Fungal Genetics and Biology 48 (2011) 35–48

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Review

Compartmentalization and molecular traffic in secondary metabolism: A new understanding of established cellular processes

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ARTICLE INFO

Article history:
Received 16 February 2010
Accepted 12 May 2010
Available online 16 May 2010

Keywords:
Aspergillus parasiticus
Aflatoxin biosynthesis
Compartmentalization
Protein traffic
Aflatoxisomes

ABSTRACT

Great progress has been made in understanding the regulation of expression of genes involved in secondary metabolism. Less is known about the mechanisms that govern the spatial distribution of the enzymes, cofactors, and substrates that mediate catalysis of secondary metabolites within the cell. Filamentous fungi in the genus *Aspergillus* synthesize an array of secondary metabolites and provide useful systems to analyze the mechanisms that mediate the temporal and spatial regulation of secondary metabolism in eukaryotes. For example, aflatoxin biosynthesis in *Aspergillus parasiticus* has been studied intensively because this mycotoxin is highly toxic, mutagenic, and carcinogenic in humans and animals. Using aflatoxin synthesis to illustrate key concepts, this review focuses on the mechanisms by which sub-cellular compartmentalization and intra-cellular molecular traffic contribute to the initiation and completion of secondary metabolism within the cell. We discuss the recent discovery of aflatoxisomes, specialized trafficking vesicles that participate in the compartmentalization of aflatoxin synthesis and export of the toxin to the cell exterior; this work provides a new and clearer understanding of how cells integrate secondary metabolism into basic cellular metabolism via the intra-cellular trafficking machinery.

Contents

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doi:10.1016/j.fgb.2010.05.006

1. Introduction

1.1. Secondary metabolism

Secondary metabolites are an extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae, and animals (Hoffmeister and Keller, 2007; Keller et al., 2005). One school of thought views secondary metabolism as a means to safely store or eliminate "waste" metabolites that result from primary metabolism; these secondary metabolites frequently are produced at highest levels during a transition from active growth to stationary phase and the producer organism can grow in the absence of their synthesis suggesting that secondary metabolism is not essential at least for short term survival. A second view proposes that the genes involved in secondary metabolism provide a "genetic playing field" that allows mutation and natural selection to fix new beneficial traits via evolution. A third view characterizes secondary metabolism as an integral part of cellular metabolism and biology; it relies on primary metabolism to supply the required enzymes, energy, substrates, and cellular machinery and contributes to the long term survival of the producer. This review adopts portions of each of these views to present a broader picture of the function and evolutionary role of secondary metabolism.

1.2. The "molecular switch" and temporal regulation

Because many secondary metabolites are synthesized at highest levels at specific times during the life cycle of the organism, it became apparent that cells have evolved a complex "molecular switch" that activates the genes involved in secondary metabolism and controls the flow of primary metabolites (carbon and nitrogen) through these pathways. To date, many details of the "molecular switch" have been discovered and it is clear that the switch helps explain, at least in part, the mechanisms by which cells control when (temporal switch) and to what extent pathway genes in secondary metabolism are activated. Many important aspects of the role of signal transduction pathways, pathway and global regulators, and the role of chromatin remodeling in the regulation of gene expression at the level of a single gene and of the gene cluster have been elucidated (please see excellent work on these topics (Bhatnagar et al., 2006; Cary et al., 2006; Ehrlich et al., 2004, 2005; Georgianna and Payne, 2009; Hoffmeister and Keller, 2007; Miller and Linz, 2006; Yabe and Nakajima, 2004).

1.3. Spatial regulation and translocation

It was recently discovered that aflatoxisomes, a group of specialized trafficking vesicles, participate in the biosynthesis, sequestering, and export of aflatoxin to the cell exterior in *Aspergillus parasiticus* (Chanda et al., 2009). The spatial distribution of key elements in secondary metabolism throughout the cell is also observed in other filamentous fungi and plants (Hong and Linz, 2008, 2009; Hoppert et al., 2001; Kutchan, 2005; Lendenfeld et al., 1993; Lunn, 2007; Saikia and Scott, 2009). It then became clear that the initial "temporal switch" model failed to take into account the sub-cellular site for the synthesis of the substrates, enzymes, cofactors, and intermediates involved in secondary metabolism and how these materials are translocated (brought together) to allow efficient operation of the metabolic pathway. The key themes of spatial distribution and translocation appear on a recurring basis throughout this review. Toward the end of this presentation, we propose a modified "molecular switch" model that summarizes our view of the mechanisms cells utilize to accomplish the temporal and spatial regulation of secondary metabolism.

1.4. Overview

The current review focuses on sub-cellular compartmentalization, intra-cellular molecular traffic, and how these processes contribute to the initiation and completion of secondary metabolism within the cell. Aflatoxin biosynthesis serves to illustrate many of the key principals discussed because of recent breakthroughs in analysis of this secondary metabolic pathway. Aflatoxin synthesis will be presented as an example of the intricate spatial and temporal organization in secondary metabolism and data will be introduced and discussed from several different organisms in support of this discussion. As an important part of this presentation, we discuss the origin and biogenesis of aflatoxisomes and a role of aflatoxisomes in fungal secondary metabolism and export. Our studies on aflatoxisomes also suggest that aflatoxin synthesis "borrows" sub-cellular organelles (vesicles and other established machinery) initially utilized for vacuole biogenesis as part of primary metabolism; the cell adds new functions to these structures as toxin synthesis and export proceed. We discuss what is known about this sequence of events and the implications to secondary metabolism specifically and to cell biology in general. We then make predictions about specific details of the mechanisms that mediate the temporal and spatial regulation of secondary metabolism and formulate hypotheses to promote filling of "knowledge gaps" in the literature. Finally, we speculate on the future directions of this field of study to address the proposed hypotheses.

2. Secondary metabolism is an integral part of cellular metabolism

Secondary metabolism in plants, fungi, and algae exhibits several common features that promote integration with primary metabolism at the molecular and cellular levels. First, secondary metabolic pathways use substrates (e.g. acyl-CoA, amino acids, nucleotides, and carbohydrates), cofactors, metabolites and energy that are formed during primary metabolism. This statement is supported by the observation that a genetic block in secondary metabolism in *A. parasiticus* results in re-direction of carbon flow within fungal cells (Roze et al., 2010). The most dramatic changes were observed after disruption of *veA* that encodes a global regulator of secondary metabolism and development (Δ*veA*); VeA is a unique fungal regulator of secondary metabolism that is not present in yeast and plants (Calvo, 2008). *A. parasiticus* Δ*veA* grown in liquid shake culture (under conditions where the block in secondary metabolism is observed but no fungal development occurs) produced significantly higher levels of ethanol and volatile compounds derived from branched chain amino acids in comparison to the wild type.

Second, an evolutionary link between secondary and primary metabolism is supported by studies that suggest that secondary metabolic pathways "borrow" enzymes from primary metabolism

and establish novel cellular functions for these existing enzymes via random mutation (Firn and Jones, 2009; Greenhagen et al., 2006; Vining, 1992).

Third, genes for secondary metabolic pathways frequently are clustered and these clusters consist of two to many non-homologous genes. Clustering provides the organism with one mechanism to coordinate temporal regulation of gene expression in an orderly manner (Bok et al., 2009; Lee et al., 2009; Palmer et al., 2008; Reyes-Dominguez et al., 2010; Roze et al., 2007; Trail et al., 1995). To accomplish this regulation, the cell employs conserved cellular regulatory "tools"; these include signal transduction pathways [heterotrimeric G-proteins, protein kinase A (PKA), PI3-kinase, Ras, MAP kinase, and cAMP (Bok and Keller, 2004; Hicks et al., 1997; Lee et al., 2007; Roze et al., 2004a; Shimizu and Keller, 2001; Shimizu et al., 2003; Valiante et al., 2009)], cyclic AMP response element binding proteins like CRE1 bp (Roze et al., 2004b), and global transcription factors such as AreA (Ehrlich and Cotty, 2002), and PacC (Espeso et al., 1993). In addition, unique pathway and global regulators of secondary metabolism evolved including AflR (Woloshuk et al., 1994), VeA (Calvo, 2008), and LaeA (Bayram et al., 2008; Bok and Keller, 2004). Our group and others recently discovered that certain pathway and global transcriptional regulators of secondary metabolism can modulate gene expression at the level of chromatin remodeling (Roze et al., 2007); in the case of aflatoxin biosynthesis, the level of acetylation of histone H4 in nucleosomes was directly correlated with the order of activation of the aflatoxin genes (Roze et al., 2007). Additionally, many secondary metabolite gene clusters are located in the telomeric regions of chromosomes and these are linked with a high frequency of single nucleotide polymorphisms (SNPs) (Cuomo et al., 2007). This observation provides important supporting evidence related to the "genetic playing field" view of secondary metabolism. The telomeric location is also associated with regulation by chromatin remodeling in *Aspergillus* (Shwab et al., 2007).

Another common characteristic of secondary metabolism is that enzymes, intermediates and end products of secondary metabolic pathways, are often localized in different sub-cellular membranous organelles (vacuoles, vesicles, peroxisomes, cytoplasm), in highly specialized cells, or in different plant tissues. Also, the enzymes involved in secondary metabolic pathways are organized into multifunctional enzyme complexes in which efficient catalysis is promoted by metabolic channeling (Burbulis and Winkel-Shirley, 1999; Jorgensen et al., 2005; Pelletier et al., 1999; Winkel-Shirley, 2001; Winkel, 2004). The spatial distribution of the biosynthetic enzymes in secondary metabolism requires translocation of these proteins and their substrates from one location to another within the same cell or between different cells (molecular traffic); translocation is also a central feature of primary metabolism (Jorgensen et al., 2005; Kutchan, 2005; Ovadi and Saks, 2004; Winkel, 2004). Intra-cellular compartmentalization of enzymes into or on membranous organelles also facilitates metabolic channeling (Jorgensen et al., 2005; Kutchan, 2005).

Finally, studies in a number of fungi, plants, and bacteria contribute to our understanding of the biochemical and physiological benefits that are derived from the ability to carry out secondary metabolism. For example, secondary metabolism helps maintain cellular homeostasis by regulating carbon and nitrogen flow in the cell (Price-Whelan et al., 2007; Roze et al., 2010), by re-generating intra-cellular NAD + concentrations (Dietrich et al., 2008; Price-Whelan et al., 2006, 2007), and by relieving cellular oxidative stress (Huang et al., 2009). Secondary metabolism also participates in cellular defense (Schroeckh et al., 2009), development (Keller et al., 2005), and promotes survival during conditions of nutrient deprivation (Dietrich et al., 2008; Price-Whelan et al., 2006, 2007). In plants, secondary metabolites have also been shown to carry out important functions including defense against herbivores and microbial pathogens, allelopathy, UV protection, fertility, and modulation of auxin transport (see reviews by (Belz, 2007; Buer et al., xxxx; Frey et al., 2009; Peer and Murphy, 2007). However, the function of many secondary metabolites is unknown.

In summary, data accumulated to date strongly suggest that secondary metabolism is an important part of a continuum with primary metabolism; the secondary metabolic pathways are intimately integrated into the biochemical and cellular machinery established as part of primary metabolism. The biosynthetic pathways also have been shaped by selective pressure to provide biochemical and physiological advantages to the producing cell.

3. Substrates for secondary metabolism are spatially distributed in the cell

Secondary metabolic pathways utilize substrates generated in different branches of primary metabolism; these metabolites are compartmentalized into metabolic pools that are distinct based on origin and destination. It is often not known which metabolic pool provides the substrate required for synthesis of a given secondary metabolite, whether different intra-cellular pools of the same metabolite interchange, and how metabolite translocation occurs. We discuss compartmentalization of acyl-CoA and amino acids, which serve as starter units for the biosynthesis of polyketides, alkaloids, phenylpropanoids, and β-lactam antibiotics to illustrate this idea.

3.1. Substrates for polyketide secondary metabolites

Polyketide synthases (PKS) utilize a variety of acyl-CoAs as starter units for the biosynthesis of polyketides, members of a large family of structurally diverse secondary metabolites produced by plants, fungi and bacteria. Acetyl-CoA, propionyl-CoA, malonyl-CoA and branched chain acyl-CoAs derived from the branched chain amino acids leucine, isoleucine, and valine, are the most common starter units for PKS I, II, and III. Type I PKS consist of one or more large proteins. These proteins contain multiple active sites, each of which catalyzes a different reaction involved in polyketide biosynthesis. Type II PKS consist of complexes carrying several monofunctional enzymes. Type III PKS are homodimeric enzyme complexes that act independently of acyl carrier protein (Moore and Hertweck, 2002; Staunton and Weissman, 2001).

Acetyl-CoA is also a starter unit in the mevalonate or isoprenoid pathway, which results in the synthesis of farnesyl phosphate (Daum et al., 1998); the latter leads to the synthesis of sterols and terpene secondary metabolites including carotenoids and trichothecenes (Daum et al., 1998; Desjardins and Proctor, 2007; Desjardins, 2009; Hoffmeister and Keller, 2007; Rau and Mitzka-Schnabel, 1985). Acetyl-CoA and the amino acids alanine, methionine, serine, and glutamic acid, are the precursors of fumonisins which are structurally related to sphingolipids (ApSimon, 2001). Fumonisins are mainly produced by *Fusarium* spp. (ApSimon, 2001; Desjardins, 2009; Rheeder et al., 2002).

Acetyl-CoA can be produced in the cell by various biochemical processes in distinct sub-cellular locations including mitochondria, plastids (plants), peroxisomes, and the cytosol. Acetyl-CoA is produced in mitochondria by conversion of pyruvate (delivered from the cytosol) by a pyruvate dehydrogenase complex and by beta-oxidation of short-chain fatty acids. In yeast, cytosolic pyruvate is converted into a cytosolic pool of acetyl-CoA with the help of pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase, and acetyl-CoA synthase (Boubekeur et al., 1999, 2001; Pronk et al., 1996). Acetyl-CoA synthase is localized in the cytosol and nucleus (Takahashi et al., 2006). In plants, mammals, and filamentous fungi, cytosolic acetyl-CoA is produced by a multiple subunit enzyme, ATP-citrate lyase (ACL) that converts citrate, ATP and CoA

to acetyl-CoA, oxaloacetate, ADP and inorganic phosphate (Fatland et al., 2000, 2002). ACL localizes to the cytosol and nucleus (Wellen et al., 2009). There is no apparent ATP-citrate lyase in yeast (Adams et al., 2002; Sheridan et al., 1990). An additional source of acetyl-CoA is catabolism of amino acids such as leucine, tryptophan, lysine, phenylalanine, tyrosine, and isoleucine.

Acetyl-CoA cannot penetrate organelle membranes and requires specific shuttle mechanisms to gain entry. First, acetyl-CoA can enter the glyoxylate cycle, which produces citrate and succinate; these can then penetrate the peroxisome and mitochondrion and subsequently generate acetyl-CoA (Antonenkov et al., 2009; Boubekeur et al., 2001; van Roermund et al., 1999). The formation of acetylcarnitine from acetyl-CoA and carnitine by carnitine acetyltransferase, provides another mechanism to shuttle acetyl-CoA across membranes that envelop peroxisomes, nuclei, and mitochondria. Acetyl-CoA is then released by the reverse reaction with the help of carnitine acetyltransferase, which is present in the cytosol, mitochondria, and peroxisomes (Madiraju et al., 2009; van Roermund et al., 1999). Different sources of acetyl-CoA are used for different subsequent processes. For example, in mammalian cells, acetyl-CoA generated in mitochondria and transported in a form of acetylcarnitine to the nucleus, is used for histone acetylation (Madiraju et al., 2009). Another source of acetyl-CoA utilized for histone acetylation in mammalian cells, is acetyl-CoA produced from citrate by ATP-citrate lyase (ACL), which catalyzes cleavage of citrate in the presence of ATP and CoA to produce acetyl-CoA and oxaloacetate; the second source is used preferentially. In *Saccharomyces cerevisiae*, a nuclear acetyl-CoA synthetase *ACS2* that generates acetyl-CoA from acetate is a rate limiting step for histone acetylation (Takahashi et al., 2006).

In filamentous fungi, acetyl-CoA can be formed in the cytosol through formation of pyruvate by a similar scheme as observed in yeast, in mitochondria from beta-oxidation of short-chain fatty acids, and in peroxisomes from beta-oxidation of long and very long-chain fatty acids (Maggio-Hall and Keller, 2004; Maggio-Hall et al., 2005). Most filamentous fungi, in contrast to yeast, possess ACL (Adams et al., 2002; Sheridan et al., 1990). In fungi, acetyl-CoA synthase is involved in acetate utilization (Sandeman and Hynes, 1989). An additional source of cytosolic acetate-CoA is the pentose phosphate pathway due to the presence of phosphoketolase genes in fungi (Thykaer and Nielsen, 2007). Secondary metabolites in the aflatoxin family are polyketide-derived furanocoumarins; the initial starter unit, acetyl-CoA, is converted to malonyl-CoA followed by generation of hexanoyl-CoA by a specialized fatty acid synthase; this intermediate is then extended to a decaketide by polyketide synthase A (PksA) (Keller et al., 2005; Trail et al., 1995).

It is reasonable to suggest that the biosynthesis of aflatoxin starts in sub-cellular locations where the substrate, acety-CoA, accumulates; however, it is unclear which acetyl-CoA pool is utilized. Recent evidence suggests that peroxisomes are the site of the initial steps of aflatoxin biosynthesis (Maggio-Hall et al., 2005). This group demonstrated that at least part of the acetyl-CoA required for aflatoxin synthesis is supplied by beta-oxidation of fatty acids in peroxisomes; the authors also showed that an early intermediate in aflatoxin biosynthesis, norsolorinic acid (NA), accumulates in peroxisomes in mutant strains of *Aspergillus nidulans*, *Aspergillus flavus*, and *A. parasiticus*; all three strains carry a defective *nor*-1 and accumulate non-physiologically high levels of NA.

3.2. Substrates for amino acid-derived secondary metabolites

In. *S. cerevisiae*, the vacuole represents the primary sub-cellular location where proteins are degraded and amino acids (primarily basic amino acids) accumulate (Kitamoto et al., 1988). The vacuole therefore plays an important role in maintaining cellular amino acid homeostasis (Sekito et al., 2008). The catabolism of branched chain amino acids is catalyzed by a branched chain amino acid aminotransferase that forms a 2-keto acid; this reaction controls the flow of carbon through the catabolic pathway (Hazelwood et al., 2008). In *S. cerevesiae*, the branched chain amino acid aminotransferase BAT1 (BCATm) is located in mitochondria whereas BAT2 is cytosolic which suggests that the accumulation of BCAA may occur in these sub-cellular locations (Islam et al., 2010). *Streptomyces* spp. synthesize a variety of polyketide antibiotics using complex starter units including isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA; the latter are derived from catabolism of the branched chain amino acids valine, leucine, and isoleucine (Denoya et al., 1995; Stirrett et al., 2009). The connection between BCAA catabolism and secondary metabolism in fungi is currently unknown.

4. Enzymes for secondary metabolism are compartmentalized within the cell

A large number of sub-cellular localization studies indicate that the enzymes, substrates, intermediates and end products of secondary metabolism often accumulate at different sub-cellular locations (e.g. vacuoles, vesicles, peroxisomes, cytoplasm, ER); this occurs regardless whether the biosynthetic pathway consists of only three enzymes as in penicillin biosynthesis, or involves more than 17 enzyme activities as in aflatoxin biosynthesis (Chanda et al., 2009; Evers et al., 2004; Hong, 2008; Hong and Linz, 2008; Hoppert et al., 2001; Kutchan, 2005; Lendenfeld et al., 1993; Maggio-Hall and Keller, 2004; Sirikantaramas et al., 2008; Ziegler and Facchini, 2008). This observed dispersal of biosynthetic machinery is a common feature of secondary metabolism in both plants and fungi. Methods suitable for localization studies in plants and fungi were recently reviewed (Lunn, 2007). Many plants exhibit a variation on this same theme whereby secondary metabolites (e.g. oils, alkaloids, flavonoids, terpenes) are frequently produced by specialized cells or specialized tissues (Kutchan, 2005; Weid et al., 2004). Accumulation of secondary metabolites in cells, tissue, cell culture and the growth medium, correlates in general with the coordinated expression of genes that encode enzymes in the corresponding biosynthetic pathway.

Intriguingly, experiments designed to track enzyme localization helped to demonstrate that early, middle, and late reactions in a biosynthetic pathway are often performed in different sub-cellular locations; synthesis of alkaloids, flavonoids, and monoterpenes in plants, penicillin biosynthesis in *Penicillium chrysogenum*, and aflatoxin biosynthesis in *Aspergilli* serve as important illustrations for this concept.

4.1. Plant secondary metabolites

4.1.1. Alkaloids

Alkaloids are low molecular weight compounds characterized by a heterocyclic backbone containing nitrogen; alkaloid biosynthesis has been reviewed extensively (Blunt et al., 2007; Facchini, 2001; Facchini and St-Pierre, 2005; Kutchan, 2005; Wang et al., 2010; Ziegler and Facchini, 2008). Examples of alkaloids with beneficial activities include the well-known anticancer drug, vinblastine, the analgetics morphine and codeine, the muscle relaxant, papaverine, as well as compounds such as caffeine, cocaine, nicotine, and colchicine (Facchini and St-Pierre, 2005; Ziegler and Facchini, 2008). Biosynthesis of alkaloids relies heavily on amino acid substrates that include ornithine, lysine, proline, phenylalanine, tyrosine and tryptophan (Mann, 1987). The alkaloid biosynthetic machinery in plants demonstrates common characteristics: these

include: (1) alkaloids accumulate within multiple cell types and tissues, (2) pathway enzymes localize to multiple sub-cellular compartments, and (3) multienzyme complexes form. These common characteristics suggest that the intermediates, end products and pathway enzymes involved in alkaloid biosynthesis, are translocated not only within a cell but also between cells that belong to different tissue types (St-Pierre et al., 1999). For example, monoterpenoid indole alkaloids generated in Madagascar periwinkle (*Catharanthus roseus*) accumulate in specialized cells called idioblasts and laticifers; however the enzymes involved in the biosynthetic pathways are found in roots, photosynthetic organs, in the epidermis of young leaves and stems, and in flower buds (St-Pierre et al., 1999). The biosynthetic enzymes localize to different subcellular compartments including cytosol, vacuole, ER, vesicles, and thylakoid membrane (Deus-Neumann and Zenk, 1986; Deus-Neumann, 1984; McCaskill et al., 1988; Roytrakul and Verpoorte, 2007; Ziegler and Facchini, 2008). Interestingly, the enzymes involved in the condensation reaction of monomers, strictosidine synthase and peroxidase, are localized to vacuoles, which suggests that cells compartmentalize the reactions that produce harmful end products, thereby protecting the cell from self-toxicity (Deus-Neumann and Zenk, 1986; Deus-Neumann, 1984; McCaskill et al., 1988; Roytrakul and Verpoorte, 2007). Examples of alkaloids sequestered in vacuoles include berberine in cell culture of *Coptis japonica* (accumulation is mediated by an ABC transporter and an H+/berberine antiporter) (Otani et al., 2005) and nicotine in tobacco (*Nicotiana rustica*) (Saunders, 1979). The biosynthesis of sanguinarine, a quaternary benzo[c]phenanthridine alkaloid with pro-apoptotic effects occurs in opium poppy (*Papaver somniferum*) cells; its transport to the vacuole is associated with ER-derived vesicles (Alcantara et al., 2005). An analogous vesicle-mediated mechanism for transport to vacuole and secretion into the culture medium was reported for camptothecin, a pentacyclic alkaloid synthesized in roots of *Ophiorrhiza pumila* (Sirikantaramas et al., 2007); camptothecin derivatives are used for treatment of human cancers.

4.1.2. Flavonoids

Flavonoids are polyphenolic secondary metabolites with pharmacological (anticancer) and biological activities (Tanaka et al., 2008). Flavonoid biosynthesis initiates from phenylalanine and acetyl CoA to form coumaryl CoA. Then a Type III polyketide synthase, chalcone synthase, catalyzes condensation of one molecule of 4-coumaryl-CoA with three molecules of malonyl-CoA to form a chalcone [tetrahydroxychalcone]. Different flavonoids with related chemical structures including anthocyanins, apigenin (flavone), naringenin (falvanone), kaemferol (flavonol), aureusidin 6-glucoside and isosalipurposide (chalcone), are formed by multiple biosynthetic branches that originate from chalcone. Flavonoid metabolism is catalyzed by an enzyme complex localized on the cytoplasmic side of the endoplasmic reticulum (Burbulis and Winkel-Shirley, 1999; Hrazdina et al., 1987). Burbulis and Winkel-Shirley (1999) showed that at least three enzymes involved in flavonoid biosynthesis in *Arabidopsis*, chalcone synthase, chalcone isomerase, and dihydroflavonol 4-reductase, physically interact in an orientation-dependent manner (Burbulis and Winkel-Shirley, 1999).

Several studies demonstrated that the products of flavonoid biosynthetic pathways are carried from biosynthetic sites in the cytoplasm to the vacuole with the involvement, at least in some species, of specific transporters. Anthocyanins produced in lisianthus (*Eustoma grandiflorum*) petals are transported to the central vacuole with the help of intra-cellular vesicles, or pre-vacuole compartments, derived directly from the endoplasmic reticulum (Zhang et al., 2006). In *Arabidopsis*, anthocyanin and proanthocyanidin transport to the vacuole is mediated by glutathione S-transferase TT19 (Alfenito et al., 1998; Kitamura et al., 2004; Marrs et al., 1995) and by a vacuolar flavonoid/H+-antiporter TT12 (Marinova et al., 2007). A multidrug resistance-associated protein, ZmMrp3, is required for the transport of the anthocyanin pigment into the vacuole in maize (*Zea mays*) (Goodman et al., 2004).

4.1.3. Cannabinoids

A unique group of terpenophenolics called cannabinoids, is synthesized in marijuana (*Cannabis sativa* L.). Among the approximately 70 cannabinoids characterized to date, delta (1)-tetrahydrocannabinol (THC) is the best-known psychoactive compound. THC biosynthesis occurs in glandular trichomes and begins with condensation of geranyl pyrophosphate with olivetolic acid to produce cannabigerolic acid (CBGA); the reaction is catalyzed by an enzyme called geranylpyrophosphate:olivatolate geranyltransferase. CBGA then undergoes oxidative cyclization to generate tetrahydrocannabinolic acid (THCA) in the presence of THCA synthase. THCA is then transformed into THC by non-enzymatic decarboxylation. Sub-cellular localization studies using RT-PCR and enzymatic activity analyses demonstrate that THCA synthase is expressed in the secretory cells of glandular trichomes, and then is translocated into the secretory cavity where the end product THCA accumulates (Sirikantaramas et al., 2005). Of particular interest, the enzyme present in the secretory cavity is functional, which suggests that the storage cavity is the site for THCA biosynthesis and storage.

4.2. Fungal secondary metabolites

Fungal secondary metabolites are broadly divided into five diverse categories (Hoffmeister and Keller, 2007): polyketides, polyketide–peptide hybrids, fatty acid derived compounds, amino-acid-derived compounds and non-ribosomal peptides. Localization studies on enzymes in fungal secondary metabolism are summarized in Table 1.

4.2.1. Polyketides

Sub-cellular localization of enzymes involved in aflatoxin biosynthesis has been studied extensively in our laboratory; to date, this work represents the most thorough sub-cellular localization studies on fungal polyketide biosynthesis. In early work, Lawellin et al. (1977) used anti-aflatoxin antibodies and an enzyme-linked immunochemical technique to detect discreet spots in the mycelium; these data suggest that aflatoxin is deposited in granules within 72 h old *A. parasiticus* mycelia grown in submerged culture. Later, we used a novel time-fractionated fungal colony technique, Western blot analysis, and immunofluorescence microscopy to detect Nor-1, Ver-1, and OmtA in *A. parasiticus* grown under aflatoxin-inducing conditions on a solid growth medium (YES). These three enzymes were not present between 0 and 24 h after inoculation, but were detected in large quantity in 24–48 h old mycelium (Lee et al., 2002, 2004). Immunoelectronmicroscopy showed that the enzymes first appear in the cytoplasm (between 24 and 36 h). Later in growth (48–72 h), the enzymes are found in vesicles and vacuoles; these organelles were concentrated on the substrate surface of the colony. VBS, the only glycosylated enzyme in the aflatoxin pathway (Silva and Townsend, 1997), was also detected between 24 and 48 h after inoculation. However, using immunofluorescence confocal laser scanning microscopy and immunogold transmission electron microscopy, VBS was detected throughout the cytoplasm in patches and in ring-like structures surrounding nuclei suggesting the enzyme is transported by vesicles to vacuoles via the classical endoplasmic reticulum/Golgi secretory pathway (Chiou et al., 2004).

Recently, Hong (Hong and Linz, 2008, 2009) established a monitoring system to track localization of aflatoxin enzymes in real

Table 1
Published studies showing localization of enzymes in fungal secondary metabolism.

Class	Biosynthetic pathway	Enzymes	Sub-cellular location	Method of analysis	References
1. Polyketides	Aflatoxin biosynthetic pathway*	Fatty acid synthases and polyketide synthase	Peroxisomes	M	Maggio-Hall et al. (2005)
		Norsolorinic acid reductase [Nor-1]	Cytoplasm, vesicles and vacuoles	I, R	Hong and Linz (2009), Lee et al. (2004)
		Versicolorin B synthase [Vbs]	Cytoplasm, vesicles and vacuoles	I, S	Chanda et al. (2009), Chiou et al. (2004)
		Versicolorin A reductase [Ver-1]	Cytoplasm, vesicles and vacuoles	I, R, S	Chanda et al. (2009), Hong and Linz (2008), Lee et al. (2004)
		O-methyl transferase A [OmtA]	Cytoplasm, vesicles and vacuoles	I, S, F	Chanda et al. (2009), Lee et al. (2004)
		Oxidoreductase A [OrdA]	Vesicles and vacuoles	F	Chanda et al. (2009)
	Sterigmatocystin biosynthetic pathway	Fatty acid synthases and polyketide synthase	Peroxisomes	M	Maggio-Hall et al. (2005)
2. Amino acid-derived secondary metabolites and non-ribosomal peptides	Penicillin and cephalosporin	ACVS [first pathway enzyme]	Golgi derived vesicles	S	Kurylowicz et al. (1987)
			Vacuole	I	Lendenfeld et al. (1993)
			Cytosol	I	van de Kamp et al. (1999), van der Lende et al. (2002)
		IPNS [second pathway enzyme]	Cytosol	I	van de Kamp et al. (1999), van der Lende et al. (2002)
		IAT [final pathway enzyme]	Peroxisomes**	I	Muller et al. (1992), van de Kamp et al. (1999), van der Lende et al. (2002), Sprote et al. (2009)
	Cyclosporin	Cyclosporin synthase [key enzyme]	Vacuole membrane	I	Hoppert et al. (2001)
3. Terpenes	Paxilline biosynthesis	GgsA	Punctuate organelles	R	Saikia and Scott (2009)
		GgsB(PaxG)	Peroxisomes	R	Saikia and Scott (2009)
	Carotenoid biosynthesis	Prenyltransferase(s)	Soluble fraction	S	Rau and Mitzka-Schnabel (1985)
		Carotegenic enzymes	Membranes of ER	S	Rau and Mitzka-Schnabel (1985)

* Only published data are included. Our proteomic analysis data on aflatoxisomes (Chanda et al., 2010b) show the presence of 13 aflatoxin enzymes out of 17 (Fas1, Fas2, PksA, Nor-1, NorA, Ver-1, Vbs, AvnA, OrdA, OmtA, OmtB, EstA, AflT).
** In P. chrysogenum, preventing IAT localization in peroxisomes blocks penicillin synthesis but in A. nidulans, blocking peroxisomal localization of IAT reduces penicillin biosynthesis but does not shut it down completely (Sprote et al., 2009). M: microscopy of mutants to detect accumulation of intermediates; R: sub-cellular tracking of EGFP fusions in real-time; I: immunogold labeling; S: detection of enzymes in isolated sub-cellular fractions; F: direct demonstration of the presence and functionality of enzymes by feeding studies with isolated organelle fraction.

time in living tissue. He fused enhanced green fluorescent protein (EGFP) to the early aflatoxin enzyme Nor-1 or the middle enzyme Ver-1; expression of the fusions was under the control of the corresponding wild-type promoter. The reporter construct integrated into the homologous site in the aflatoxin gene cluster. Using confocal laser scanning microscopy (CLSM), he demonstrated that EGFP-tagged Ver-1 or Nor-1 localized to the cytoplasm, vacuoles, and vesicle-like structures in 48 h and 72 h old *A. parasiticus* mycelia grown in slide culture under aflatoxin-inducing conditions; the fusions were not detected under conditions that restricted aflatoxin biosynthesis.

4.2.2. β-Lactam antibiotics

The fungal β-lactam antibiotics penicillin G, produced by *P. chrysogenum* and *A. nidulans*, and cephalosporin C, produced by *Cephalosporium acremonium*, originate from amino acids L-α-aminoadipic acid, L-cystein, and L-valine. The biosynthetic pathways for these two antibiotics share the first two enzymatic steps, and then the pathways diverge (Brakhage et al., 2004; Kurylowicz et al., 1987; van de Kamp et al., 1999). To complete biosynthesis of penicillin, the catalytic activity of three enzymes is needed, including δ-(L-a-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase (ACVS), isopenicillin N (IPN) synthase (IPNS) and acyl-coenzyme A:isopenicillin N (acyl-CoA:IPN) acyltransferase (IAT). Cephalosporin biosynthesis requires activities of a different set of enzymes after formation of IPN, including IPN epimerase (IPNE), the bifunctional deacetoxycephalosporin (DAOC) synthase/deacetylcephalosporin (DAC) synthase (DAOCS/DACS; expandase/hydroxylase), and acetyl-CoA:DAC acetyltransferase (DAT). ACVS and IPNS are located in the cytosol (van de Kamp et al., 1999; van der Lende et al., 2002). Despite this cytosolic enzyme location, a primary source of the precursors for the pathway is the vacuolar pool of amino acids. The last enzyme in penicillin the biosynthetic pathway, IAT, is located in peroxisomes (Muller et al., 1992; van de Kamp et al., 1999; van der Lende et al., 2002). Of note, peroxisomal localization of IAT in *P. chrysogenum* was essential for completion of penicillin biosynthesis. However, in *A. nidulans*, mislocalization of IAT due to mutations or deletion in the peroxisomal targeting signal PTS1, or due to mutations in proteins that mediate PTS1-dependent protein import into peroxisomes, did not block penicillin produc-

tion but reduced it in comparison to the wild-type strain (Sprote et al., 2009). Moreover, in a strain lacking peroxisomes, penicillin production was not abolished but was reduced by approximately 50%. These data indicate that cytosolic IAT is also functional.

Based on localization studies, it appears that cephalosporin biosynthesis in *C. acremonium* takes place in the cytosol because all the enzymes are cytosolic (van de Kamp et al., 1999). However, a recent study reports that the epimerization of IPN to penicillin N (PenN) occurs in peroxisomes (Teijeira et al., 2008). Disruption of *cefM* that encodes a protein of the major facilitator superfamily (MFS) resulted in accumulation of large quantities of PenN; in addition, the *cefM* disruptant strain was unable to make cephalosporin. These data suggest that *cefM* is involved in the translocation of the cephalosporin intermediate PenN from the peroxisome to the cytosol, where cephalosporin biosynthesis is completed.

4.2.3. Cyclosporin

Cyclosporin, an undecapeptide with immunosuppressive properties, is synthesized in *Tolypocladium inflatum* by a non-ribosomal peptide synthetase (NRPS), cyclosporine synthetase. This enzyme consists of a large, multi-domain, single polypeptide chain protein with the size of 1.6 MDa that possesses 11 modules (Weber et al., 1994). A module is a portion of the NRPS peptide that selects one amino acid, activates it to an amino acyl adenylate in the presence of ATP, and then forms a peptide bond followed by cyclization. Cyclosporin is composed of residues of L-valine, L-leucine, L-alanine, glycine, 2-aminobutyric acid, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-L-threonine, and D-alanine. Localization studies using sucrose density gradient centrifugation and electron microscopy in *T. inflatum* demonstrated that cyclosporine synthetase and D-alanine racemase that catalyze racemization of L-alanine to D-alanine, are loosely associated with the outer surface of the vacuolar membrane; the cytosolic amino acids are apparently used as substrates. Cyclosporine accumulates in the vacuole lumen (Hoppert et al., 2001). Consistent with data presented by Hoppert, carbon flow in cyclosporin synthesis is directed from the cytosol towards the vacuolar lumen. In contrast, carbon flow during penicillin biosynthesis is directed from vacuoles to cytosol to peroxisomes prior to penicillin excretion to the cell exterior.

Sub-cellular compartmentalization associated with biosynthesis of secondary metabolites generates several logistical problems. For example, to accomplish the early steps in biosynthesis, the substrate must be transported to the site that houses the early pathway enzymes or the enzymes must be moved to the locations of substrate accumulation. Then, either pathway intermediates must be transported to later pathway enzymes (such as for penicillin biosynthesis) or the enzymes must be transported to a common location allowing the formation of multienzyme complexes which promote efficient synthesis and provide advantages associated with the metabolic channeling of the intermediates over very short distances. For many fungal secondary metabolites, the end product is exported to the cell exterior which means that the site of synthesis must come in contact with the cytoplasmic membrane. Compartmentalization and export promote sequestering of potentially toxic metabolites and provide a means for cells to affect competitors in their environment without harming themselves. To solve these logistical problems, cells evolved unique and interesting schemes to accomplish intra-cellular transport of the biosynthetic enzymes as well as pathway intermediates and endproducts; we call this transportation process translocation.

How do enzymes, substrates, intermediates, and endproducts move to the appropriate sub-cellular location at the correct time and in the correct quantity? The available data suggest that the cellular machinery for protein traffic may play an important role in this translocation process. Below we discuss relevant cellular mechanisms of protein traffic. Then, using aflatoxin biosynthesis as an example we provide a model that shows how a cell can modify and use existing protein traffic and sorting machinery to translocate required materials for synthesis and accomplish the storage and export of secondary metabolites.

5. Protein traffic, fungal vesicles and vacuole biogenesis

Protein trafficking machinery is responsible for moving proteins within the cell from the site of their biosynthesis to their final destination. Our current understanding of protein traffic helps to correlate the distribution of secondary metabolic pathway enzymes inside the cell with intra-cellular protein dynamics.

The intra-cellular endomembrane system (EMS) consists of the endoplasmic reticulum (ER), the Golgi complex, vacuoles, endosomes, peroxisomes, autophagosomes, and vesicles. We focus our attention on vesicles and vacuoles because recent data suggest that they contribute to aflatoxisome development and the synthesis and accumulation of aflatoxin.

The vacuole, a single-membrane bound organelle, is an essential multi-functional component of the EMS (Baba et al., 1997; Kim and Klionsky, 2000; Klionsky et al., 1990; Klionsky, 1998; Kucharczyk and Rytka, 2001; Teter and Klionsky, 2000). Vacuoles are associated with protein turnover, maintenance of intra-cellular pH, and the storage of a variety of primary metabolites including amino acids and cofactors (S-adenosyl-methionine, SAM). Vesicles are double membrane bound organelles that are formed via "budding" of the ER, Golgi complex, cytoplasmic membrane, and from other sub-cellular organelles such as peroxisomes, mitochondria, and nucleus. These vesicles maintain the flow of protein and other cargo in the cell; they also carry protein cargo to the vacuole. Endosomes are generated by fusion of specialized vesicles that form during endocytosis by invagination of the cytoplasmic membrane. Vesicles and endosomes fuse and generate small vacuoles and eventually large vacuoles.

Fungi utilize several distinct pathways to target proteins to the vacuole; our current understanding of these processes is based on studies performed with yeast (Baba et al., 1997; Kim and Klionsky, 2000; Klionsky et al., 1990; Klionsky, 1998; Teter and Klionsky, 2000). The two most relevant pathways to this discussion are the secretory pathway and the cytoplasm to vacuole transport (CVT) pathway (Klionsky, 1998); these pathways as described below, participate in trafficking of the aflatoxin enzymes. Carboxypeptidase Y (CpY), the most thoroughly studied vacuolar hydrolase, is targeted to the vacuole through the secretory pathway. CpY is synthesized as an inactive pro-enzyme on ribosomes in the cytoplasm. An N-terminal signal peptide targets the protein to the endoplasmic reticulum (ER) where it undergoes post-translational modification. A cargo vesicle carries CpY p1 to the Golgi complex where it is fully glycosylated generating a 69 kDa p2 protein. Another cargo vesicle buds from the Golgi and carries CpY p2 to the vacuole, where the 61 kDa active enzyme is generated. The aflatoxin enzyme, VBS, is thought to be targeted to the vacuole through the secretory pathway (see below) (Chiou et al., 2004; Silva et al., 1996; Silva and Townsend, 1997).

Another trafficking mechanism for delivery of cytoplasmic cargo (including proteins) to the vacuole is the CVT pathway which is similar to the autophagy pathway. These two pathways operate under different physiological conditions, display certain morphological similarities and possess overlapping functions (Huang and Klionsky, 2002). The CVT pathway is constitutively active; an essential characteristic of autophagy is induction by nutrient starvation. One example of a protein delivered to vacuole via the CVT pathway is aminopeptidase 1 (Ape1). Ape1 is synthesized on cytoplasmic ribosomes as a 61 kDa precursor that quickly oligomerizes

into homododecameric units; these are then packaged in double membrane-bound CVT vesicles via a mechanism that is similar to the autophagy pathway (Oda et al., 1996; Teter and Klionsky, 2000). The cargo protein is carried to the vacuole and becomes incorporated via fusion of the vesicle with the developing vacuole. At least three aflatoxin enzymes, Nor-1, Ver-1, and OmtA, are believed to be transported via the CVT pathway (see below).

Protein traffic between organelles occurs with the involvement of trafficking vesicles. These form by membrane budding of the precursor organelle and then the resulting vesicles fuse with the target organelle. In the yeast *S. cerevisiae*, the homotypic fusion (fusion of like vesicles) of transport vesicles to the vacuole proceeds through an orderly series of events that includes priming, tethering (docking) and fusion (Whyte and Munro, 2002; Wichmann et al., 1992). A Class C Vps complex (consists of Vps11, Vps16, Vps18, and Vps33) in association with Class B Vps proteins (vacuole-linked Vam2p and Vam6p) and SNARE proteins promote tethering of vesicles and vacuole and these events eventually lead to membrane fusion (Ostrowicz et al., 2008; Whyte and Munro, 2002). Tethering also requires the involvement of a small Rab-like GTPase called Ypt7; *ypt7* mutants exhibit a fragmented vacuole phenotype due to inability to carry out vesicle fusion. Vacuole biogenesis in filamentous fungi has been recently observed in *A. nidulans*; disruption of *avaA*, a homolog of *ypt7*, blocks tethering and generates a "fragmented vacuole" morphology characterized by an increased number of vesicles (Ohsumi et al., 2002).

Two approaches were utilized to demonstrate a role for vesicle/vacuole tethering and fusion in aflatoxin biosynthesis (pathway described below in more detail). As *A. parasiticus* undergoes a transition from primary to secondary metabolism, there is a 5–8-fold increase in the number of vesicles (a shift from a low vesicle number [LVN] phenotype to a high vesicle number [HVN] phenotype); under aflatoxin non-inducing conditions the fungus demonstrated a LVN phenotype (Chanda et al., 2009). In the first approach, disruption of an *avaA* homolog in *A. parasiticus* generated a HVN phenotype, even on aflatoxin non-inducing growth medium (YEP). In the second approach, *A. parasiticus* was treated with protein sorting inhibitor 3 (Sortin3) that also generated an HVN phenotype on YEP. Sortin3 interferes with the function of Vps16, although the mechanism of this interference is not clear (Zouhar et al., 2004). Importantly, under aflatoxin-inducing conditions (YES), both treatments increased aflatoxin accumulation and aflatoxin export to the cell exterior. In addition, both treatments resulted in an increase in aflatoxin enzyme accumulation but did not affect aflatoxin gene expression at the transcript level. These data strongly implicated vesicles as the primary site for aflatoxin biosynthesis, aflatoxin compartmentalization and export. These vesicles were designated aflatoxisomes.

What are the molecular mechanisms that underlie a shift to the HVN phenotype and formation of aflatoxisomes under aflatoxin-inducing conditions? An observed decrease in expression of *vps16* and *avaA* coincided with the LVN to HVN shift and the peak in aflatoxin biosynthesis suggesting that downregulation of tethering activity may be one driving force that diverts aflatoxisomes from fusing with the vacuole and results in the HVN phenotype (Chanda et al., 2009). The mechanism which targets aflatoxisomes to the cell membrane for aflatoxin export is unclear.

6. Aflatoxin biosynthesis and aflatoxisomes; an example of how cells integrate secondary metabolism into the established cellular machinery

Aflatoxins are synthesized as a small family of related compounds including aflatoxins B_1, B_2, G_1, and G_2, by several aflatoxigenic fungal species in the genus *Aspergillus* including *A. parasiticus*, *A. flavus*, *A. nomius*, and *A. nidulans* (carries a similar gene cluster and synthesizes sterigmatocystin, a late pathway intermediate in aflatoxin biosynthesis). Aflatoxin B_1 (AFB_1) is the most abundant, mutagenic, and carcinogenic aflatoxin synthesized in culture and on plants (Stora and Dvorackova, 1987). Since it was discovered that aflatoxin was the causative agent of "Turkey X disease" in 1960 (Lancaster et al., 1961), the biosynthetic pathway for this mycotoxin has been intensively studied; as a result, it is currently one of the most highly characterized secondary metabolic pathways and serves as an important model system to study secondary metabolism in eukaryotes and to develop safe and effective strategies to eliminate aflatoxin from food and feed.

At least 17 enzyme activities catalyze aflatoxin biosynthesis (Keller et al., 2005; Miller and Linz, 2006); 25 or more genes clustered in a 75-Kb region on one chromosome encode these enzymes. The effect of light on aflatoxin accumulation is dependent on medium composition, initial pH, and temperature (Bennett et al., 1978; Joffe and Lisker, 1969). As a general trend, *Aspergillus* spp. produce aflatoxin in larger quantities in the dark as compared to the light; however, the effect varies in regard to the absolute quantities of aflatoxin produced and the level of the inhibition. Biosynthesis of aflatoxins appears to fulfill multiple biochemical and biological functions for the producer including removal of acetate, protection of the genome from UV damage (Ehrlich, 2006), quenching oxidative stress (Huang et al., 2009; Kim et al., 2008; Narasaiah et al., 2006), protection from insects (Bhatnagar et al., 2003; Rohlfs et al., 2007), and regulation of conidiation, and sclerotia development (Calvo et al., 2004; Cary et al., 2002; Mahanti et al., 1996; Trail et al., 1995).

During the past 25 years, intensive study has generated a significant body of information on aflatoxin biosynthesis including a clearer understanding of the genes involved in the biosynthetic pathway, of the signal transduction pathways that control aflatoxin biosynthesis, and of the regulation of aflatoxin biosynthesis at the level of the individual gene and the entire aflatoxin gene cluster. These processes have been reviewed recently (Bhatnagar et al., 2003; Calvo et al., 2004; Cary et al., 2002; Ehrlich, 2006; Huang et al., 2009; Kim et al., 2008; Mahanti et al., 1996; Narasaiah et al., 2006; Trail et al., 1995).

Recently, we demonstrated that specialized multifunctional vesicles, called aflatoxisomes (see above), carry out aflatoxin biosynthesis, sequester aflatoxin, and also export aflatoxin to the cell exterior (Chanda et al., 2009). In order to carry out aflatoxin biosynthesis, functional aflatoxin enzymes must either enter these organelles or the proteins must be present on their surface. Here we present a summary of available evidence that argue that aflatoxisomes originate through fusion of vesicles derived from at least two of the trafficking pathways described above, CVT and the secretory pathways. (1) Western blot analysis and feeding studies conducted on a purified vesicle/vacuole (V) fraction demonstrate that aflatoxisomes carry at least three aflatoxin enzymes OmtA, Ver-1 and Vbs (Chanda et al., 2009); new data derived from proteomic profile analysis of this same V fraction suggest that at least 13 different aflatoxin enzymes (Fas1, Fas2, PksA, Nor-1, NorA, Ver-1, Vbs, AvnA, OrdA, OmtA, OmtB, EstA, AflT) including most of the early aflatoxin enzymes are present in these organelles (Chanda et al., 2010b). (2) Data strongly suggest that Vbs, a middle aflatoxin pathway enzyme, is transported within the fungal cell via the classical secretory pathway; it is the only aflatoxin enzyme which is glycosylated (Chiou et al., 2004; Silva et al., 1996; Silva and Townsend, 1997); Vbs is localized in discrete spots surrounding the nucleus and associates with the ER which indicates that the enzyme passes through the Golgi (Chiou et al., 2004). N-glycosylation of Vbs supports passage of this protein through the ER-Golgi via the classical secretory pathway (Chiou et al., 2004). (3) In contrast to Vbs, three other aflatoxin

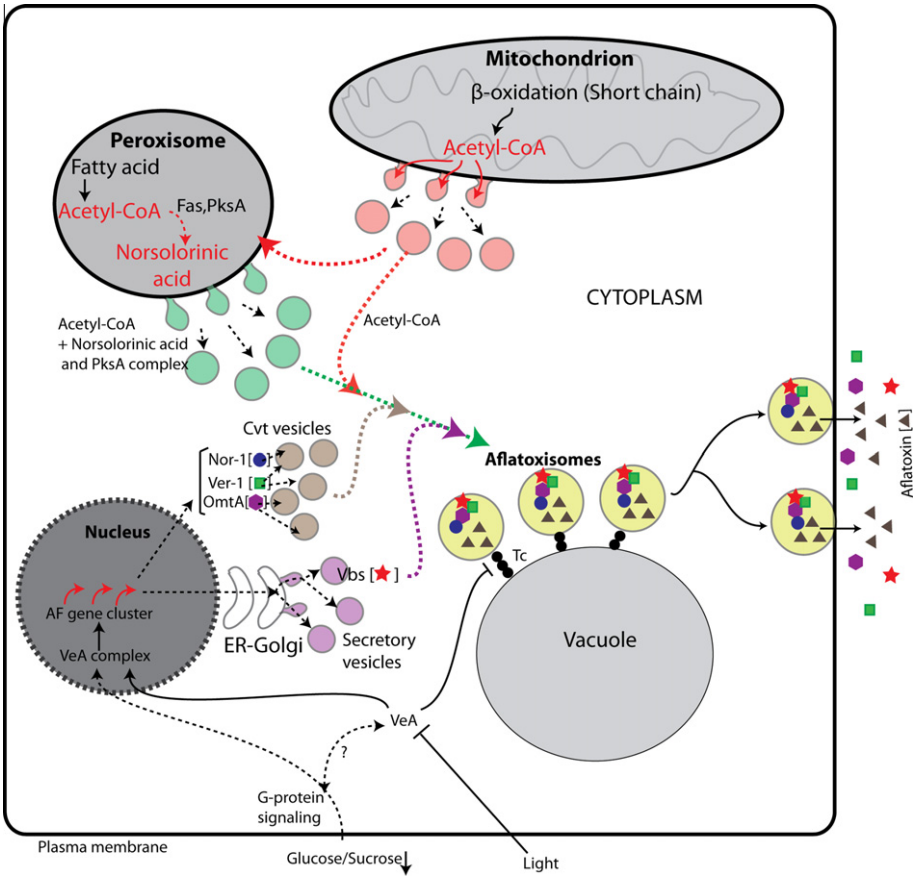

Fig. 1. A model for compartmentalization, translocation, and aflatoxisome biogenesis during biosynthesis of aflatoxin in *Aspergillus* spp. The first steps in aflatoxin biosynthesis are proposed to take place in peroxisomes, where the substrate acetyl-CoA accumulates; the early enzymes (Fas, PksA) are translocated to this site by unknown mechanisms. Vesicles that originate by budding from peroxisomes (containing norsolorinic acid, Fas, PksA) and mitochondria (containing mitochondria-derived acetyl-CoA) fuse with CVT trafficking vesicles (containing middle and late aflatoxin enzymes), and eventually with secretory vesicles (containing Vbs) to develop aflatoxisomes. VeA, via an unknown mechanism, directs aflatoxisomes to export aflatoxin and aflatoxin enzymes to the cell exterior by preventing fusion of vesicles (aflatoxisomes) with the vacuole. Aflatoxin export occurs by a mechanism similar to exocytosis. This model expands on the "two-branch model" presented in Chanda et al. (2009). Known routes are depicted by bold lines; hypothetical routes are depicted by dashed lines.

enzymes studied to date, Nor-1, Ver-1, and OmtA, appear to be transported by CVT vesicles within the cell. Evidence to support this idea is derived from the cytoplasmic location of synthesis, the lack of glycosylation (which normally occurs as part of the classical secretory pathway), and the observation that small peptides are cleaved from one or both ends of each of these proteins during the maturation process consistent with entry into a CVT vesicle (Baba et al., 1997; Hong and Linz, 2008). Based on these observations, it is reasonable to suggest that vesicles derived from the classical secretory and CVT pathways must to fuse in order to form aflatoxisomes to bring components of the biosynthetic machinery together. However, the occurrence of this fusion process must be verified. Aflatoxisomes appear to fulfill multiple tasks in biosynthesis of aflatoxin. First, they contain active aflatoxin enzymes and provide an environment conducive for carrying out at least the last two biosynthetic steps (Chanda et al., 2009). Second, aflatoxisomes sequester pathway intermediates, the endproduct, aflatoxin, and cofactors (S-adenosylmethionine, NADH). And finally, aflatoxisomes participate in export of the toxin to the cell exterior.

An important question remains. How are aflatoxisomes brought together with peroxisomes, the proposed site of initiation for aflatoxin biosynthesis? Below we present a model which proposes that vesicles bud from peroxisomes and mitochondria and contribute to these processes (Fig. 1). Mitochondria-derived vesicles that deliver cargo to peroxisomes have been identified (Neuspiel et al., 2008). Subsequently, mature aflatoxisomes may develop by fusion of trafficking vesicles of different origins. However, the detailed mechanisms of this trafficking cross-talk remain to be investigated.

7. Temporal and spatial organization in secondary metabolism; VeA is a master coordinator of aflatoxisome biogenesis

In order to understand how secondary metabolism is orchestrated in the cell, we must know how the enzymes, substrates/intermediates and cofactors are delivered at the appropriate time, in the appropriate quantities, and to the appropriate sub-cellular compartments to trigger efficient operation of the pathway (temporal and spatial regulation). This process also requires the balanced flow of materials. Aflatoxin biosynthesis provides a useful example of the ability of the cell to coordinate temporal and spatial regulation. Based on data derived from recent studies on aflatoxin biosynthesis, a "two-branch model" was introduced to illustrate our current understanding of aflatoxin synthesis (Chanda et al., 2009). The model proposes that two branches are necessary to carry out aflatoxin synthesis. Branch one is the most thoroughly understood and controls synthesis of the aflatoxin biosynthetic enzymes. A heterotrimeric G-protein/cAMP/PKA signal transduction pathway (Hicks et al., 1997; Roze et al., 2004a; Shimizu and Keller, 2001; Shimizu et al., 2003), a phosphatidyl inositol (PI)-3 kinase signal transduction pathway (Lee et al., 2007), chromatin remodeling (Brosch et al., 2008; Lee et al., 2009; Reyes-Dominguez et al., 2010; Roze et al., 2007; Shwab et al., 2007), and specialized

transcription factors including AflR, AflJ, and CRE1 bp (Chang, 2003, 2004; Payne et al., 1993; Roze et al., 2004b), are important players in activation of genes that control aflatoxin enzyme biosynthesis and activity (Fig. 1).

Branch two controls vesicle fusion, formation of aflatoxisomes, and the proper localization of aflatoxin enzymes, intermediates, and cofactors to aflatoxisomes. Others previously demonstrated that VeA, a global regulator of *Aspergillus* development and secondary metabolism, plays an important role in regulation of branch 1 (Calvo, 2008). We recently demonstrated that VeA, also, at least in part, regulates branch two (Chanda et al., 2009). Disruption of *veA* in *A. parasiticus* blocked accumulation of aflatoxin (as observed previously (Calvo et al., 2004) but also blocked the down regulation of expression of genes (*avaA* and *vps16*) encoding two key components of the tethering complex and strongly inhibited the LVN to HVN shift, even under aflatoxin-inducing conditions (Chanda et al., 2009). These data strongly suggest the VeA is a key player that coordinates aflatoxin gene expression with aflatoxisome development. A current revised model that includes both branches and aflatoxisome biogenesis is presented in Fig. 1.

It was previously established that aVeA protein complex mediates the light-dependent regulation of sexual reproduction and secondary metabolism (Bayram et al., 2008); the discovery and functional analysis of aflatoxisomes helped to uncover new responsibilities for VeA in secondary metabolism. Based on these studies, it appears likely that a VeA protein complex controls a functional connection between carbon flow through primary and secondary metabolism (chemical development), organelle development (aflatoxisomes), as well as development of reproductive structures. One clue about the mechanism by which VeA carries out these functions derives from the observation that disruption of *veA* causes significant changes in the volatile profile observed in cells of the mutant; these changes indicate disturbances in carbon flow through several metabolic pathways (e.g. ethanol biosynthesis and catabolism of branched chain amino acids) (Roze et al., 2010). This study also demonstrated a strong association between the accumulation of biologically active volatile compounds in Δ*veA* and inhibition of asexual conidiation and sclerotia formation. Therefore, VeA appears to be a "master coordinator" of the temporal and spatial regulation of chemical and morphological development (Fig. 2).

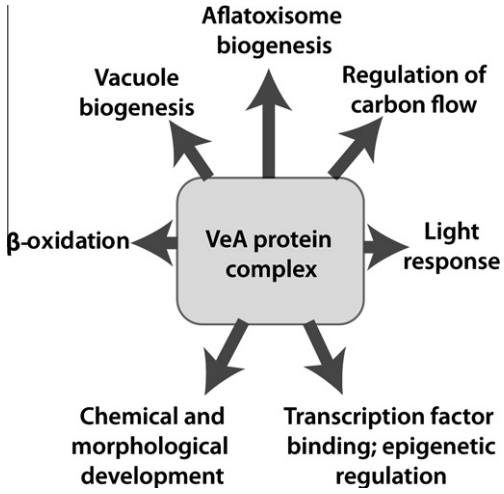

Fig. 2. Multiple roles of VeA. The schematic summarizes known functions of VeA, a master-coordinator of many processes in *Aspergillus* spp.; VeA is thought to exert its functions through protein–protein interaction. A VeA protein complex was previously shown to mediate light-dependent regulation of secondary metabolism with asexual/sexual development and sclerotia formation (Bayram et al., 2008; Calvo, 2008). VeA was implicated in control of gene expression via chromatin remodeling and/or effects on transcription factor function (including AflR, CRE1 bp, and AflJ). Recent work uncovered a functional connection between carbon flow through primary (ethanol biosynthesis and catabolism of branched chain amino acids) and secondary metabolism (chemical development), organelle (aflatoxisomes and vacuole) development through expression of tethering complex components *avaA* and *vps16*, as well as development of reproductive structures (asexual conidiation and sclerotia development) (Chanda et al., 2009; Roze et al., 2010).

8. Conclusions and implications for future research

Significant breakthroughs in our understanding of secondary metabolism at the sub-cellular level have been achieved in recent years. These studies strongly suggest that *Aspergillus* spp., filamentous fungi with relatively small genomes, utilize conserved cellular protein trafficking machinery to conduct new cellular functions that include the biosynthesis and export of aflatoxin (Chanda et al., 2010a).

Watanabe and Townsend (2002) purified a multienzyme complex called norsolorinic acid synthase (NorS) that was composed of PksA, Fas1, and Fas2, the first enzymes in aflatoxin biosynthesis. The complex synthesizes norsolorinic acid in the presence of acetyl-CoA, malonyl-CoA, and NADPH; NorS had a mass of 1.4×10^6 Da that suggested a $\alpha 2\beta 2\gamma 2$ complex composition. Recent co-immunoprecipitation studies conducted in our laboratory (unpublished) indicate that at least Ver-1, OmtA, Nor-1, and Vbs form multienzyme complexes within the cell. Proteome profile analysis of the vesicle/vacuole (V) fraction also suggests that other aflatoxin enzymes could participate in an even larger complex in aflatoxisomes, and imply that metabolic channeling promotes efficient catalysis of aflatoxin biosynthesis; this process needs to be characterized in more detail.

Interestingly, proteome profile analysis of the (V) fraction suggests that at least one additional polyketide synthase (FluP) localizes to the aflatoxisomes. FluP exhibits strong identity to 6-methyl salicylic acid synthase in *Penicillium patulum* which catalyzes patulin production (another polyketide mycotoxin); these data point out that synthesis of other secondary metabolites may occur in aflatoxisomes. Of particular interest, disruption of *fluP* completely eliminated conidia development in *A. parasiticus* supporting a possible link between synthesis of specific polyketides and fungal development (Zhou et al., 2000). To date, we have not demonstrated the presence of patulin in (V) fraction, although it is clear that several species of *Aspergillus* are capable of patulin synthesis; more work remains to address this issue.

Several enzymes with functional connection to secondary metabolism in eukaryotes and some bacteria were also detected as part of the proteome profile of (V) fraction. For example, phosphomevalonate kinase (mevalonate/isoprene pathway), pentafunctional AROM protein (shikimate pathway), myo-inositol-1-phosphate synthase (streptidine biosynthetic pathway), and acetyl-CoA carboxylase (Chanda et al., 2010b) were identified consistent with multiple functions of these organelles. We are currently conducting preliminary LC/MS/MS analysis of (V) fraction to characterize their metabolome and fatty acid composition to help determine the sub-cellular origin of the vesicles and to identify possible end products of secondary metabolite pathways.

The model for temporal and spatial regulation of aflatoxin synthesis presented above identifies several "knowledge gaps" and generates questions that still must be answered. Are there multiple classes of aflatoxisomes, each with a unique function, or is each aflatoxisome capable of synthesizing a wide array of secondary metabolites? By what specific mechanism does VeA influence temporal and spatial regulation of secondary metabolism and development? Much remains to be elucidated at the molecular level.

The story of aflatoxisome biogenesis raises an interesting and important question relative to the translocation of Vbs to vesicles.

Why is this particular enzyme transported to vesicles via the classical secretory pathway while most if not all of the other enzymes are transported either from peroxisomes (early enzymes) or from the cytoplasm via the CVT pathway (middle and late enzymes)? We know that Vbs catalyzes the formation of versicolorin B, the last non-toxic intermediate in aflatoxin biosynthesis. This reaction is a key step in the subsequent creation of versicolorin A (via a desaturase) and eventually aflatoxin B_1 (via several additional enzyme activities). We also know that the pathway intermediates after versicolorin B and the end product aflatoxin B_1 are extremely toxic (Silva et al., 1996). Therefore, we hypothesize that the delivery of this key enzyme to aflatoxisomes through a different trafficking pathway signals the completion of aflatoxisome assembly and assures that the reactions that generate subsequent toxic intermediates are safely compartmentalized. Further study is required to address this hypothesis.

Recent experimental evidence suggests that aflatoxisomes export aflatoxin to the cell exterior by a "blast" mechanism reminiscent of exocytosis. The involvement of AflT, an MSF-like transporter, in export is currently unclear; however, the available data do not exclude a mixed pump/blast (exocytosis) mechanism. We hypothesize that initially as aflatoxisomes comes into a physical contact with the plasma membrane, AflT and/or other transporters residing in the plasma membrane participate in the pumping of aflatoxin through the membrane. Later, aflatoxisomes fuse with the cell membrane that protrudes outward through the cell wall into the surrounding environment and finally discharge (blast) the content outside the cell; the aflatoxisome membrane patches the rupture in the cell membrane. Apparently, the fungus releases large quantities of aflatoxin via this "blast" mechanism and is able to compensate for the absence of the "pump" since the deletion of *aflT* (a gene located in the aflatoxin gene cluster) has no effect on aflatoxin accumulation (Chang et al., 2004). Details of this intriguing export mechanism await further studies.

Another interesting question associated with this discussion is whether all fungal cells make aflatoxin or do specialized cells within the mycelium and colony carry out synthesis of aflatoxin and possibly other secondary metabolites? Using GFP fusions with the aflatoxin enzymes Nor-1 and Ver-1, Hong tracked intra-cellular localization of these proteins in real time. Accumulation of fluorescence was frequently observed in one cell but not at all in the adjacent cell (Hong and Linz, 2008, 2009). This observation strongly suggests that either the adjacent cell does not synthesize aflatoxin, or it synthesizes aflatoxin at earlier or later times during growth of a single hyphal fragment. This observation prompts us to hypothesize that *A. parasiticus* has evolved mechanisms to achieve temporal and spatial regulation of aflatoxin synthesis at the level of the single cell, the hyphal fragment, the mycelium and the colony. We propose to utilize laser capture microdissection (LCM) technology combined with real-time RT-PCR to analyze aflatoxin gene expression at the level of individual cells to address this hypothesis.

One basic question in the field of secondary metabolism regards the evolutionary significance of the acquisition of the ability to synthesize secondary metabolites (Firn and Jones, 2000, 2009; Vining, 1992). To begin to address this question, an interesting comparison can be made between yeast (which are not noted for synthesis of a wide variety of secondary metabolites) and their distant relatives, the filamentous fungi, which are particularly renowned for their abilities to synthesize a vast array of natural products. The reproductive mechanisms of filamentous fungi are significantly more complicated than those observed in yeast. A close relationship between secondary metabolism and fungal development is well established (Calvo et al., 2002). Based on sequence and metabolic analysis, the yeast *S. cerevisiae* does not carry genes that encode polyketide synthases (PKS) (that synthesize one diverse class of secondary metabolites), does not appear to produce other classes of secondary metabolites, and does not carry *veA* (Calvo, 2008; Kroken et al., 2003). Therefore, carbon from primary metabolism in yeast predominantly flows to ethanol biosynthesis as well as to alcohols, acids, and products derived from catabolism of branched chain amino acids (Hazelwood et al., 2008). Disruption of *veA* in *Aspergillus* spp. modulates carbon flow to a pattern reminiscent of yeast (increases biosynthesis of ethanol and products derived from catabolism of branched chain amino acids); the volatiles produced by Δ*veA* inhibit asexual sporulation and sclerotia production in *Aspergillus* spp. (Roze et al., 2010). We speculate based on these observations that acquisition of *veA*, which is found exclusively in the filamentous fungi, played a major role in the ability of this group of organisms to coordinate carbon flow between primary and secondary metabolism. Acquiring a single novel gene *veA* provided *Aspergillus* spp. with a regulatory framework for the spatial and temporal regulation of metabolic diversity which results from modulation of carbon flow and biosynthesis of secondary metabolites (chemical development); this framework also enhanced the ability of the cell to evolve reproduction mechanisms that are more complex than in yeast. Addressing this hypothesis provides a unique opportunity to study the evolution of secondary metabolic pathways.

In summary, the available data support the idea that aflatoxisomes are multifunctional organelles that carrying out the synthesis, storage and export of aflatoxin but may be involved in synthesis of several other secondary metabolites as well thus providing the fungal cell with ability to maximize the use of a limited genome.

Acknowledgments

Research in the Linz laboratory was supported by funds from NIH (RO1 52003-18), the Michigan State University (MSU) Agricultural Experimental Station, and MSU Internal Research Grant Program.

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