolites that result from different branches of a general biosynthetic pathway. Because of the multiple levels of regulation involved in production of these structurally similar but functionally different compounds, flavonoid biosynthesis has been studied extensively. The pathway has been recently reviewed in detail (Tanaka *et al.*, 2008). Biosynthesis starts from a compound called coumaryl CoA. Chalcone synthase, a polyketide synthase, catalyzes the reaction of one molecule of 4-coumaryl CoA and three molecules of malonyl CoA to form a chalcone [tetrahydroxychalcone]. From here the pathway can branch in different directions forming different products [anthocyanins, apigenin (flavone), naringenin (flavanone), kaemferol (flavonol), aureusidin 6-glucoside and isosalipurposide (chalcone)] depending on the enzyme[s] in play. From interaction studies it was proposed that multiple flavonoid biosynthetic enzymes interact to form macromolecular complexes (Burbulis & Winkel-Shirley, 1999) at the cytoplasmic face of the endoplasmic reticulum [ER] membrane (Hrazdina *et al.*, 1987). It has been suggested from many studies that flavonoids, after being synthesized in the cytosol, are transported to vacuoles for storage. A study on lisianthus (Eustoma grandiflorum) petals shows the involvement of pre-

vacuolar compartments [PVCs] derived from endoplasmic reticulum [ER] for transport of anthocyanins to vacuoles (Zhang *et al.*, 2006). Involvement of glutathione s-transferase (Alfenito *et al.*, 1998; Kitamura *et al.*, 2004; Marrs *et al.*, 1995), a multidrug resistance-associated protein (MRP), ZmMrp3 (Goodman *et al.*, 2004) and a membrane protein [TT12] of the multidrug and toxic efflux [MATE] transporter family (Marinova *et al.*, 2007) also have been demonstrated in the mediation of anthocyanin transport to vacuoles.

Marijuana (*Cannabis sativa*) is a herb that synthesizes a group of terpenophenolics called cannabinoids. Delta (1)-tetrahydrocannabinol (THC) is one such compound, the biosynthetic pathway of which has been characterized. Sub-cellular localization studies in *C. sativa* have shown that THCA synthase that synthesizes tetrahydrocannabinolic acid (THCA) is sorted from secretory cells into a storage cavity in glandular trichomes (Sirikantaramas *et al.*, 2005). In the storage cavity THCA was functional and both the substrate [CBGA] and the product [THCA] were present. Hence it was suggested that the storage cavity is not only the site for the accumulation of cannabinoids but also the site for THCA biosynthesis.

Fungi

Fungal secondary metabolites occurring in nature can be broadly divided into two major categories (Hoffmeister & Keller, 2007):

1. Polyketides and fatty acid-derived compounds, and
2. Non-ribosomal peptides and aminoacid-derived compounds

The first group includes aflatoxin/sterigmatocystin/dothistromin, ochratoxin, aurofusarin, aromatic polyketides, lovastatin/compactin, squalestatin, fumonisin, fusarin, equisetin, methylsalicylic acid and related compounds, oxylipins and spore and hyphal pigments. The second group includes penicillin and cephalosporin, cyclosporin, ergot alkaloids, tricothecenes, diketopiperazines, asterriquinones, siderophores, HC-toxin and victorin, AM-, AK-,AF- and ACT-toxins, destruxin, enniatins, peptaibols, peramine, loline alkaloids, terpenes, gibberellin, indole diterpenes, botrydial, aphidicolin and carotenoids. Unlike plants, little is known about sub-cellular organization of secondary metabolism in fungi.

Two β-lactam antibiotics, penicillin and cephalosporin (amino acid-derived metabolites) have been studied extensively at the sub-cellular level. Penicillin biosynthesis involves three enzymes – ACVS ((L-aminodipyl)-L-cysteiyl-D-valine synthetase), IPNS (isopenicillin N synthetase) and IAT (acyl-coenzyme A:isopenicillin N acyltransferase isopenicillin N acyltransferrase). In *Penicillium chrysogenum* and *A. nidulans*, ACVS and IPNS are located in cytosol (van de Kamp *et al.*, 1999; van der Lende *et al.*, 2002) whereas IAT is located in peroxisomes (Muller *et al.*, 1991; van de Kamp *et al.*, 1999; van der Lende *et al.*, 2002). So, it is thought that penicillin biosynthesis and storage occurs in peroxisomes. Precursors for this biosynthetic pathway are proposed to be withdrawn from the vacuolar amino acid pool with the help of a fraction of ACVS that is loosely bound to the vacuolar membrane (Lendenfeld *et al.*, 1993). Cephalosporin (a structural relative of penicillin) biosynthesis probably occurs in the cytosol because all enzymes of cephalosporin biosynthesis pathway in *Cephalosporium acremonium* have a cytosolic location (van de Kamp *et al.*, 1999);

however, a recent study demonstrates the involvement of peroxisomes in cephalosporin synthesis in *Acremonium chrysogenum* (Teijeira *et al.*, 2008) but the nature of this involvement is not clear.

Cyclosporin is a non-ribosomal peptide whose synthesis is catalyzed by a large enzyme complex, cyclosporin synthetase. The enzyme activates the substrate amino acids (L-valine, L-leucine, L-alanine, glycine, 2-aminobutyric acid, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-threonine(butenyl-methyl-Thr), and D-alanine) to amino acyl adenylates and binds them covalently via thioester linkages at prosthetic phosphopantetheine groups. Recent studies in *Tolypocladium inflatum* indicate that cyclosporine synthetase and another enzyme alanine racemase (that synthesizes D-alanine for cyclosporine synthesis from L-alanine) are attached loosely to the vacuolar membrane (Hoppert *et al.*, 2001). According to the model of Hoppert (Hoppert *et al.*, 2001) the metabolic flux in cyclosporine synthesis is directed from the cytosol towards the vacuolar lumen in contrast to penicillin where the metabolic flux is directed from vacuoles→cytosol→peroxisomes. Cyclosporin synthesis presumably occurs at the vacuolar membrane at cyclosporine synthetase locus and is stored in vacuoles.

Aflatoxin biosynthesis has been studied extensively by us at the cellular level (the only sub-cellular studies known for fungal polyketide biosynthesis). Specific rabbit polyclonal antibodies were developed through our previous work for an early pathway enzyme Nor-1 (Zhou, 1997), middle enzymes Ver-1 (Liang, 1996) and Vbs (Chiou, 2003) and a late enzyme called OmtA (Lee, 2003). Lee and Chiou developed a "concentric circle colony fractionation" procedure (Chiou, 2003; Lee, 2003) to determine the sub-cellular localization of Nor-1, Ver-1, Vbs and OmtA in specific fractions of *A.*

parasiticus grown on solid aflatoxin inducing media. Transmission electron microscopy (TEM) performed after immunogold labeling with antibodies against Nor-1. Ver-1, Vbs and OmtA suggested that at a relatively early time in colony growth [24- 48h], all four enzymes localize in the cytoplasm. However, in the cells on the substrate surface of colonies of the same age [24-48h old], OmtA predominately localized in vesicles and vacuoles. In a recent study, sub-cellular localization of Nor-1 and Ver-1 in *A.parasiticus* was monitored in real time (Hong, 2008). Hong developed an EGFP reporter system in which expression of Nor-1 and Ver-1 fused to EGFP reporter were driven by wild-type *nor-1* and *ver-1* promoters respectively. Confocal laser scanning microscopy (CLSM) demonstrated that these fusion proteins were synthesized in the cytoplasm but later localized in vesicles and vacuoles. Based on these data, two possible models to explain the intracellular site of aflatoxin synthesis were proposed. In the first model, aflatoxin is synthesized in the cytoplasm by aflatoxin enzymes that are present in that location. After synthesis is complete, the toxin is secreted outside the cell by an unknown mechanism [previous data demonstrated that 99% of the aflatoxin made by the cell was found in the growth medium (Roze *et al.*, 2007a)]. Aflatoxin enzymes are then transported to vacuoles for proteolytic cleavage by peptidases that also localize there. According to the second model, aflatoxin enzymes are made in the cytoplasm and transported to vesicles and vacuoles. These proteins are enzymatically functional in vesicles and vacuoles and hence, part or all of aflatoxin biosynthesis occurs in these organelles; the enzymes are eventually degraded (Chiou, 2003; Lee, 2003; Skory, 1992), likely by peptidases that are transported to these organelles. According to this model, aflatoxin is sequestered within

the vesicles and vacuoles and finally is transported outside the cell by an unknown mechanism.

My research was aimed at determining the functional significance of vesicles and vacuoles in aflatoxin biosynthesis. The hypothesis tested in this study is that **vesicles and vacuoles are the intracellular site(s) for aflatoxin biosynthesis.** Two approaches were adopted to test this hypothesis. In the first approach (as discussed in Chapter 2), I developed a method to purify a vesicles-vacuoles fraction from *A. parasiticus* during aflatoxin synthesis to conduct a functional analysis of this fraction. In the second approach (as discussed in Chapter 3), I interrupted vacuole biogenesis genetically and chemically to determine the individual roles of vesicles and vacuoles in aflatoxin biosynthesis.

Significance of this study

The current study has increased our understanding about the role of vesicles and vacuoles in aflatoxin biosynthesis and the organization of this polyketide biosynthetic pathway within the cell. Follow up studies will characterize the composition of these organelles in detail. The knowledge gained from these studies can be applied directly to solving the aflatoxin contamination problem by providing novel targets to block aflatoxin contamination. This will have positive outcomes on human and animal health and the global economy and will also help in development of new strategies to manipulate secondary metabolism in nature for the benefit of human kind.

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CHAPTER 2

VESICLES AND VACUOLES ARE ONE PRIMARY SITE FOR THE LATE STEPS IN AFLATOXIN BIOSYNTHESIS AND STORAGE IN ASPERGILLUS

ABSTRACT

Aflatoxin biosynthesis initiates in response to environmental signals, involves the tightly regulated expression of a 75kb gene cluster resulting in the coordinated activity of at least 17 enzymes, and ends with secretion of the end product(s) outside the cell. Aflatoxin synthesis is one of the most highly characterized eukaryotic secondary metabolic pathways and serves as a useful model to understand secondary metabolism in fungi and plants. Our previous studies demonstrated localization of early, middle and late aflatoxin enzymes in vesicles and vacuoles during aflatoxin synthesis; these organelles exhibited significant heterogeneity in size (20nm to 5μm) and density. In the current study, we developed methods to purify and conduct functional analyses of vesicles and vacuoles from *Aspergillus parasiticus* protoplasts generated during aflatoxin synthesis using a 'high density sucrose cushion' method. Western blot analysis demonstrated the presence of three aflatoxin enzymes (Ver-1, Vbs and OmtA) in this purified organelle fraction. Vesicles and vacuoles also were observed to represent a primary site to sequester aflatoxin. The conversion of sterigmatocystin, a late intermediate in aflatoxin biosynthesis, to aflatoxin B_1 was demonstrated in this purified fraction showing that the

last two steps of aflatoxin biosynthesis occur in or on vesicles and vacuoles. These data demonstrate a strong functional link between aflatoxin synthesis and storage, and vesicles and vacuoles in *A. parasiticus*.

INTRODUCTION

Secondary metabolites, natural products generated by filamentous fungi, plants, bacteria, and animals, have an enormous impact on humans due to their application in health, medicine and agriculture. Many secondary metabolites are beneficial (antibiotics, statins, morphine, etc.), while phytotoxins (e.g. ricin, crotin, amygdalin) and fungal poisons called mycotoxins (e.g. aflatoxin, sterigmatocystin, fumonisin) are detrimental to humans. To control or customize biosynthesis of these natural products we must understand how and where secondary metabolism is orchestrated within the cell.

Aflatoxins are polyketide-derived furanocoumarins synthesized primarily by the filamentous fungi *A. parasiticus* and *Aspergillus flavus* (Bennett & Klich, 2003; Miller MJ, 2006). Aflatoxin biosynthesis is one of the most highly characterized secondary metabolic pathways and serves as a model to understand secondary metabolism in eukaryotes. Aflatoxin B_1 (AFB_1) is the most potent naturally occurring carcinogen known (Miller MJ, 2006) and has significant health and economic impacts worldwide (Miller MJ, 2006; Yabe & Nakajima, 2004). Aflatoxin biosynthesis involves at least 17 enzyme activities encoded by 25 or more genes that are clustered in a 75-Kb region on one chromosome (Miller MJ, 2006; Yabe & Nakajima, 2004; Yu *et al.*, 2002). The molecular mechanisms that regulate aflatoxin biosynthesis have been studied extensively

by us and others [reviewed in (Keller *et al.*, 2005; Miller MJ, 2006)]. We are currently studying the spatial distribution of aflatoxin enzymes and pathway intermediates at the sub-cellular level to identify targets to control aflatoxin synthesis.

In plants, vesicles and vacuoles sequester secondary metabolites to protect host cells from self-toxicty (Facchini & St-Pierre, 2005; Tanaka *et al.*, 2008). Multi-enzyme complexes involved in synthesis of these metabolites localize either in sub-cellular compartments [alkaloid biosynthesis (Facchini & St-Pierre, 2005)] or in the cytosol [flavonoid biosynthesis (Tanaka *et al.*, 2008)]. Less is known about intracellular sites of secondary metabolism in fungi. Penicillin, a β-lactam antibiotic (amino-acid-derived fungal secondary metabolite) is thought to be synthesized in peroxisomes, where the final two enzymes in the biosynthetic pathway localize in *Penicillium chrysogenum* (van de Kamp *et al.*, 1999). Cephalosporin, a structural relative of penicillin, is thought to be made in the cytosol in *Cephalosporium acremonium* where most of the biosynthetic enzymes localize (van de Kamp *et al.*, 1999). However, a recent study in *Acremonium chrysogenum* demonstrated involvement of peroxisomes in cephalosporin synthesis (Teijeira *et al.*, 2008). In *Tolypocladium inflatum*, cyclosporin (a non-ribosomal peptide derived fungal secondary metabolite), and a key pathway enzyme (cyclosporin synthetase) were detected in vacuoles (Hoppert *et al.*, 2001); the functional significance of this location is not yet clear.

Unlike for amino acid and non-ribosomal peptide-derived secondary metabolites, the intracellular site of fungal polyketide-derived secondary metabolites was not known prior to our current work. We previously used transmission electron microscopy (TEM) and immunogold-labeled antibodies to detect OmtA (a late enzyme in aflatoxin

biosynthesis) primarily in the cytoplasm in cells grown for 24 to 48 h under aflatoxin inducing conditions. However, OmtA localized primarily to vacuole-like organelles (Lee *et al.*, 2004) in cells on the substrate surface of colonies (also at 24 h-48 h), while Nor-1 (an early enzyme in the pathway), Ver-1 and Vbs (middle enzymes) were detected primarily in the cytoplasm (Chiou *et al.*, 2004; Lee *et al.*, 2004). More recently, we demonstrated that Nor-1 or Ver-1 fused to EGFP localized to vesicles and vacuoles (Hong, 2008). Despite compelling evidence supporting the localization of multiple aflatoxin enzymes in vesicles and vacuoles, it was not clear whether aflatoxin biosynthesis occurred in these organelles or if the aflatoxin enzymes localized to the vacuole for turnover.

As a first step to determine the functional role of these organelles in Aspergillus secondary metabolism, we developed a novel 'high density sucrose cushion' method for purification of a vesicles-vacuoles fraction from *A. parasiticus* during aflatoxin synthesis (Figure 2.1). Western blot analysis showed the presence of three aflatoxin enzymes (Ver-1, Vbs and OmtA) in this fraction. This fraction also was the predominate site for sequestering aflatoxin. Significantly, the fraction converted exogenously added sterigmatocystin to aflatoxin *in vitro*, demonstrating that the final two steps in aflatoxin biosynthesis can be carried out by vesicles and vacuoles. This current work is the first to demonstrate the primary sub-cellular site(s) for the late steps in aflatoxin biosynthesis.

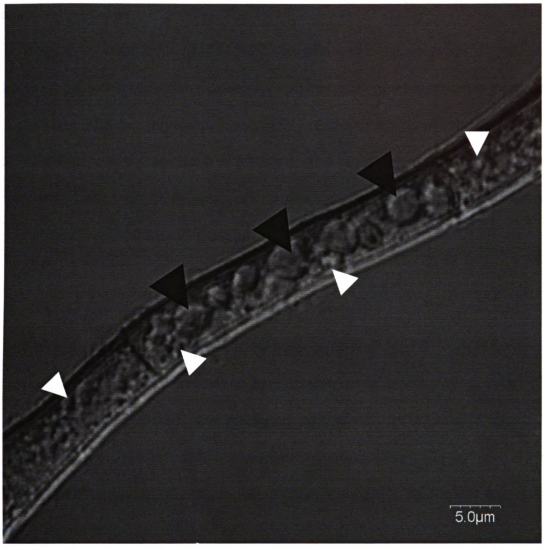

Figure 2.1. A differential interference contrast [DIC] image of *A. parasiticus* (whole cell): *A. parasiticus* was grown for 36 h under aflatoxin inducing conditions and observed by CLSM. Organelles in whole cells were divided into two subsets; organelles ≥ 2.5µm were designated 'vacuoles' (black arrows) and those <2.5µm were designated as 'vesicles' (white arrows).

MATERIALS AND METHODS

Fungal strains and growth media

A. parasiticus strain SU-1 (ATCC 56775), a wild-type aflatoxin producer, was used in this study. *A. parasiticus* SU-1 conidiospores (spores) from a frozen spore stock were inoculated into YES liquid medium [contains 2% yeast extract and 6% sucrose; pH 5.8] at 10^4 spores per ml and incubated at 30°C with shaking at 150 rpm for 36h (standard growth conditions). Western blot analysis demonstrated accumulation of aflatoxin enzymes beginning at 30 h under these growth conditions (Roze *et al.*, 2007).

Preparation of protoplasts from mycelia

Protoplasts were prepared by a published method (Birch *et al.*, 2001). Mycelia grown for 36h in YES media were harvested by filtration through miracloth [Calbiochem]. The miracloth carrying the mycelia was placed on paper-towels for 30 min to absorb the remaining medium. The mycelia were resuspended [40mg of mycelia per mL] in lysis buffer [10 mM potassium phosphate, pH 5.8] containing 1.2 M $MgSO_4$ as osmotic stabilizer and 50 mg/mL of driselase and lysing enzymes [both from Sigma, St.Louis, St. Louis, MO]. The suspension was incubated for 7 h 30 min at 30°C with shaking at 100 rpm. The protoplasts were separated from debris by filtration through a double layer of cheese cloth and then nylon mesh (30 μm mesh, 64 μm thickness) [Spectrum Labs, Rancho Dominguez, CA]. 25 mL of filtrate was overlaid with 25 mL of separation buffer [0.6 M sorbitol, 100 mM Tris-Cl, pH 7.0] in a 50 mL conical tube and centrifuged at 1500 x g at room temperature (RT) for 15 min. Protoplasts (10^6 obtained per gram of mycelia) were collected from the interface in a total volume of 5 mL, mixed with an

equal volume of protobuffer [1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5], and centrifuged at 1000 x g for 10 min (at RT) to obtain a protoplast pellet. This pellet was washed with protobuffer and centrifuged at 1000 x g for 10 min at 4°C. The protoplast pellet was finally resuspended in 0.5mL of protobuffer. The purity of the protoplast fraction was analyzed by bright field microscopy using a Nikon Eclipse E600 microscope [Nikon Inc., Melville, NY].

Purification of a vesicles-vacuoles fraction

The protoplast suspension (0.5 mL) was added to 1.5 mL of protoplast lysis solution [0.6M sorbitol, 10mM Tris-Cl, 0.025% Triton-X 100 pH 7.5]. Release of vesicles and vacuoles was observed by the use of MDY-64 (Molecular Probes, Invitrogen, Carlsbad, CA) that stains yeast vacuolar membranes green; cells were viewed under a Nikon Eclipse E600 or Labophot fluorescence microscope (Nikon Inc., Melville, NY). After approximately 15 min, when 90-95% of the protoplast lysed, 1 ml of lysis mixture was carefully overlaid on a 1mL high density sucrose cushion [60% (w/v) sucrose, 1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5] and then centrifuged at 3000 x g at RT for 45 minutes. The vesicles-vacuoles fraction was collected from the interface in a volume of 100 µL and stored on ice for further analysis.

Purity assessment of the vesicles-vacuoles fraction

Three parallel approaches were utilized to confirm purity of the vesicles-vacuoles fraction.

a. Confocal laser scanning microscopy (CLSM)

MDY-64 and CellTracker Blue CMAC (vacuolar peptidase marker) [Molecular Probes, Invitrogen, Carlsbad, CA] were used to visualize vesicles and vacuoles and to assess their purity. These dyes were utilized according to manufacturer's protocols with modifications. 2 μL of a 10 mM MDY-64 stock solution in DMSO was added to the protoplast lysis mixture (2 mL total volume) 5 min before loading on the density gradient. 1 μL of 10 mM CMAC in DMSO was added to the purified vesicles-vacuoles fraction and incubated at RT for 15 min before microscopy. Images were acquired using an Olympus FluoView 1000 confocal laser scanning microscope (CLSM) (*Olympus*, Center Valley, PA) using a 60x/1.42 oil objective and BP 430-470 emission filter set under excitation with the 405 nm diode laser line for CMAC fluorescence (353 nm excitation/466 nm emission) and BP 505-525 emission filter set under excitation with the 488 nm diode laser line for MDY-64 fluorescence (451 nm excitation/497 nm emission).

b. Transmission Electron Microscopy (TEM)

The purity of protoplasts and the vesicles-vacuoles fraction also was assessed by TEM. Protoplast samples were fixed with 2.5% glutaraldehyde overnight at 4^0C on a rotating platform shaker. Samples were post-fixed in 1% buffered osmium tetroxide overnight at 4°C, dehydrated in an acetone series (30–100%), and then infiltrated and polymerized in Poly/Bed 812 resin for 24 h at 60°C. Resin blocks were sectioned using a Power Tome-XL ultramicrotome (Boeckeler Instruments, Tucson, AZ). Protoplast sections (70 nm thick) were mounted on formvar coated copper grids and stained with 1% uranyl acetate and lead citrate. Vesicles-vacuoles samples were prepared by fixing

with 1% osmium tetroxide for 1 h at RT. A drop of the solution was then placed on a copper grid and the excess solution was removed by filter paper. The grid was air-dried and stained with 1% uranyl acetate (negative stain). Images were generated with a JEOL 100CX transmission electron microscope.

c. Enzyme assays

The activities of specific marker enzymes were examined in the vesicles-vacuoles fraction. The α-mannosidase (a marker tightly associated with vesicles and vacuoles) activity was measured by a method adopted from a published protocol (Boller & Kende, 1979). 10 μL of the sample [total protein range from 3 μg to 10 μg] was added to a reaction mix containing 0.5 mL of sodium succinate buffer [50 μM Na-succinate, pH 5.0]; then, 3 μL of 0.1 M p-nitrophenyl substrate [Sigma, St.Louis, MO] was added. The reaction was stopped by addition of 0.8 mL of 1M sodium carbonate. The absorbance was determined at 405 nm. The specific activity was expressed as nmoles of p-nitrophenol produced per min per μg total protein. Succinate dehydrogenase activity (mitochondrial marker) was assessed according to a standard protocol (Berg, 1995). 10 μL of the sample solution [total protein range from 3 μg to 10 μg] was added to a reaction mixture consisting of 0.3 M potassium phosphate, 8. mM potassium cyanide and 50 μg of DCPIP [dichlorophenolindophenol, Na salt]. 0.1 mL of sodium succinate was then added to this reaction mixture and the absorbance was recorded at 600 nm. Specific activity was expressed as μmoles DCPIP reduced per minute per μg total protein. Lactate dehydrogenase activity (cytoplasmic marker) was measured according to a standard protocol (Kuznetsov, 2006). 10 μL of the sample was added to 1 mL of pre-

incubated reaction medium (at 30°C) containing 100 mM Tris-HCl buffer, 10 mM of pyruvate and 0.3 mM NADH and the absorbance was measured at 340 nm. Enzyme specific activity was expressed as μmoles of lactate formed per min per μg total protein.

Protein Determination

The protein concentration was determined using Bio-Rad protein dye reagent (Invitrogen, Carlsbad, CA).

Enzyme-linked immunosorbent assay (ELISA) for measuring aflatoxin

Aflatoxin in feeding experiments was measured with an enzyme-linked immunosorbent assay as described previously (Roze *et al.*, 2007) using polyclonal antibodies against aflatoxin B_1 (Sigma).

Western blot analysis

Whole cell (CE) proteins were prepared from 36 h old frozen mycelium using a method similar to a procedure described by Roze (Roze *et al.*, 2007). Protoplast proteins were extracted by adding 1:1 TSA buffer (Roze *et al.*, 2007) directly to the protoplast pellet and then homogenizing the solution in a Potter homogenizer placed in ice. Proteins from the vesicles-vacuoles fraction were extracted by adding 700 μL of 1:1 TSA solution to every 500 μL of vesicles-vacuoles fraction to enable osmotic lysis. Total protein (20 mg) was separated by electrophoresis on 12% SDS polyacrylamide gels, transferred to PVDF membrane, exposed to antibody specific to OmtA, Ver-1,Vbs and Nor-1 [all generated in our laboratory (Lee *et al.*, 2004)]. Then the filter was incubated with goat

anti-rabbit secondary antibody conjugated to IRDye 800 (Invitrogen Corporation, Carlsbad, CA). Infrared fluorescence was directly detected by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

Feeding experiments

Each feeding experiment was repeated a total of three times with similar trends. Representative data from a single experiment are presented in each panel to illustrate the results. A schematic that illustrates the experimental design is presented to the left of each panel (Figure 2.7).

a. Feeding pure protoplasts.

Sterigmatocystin (ST) (Sigma, St Louis, MO) [20 μg/mL] was added (fed) to 1 mL of a pure protoplast fraction containing approximately 10^6 protoplasts and this mixture was incubated overnight at 30°C. The aflatoxin content as determined by ELISA (see below) was measured before and after incubation and normalized to total protein in the protoplast fraction. A no-ST control was conducted; protoplasts were incubated under the same conditions without added ST. Protoplasts prepared from *A. parasiticus* AFS10 [derived from SU-1, *aflR* disruption, no aflatoxin enzymes or aflatoxin accumulate] and LW1432 [*omtA* disruption] were also included as controls.

To measure aflatoxin content, protoplasts were pelleted at 1000 g, the supernatant was discarded and the pellet was suspended with 500 μL of chloroform and shaken 20 times to extract aflatoxins. The suspension was centrifuged at 10000 x g, the lower organic layer was removed, dried to evaporate chloroform and the aflatoxin residue was

dissolved in 70% methanol. Aflatoxin was measured by ELISA (Roze *et al.*, 2007) using standard methods and the values were normalized to total protein in the fraction.

To determine if aflatoxins are stored in the vesicles-vacuoles fraction, protoplasts prepared from strain SU-1 were incubated with and without added ST as described above; the vesicles-vacuoles fraction was purified from the protoplasts as described above. Once the vesicles-vacuoles fraction was removed from the gradient, we combined the remaining contents of the gradient and designated it as the non-vesicles-vacuoles fraction. Aflatoxins from both the vesicles-vacuoles and the non-vesicles-vacuoles fractions were extracted with chloroform (500μL chloroform per 100μL of volume fraction) after which the chloroform was evaporated. Aflatoxin was dissolved in 70% methanol, measured by ELISA, and the data normalized to the total protein in the appropriate fraction.

b. Feeding vesicles-vacuoles and non-vesicles-vacuoles fractions

The vesicles-vacuoles and the non- vesicles-vacuoles fractions prepared from 1mL of pure protoplasts (containing approximately 10^6 protoplasts) were incubated in parallel, with or without added ST (20μg/mL) at 30°C overnight. Aflatoxin (measured by ELISA) in each fraction was normalized to the total protein in the appropriate fraction.

RESULTS AND DISCUSSION

Protoplast isolation

Protoplast preparation from mycelia harvested during aflatoxin synthesis (during a transition from exponential growth to stationary phase) is challenging as aging

negatively affects cell wall digestion. Our previous studies showed that aflatoxin gene transcripts and enzymes (Nor-1, Ver-1,VbsA and OmtA) were first detected between 24 and 40 h of growth (Roze *et al.*, 2007). Aflatoxin enzymes and transcripts reached peak levels at 48 h and then declined. We determined that 36h was an optimum time point because we could detect aflatoxin enzymes and aflatoxin in the mycelia and still digest the cell wall to produce protoplasts. Protoplasts were isolated and their purity assessed by bright field microscopy; this analysis showed no detectable contamination by mycelial debris (Figure 2.2A). The presence of the vesicles-vacuoles population in protoplasts was observed by CLSM (Figure 2.2B-D) and TEM (Figure 2.2E.). Throughout this current work we define the size range of vesicles and vacuoles as $\geq$ 2.5 μm and $<$ 2.5 μm respectively. Vesicles and vacuoles with similar size range were observed in purified protoplasts and whole cells.

Purification of a vesicles-vacuoles fraction

Vesicles and vacuoles were released from protoplasts (see Methods). Within 15 minutes after addition of Triton X 100, most protoplasts (> 90%) released vesicles and vacuoles (Figure 2.3). Storing the released vesicles and vacuoles in the presence of Triton X-100 for longer periods of time negatively affected the stability of the vesicles and vacuoles; after 30 min few intact vesicles and vacuoles could be observed in the lysis mixture.

Isolation of a vacuolar fraction has been reported for filamentous fungi (Martinoia *et al.*, 1979), yeast (Rieder & Emr, 2001), and plants (Robert *et al.*, 2007). However, the high degree of size and density heterogeneity in the vesicles and vacuoles population of

A. parasiticus during aflatoxin synthesis prevented us from directly applying published procedures to obtain a purified vesicles and vacuoles fraction. The 'high density sucrose cushion' method to fractionate vesicles and vacuoles developed in this study (see methods) caused the vesicles-vacuoles population to migrate downward to the sucrose cushion forming a thin but distinct band at the interface; the remaining cell debris banded either within the cushion (small protoplast fragments) or pelleted at the bottom of the cushion (large protoplast fragments). Experimental conditions were optimized empirically. This procedure routinely yielded sufficient vesicles and vacuoles to generate 30 μg of protein from 4 g of mycelia (wet weight).

Figure 2.2. Microscopic analysis of purified protoplasts.

A. parasiticus was grown for 36 h under aflatoxin inducing conditions and protoplasts were prepared and purified as described in Methods.

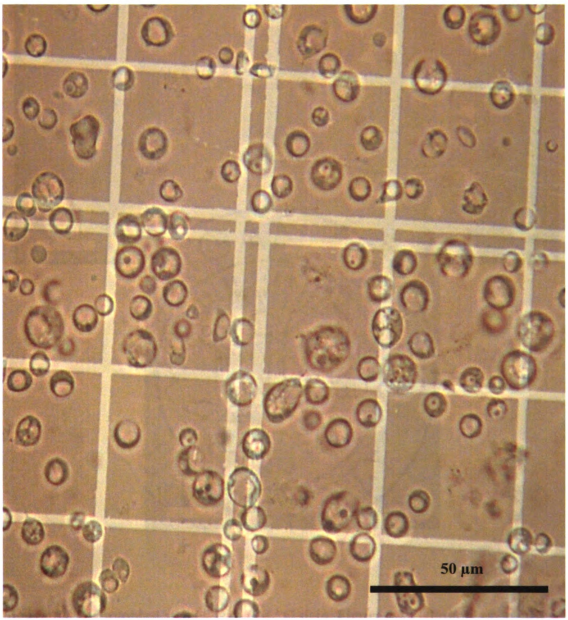

Figure 2.2 A. Bright field microscopy image of a pure protoplast fraction.

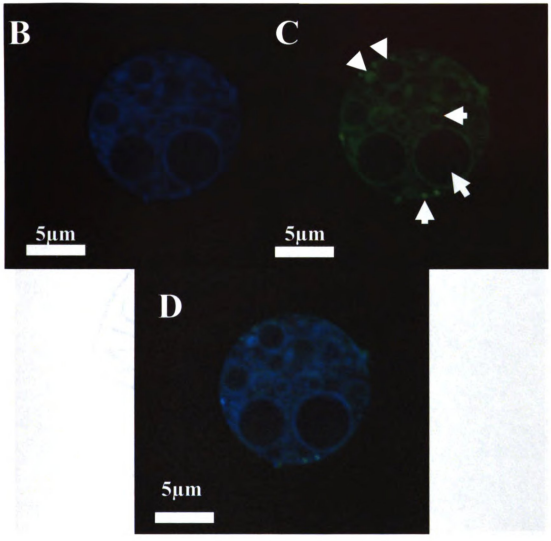

Figure 2.2 B. Protoplast stained with CMAC stain. **Figure 2.2 C.** Protoplast stained with MDY-64. **Figure 2.2 D.** Digital overlay of B and C. B and C were acquired with CLSM under dark field. The white arrows in C illustrate the various sized organelles in the vesicles -vacuoles population in the protoplast.

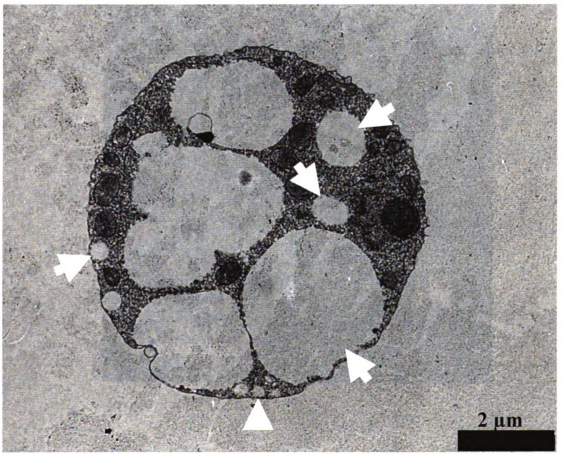

Figure 2.2 E. Protoplast observed under TEM as described in Methods. Image was acquired on the JEOL 100CX transmission electron microscope. White arrows illustrate the various sized organelles in the vesicles-vacuoles population in the protoplast

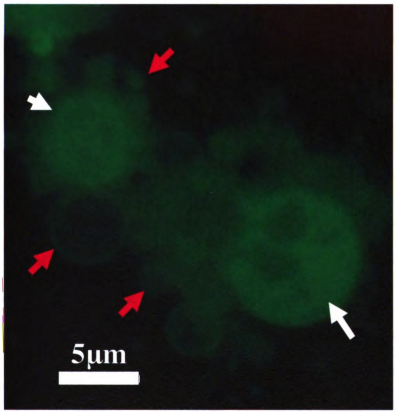

Figure 2.3. Release of vesicles and vacuoles from protoplasts after addition of Triton-X 100 (lysis) solution. Red arrows illustrate vesicles and vacuoles of various sizes released from protoplast. White arrows illustrate the protoplasts undergoing lysis.

Fraction purity

Three methods were employed to test the purity of the vesicles and vacuoles fraction. Alpha(α)-mannosidase has been used frequently as a vacuolar marker enzyme in plants (Boller & Kende, 1979), filamentous fungi (Hoppert *et al.*, 2001), and yeast (Yoshihisa *et al.*, 1988); the enzyme also localizes to vesicles of yeast (Campbell & Rome, 1983) and filamentous fungi (Akao *et al.*, 2006). We therefore measured α-mannosidase specific activity in the vesicles-vacuoles fraction and compared it with specific activity in protoplasts and in whole-cells. Succinate dehydrogenase activity, a mitochondrial marker enzyme (Pycock & Nahorski, 1971), allowed us to detect the presence of mitochondria in the vesicles-vacuoles fraction. We also measured activity of lactate dehydrogenase, a cytosolic marker enzyme (Kobayashi *et al.*, 2006), to track the successful elimination of this potential contaminant during the purification process. We observed that α-mannosidase specific activity increased markedly at each successive purification step (whole cells, protoplasts, vesicles and vacuoles) accompanied by elimination of detectable succinate dehydrogenase and lactate dehydrogenase activities (Figure 2.4).

Peroxisomes have been shown previously to sediment at centrifugal forces >50000xg with maximum quantities in 140000xg supernatant, that is significantly higher than that required to sediment mitochondria (20000xg) (Liang, 1996; Zhou, 1997) and vesicles-vacuoles (3000xg) (current work). We reasoned that like mitochondria, peroxisomes do not co-purify with vesicles-vacuoles fraction.

An independent assessment of purity of the vesicles and vacuoles fraction by CLSM showed no protoplast debris (Figures 2.4A-C).The vacuolar membrane stain

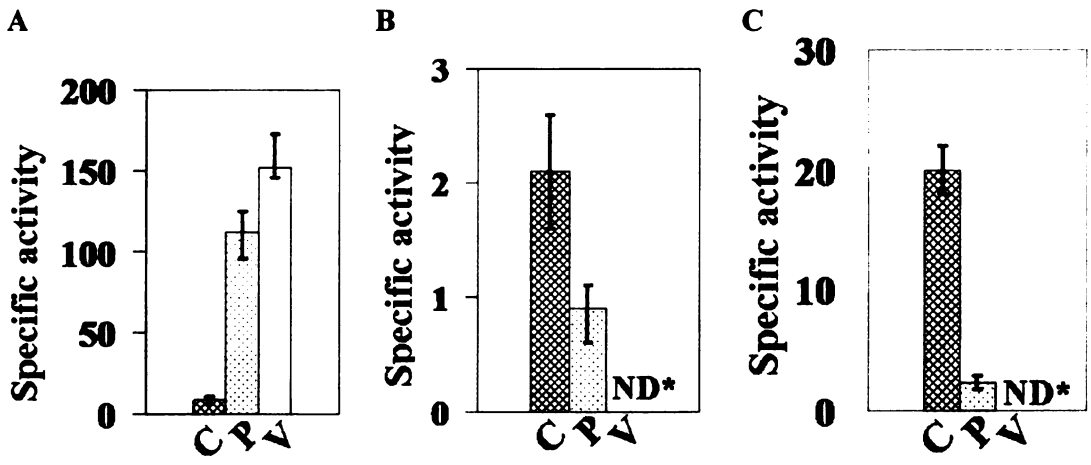

Figure 2.4. Measurement of marker enzyme specific activities during purification of vesicles-vacuoles; protein extracts from whole cells, protoplasts and vesicles-vacuoles. Protein extracts were prepared from whole cells (CE), pure protoplasts (P), and pure vesicles-vacuoles (V). The specific activities of α-mannosidase (A) (vacuole marker; specific activity is expressed as nmoles of p-nitrophenol produced per min per μg of total protein), succinate dehydrogenase (B) (mitochondrial marker; specific activity is expressed as μmoles of DCPIP reduced per minute per μg of total protein) and lactate dehydrogenase (C) (cytoplasmic marker; specific activity is expressed as μmoles of lactate formed in the reaction per min per μg of total protein) were measured. Data summarize three independent experiments. Specific activities are reported as the mean ± standard error. ND = not detectable.

Figure 2.5. Microscopic analysis of a vesicles-vacuoles fraction

The vesicles-vacuoles fraction was purified and prepared for microscopic analyses as described in Methods.

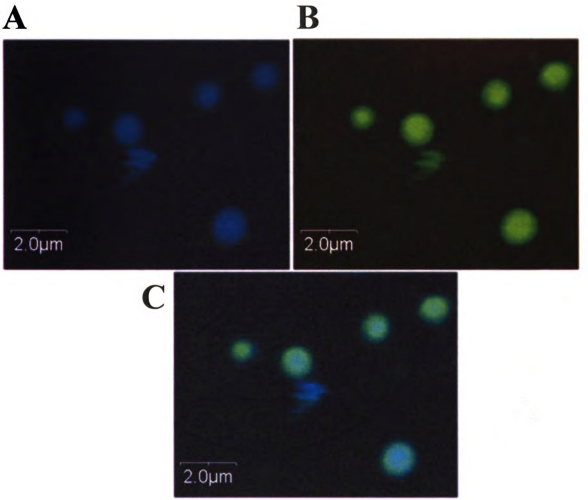

Figure 2.5 A – C. CLSM images of a vesicles-vacuoles fraction. **Panel A**. Vesicles-vacuoles stained with CMAC. **Panel B.** Vesicles-vacuoles stained with MDY-64 stain. **Panel C.** Digital overlay of A and B.

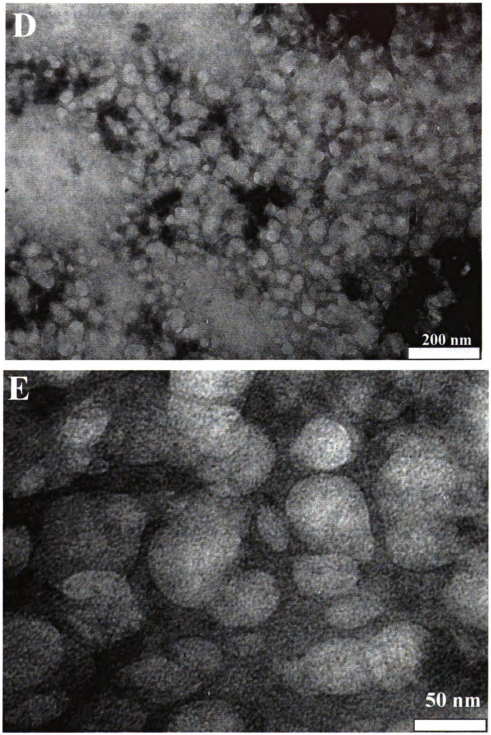

Figure 2.5 D-E. TEM images of a vesicles-vacuoles fraction, two different magnifications

MDY-64 and the vacuolar peptidase stain CMAC demonstrated great affinity for all organelles in the vesicles-vacuoles preparation. These organelles were spherical in shape and were highly diverse in size (range approximately 20nm to 5μm). We observed ten different fields under CLSM with at least 50 vesicles-vacuoles in each field and found no protoplast debris or unbroken protoplasts associated with that fraction.

TEM images (Figure 2.5D-E) supported the CLSM data showing no contamination of the vesicles-vacuoles fraction with mitochondria or other organelles. As in protoplasts, CLSM and TEM images of the organelles in the fraction demonstrated significant size heterogeneity

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The vesicles-vacuoles fraction carries the aflatoxin enzymes, Ver-1, Vbs and OmtA

The aflatoxin enzymes Ver-1, Vbs and OmtA were detected in the vesicles-vacuoles fraction by Western Blot (Figure 2.6). The presence of Ver-1 confirms data from recent studies (Hong, 2008; Hong & Linz, 2008; Hong & Linz, 2009) that suggest that Ver-1 and Nor-1 localize to vesicles and vacuoles. No evidence of aflatoxin enzyme turnover was observed in any fraction tested. Surprisingly, Nor-1 could not be detected (Western Blot) in protoplasts or the vesicles-vacuoles fraction. This could mean either that Nor-1 is not stable in isolated protoplasts or the vesicles-vacuoles fraction or that the signal from antibody bound to Nor-1 was below the detection limit.

Figure 2.6. Western blot analysis of aflatoxin enzymes present in a vesicles-vacuoles fraction purified from *A. parasiticus* during aflatoxin biosynthesis. A total of 10^6 spores were inoculated into 100 ml YES liquid medium and incubated at 30°C with shaking for 36h. Vesicles-vacuoles fraction was purified from protoplasts generated from whole cells as described in methods. Protein extracts were prepared from whole cells (CE), pure protoplasts (P), and pure vesicles-vacuoles (V) as described in methods.

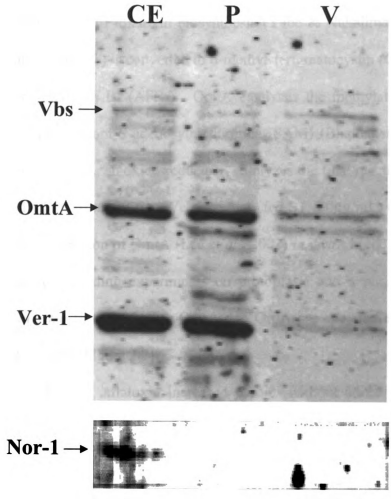


Figure 2.6.

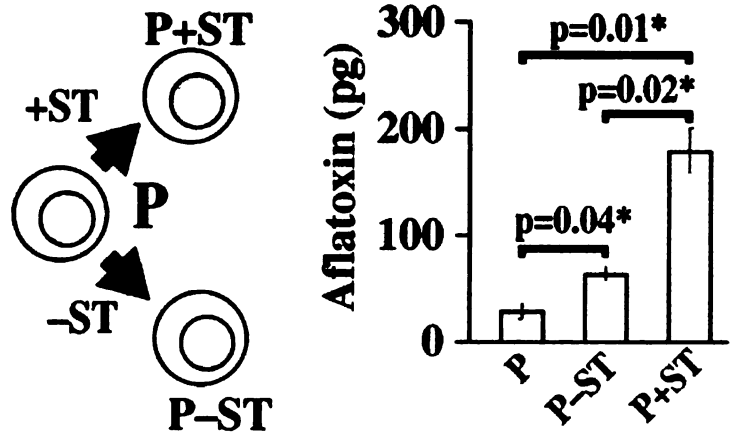
Protoplasts and vesicles-vacuoles convert exogenously added sterigmatocystin to aflatoxin

In the final two steps of aflatoxin biosynthesis (Yabe & Nakajima, 2004; Yu *et al.*, 2002) sterigmatocystin (ST) is converted to o-methylsterigmatocystin (OMST) which is then converted to aflatoxin B_1 (AFB_1). OmtA catalyzes the methylation of ST by transfer of a methyl group from S-adenosylmethionine (SAM) (Bhatnagar *et al.*, 1987; Cleveland *et al.*, 1987). OrdA, an oxido-reductase, catalyzes the final reaction step in the presence of NADPH (Yu *et al.*, 1998). We previously fed ST (20μg/mL) to whole cells to confirm the catalytic function of OmtA (Lee *et al.*, 2002) *in vivo*. In the current work, we performed analogous feeding experiments on protoplasts and a purified vesicles-vacuoles fraction *in vitro* to test whether OmtA and OrdA are enzymatically active in these sub-cellular locations (Figure 2.7).

In protoplasts fed ST, aflatoxin increased 6 fold; without added ST, aflatoxin increased only 2-fold (Figure 2.7A). When the vesicles-vacuoles fraction was purified from protoplasts fed with ST, this fraction carried the vast majority of the aflatoxin when compared to the non-vesicles-vacuoles fraction (contains all remaining cell materials besides vesicles-vacuoles) (Figure 2.7B). These data strongly suggest that aflatoxin was synthesized and accumulated in the vesicles-vacuoles fraction and that vesicles-vacuoles are the major site for AFB_1 accumulation in the cell. Secondary metabolites including anthocyanins (Marrs *et al.*, 1995), berberine (Otani *et al.*, 2005), nicotine (Saunders, 1979) sanguinarine (Alcantara *et al.*, 2005), camptothecin (Sirikantaramas *et al.*, 2007) and many alkaloids in *Catharanthus roseus* (Roytrakul S, 2007) are commonly sequestered in vacuoles or vesicles in plants. To date, the sole example of vacuolar

Figure 2.7 Feeding experiments: Each feeding experiment was conducted three times with similar trends. A schematic illustrating the experimental design for feeding experiments is included at the left of each panel. Representative results from one experiment are presented in the graph at the right in each panel. **Panel A.** Feeding experiment with protoplast (P) fraction. +ST represents addition of sterigmatocystin and –ST represents a control experiment with no sterigamtocystin. P+ST = protoplasts fed with ST; P-ST = protoplasts not fed with ST. **Panel B**. Feeding experiment to test whether vesicles-vacuole fraction (V) can sequester aflatoxin made in protoplasts. NV represents non-vesicle-vacuole fraction. V(P+ST) and NV(P+ST) represent the vesicle-vacuole fraction and non-vesicle-vacuole fraction obtained from protoplasts fed with ST, respectively. V(P-ST) and NV(P-ST) represent the same fractions obtained from protoplasts not fed with ST. **Panel C** Feeding experiments with fraction V and NV. V+ST and V-ST represent the vesicle-vacuole fraction fed and not fed with ST, respectively; NV+ST and NV-ST represent non-vesicle-vacuole fraction fed and not fed with ST. Aflatoxin is expressed as pg per μg of total protein. The two-tailed p-values used to determine statistical significance were calculated using an unpaired t-test with sample size = 3 (aflatoxin measurements in each experiment were conducted in triplicate).

A.



B.

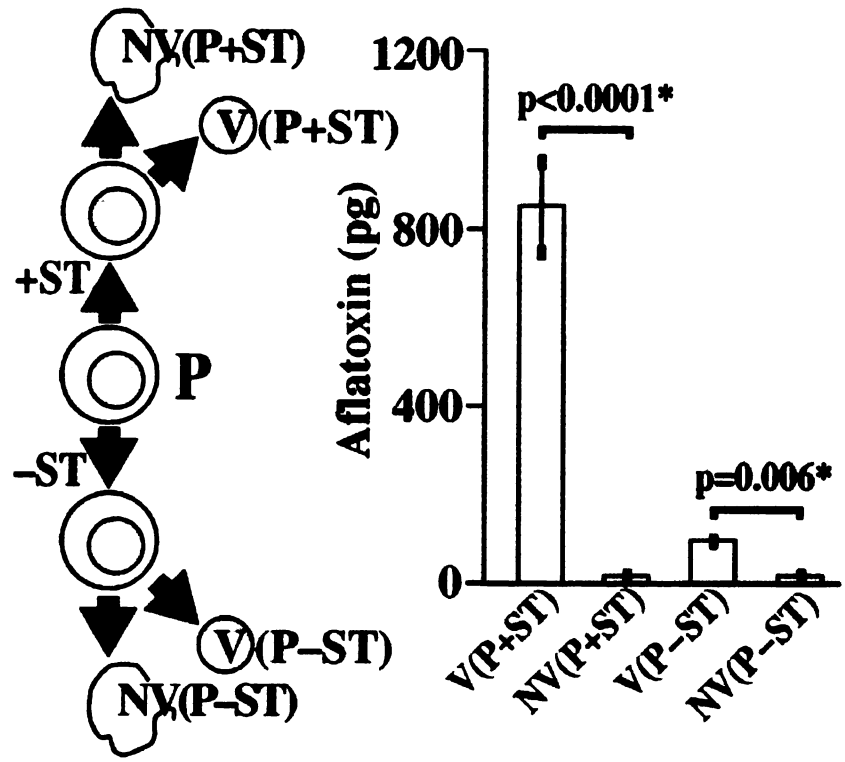


Figure 2.7 A-B

C.

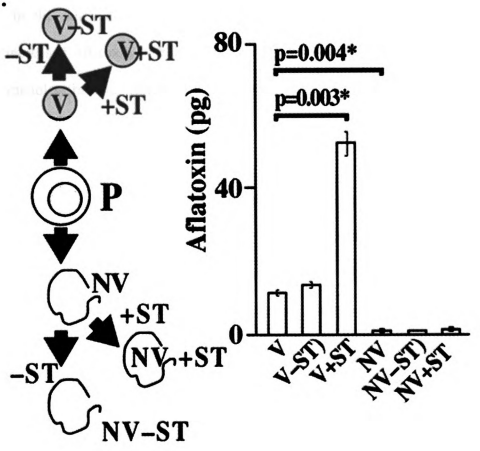


Figure 2.7 (continued)

sequestration of fungal secondary metabolites (to our knowledge) is cyclosporin in *Tolypocladium inflatum* (Hoppert *et al.*, 2001).

The hypotheses stated above were tested in independent feeding experiments using a modification of the original protocol. Here, ST was fed directly to vesicles and vacuoles in the purified fraction. Addition of ST to the purified vesicles-vacuoles fraction increased aflatoxin > 5 fold (Figure 2.7C). The increase in aflatoxin in the vesicles-vacuoles fraction without added ST was much smaller and not significant. In contrast, feeding of ST to the non-vacuolar fraction resulted in nearly undetectable levels of aflatoxin. These data combined with data obtained by Western blot analysis on the purified vesicles-vacuoles fraction, confirm that OmtA is present and enzymatically functional in this sub-cellular location. The data also strongly suggest that OrdA is present and functional at this location. We do not currently possess anti-OrdA antibodies that could be used to help confirm this hypothesis. However, since OrdA is the only enzyme in *A. parasiticus* reported to catalyze the NADPH-dependent reduction of OMST to AFB_1 (Yu *et al.*, 1998), accumulation of AFB_1 in the vesicles-vacuoles fraction strongly suggests that both OmtA and OrdA are functional in that sub-cellular location.

The data also suggest that SAM and NADP are contained in the vesicles-vacuoles fraction since these compounds were not added during the feeding studies but are required for these reactions to occur. It is possible that even higher levels of AFB_1 could be obtained by feeding ST, SAM, and NADP to the vesicles-vacuoles fraction if these compounds normally are present in rate-limiting quantities.

Intracellular site of aflatoxin biosynthesis in *A.parasiticus*

We initiated these studies to test two possible models for the sub-cellular location of aflatoxin synthesis. According to the first model, aflatoxin is synthesized in the cytoplasm by aflatoxin enzymes that are present in that location. After synthesis is complete, the toxin is secreted outside the cell by an unknown mechanism [previous data demonstrated that 99% of aflatoxin made by the cell was found in the growth medium (Roze *et al.*, 2007)]. Aflatoxin enzymes are then transported to vacuoles for proteolytic cleavage by peptidases that also localize there.

The second model proposes that aflatoxin enzymes are made in the cytoplasm and transported to vesicles-vacuoles. These proteins are enzymatically functional in vesicles-vacuoles and hence, part or all of aflatoxin biosynthesis occurs in these organelles; the enzymes are eventually degraded (Chiou, 2003; Lee, 2003; Skory, 1992), likely by peptidases that are transported to these organelles. According to this model, aflatoxin is sequestered within the vesicles-vacuoles and finally is transported outside the cell by an unknown mechanism.

The observation that the vesicles-vacuoles fraction efficiently converts ST to AFB_1 while the non-vacuolar fraction does not carry out this conversion at detectable levels strongly supports model 2 (see Figure 2.8). Our data demonstrate that OmtA and likely OrdA are active predominantly in the vesicles-vacuoles fraction. Direct comparison of the size range of vesicles and vacuoles in whole cells, protoplasts and in the purified vesicles-vacuoles fraction allow us to conclude that organelles in the purified fraction are representative of the organelles existing in whole cells. Further characterization of these organelles is a subject for future research.

Figure 2.8. Model depicting vesicles-vacuoles as the primary intracellular site for aflatoxin synthesis and storage. Several studies suggest that early, middle and late enzymes in aflatoxin biosynthesis are synthesized initially in the cytoplasm. During peak levels of aflatoxin synthesis, some or all of the aflatoxin enzymes are transported to vacuoles via a direct route (a) or a vesicle-mediated route (b). Recent data obtained with Nor-1 or Ver-1 EGFP fusions combined with Western blot analysis in the current study support transport via route (b). Transport via route (a): the model proposes that early and middle aflatoxin enzymes are functional in the cytoplasm and transported to vacuoles for turnover; this model predicts that ST is present initially in the cytosol and then transported to vesicles by an unknown mechanism – here, only the late pathway steps occur in vesicles-vacuoles. Transport via route (b). If most or all aflatoxin enzymes localize to vesicles-vacuoles and are functional in that location, the model predicts that most or all of aflatoxin synthesis occurs in vesicles-vacuoles. ST = sterigmatocystin, OMST = *o*-methylsterigmatocystin, AF = aflatoxin

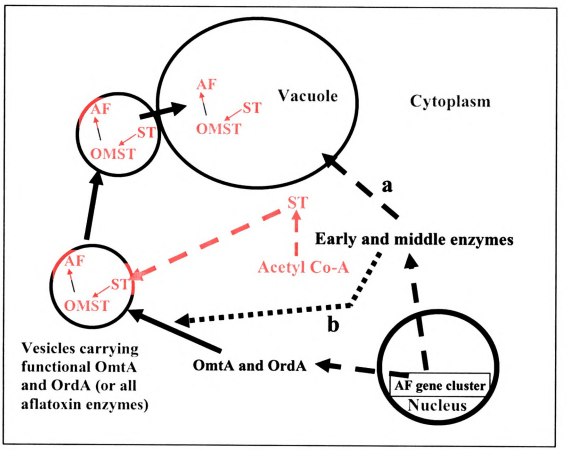

AF
ST
OMST
Vacuole
Cytoplasm
AF
ST
OMST
a
ST
Early and middle enzymes
AF
ST
OMST
Acetyl Co-A
b
Vesicles carrying functional OmtA and OrdA (or all aflatoxin enzymes)
OmtA and OrdA
AF gene cluster
Nucleus

Figure 2.8.

In related work, norsorolinic acid (NA, an early intermediate in the pathway) was detected in peroxisomes of Aspergillus strains carrying a genetic block in *nor-1* (Maggio-Hall *et al.*, 2005). We subsequently showed that the enzyme responsible for NA synthesis (Nor-1) is part of a large protein complex that is different from the complex that includes Ver-1, Vbs and OmtA (Appendix II). Together, these data may suggest that peroxisomes carry a protein complex that contains enzymes (FAS, PKS, and Nor-1) required to conduct the early steps in aflatoxin biosynthesis; we propose that vesicles and vacuoles, responsible for the late steps in aflatoxin biosynthesis, carry a separate complex that includes Ver-1,Vbs, and OmtA. Details of this model and the proposed interaction between peroxisomes and vesicles-vacuoles in Aspergillus remain to be elucidated.

In summary, our studies prompt us modify our existing model (Roze *et al.*, 2007) of the genetic switch that controls the shift from primary to secondary metabolism in *A. parasiticus*. During active growth vacuoles participate in primary metabolism; they store required substrates and ions and maintain pH balance and cell homoeostasis; these vacuolar functions have been characterized in detail (Klionsky *et al.*, 1990). The modified model proposes that vacuoles undergo morphological, chemical and functional development during this transition. In support of this model, our data demonstrate that as cells switch to secondary metabolism, vacuoles gain additional functions which include synthesis, storage and secretion of secondary metabolites. The model further proposes that late in this developmental process vacuoles predominantly engage in protein turn-over and eventually cell death occurs. Future studies will focus on understanding how vacuolar function correlates with the population of proteins that are present in vacuoles at different phases during development of this organelle.

Compartmentalization of enzymes, intermediates and end products appears to be a common feature of secondary metabolism in plants and fungi. In a recent study, we demonstrated that interfering with vacuolar biogenesis or protein trafficking to the vacuole strongly affected aflatoxin biosynthesis in *A. parasiticus* (Chapter 3 of the current study). Therefore, regulating protein traffic into vacuoles or between vesicles and vacuoles presents one promising mechanism to manipulate secondary metabolism. In just one potential application, we are hopeful that this general strategy will enable effective control of aflatoxin contamination on susceptible plant materials.

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CHAPTER 3

AFLATOXIN BIOSYNTHESIS IS FUNCTIONALLY RELATED TO VACUOLE BIOGENESIS

ABSTRACT

Aflatoxin biosynthesis is a multi-step secondary metabolic pathway involving at least 17 enzyme activities encoded by 25 or more genes clustered in a 75-Kb region on one chromosome; this highly characterized pathway serves as a model to understand secondary metabolism in eukaryotes. Recent sub-cellular studies provided a functional tie between vesicles and vacuoles and aflatoxin synthesis. Despite increasing evidence that demonstrates the importance of vesicles and vacuoles in secondary metabolism, the role of vacuole biogenesis in eukaryotic secondary metabolism was previously unclear. In the current study, we demonstrate that, vesicles (and not vacuoles) are the primary sites for aflatoxin synthesis. We interrupted vesicle-vacuole fusion (a late event in vacuole biogenesis) in *Aspergillus parasiticus* by disruption of the Rab7/Ypt7 GTPase homologue, *vb1* and by application of Sortin3 (that produces a Vps16 phenotype in yeast) during aflatoxin synthesis. Both treatments resulted in accumulation of vesicles and significantly higher levels of aflatoxins compared to wild-type. This increase in aflatoxin was not due to up-regulation in aflatoxin gene expression but due to significantly higher accumulation of functional enzymes caused by elevated vesicles accumulation. An increase in vesicles number was functionally linked to down-regulation of *A. parasiticus vb1* and *vps16* expression and to up-regulation of aflatoxin synthesis. These data provide

the first evidence that manipulation of the vesicle transport machinery in Aspergillus affects secondary metabolism.

INTRODUCTION

Proteins involved in secondary metabolism are often found in vesicles and vacuoles. Multiple studies indicated the importance of vacuoles in sequestration, and storage of plant secondary metabolites (e.g. anthocyanins (Tanaka *et al.*, 2008), sanguinarine (Alcantara *et al.*, 2005)). Significantly, localization studies suggest that vacuoles are also involved in biosynthesis of secondary metabolites (Costa et al. (Costa *et al.*, 2008), Ono et. al. (Ono *et al.*, 2006), Saslowsky et. al. (Saslowsky & Winkel-Shirley, 2001)). The few sub-cellular studies conducted on fungi, primarily on penicillin and cephalosporin biosynthesis (amino acid derived secondary metabolites), suggest that the precursors for their biosyntheses are withdrawn from vacuolar amino acid pools (Lendenfeld *et al.*, 1993; Muller *et al.*, 1991; Teijeira *et al.*, 2008; van de Kamp *et al.*, 1999; van der Lende *et al.*, 2002). In *Tolypocladium inflatum* the synthesis of cyclosporin (a non-ribosomal peptide) is associated with vacuoles. Cyclosporin synthesis presumably occurs at the vacuolar membrane catalyzed by cyclosporin synthetase and the final product is stored in vacuoles (Hoppert *et al.*, 2001).

Eukaryotes employ highly conserved vesicle transport machinery to deliver proteins to vacuoles (plants and fungi) and lysosomes (mammals) (Conibear & Stevens, 1995; Ferro-Novick & Jahn, 1994); separate reviews are available for yeast (Bowers & Stevens, 2005; Bryant & Stevens, 1998; Conibear & Stevens, 1998; Klionsky, 1997; Klionsky & Ohsumi, 1999; Teter & Klionsky, 2000; Wickner, 2002), plants (Mo *et al.*,

2006; Ueda & Nakano, 2002), filamentous fungi (Shoji *et al.*, 2008) and mammals (Mullins & Bonifacino, 2001). A small GTPase, called Rab7 in mammals and Ypt7 in yeast(Bucci *et al.*, 2000; Cantalupo *et al.*, 2001; Vitelli *et al.*, 1997) (Haas *et al.*, 1995; Ostrowicz *et al.*, 2008; Schimmoller & Riezman, 1993; Wichmann *et al.*, 1992) (Agarwal *et al.*, 2008; Nahm *et al.*, 2003), has been shown to interact with a tethering protein complex (HOPS) to mediate the physical contact of late endosomes to vacuoles eventually leading to membrane fusion with the assistance of SNARE proteins (Ostrowicz *et al.*, 2008; Whyte & Munro, 2002). The involvement of Rab7/Ypt7 in vacuolar biogenesis in filamentous fungi has been recently demonstrated in *Aspergillus nidulans*; the disruption of *avaA*, a homologue of Rab7/Ypt7, resulted in a fragmented vacuolar morphology (Ohsumi *et al.*, 2002).

Aflatoxin biosynthesis (a pathway involving at least 17 enzyme activities encoded by 25 or more genes clustered in a 75-Kb region on a chromosome (Miller MJ, 2006; Yabe & Nakajima, 2004; Yu *et al.*, 2002)), is one of the most highly characterized secondary metabolic pathways and serves as a model to understand secondary metabolism in eukaryotes. *A. parasiticus* initiates aflatoxin synthesis between 24h and 30h of growth on sucrose (YES medium) (Roze *et al.*, 2007). Aflatoxin accumulation increases exponentially until 48h and then declines(Liang, 1996). Aflatoxin enzymes and transcripts appear between 24h and 40h in roughly the same order as the genes in the cluster(Roze *et al.*, 2007). As aflatoxin synthesis increases, early (Nor-1), middle (Ver-1) and late (OmtA) pathway enzymes localize to two primary sub-cellular locations: i) cytoplasm; and ii) vesicles and vacuoles (Chiou *et al.*, 2004; Hong & Linz, 2008).

As discussed in chapter 2 of this dissertation, we first obtained a highly pure vesicle-vacuole fraction and demonstrated the presence of late aflatoxin enzymes in this fraction by Western blot consistent with our previous TEM and CLSM data(Hong & Linz, 2008; Lee *et al.*, 2004). Next, we performed feeding experiments with protoplasts, the vesicles-vacuole fraction, and the non-vacuole fraction (contains the remaining cell materials) in *vitro* with a late pathway intermediate sterigmatocystin (ST) to test whether these could convert ST to aflatoxin B_1 (AFB_1) as described in a previous study(Lee *et al.*, 2002). In protoplasts fed ST, aflatoxin increased 6 fold; without added ST, aflatoxin increased 2-fold. The vesicles–vacuole fraction (purified from protoplasts which had been fed ST) carried the vast majority of the aflatoxin as compared to the non-vacuolar fraction. ST fed directly to the vesicles-vacuole fraction increased aflatoxin more than 5 fold. In contrast, ST fed to the non-vacuole fraction resulted in nearly undetectable levels of aflatoxin. These data demonstrated that the vesicles-vacuole fraction carry functional aflatoxin enzymes and compartmentalize aflatoxin.

This work was initiated to clarify which compartments (vesicles or vacuoles) associate functionally with aflatoxin synthesis. We targeted the Class C Vps tethering complex that mediates fusion of vesicles and other pre-vacuolar compartments to vacuoles (Whyte & Munro, 2002). We disrupted *A. parasiticus vb1* that encodes a homolog of *ypt7* in yeast (Ypt7 is one member of Class C Vps complex) and *avaA* (vacuole biogenesis gene) in *A. nidulans* ; *ypt7* and *avaA* disruption results in "fragmented vacuoles" (Ohsumi *et al.*, 2002; Wichmann *et al.*, 1992). Vb1 contains 205 amino acids and is 70% identical to yeast Ypt7, 74% identical to mammalian Rab7 GTPases, and 94% identical to *A. nidulans* AvaA. In addition we treated *A. parasiticus*,

SU-1 (wild type) with Sortin3, a low-mass compound, that inhibits Vps16, another protein in the Class C Vps tethering complex in yeast (Zouhar *et al.*, 2004). Our data demonstrate that *vb1*- and Sortin3- dependent defects in trafficking to the vacuole affect aflatoxin biosynthesis. We hypothesize that vesicles represent the primary intracellular site for the late steps of aflatoxin biosynthesis, whereas the vacuole is primarily responsible for aflatoxin storage and aflatoxin enzyme turnover.

MATERIALS AND METHODS

Strains and culture conditions

A. parasiticus NRRL 5862 (SU-1) served as the aflatoxin-producing wild-type strain. *A. parasiticus* NR-1 (*niaD*, encodes nitrate reductase), derived from *A. parasiticus* NRRL 5862 (SU-1; ATCC 56775) served as the recipient for fungal transformation. *Escherichia coli* DH5α F'^{e} [F'/*endA1 hsdR17* (r_k^- m^{k-}) *supE44 thi-1 recA1 gyrA* (Nal^r)) *relA1* (*lacZYA argF*) $_{u169}$:(m80)*lacZ M15*) (Invitrogen, Carlsbad, CA) was used to amplify plasmid DNA using standard procedures (Ausubel & Struhl., 2003). Fungal strains SU-1 and NR-1 were maintained on potato dextrose agar (PDA) and the *vb1* disrupted strains, AC5, AC7 and AC11, constructed as part of this study, were maintained on Czapek-Dox agar (CZA), a selective growth medium. *A. parasiticus* was cultured in YES liquid medium (2 % yeast extract, 6 % sucrose, pH 5.8), a rich aflatoxin inducing media, at 30°C in the dark with shaking at 150 rpm (batch fermentation) for RNA isolation, for total protein extraction, to measure mycelial dry weight and aflatoxin produced in the growth medium, and for microscopy. For DNA isolation from AC5, AC7 and AC11 and for Southern hybridization analysis, CZ, a defined growth medium was

used instead of YES. YEP liquid medium (2% yeast extract, 6% peptone, pH 5.8), a rich aflatoxin non-inducing medium, was used for comparisons of vacuole-vesicle morphology of SU-1 in aflatoxin inducing and non-inducing conditions and also for analyzing gene expression at the RNA level.

Microscopy

Forceps were used to remove hyphal filaments from a mycelial pellet harvested from the growth medium. These filaments were placed on a microscope slide and bright field images were obtained using a Nikon Eclipse E600 microscope [Nikon Inc., Melville, NY].

Sortin treatment of *A. parasiticus*

Sortins were added (DMSO stock solutions) to 50ml growth medium at concentrations (25μg/mL for Sortin1, 10μg/mL for Sortin2 and 10μg/mL for Sortin3) optimized in yeast (Zouhar *et al.*, 2004). SU-1 spores were inoculated (10^6 spores/mL) after addition of Sortins and incubated at 30^{O}C in a rotary shaker (150rpm). SU-1 grown in untreated growth medium and DMSO treated medium were used as controls.

Identification and Cloning of *A. parasiticus vb1*

We performed a database search for homologues of *avaA* of *A. nidulans* (Ohsumi *et al.*, 2002)) using the *A. flavus* genome sequence (Yu *et al.*, 2008). Gene 92.m03481 was identified in this blast search. Using the sequence of 92.m03481, forward (5'-

TCGGCGCGGATTTCCTTAC-3') and reverse ((5'- GGCTTCCTTGGCAC TGGTTTC-3') primers were designed to amplify a 0.5 Kb PCR product in the *avaA* open reading frame (ORF) using *A.parasiticus* genomic DNA as template. This PCR product was used as a probe to screen an *A. parasiticus* cosmid library. Cosmid C31 was isolated in this screen and it harbored 92.m03481, the *avaA* homologue. We named this homologue *vb1* ("vb" stands for vacuolar biogenesis).

On the basis of *A. flavus* genome sequence, we designed forward (5'- AGTTAAC CTTAGGAAATCAGATG-3') and reverse (5'-AAGCAGAGGGAATATTGGTC-3') primers to amplify a 5Kb fragment using cosmid C31 as template. This fragment contained *vb1* with approximately 2Kb flanking sequence on either side of the gene. A 4.4Kb *XbaI/XmaI* fragment was excised from this 5Kb PCR fragment and sub-cloned into the XbaI/*XmaI* sites of pUC19 (New England Biolabs, Beverly, MA) resulting in plasmid pC4k. This plasmid was used for construction of the disruption plasmid (discussed below).

For nucleotide sequence analysis, we amplified a 1.1Kb fragment (forward primer 5'-ATCTCCCACCGCGCATACCGCC-3' and reverse primer 3'-AAACAACCCGTTC CCCAAGCCCG-3'; based on the *A.flavus* genome sequence) from *A.parasiticus* genomic DNA containing the *vb1* gene and subcloned this PCR product into a pGTEMeasy vector. Nucleotide sequence analysis was conducted on the 1.1 Kb fragment using an automated nucleotide sequencer (ABI robotic catalyst and 373A DNA sequencer) at the Plant Research Laboratory at Michigan State University. A complementary DNA sequence was analyzed using a SMART RACE cDNA Amplification Kit (Clonetech) following the manufacturer's instructions. The nucleotide

sequence accession number for *vb1* is AY520458. Comparison of the amino acid sequence of Vb1 (deduced from the cDNA sequence) with mammalian Rab7, yeast Ypt7 and *A. nidulans* AvaA was performed using the EMBL database.

Disruption of *A. parasiticus vb1*

The *vb1* disruption plasmid pVB was constructed (shown in Figure 3A) by inserting a 6.3Kb *HindIII* fragment from plasmid pSL82 (Horng *et al.*, 1990) (blunt ended with Klenow fragment of DNA PolI) into a *ClaI* site of pC4K (also blunt ended by Klenow enzyme) using standard methods (Ausubel, 1998). The blunt ended *HindIII* fragment carried the *niaD* selectable marker. In pVB, this marker was inserted into the middle of *vb1*.

Transformation of *A. parasiticus* protoplasts was performed as described by Horng et al. (Skory *et al.*, 1990). Two to four micrograms of DNA (*SphI/NdeI* fragment from pVB carrying the disruption construct (Figure 3.3B)) and approximately 10^7 protoplasts resulted in approximately 100 transformants. Transformants were screened for the *vb1* disruption by a PCR-based screening method using forward (5'-ATCTCCCACCGCGCATACCGCC-3') and reverse (5''-GGAGGGACAGGGATG GTACC-3') primers and crude genomic DNA (as described below) obtained from the transformants as template. Strains carrying the *vb1* gene disruption were confirmed by Southern blot analysis.

Isolation of crude genomic DNA from transformants

Spores (from a 10^6 spores/mL spore stock) were inoculated in 100mL of YES growth medium for 48h. The mycelium was harvested and ground in liquid nitrogen. 1gm of ground mycelium was mixed with 10mL of DNA extraction buffer (100 mM Tris-. HC1, 150 mM NaC1, 100 mM EDTA, pH 8.0) containing 300µL of 20% SDS and 60µL of proteinase-K(20mg/mL) and incubated for 16h at 50°C. The lysate was extracted with 10mL of 1:1 phenol:chloroform and the crude genomic DNA precipitated from the aqueous phase with 5M sodium acetate and two volumes ice-cold 100% ethanol. The precipitate was collected by centrifugation (13000xg for 10min) and the pellet was dissolved in TE buffer to be used as template for PCR screening.

Southern hybridization analysis

Spores (10^6) of individual fungal isolates (obtained by two rounds of single spore isolation (Skory *et al.*, 1992)) were inoculated in 100mL of YES liquid medium and incubated on a rotary shaker (150 rpm) at 30°C for 48h. Genomic DNA was then isolated from the harvested mycelia according to our standard protocol (Skory *et al.*, 1990). Restriction enzyme *SalI* was purchased from New England Biolabs (Beverly, MA) and used according to the manufacturer's instructions. Enzyme digestion, agarose gel electrophoresis, and Southern hybridization analyses were performed according to standard procedures (2). A radiolabeled *vb1* probe was generated with a random-primed DNA labeling kit (Roche, Indianapolis, IN) by incorporation of [α-32P]dCTP (DuPont) into a 0.5kb PCR fragment obtained using pAVD as template and primers described above.

RT-PCR analysis of gene expression

Total RNA was isolated from mycelia in duplicate samples by the Trizol method (Trizol Reagent; Invitrogen, Carlsbad, CA) as described previously (Roze *et al.*, 2007). 2 μL of cDNA was used as a template in the subsequent PCR using the following robocycler (Stratagene, La Jolla, CA) parameters: initial denaturation at 94°C for 5 min; 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; final extension at 72°C for 10 min. Primer pairs for PCRs are shown in Tables 3.1 and 3.2. The PCR products were separated by electrophoresis on a 0.8% agarose gel.

Western blot analysis

Fungal cells were cultured for 40 h in appropriate growth media and proteins for Western blot were prepared by grinding the mycelial samples in liquid N_2 in a mortar with a pestle, and the powdered mycelium was re-suspended 1:1 (w/v) in TSA buffer (0.01 M Tris, 0.15 M NaCl, 0.05% NaN3, pH 8.0) containing 1 tablet of Complete Mini Protease Inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 50 ml Proteinase Inhibitors mix (Sigma, St Louis, MO) and 0.125 mM phenylmethylsulphonyl fluoride per 10 ml. Total protein (20mg) was separated by electrophoresis on 12%SDS polyacrylamide gels, transferred to PVDF membrane, exposed to antibody specific to OmtA, Ver-1,Vbs and Nor-1 (all generated in our laboratory (Lee *et al.*, 2004)). Then the filter was incubated with goat anti-rabbit secondary antibody conjugated to IRDye 800 (Invitrogen Corporation, Carlsbad, CA). Infrared fluorescence was directly detected by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). For

comparison of protein levels, we measured the band intensities in Western blot using Odyssey software (Li-Cor Biosciences, Lincoln, NE).

Protein Determination

The protein concentration was determined using Bio-Rad protein dye reagent (Invitrogen, Carlsbad, CA)(Roze *et al.*, 2007).

Aflatoxin estimation

Aflatoxin accumulated in the growth medium was estimated by ELISA (Pestka, 1988) using polyclonal antibodies against aflatoxin B_1 (Sigma) as described previously (Liang, 1996).

Dry weight measurements

Mycelial dry weight was estimated according to our standard protocol (Liang, 1996).

RESULTS

Effect of growth medium on vesicles-vacuoles morphology in *A. parasiticus*

Yeast-extract sucrose (YES) is an aflatoxin inducing growth medium and yeast extract peptone (YEP) is a non-inducing medium (Cary *et al.*, 2000). Under standard growth conditions (100mL YES liquid shake culture) *A. parasiticus* initiates aflatoxin synthesis between 24 and 30 h. Synthesis increases exponentially until 48h and then slows (Liang, 1996). Aflatoxin enzymes and transcripts are first detected under the same

conditions between 24h to 40h (Roze *et al.*, 2007). We compared the morphology of vesicles-vacuoles in SU-1 (a wild type aflatoxin producing strain) grown in YES at 24h (when aflatoxin synthesis initiates) and 40h (when the cells are actively making aflatoxin). A similar comparison was conducted with SU-1 grown in YEP medium. At 40h in YES, we observed a significant increase in vesicles as compared to 24h. An increase in vesicles was not observed in YEP (Figure 3.1). We also conducted a nutritional shift experiment. SU-1 was grown in YES or YEP for 40h and transferred to the opposite medium. Within 6h after transfer we observed a significant increase in vesicles when shifted from YEP to YES but not from YES to YEP.

To examine whether changes in vesicles depended on aflatoxin biosynthesis, we conducted a similar experiment with strain AFS10 [disruption of a pathway regulator *aflR* in this SU-1derivative eliminates aflatoxin biosynthesis]. AFS10 also demonstrated an increase in vesicles in YES at 40h similar to that in SU-1. Based on these data, we conclude that changes in vesicles number resulted from changes in the growth environment (growth medium) and were not dependent on aflatoxin biosynthesis.

Figure 3.1. Effect of growth medium on vesicles-vacuoles morphology: A total of 10^6 spores of wild type *A. parasiticus* SU-1 were inoculated separately into 100 ml YES liquid medium (aflatoxin inducing medium) and 100mL YEP liquid medium (aflatoxin non-inducing medium) and incubated at 30°C with shaking (150 rpm) for 40h. A proportion of vacuoles (compartments $\geq$ 2.5μm) and vesicles (compartments < 2.5μm) was estimated at 24h and 40h time points. **Panel A**: Vesicles-vacuoles morphology was compared between SU-1 growing in YEP and YES growth media, using bright field microscopy. Black arrowheads show examples of vacuoles and white arrows point to vesicles. The reversibility of the morphologies as demonstrated by nutritional shift experiments is represented by forward and backward arrows. Size Bar = 5μm. **Panel B:** Vacuole (VAC) and vesicles numbers (VES) of SU-1 grown in YES medium at 24h and 40h. **Panel C:** Vacuole and vesicles numbers of SU-1 grown in YEP medium at 24h and 40h. **Panel D:** Nutritional shift of SU-1 from YES at 40h to YEP medium. B (for panels D and E) represents "before shift" i.e. 40h time point and A (for panels D and E) represents "after shift" which is 6h after the shift was made. **Panel E:** Nutritional shift of SU-1 from YEP at 40h to YES medium.

N (for panels B-E) equals the average number of vacuoles (VAC) or vesicles (VES) observed per mycelium in focus per field observed under bright field microscopy (from 10 different fields per biological replicate. Two biological replicates were used.

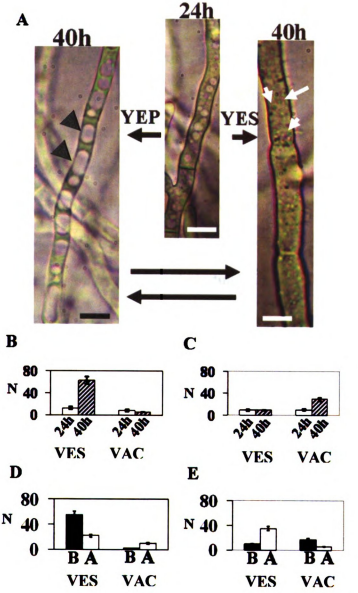


Figure 3.1

Treatment of *A. parasiticus* with Sortin3

In a recent study, three chemicals were identified as protein sorting inhibitors (Sortins) in yeast (Zouhar *et al.*, 2004). Sortin1, Sortin2 and Sortin3 caused carboxy peptidase Y (CPY) secretion to the growth medium; normally CPY is transported to vacuoles. Sortin3 showed a weaker effect on CPY secretion, but, unlike Sortins1 and 2, showed an *altered vacuolar morphology* (*vam*) phenotype that was similar to a yeast strain carrying a mutation in *vps16* (*vps16*Δ). The mechanisms underlying the effects of Sortins are not understood. To detect a possible link between vacuole biogenesis and aflatoxin synthesis, we treated *A. parasiticus* with Sortins1, 2 and 3 with doses optimized by Zouhar in yeast (Zouhar *et al.*, 2004).

Growth rate of SU-1 was severely reduced with Sortins1 and 2 treatments. In contrast, Sortin3, did not show any significant effect on growth rate of SU-1 as compared to untreated SU-1 (YES only) in the vehicle control (YES plus DMSO) (Figure 3.2 C). Due to severe effects of Sortin1 and 2 treatments on growth, their effects on vacuolar morphology and aflatoxin production were not studied further.

To determine the effect of Sortin3 treatment on aflatoxin synthesis, SU-1 was grown in YES medium for 40h. At this time, SU-1 synthesizes aflatoxin at peak levels and exports 99% of the toxin into the growth medium (Roze *et al.*, 2007). To measure the effect of Sortin3 treatment on vesicles-vacuole morphology, SU-1 was grown in YEP medium for 40h (at this time, SU-1 showed significantly lower numbers of vesicles (<2.5μm) than in YES. We reasoned, if Sortin3 treatment inhibited vacuole biogenesis to produce increased vesicles numbers, this effect could be better observed if SU-1 is grown in YEP medium.

Bright field microscopy demonstrated that Sortin3 treated SU-1 carried a significantly higher number of vesicles in YEP as compared to untreated SU-1 (Figure 3.2A-B). Sortin3 treatment also resulted in aflatoxin accumulation (ELISA) in YES growth medium that was approximately 5 fold higher as compared to untreated or vehicle controls (Figure 3.2 D). To determine whether the increase in aflatoxin was associated with an increase in aflatoxin enzymes, we compared the levels of three enzymes, Ver-1, Vbs and OmtA, in SU-1 grown in YES with or without Sortin3 treatment using Western blot analysis (Figure 3.2E). Sortin3 increased Ver-1, OmtA, and Vbs levels significantly but not to the extent that aflatoxin accumulation increased. These data demonstrate that interruption of vesicle-vacuole fusion increases vesicles accumulation that results in accumulation of functional aflatoxin enzymes and hence increases aflatoxin production.

Sequence analysis of *vb1* in *A. parasiticus*

A.parasiticus vb1 encodes a protein containing 205 amino acids. The Vb1 amino acid sequence is 74% identical to mammalian Rab7, 70% identical to yeast Ypt7 and 94% identical to AvaA in *A.nidulans*. All five regions involved in GTP-binding and hydrolysis in Ypt7/Rab7 GTPases along with a isoprenoid binding motif with two characteristic cysteine residues at the c-terminus (Lazar *et al.*, 1997; Ohsumi *et al.*, 2002) are conserved. These similarities in amino acid sequence suggest functional similarity (homology) of Vb1 with Ypt7/Rab7 GTPases.

Figure 3.2 Effect of Sortin3 on SU-1

Panel A-B: Comparison of vesicle-vacuole morphology in presence (panel B) and absence (panel A) of Sortin3. Size Bars = 5μm. **Panel C:** Dry weight comparisons between SU-1 grown in YES medium in presence (+) and absence (-) of Sortin3. **Panel D:** Comparison of aflatoxin accumulated in the media in presence (+) and absence (-) of Sortin3. **Panel E:** Comparison of aflatoxin enzymes Ver-1, Vbs and OmtA accumulated in SU-1 at 40h grown in presence (+) and absence (-) of Sortin3.

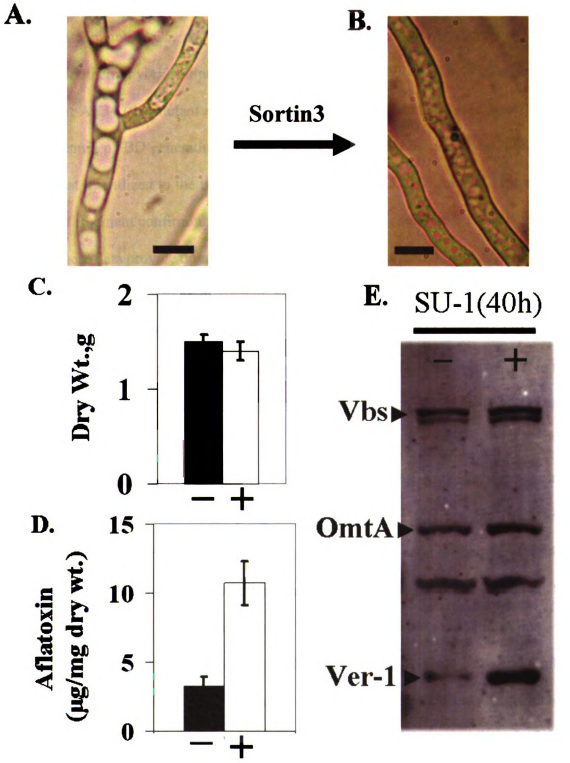

A.
B.
Sortin3
C.
Dry Wt.g
2
1
0
− +
D.
Aflatoxin
(µg/mg dry wt.)
15
10
5
0
− +
E.
SU-1(40h)
− +
Vbs
OmtA
Ver-1

Figure 3.2.

Role of *vb1* in *A. parasiticus* vacuole biogenesis and aflatoxin synthesis

Transformants AC5, AC7 and AC11 were identified as *vb1* disruptant strains based on Southern hybridization analysis (Figure 3.3C). NR-1 (the host strain) and transformant AC34 (the mutant *niaD* allele in the host strain was replaced by wild type *niaD* in plasmid pVBD generating a *niaD*+ phenotype) carried the wild type 5.8Kb *SalI* fragment that hybridized to the *vb1* probe; transformants AC5, AC7 and AC11, carried a 12.8Kb *SalI* fragment confirming the presence of a 6Kb *niaD* selectable marker inserted into *vb1*. These data provide direct evidence of vb1 gene disruption in AC5, 7, and 11.

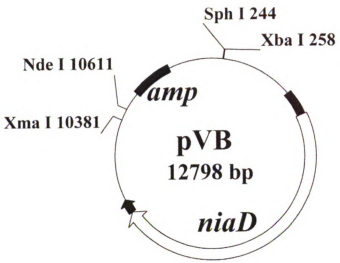
To determine whether disruption of *vb1* affects vacuole biogenesis, vesicles-vacuole morphology in the *vb1* disruptants was compared with wild-type SU-1 at 40h in YEP medium. *vb1* disruption resulted in a large increase in vesicles number in YEP in the transformants. Figure 3.4 shows the comparison of one of the transformants (AC11) with SU-1 and NR-1.

To determine whether disruption of *vb1* affects aflatoxin synthesis, we measured aflatoxin synthesized in the medium per gram (dry weight) of AC11 and SU-1 (ELISA). AC11 accumulated approximately 15 fold more aflatoxin as compared to SU-1 at 40h (Figure 3.5A b). To determine whether the increased aflatoxin accumulation in the growth medium by AC11 was due to higher aflatoxin enzyme levels in the mycelium, we performed Western blot analysis with antibodies against Nor-1, Ver-1, Vbs and OmtA. The data (Figure 3.5B) show that all four aflatoxin enzymes were detected at significantly higher levels (Vbs and OmtA by approximately 2 fold, Ver-1 by approximately 3 fold and Nor-1 approximately 2.5 fold) in AC11 as compared to wild-type SU-1. Using Oddesey software, the approximate mean intensities of the bands representing aflatoxin

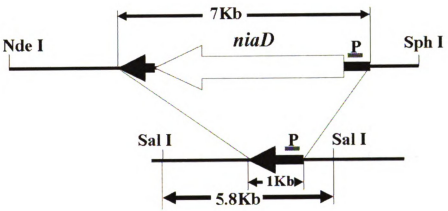
Figure 3.3 Disruption of *A. parasiticus vb1* gene

Panel A. Plasmid map of pVB used for disruption of *vb1* gene **Panel B.** Scheme of integration of the *SphI/NdeI* linear fragment (10kB) leading to replacement of wild type *vb1* with the disruption construct carrying Δ*vb1*::*niaD*. **Panel C.** Southern blot analysis for confirmation of the disruption of *A. parasiticus vb1* in strains AC5, AC7 and AC11. P represents a 0.3Kb probe for *vb1*. Disruption of *vb1* was demonstrated by a 11.8 Kb Sal I fragment; 5.8 Kb Sal I fragment represents wild type *vb1*.

A.



B.



C.

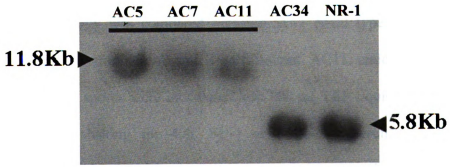


Figure 3.3

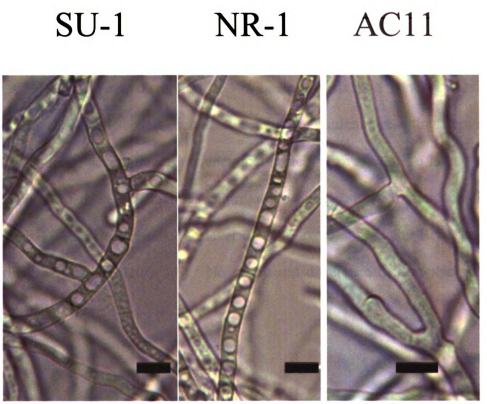


Figure 3.4 Effect of *A.parasiticus vb1* on vacuole-vesicles morphology

Vacuole-vesicuar morphology was compared between SU-1 (wild type strain), NR-1 (host strain for fungal transformation) and a *vb1* mutant AC11 under bright field microscopy. A total of 10^6 spores were inoculated into 100 ml YEP liquid medium and incubated at 30°C with shaking for 40h. SU-1 and NR-1 represented a similar morphology (predominance of vacuoles compared to vesicles). AC11 showed less than 5% vacuoles at this time point grown under similar conditions demonstrating a "high vesicles number" phenotype. Size Bars = 5μm.

proteins were compared between SU-1 grown in presence (+) and absence (-) of Sortin3. A 10 fold higher mean intensity was observed for Ver-1 protein in (+) than (-). A 25% higher mean intensity was observed for Vbs and 20% higher mean intensity was observed for OmtA protein in (+) compared to (-).

There are at least two possible explanations for the observed increase in aflatoxin enzymes in AC11. First, disruption of *vb1* increases aflatoxin gene expression by an unknown mechanism. Second, increased vesicles accumulation resulting from disruption of *vb1* leads to an accumulation of functional proteins in vesicles. To help determine if either or both of these explanations contribute to increased aflatoxin accumulation, we performed RT-PCR to measure the level of gene expression at the RNA level for the aflatoxin gene, *ver-1* (Figure 3.6). No significant difference in the expression of this gene was observed

Figure 3.5 Effect of *vb1* disruption on aflatoxin and aflatoxin enzymes

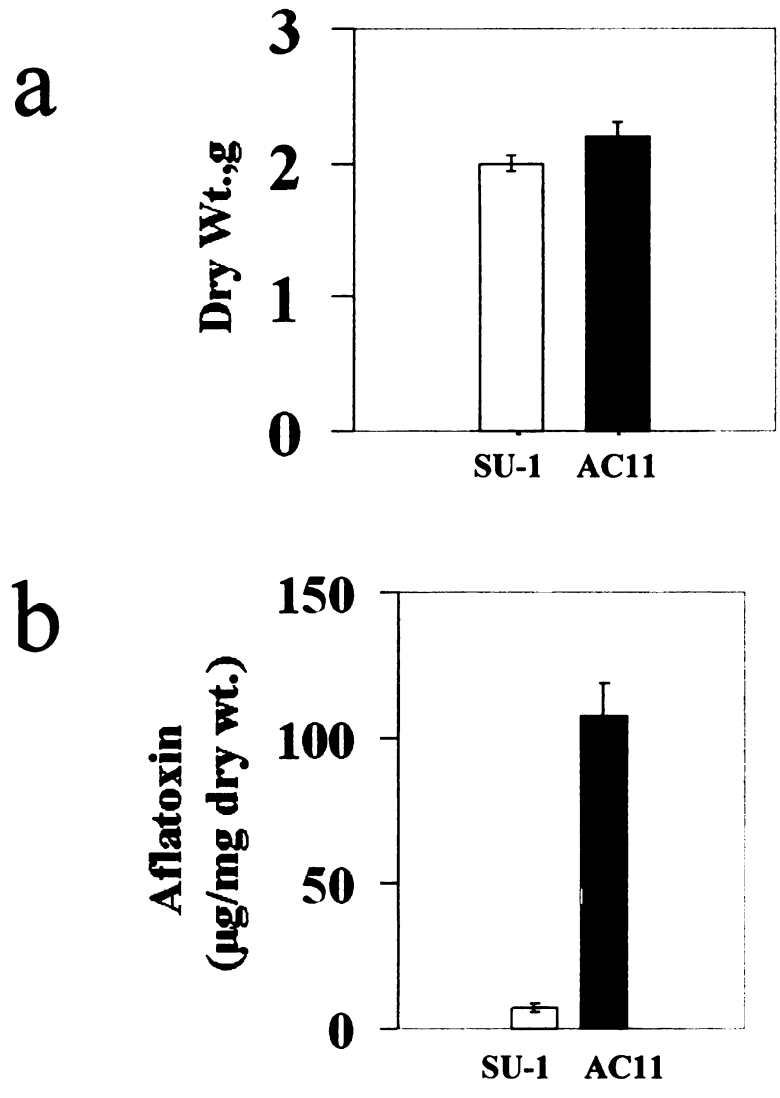


Figure 3.5 A. a. Dry weight comparisons between SU-1 and AC11 (a *vbl* disruptant) grown in YES medium for 40h. **b:** Comparison of aflatoxin accumulated in the media by AC11 and SU-1. Aflatoxins in the media was estimated by ELISA and normalized to the dry weight of the mycelium.

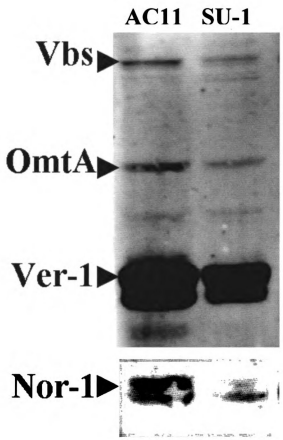


Figure 3.5B: Comparison of aflatoxin enzymes Nor-1, Ver-1, Vbs and OmtA accumulated in AC11 and SU-1 at 40h.

Figure 3.6 Comparison of transcript levels by RT-PCR. 10^6 conidiospores of each strain were inoculated into 100 ml YES liquid medium and incubated at 30°C with shaking for 40h. Total RNA was extracted. RT-PCR was performed on total RNA treated with RNAse-free DNAse I with primers specific for the coding region of each gene. PCR products were separated by electrophoresis on a 0.8% agarose gel.

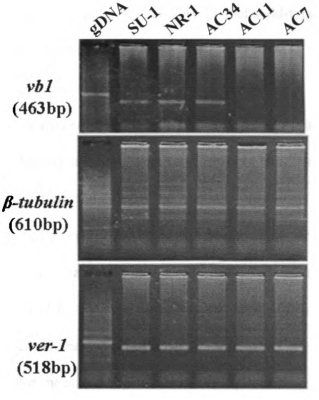


Figure 3.6

Table 3.1. List of primers used for RT-PCR (Figure 3.6)

Gene	Primers	PCR product		Introns
		gDNA	cDNA	
vb1	F-5' CAACAAGTTCAGCGCCAGCTAC 3' R-5' AAACTCTTCGGCCTCTTCTTGC 3'	689bp	463bp	3
β-tubulin	F-5' TTCCGTCCCGACAACTTC 3' R-5' TCAGGAACGGAGACGGC 3'	610bp	610bp	0
ver-1	F-5' AACAGATCAAGGCCAATGGT 3' R- 5' AGGAGAGAGCCAAGCGG 3'	628bp	518bp	2

between the *vb1* disruption strains AC7 and AC11 and wild-type SU-1. The RT-PCR data tend to support the second explanation.

Comparison of the expression (RT-PCR) of *A. parasiticus vb1* and the *vps16* homologue under aflatoxin inducing and non-inducing conditions

To better understand the role of the vacuole biogenesis gene *vb1* in aflatoxin synthesis, we compared expression of *vb1* at the level of RNA by RT-PCR in *A. parasiticus* grown for 24, 30 and 40h in aflatoxin inducing medium (YES) and aflatoxin non-inducing medium (YEP). Because Sortin3 treatment results in a Vps16 phenotype in yeast and because vesicle transport machinery is conserved in eukaryotes (Bennett & Scheller, 1993), we also measured expression of the *A.parasiticus vps16* homologue. The data demonstrate that the expression of *vb1* and the vps16 homologue remained the same at all time points in YEP. However, in YES, expression of both genes declined as aflatoxin synthesis increased from 24 to 40h (Figure 3.7). This decline in expression correlates with a significant rise in vesicles in SU-1 observed between 24 and 40h in YES medium.

Figure 3.7. Comparison of time course transcript levels between SU-1 grown in aflatoxin inducing conditions (YES growth medium) and non-inducing medium (YEP growth medium) by RT-PCR. 10^6 conidiospores of SU-1 wild type strain was inoculated into 100 ml YES and YEP liquid medium and incubated at 30°C with shaking for three different time points (24h, 30h and 40h). Total RNA was extracted. RT-PCR was performed on total RNA treated with RNAse-free DNAse I with primers specific for the coding region of each gene. PCR products were separated by electrophoresis on a 0.8% agarose gel.

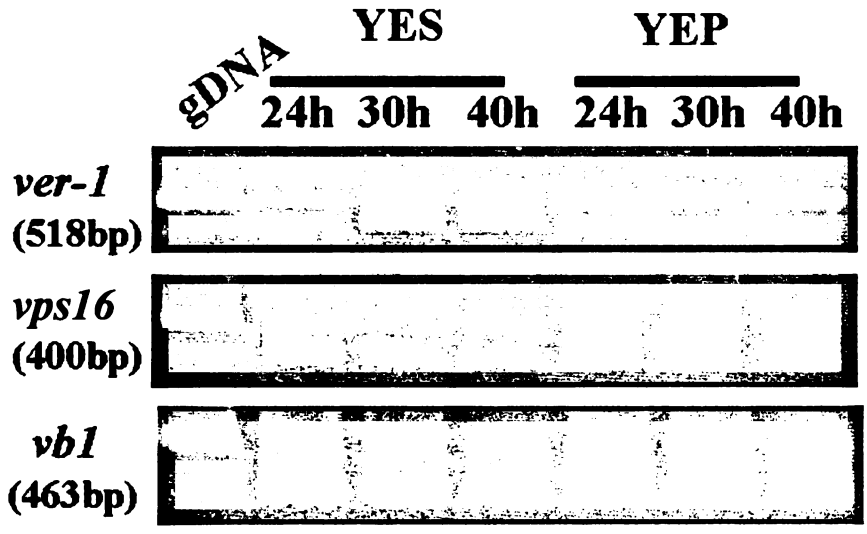


Figure 3.7

Table 3.2. List of primers used for RT-PCR (Figure 3.7)

Gene	Primers	PCR product		Introns
		gDNA	cDNA	
vb1	F-5' CAACAAGTTCAGCGCCAGCTAC 3' R-5' AAACTCTTCGGCCTCTTCTTGC 3'	689bp	463bp	3
vps16	F-5' TGAAGATGTTGAACTAGAGAAC 3' R-5' GGGCTCGCTATAGTTAGAC 3'	500bp	400bp	3
ver-1	F-5' AACAGATCAAGGCCAATGGT 3' R- 5' AGGAGAGAGCCAAGCGG 3'	628bp	518bp	2

DISCUSSION

Fusion of vesicles with vacuoles is a frequent occurrence in *A. parasiticus* (Figure 3.8). As discussed in Chapter 2 of this dissertation, we functionally linked aflatoxin synthesis to a pure vesicles-vacuoles fraction. Analysis of the organelles in this fraction demonstrated the presence of Ver-1, Vbs and OmtA. Another recent study demonstrated the presence of Nor-1 in these same organelles (Hong, 2008). These organelle fraction also could convert the late pathway intermediate sterigmatocystin (exogenously added) to aflatoxin, demonstrating directly that vesicles-vacuoles serve as the primary sub-cellular site(s) for the late steps in aflatoxin synthesis. The current study demonstrates that one can increase aflatoxin accumulation by interfering with fusion of vesicles with vacuoles.

The data strongly support a model which proposes that aflatoxin enzymes are made in the cytoplasm and transported in vesicles to vacuoles. At least the last two enzymatic steps in aflatoxin biosynthesis are completed in vesicles and these organelles also participate in compartmentalization and probably in export of the end-product, aflatoxin, into the growth medium. Characterization of these aflatoxin synthesizing vesicles ("aflatoxisomes") will be addressed in our future studies. We hypothesize that aflatoxin enzymes likely continue to make aflatoxin until they are eventually turned over in vacuoles by peptidases that are also transported there. We also propose that during aflatoxin synthesis, signals from the growth environment not only turn on gene expression (a model described by us previously (Roze *et al.*, 2007)) but also result in a decrease in vacuole biogenesis resulting in accumulation of aflatoxisomes that carry

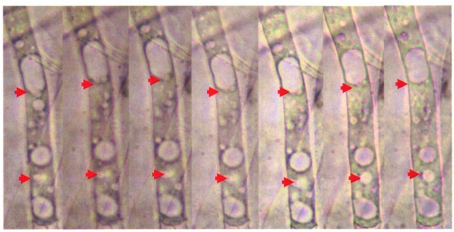


Figure 3.8. Bright field microscopy representing vacuole biogenesis in *A.parasiticus*. A mycelium of SU-1 grown on solid culture for 3 days was observed in an independent study. A series of images of a mycelium was acquired with 2 to 5 min intervals. The series of red arrows on the top shows the fusion of a vesicle into a vacuole. The lower series of red arrows show the fusion of two vesicles to form single vesicles.

Figure 3.9A-B. Model depicting the role of vacuole biogenesis in aflatoxin biosynthesis

3.9 A: Several studies suggest that early, middle and late enzymes in aflatoxin biosynthesis are synthesized initially in the cytoplasm. During peak levels of aflatoxin synthesis, some or all of the aflatoxin enzymes are transported to vacuoles via a direct route (a) or a vesicles-mediated route (b). Transport via route (a): the model proposes that early and middle aflatoxin enzymes are functional in the cytoplasm and transported to vacuoles for turnover; this model predicts that ST is present initially in the cytosol and then transported to vesicles by an unknown mechanism – here, only the late pathway steps occur in vesicles-vacuoles. Transport via route (b). If most or all aflatoxin enzymes localize to vesicles-vacuoles and are functional in that location, the model predicts that most or all of aflatoxin synthesis occurs in vesicles-vacuoles. ST = sterigmatocystin, OMST = *o*-methylsterigmatocystin, AF = aflatoxin. The green dashed arrow proposes site of action of Vb1 and Sortin3. The increased aflatoxin accumulation on interruption of vacuolar biogenesis support transport via route (b).

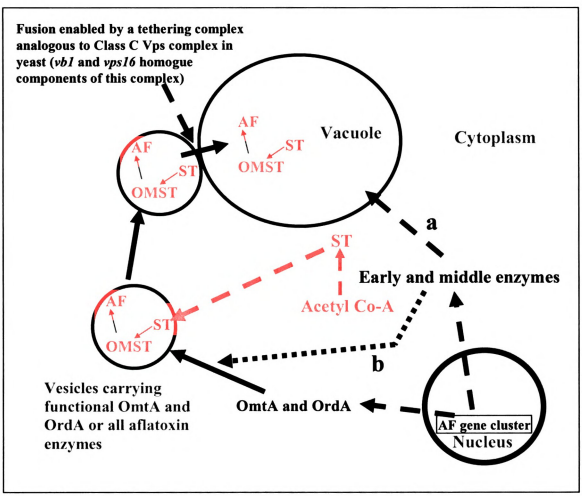
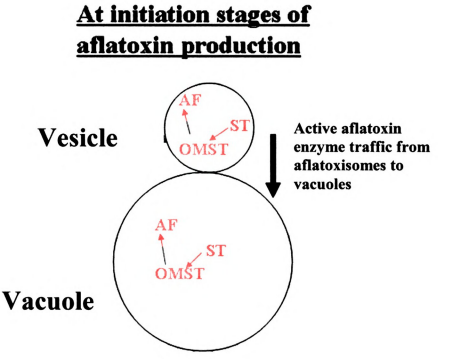

Fusion enabled by a tethering complex analogous to Class C Vps complex in yeast (*vb1* and *vps16* homogue components of this complex)
AF
ST
OMST
Vacuole
Cytoplasm
a
Early and middle enzymes
Acetyl Co-A
b
Vesicles carrying functional OmtA and OrdA or all aflatoxin enzymes
OmtA and OrdA
AF gene cluster
Nucleus

Figure 3.9 A

3.9 B. Panel a. At the start of aflatoxin synthesis vacuole biogenesis enables functionally active aflatoxin enzymes to reach vacuoles. Vacuolar peptidases also transported to vacuoles probably lead to degradation of aflatoxin enzymes keeping the aflatoxin synthesis at low level. **Panel b.** Between 24 to 48h vacuole biogenesis is downregulated (suggested by time course gene expression levels for *vb1* and *vps16 homologue* in *A. parasiticus*) vesicles containing functional aflatoxin enzymes accumulate leading to accumulation of aflatoxins at maximum levels. We designate these aflatoxin synthesizing vesicles as aflatoxisomes (see discussion).

a.



b.

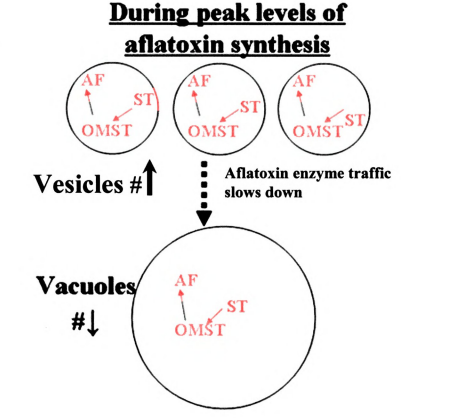


Figure 3.9 B

active aflatoxin enzymes. Blocking vacuole biogenesis by genetic or chemical means therefore results in further accumulation of aflatoxisomes leading to an even larger increase in aflatoxin accumulation. Fusion of vesicles to vacuoles therefore appears to be one important mechanism by which the fungus regulates the level of accumulation of aflatoxin enzymes and aflatoxin in the cell. For a schematic of our model (the role of vesicle-vacuole fusion in aflatoxin synthesis) see Figure 3.9. The observation that early (Nor-1), middle (Ver-1 and Vbs) and late (OmtA) aflatoxin enzymes all increase with increased accumulation of vesicles in *vb1* disruption mutants suggests that most of, or the entire aflatoxin biosynthetic pathway is carried in or on vesicles and vacuoles.

The N-glycosylation motif in Vbs combined with TEM and immunofluoresence data demonstrate the localization of Vbs in ring-like structures around nuclei as well as in the cytoplasm (Chiou, 2003). The current data (Western blot analysis suggests the presence of Vbs in vesicles-vacuoles fraction and interruption of vesicle-vacuole fusion increases Vbs accumulation in the cell) suggest strongly that Vbs is transported to vacuoles through a secretory pathway (ER→Golgi→vesicles→vacuoles). Recent studies (Hong & Linz, 2008; Lee, 2003) demonstrate that Nor-1, Ver-1, and OmtA (proteins that do not have N-glycosylation motif) are localized in cytoplasm when aflatoxin synthesis initiates; then localizes in the cytoplasm, vesicles and vacuoles when aflatoxin synthesis occurs at peak levels. We propose based on these observations that these proteins also reach vacuoles through vesicles; but they probably take a different route (cytoplasm-to-vacuoles-targeting pathway or Cvt) to reach vacuoles. Why should the fungus employ a different route for Vbs trafficking? Vbs catalyzes the bisfuran ring closure in versiconal hemiacetal (a reaction near the middle of the pathway) to form versicolorin B (the first

toxic intermediate in aflatoxin biosynthetic pathway) (Chiou *et al.*, 2004). We propose that a different route for Vbs protein traffic is a mechanism by which the fungus ensures prevention of any aflatoxin synthesis in the cytoplasm, thus avoiding self-toxicity. Our preliminary data suggest that Vbs forms a complex with Ver-1 and OmtA. We hypothesize that vesicles form aflatoxisomes only when functional enzymes involved in aflatoxin biosynthesis co-localize to a form complex that can complete the pathway. When and how the enzymes co-localize to aflatoxisomes to carry out their functions remain to be elucidated. Most aflatoxin that accumulates in the *vb1* disruption strain can be transported outside the cell; this observation demonstrates that the aflatoxin secretion machinery is not vacuole dependent. This may suggest then that aflatoxin synthesis and export may both be primary functions of aflatoxisomes, while aflatoxin storage and aflatoxin enzyme turnover may take place primarily in vacuoles. Whether or not aflatoxisomes mediate this export (exocytosis) needs to be elucidated in our future studies.

In yeast, at least six different pathways can be used to transport proteins to vacuoles; many of these pathways are mediated by vesicles (Bowers & Stevens, 2005; Bryant & Stevens, 1998; Kucharczyk & Rytka, 2001; Martin *et al.*, 2005; Ostrowicz *et al.*, 2008; Takegawa *et al.*, 2003; Teter & Klionsky, 2000; Weisman, 2003; Weisman, 2006; Wickner, 2002). One observation during our work with Sortin1 suggests the importance of a CPY trans-golgi to vacuole route in mediating aflatoxin enzyme delivery to vacuoles. Sortin1 treatment results in missorting of CPY outside the yeast cell instead of sorting to vacuoles (Zouhar *et al.*, 2004). Sortin1 treatment severely inhibited growth in *A. parasiticus* However, in a preliminary study using strain V-86 (expresses

Ver-1 protein fused to EGFP), we observed that Sortin1 treatment caused Ver-1 fused to EGFP (contained in vesicles) to be secreted outside the cells into the medium. Follow up studies will try to understand why Sortin1 affects growth in *A. parasiticus* and why it stimulates the export of aflatoxin enzymes into the medium.

The enormous impact of vacuole biogenesis on growth in filamentous fungi makes it difficult to obtain vacuolar sorting mutants (Shoji *et al.*, 2008). In support of this we observed that when *vb1* disruption mutants were grown in non-selective conditions, 15-20% of nuclei appeared to revert to wild type by elimination of the disruption construct (data not shown). Our work demonstrates that *A. parasiticus* Vb1 and *A. nidulans* AvaA share a high similarity not only in protein sequence (94% identity) but also in their function (both affect vacuole biogenesis). Based on these data, we decided to re-designate *A. parasiticus vb1* gene as *A. parasiticus avaA* in our future studies to avoid any unnecessary confusion in gene designation.

Of primary importance, our studies support the idea that Aspergilli, like other organisms with small genomes, utilize existing conserved cellular machinery (vesicle trafficking) to conduct new cellular functions (aflatoxin synthesis). Understanding the mechanisms that co-regulate the activation of aflatoxin synthesis and the shift to form aflatoxisomes is a key to development of efficient strategies to manipulate secondary metabolism in general and aflatoxin synthesis specifically.

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APPENDIX I

TIME-COURSE OF AFLATOXIN PROTEIN AND AFLATOXIN ACCUMULATION IN *ASPERGILLUS PARASITICUS*

INCLUDED AS A PART OF:

Roze, L. V., Arthur, A. E., Hong, S. Y., Chanda, A. & Linz, J. E. (2007a). The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. *Mol Microbiol* **66**, 713-726.

MATERIALS AND METHODS

Western blot analysis

Mycelial samples for Western blot analysis were collected and frozen in liquid N_2. Frozen mycelium was ground in liquid N_2 in a mortar with a pestle, and the powdered mycelium was re-suspended 1:1(w/v) in TSA buffer (0.01 M Tris, 0.15 M NaCl, 0.05% NaN3,pH 8.0) containing 1 tablet of Complete Mini Protease Inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 50 ml Proteinase Inhibitors mix (Sigma, St Louis, MO) and 0.125 mM phenylmethylsulphonyl fluoride per 10 ml. Total protein (30–60 mg) was separated by electrophoresis on 12% or 4–20% SDS polyacrylamide gels, transferred to PVDF membrane, exposed to antibody specific to OmtA, Ver-1, Nor-1 (all generated in our laboratory; Lee, 2003). Then the filter was incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma, St Louis, MO), and developed in the presence of NBT/BCIP (Roche, Penzberg, Germany).

Detection of aflatoxin B_1

Aliquots of growth medium (10 ml) for aflatoxin analysis were collected and extracted three times with 10 ml chloroform each. Aflatoxins were detected by ELISA (Liang, 1996) using polyclonal antibodies against aflatoxin B_1 (Sigma).

RESULTS

Aflatoxin proteins were not detected at 24h of growth but were detected at 30h; Vbs was barely detected at 24 and 30h. Then, the accumulated levels of the proteins increased in the 40h mycelial samples (Figure AI). Accumulation of aflatoxin in the medium (TableAI) correlated well with the appearance of the aflatoxin proteins. No aflatoxins were detected at 24 or 30h, but clearly were detected at 40h. In liquid shake culture, we observed that approximately 99% of the synthesized toxin was detected in the growth medium, while only 1% was found in the mycelium. A similar observation was made previously for fungal colonies grown on YES agar medium (Lee, 2003).

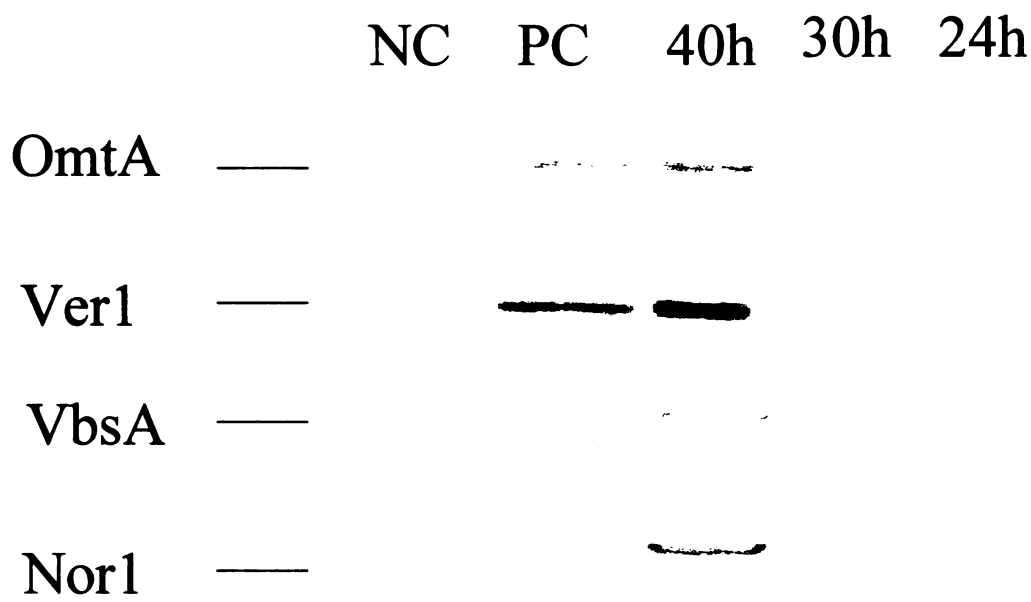

Figure AI. Western blot analysis of aflatoxin proteins in *A. parasiticus* SU-1 during a transition from exponential growth to stationary phase.

Table AI. Aflatoxin levels in *A. parasiticus* SU-1 grown in YES liquid medium.

Time	Mycelium	Medium
24h	Undetected	0.55
30h	Undetected	0.78
40h	0.018	6.0
68h	2.83	550

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APPENDIX II

INTERACTION OF AFLATOXIN PROTEINS (A PRELIMINARY STUDY)

INTRODUCTION

The organization of enzymes into macromolecular complexes localized to specific subcellular sites is a central feature of cellular metabolism. Recent studies in plant secondary metabolism suggest that enzymes involved in biosynthesis of secondary metabolites co-localize in cellular compartments to form multi-enzyme complexes e.g. polyamine metabolism (Panicot *et al.*, 2002) and flavoinoid metbaolism (Burbulis & Winkel-Shirley, 1999). Such metabolic complexes/channels (also called 'metabolons') enable direct transfer of a pathway-intermediate from one enzyme to another thereby maintaining a high local substrate concentration (Facchini & St-Pierre, 2005)

Our previous and current studies show that more than one aflatoxin proteins are expressed at the same time range (Roze *et al.*, 2007) and they localize at the same cellular location (vesicles and vacuoles) (Hong, 2008; Hong & Linz, 2008; Lee *et al.*, 2004). We hypothesized that since these proteins are expressed almost at the same time range and at the same cellular location, there is a strong possibility that the proteins physically interact with each other to from a complex which helps the proteins to complete the biosynthetic pathway.

MATERIALS AND METHODS

Strains, media and growth conditions

A. parasiticus strain SU-1 (ATCC 56775), a wild-type aflatoxin producer, was used in this study. *A. parasiticus* SU-1 conidiospores (spores) from a frozen stock were inoculated into YES liquid medium [contains 2% yeast extract and 6% sucrose; pH 5.8] at 10^4 spores per ml and incubated at 30°C with shaking at 150 rpm for 40h.

Native gel electrophoresis and Western blot analysis

Proteins for native gel analysis were extracted from 36h old frozen mycelium by grinding in liquid N_2 in a mortar with a pestle, and the powdered mycelium was re-suspended 1:1(w/v) in native gel solubilization buffer (5% (w/v) sucrose containing 1 tablet of Complete Mini Protease Inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 50µl Proteinase Inhibitors mix (Sigma, St Louis, MO), 0.125mM phenylmethylsulphonyl fluoride and 0.1mg of bromophenol blue per 10 ml of the buffer). Four different lanes were loaded with total protein 40µg (each), separated on a 12.5% non denaturing gel (Ausubel, 2003) and transferred to PVDF membrane. The lanes were separately exposed to antibody specific to Nor-1, Ver-1, Vbs and OmtA respectively (all generated in our laboratory; Chiou, 2003; Lee, 2003). The filters were finally incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma, St Louis, MO), and developed in the presence of NBT/BCIP (Roche, Penzberg, Germany).

Coimmunoprecipitation studies

Proteins from whole cells were prepared from 36 h old frozen mycelium similar to a procedure described in Appendix I. To 1 mL of this protein solution (containing 2mg of total proteins), 15μL of anti-rabbit IgG/protein-A agarose complex (Oncogenic science, Uniondale, NY) and 10μL of rabbit IgG (0.4 μg/μL) were added. The solution was incubated on a platform shaker (100 rpm) at 4°C overnight followed by centrifugation at 2500xg for 15min to precipitate the proteins that bind non-specifically to the rabbit IgG (a preabsorption procedure). The precipitate was discarded. The supernatant was divided into two equal volumes. To the first volume 4μg of anti-OmtA was added along with 15μL of anti-rabbit IgG/protein-A agarose complex (Oncogenic Science, Uniondale, NY) to immunoprecipitate the complex involving OmtA. To the other volume, 4μg of rabbit IgG was added as a negative control. The solutions were incubated overnight at 4°C with shaking followed by centrifugation as described above. The precipitates and supernatants were analyzed for the presence of middle and late aflatoxin proteins OmtA, Ver-1 and Vbs by Western blot analysis as described above.

RESULTS

Native gel

Native gel analysis (Figure AII-a) showed that protein complexes recognized by antibodies against Vbs, Ver-1 and OmtA aligned at the same level relative to the loading point suggesting that these three proteins might participate in a multi-protein complex. Nor-1 antibody recognized a protein complex that was larger in size compared to the Ver-1,Vbs, OmtA complex.

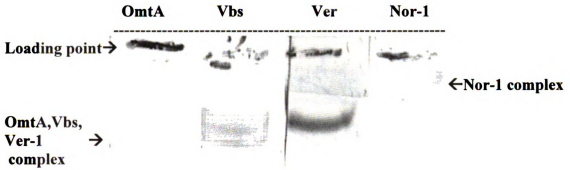

Figure AII-a. Native gel and Western blot analysis. Proteins were extracted from 36h old frozen mycelium as described in methods. Four different lanes were loaded with total protein 40μg (each), separated on a 12.5% non denaturing gel and then transferred to PVDF membrane. The membrane was cut to separate the lanes which were then separately exposed to antibody specific to Nor-1, Ver-1, Vbs and OmtA respectively for Western blot analysis

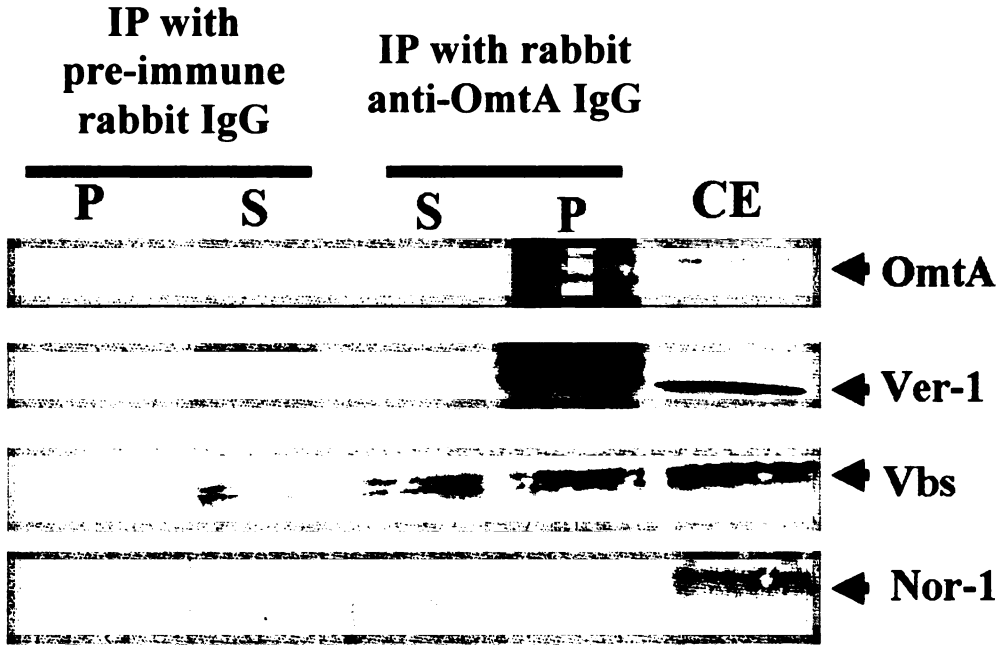

Figure AII b : Immunoprecipitation (IP) with anti-OmtA. Proteins from whole cells were prepared from 36 h old frozen mycelia as described. After a preabsorption step as described in the methods, the protein solution was divided into two equal volumes. The first volume was precipitated with anti-OmtA (made in rabbit(Lee *et al.*, 2002)). The second volume was precipitated with rabbit IgG (Sigma, St Louis, MO) (immunoprecipitation described in methods). Precipitates (P) and supernatants(S) were analyzed for the presence of aflatoxin proteins Nor-1, Ver-1, Vbs and OmtA by Western blot analysis as described above. CE= whole cell protein extract used a positive control.

Coimmunoprecipitation

Native gel analysis suggested possible of complex formation between three aflatoxin enzymes (Ver-1, Vbs and OmtA). As a first step to determine whether such a protein interaction occurs between middle (Vbs) and late (Ver-1 and OmtA) aflatoxin enzymes, an immunoprecipitation experiment was done with antibody against one of the proteins (OmtA). A similar "pull down" was done with rabbit IgG (negative control). As shown in figure AII b, α-OmtA was able to pull down Ver-1 and some Vbs proteins along with OmtA. Nor-1 as expected (from native gel results) was not obtained as immunoprecipitate.

Discussion

The results suggest strongly that during aflatoxin biosynthesis middle and late aflatoxin proteins participate in a multi-protein complex. The exact location and significance of this complex is yet unknown and could be a possible area to explore in future.

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APPENDIX III

AFLATOXIN SECRETION: PRELIMINARY EVIDENCE FOR INVOLVEMENT OF SECRETORY VESICLES

INTRODUCTION

Secondary metabolites are often toxic to the microorganisms that produce them. To protect the host strain from self-toxicity, the producer strains have developed means to export the end-products of secondary metabolism into the environment. Along with using transporter proteins (ATP-binding cassette(ABC) and major facilitator superfamily (MFS) transporters) (Del Sorbo *et al.*, 2000; Martin *et al.*, 2005) recent studies revealed the use of vesicle transport mechanisms that help to secrete the toxic compounds out of the cell (e.g. exudation of secondary metabolites in the marine red alga *Laurencia obtusa* (Salgado *et al.*, 2008).

Our studies have shown that *A. parasiticus* exports 80% (solid culture studies; Lee, 2003) to 99% (liquid culture studies; Roze *et al.*, 2007) of total aflatoxins made by the cell to the growth medium. The model of Roze for aflatoxin secretion proposes the use of protrusions from the cell surface (Roze *et al.*, 2007). It was proposed that aflatoxins are exocytosed into the environment by vesicles containing aflatoxins that fuse with the membrane surface (observed as protrusions under a microscope). Recent transmission electron microscopy with protoplasts from aflatoxin producing *A. parasiticus* (methods described in chapter 2) shows similar protrusions from the

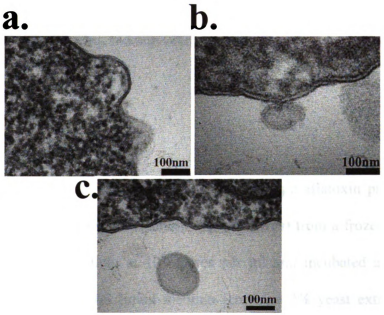

Figure AIII. 1. Protrusions and secretory vesicles observed in TEM images of pure protoplasts. **Panel a.** Two protrusions seen on plasma membrane **Panel b.** A secretory vesicle-like-structure budding out of a plasma membrane. **Panel c.** A secretory vesicle like structure observed outside the cell near the plasma membrane. Panel a, b and c represent three different locations in the same protoplast.

membrane surface and also shows vesicles budding out of the membranes of protoplasts (Figure AIII. 1).

As a preliminary study to test whether secretory vesicles might be involved in aflatoxin export we developed a novel surface immunofluoresence assay to compare the pattern of immunofluorescence imparted by anti-aflatoxin B_1 on the *A. parasiticus* cell surface during aflatoxin producing and non-producing conditions.

Materials and methods

Strains, growth media and antibodies

A. parasiticus strain SU-1 (ATCC 56775), a wild-type aflatoxin producer, was used in this study. *A. parasiticus* SU-1 conidiospores (spores) from a frozen stock were inoculated into the growth medium at 10^4 spores per ml and incubated at 30°C with shaking at 150 rpm for 40h. YES liquid medium [contains 2% yeast extract and 6% sucrose; pH 5.8] was used as aflatoxin inducing medium and YEP liquid medium [contains 2% yeast extract and 6% peptone; pH 5.8] was used as aflatoxin non-inducing medium. Antibodies against aflatoxin B_1 made in rabbit (Sigma, St.Louis ,MO) were used for recognition of aflatoxin on the surface being exocytosed. Anti-IgG labeled with FITC (Sigma, St.Louis ,MO) was used as secondary antibody for detection of the binding pattern. *A. parasiticus* AFS10 [derived from SU-1, *aflR* disruption, no aflatoxin enzymes or aflatoxin accumulation] was used a control strain. An antibody against satratoxin (kindly provided by Pestka lab) was used in one control experiment for determining the specificity of this immunoassay.

Surface immunofluoresence assay

A pellet of mycelia harvested from liquid growth medium was untangled with sterile forceps until a visible thread was obtained. The threads of mycelia were added to 50uL of fresh YES with α-AFB_1 antibodies (Sigma, St.Louis ,MO) [dilution 1:10] and incubated for 30min at 30deg The mycelia were centrifuged at 13,000 rpm and washed three times with fresh YES medium before a 15min treatment with α-Ig-rabbit with FITC label [dilution 1:50 in fresh YES]. Finally the mycelia were centrifuged and washed three times with fresh YES media before observing under Labophot fluorescence microscope (Nikon Inc., Melville, NY).

Results

Antibodies against aflatoxin B_1 binds in a specific pattern on cell surface in aflatoxin producing conditions

As shown in Figure AIII-2 a, anti-AFB1 (recognized by FITC labeled secondary antibody) binds to the surface of the *A.parasiticus* cell grown in aflatoxin inducing medium (YES) for 48h. To check for the specificity of this binding pattern we employed a series of control experiments. First, we compared the binding pattern of the wild type strain SU-1 in YES (aflatoxin inducing medium) and YEP (aflatoxin non-inducing medium). Second we compared the binding pattern between wild type SU-1 and a mutant strain AFS10 (genetically blocked for aflatoxin biosynthesis). Third we conducted two experiments two check the specificity of the antibody binding to the cell surface. In the

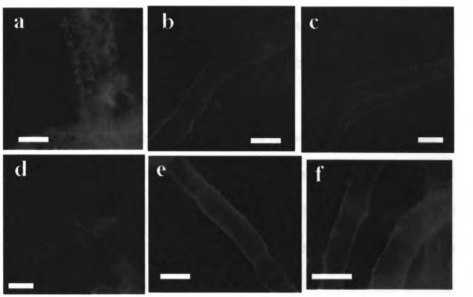

Figure AIII-2. Anti-aflatoxin B_1 (AFB_1) binds on cell surface of *A.parasiticus* in a specific pattern in aflatoxin producing conditions. **Panel a**. A pellet of 48h old SU-1 mycelia harvested from liquid YES medium was untangled with sterile forceps until a visible thread was obtained. The threads of mycelia were used for surface immunoassay (desribed in methods). **Panel b.** Similar treatment was done with SU-1 grown in YEP. **Panel c.** Surface immunoassay using mycelial fragments obtained from AFS10 strain grown for 48h in YES. **Panel d.** Surface immunoassay using mycelial fragments obtained from SU-1 grown for 48h in YES using anti-FITC only. **Panel e.** Surface immunoassay using mycelial fragments obtained from SU-1 grown for 48h in YES using anti-satratoxin instead of anti-AFB_1. **Panel f.** Surface immunoassay using mycelial fragments obtained from SU-1 grown for 48h in YES using neutralized anti-AFB_1. Size bars = 5μm.

first experiment we compared the binding pattern of anti-AFB_1 with anti-satratoxin and second we neutralized anti-AFB_1 with aflatoxin B_1 and then applied netralized antibodies to see whether the binding pattern is affected.

Our results demonstrate that antibodies bind to the surface only during conditions when aflatoxin is produced actively by the cell (Figure AIII-2). On the basis of the characteristic fluorescence pattern observed we propose that anti-AFB1 binds to aflatoxin in the vesicles that participate in transport of aflatoxin outside the cell during exocytosis.

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APPENDIX IV

FUTURE STUDIES

A. Studies to understand vacuolar development in detail

The studies included in this dissertation demonstrated that vesicles and vacuoles are functionally linked with aflatoxin biosynthesis. We propose that vacuoles undergo morphological, chemical and functional development as fungal cells switch to secondary metabolism. As a result vacuoles gain additional functions which include synthesis, storage and secretion of secondary metabolites. The model further proposes that late in this developmental process vacuoles predominantly engage in protein turn-over and eventually cell death occurs. To understand how vacuolar function correlates with population of proteins present in vacuoles at different phases during development of this organelle it is essential to characterize the biochemical composition of vacuoles over time. A time course vacuole proteome analysis could be very helpful to understand this correlation.

B. Studies to understand details of the involvement of vesicle transport machinery in aflatoxin biosynthesis

Our studies linked aflatoxin biosynthesis to vacuolar biogenesis. We propose that blocking vesicle to vacuole fusion facilitates accumulation of aflatoxisomes carrying functional aflatoxin enzymes resulting in increased aflatoxin accumulation. Studies involving interruption of aflatoxin protein traffic (either chemically or genetically) at

various points in the vesicular transport machinery could provide novel targets to manipulate secondary metabolism at the cellular level