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Characterization of the *Neurospora crassa* DHN melanin biosynthetic pathway in developing ascospores and peridium cells

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ABSTRACT

Neurospora crassa contains all four enzymes for the synthesis of DHN (dihydroxynaphthalene), the substrate for melanin formation. We show that the DHN melanin pathway functions during *N. crassa* female development to generate melanized peridium and ascospore cell walls. *N. crassa* contains one polyketide synthase (PER-1), two polyketide hydrolases (PKH-1 and PKH-2), two THN (tetrahydroxynaphthalene) reductases (PKR-1 and PKR-2), and one scytalone dehydratase (SCY-1). We show that the PER-1, PKH-1, PKR-1 and SCY-1 are required for ascospoer melanization. We also identified the laccase that functions in the conversion of DHN into melanin via a free radical oxidative polymerization reaction, and have named the gene *lacm-1* (laccase for melanin formation-1). In maturing perithecia, we show that LACM-1 is localized to the peridium cell wall space while the DHN pathway enzymes are localized to intracellular vesicles. We present a model for melanin formation in which melanin is formed within the cell wall space and the cell wall structure is similar to "reinforced concrete" with the cell wall glucan, chitin, and glycoproteins encased within the melanin polymer. This arrangement provides for a very strong and resilient cell wall and protects the glucan/chitin/glycoprotein matrix from digestion from enzymes and damage from free radicals.

1. Introduction

A variety of fungi have been shown to produce melanin pigments, which are generally associated with the cell wall. Melanins, along with carotenoids, are responsible for the pigmentation of fungal hyphae and spores. A large body of literature demonstrates that the melanins confer protection against reactive oxygen species (ROS), UV irradiation, desiccation, elevated temperatures, freezing, and attack from cell wall hydrolytic enzymes (Eisenman and Casadevall, 2012; Langfelder et al., 2003; Nosanchuk et al., 2015; Pihet et al., 2009; Rehnstrom and Free, 1996). For both plant and animal pathogenic fungi, melanin has been shown to play an important role in the infection process and to be a virulence determinant. For the human pathogen *Aspergillus fumigatus*, melanin has been reported to play a role in the survival of fungal spores, and mutants lacking melanization are less virulent than normal spores (Langfelder et al., 1998; Pihet et al., 2009). Cell wall melanization has also been shown to play a vital role in plant pathogens. For example, melanin is vital to the functioning of the *Magnaporthe grisea* appressorium, a cell type that uses elevated turgor pressure to drive a penetration peg through the plant cell wall into the host cell during the infection process. Appressorium melanization is required for the cell to withstand the high turgor pressure (Chumley and Valent, 1990; Talbot, 2003). For *Monilia fructicola*, we showed that melanization of conidia is important for survival during a variety of different environmental stresses and protects the conidia from cell wall digestive enzymes (Rehnstrom and Free, 1996).

There are two well-characterized pathways for melanin biosynthesis, the DOPA pathway and the DHN pathway (Eisenman and Casadevall, 2012; Langfelder et al., 2003; Nosanchuk et al., 2015; Pal et al., 2014; Pombeiro-Sponchiado et al., 2017). The DOPA pathway is present in both animals and fungi and uses the key enzyme tyrosinase to convert tyrosine to dihydroxyphenylalanine (DOPA), which is then converted to a melanin polymer. In the animal pathway, the DOPA melanin is formed within a small membrane-bound intracellular organelle called the melanosome. In *Crytococcus neoformans*, DOPA melanin is produced in intracellular vesicles and released to form a layer of melanin next to the plasma membrane (Eisenman et al., 2005).

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A variety of fungi synthesize 1,8-dihydroxynaphthalene (DHN) melanin as a component of their cell walls. Fungal cell wall-associated DHN-derived melanin is generally localized to the outer portion of the fungal cell wall, and represents a very different situation than the intracellular DOPA melanin seen in animals. The DHN melanin pathway is found in many, but not all, ascomycetes, but not in animal cells. The DHN pathway begins with the formation of a polyketide, which is subsequently converted to DHN. The DHN is acted on by a laccase to generate a DHN free radical, and high molecular weight melanin is formed by oxidative polymerization. The enzymes needed to make DHN lack signal peptides and would therefore be predicted to be localized in the cytosol. However, it has been recently shown that the DHN pathway enzymes are associated with endosomal vesicles in *A. fumigatus* (Upadhyay et al., 2016a, 2016d). Interestingly, the *Neurospora crassa* 2206 amino acid polyketide synthase PER-1 has a palmitoylation site at cysteine 592, and could therefore be associated with a membrane. It's likely that all of the enzymes in the pathway form a vesicle-associated enzyme complex and transfer DHN into an intracellular vesicle. The laccase, which functions as the last step in melanin biosynthesis, has a signal peptide and would be predicted to travel as a luminal protein through the canonical vesicular secretory pathway.

Enzymes involved in the DHN melanin biosynthetic pathway have been identified and characterized in several fungi (Eisenman and Casadevall, 2012; Engh et al., 2007; Langfelder et al., 2003; Pombeiro-Sponchiado et al., 2017; Schumacher, 2016). The first step in the pathway is catalyzed by a polyketide synthase, a large multi-domain enzyme that uses acetyl-CoA and malonyl-CoA to generate a large polyketide (Langfelder et al., 1998; Tsai et al., 2001). The polyketide is then cleaved by a hydrolase to produce 1,3,6,8-tetrahydroxynaphthalene (THN) (Tsai et al., 2001). A reductase and a dehydratase then convert the THN to DHN. A laccase then functions to generate a DHN free radical, which participates in a reaction to cross-link the DHN molecules together to generate melanin (Sugareva et al., 2006). In this reaction, the free radical is regenerated during the cross-linking reaction and multiple DHN molecules are cross-linked together to create DHN melanin. The genes encoding the various enzymes involved in the pathway are well-conserved in the ascomycetes.

During *N. crassa* sexual development, the developing ascospores become highly melanized. This protective melanization has been shown to be important for the survival of the ascospores, which can survive in the soil for decades before germinating (Davis and DeSerres, 1970). The outer layer of the cells in the perithecium (female mating structure) is also melanized. This layer of cells is called the peridium, and functions to surround and protect the developing ascospores. We report that the seven step DHN melanization pathway is used in the developing ascospores and peridium cells. *N. crassa* contains a single polyketide synthase gene called *per-1* (NCU03584), which is required for DHN melanin formation. We showed that the *N. crassa* genome encodes 2 polyketide hydrolases, PKH-1 (NCU01903) and PKH-2 (NCU05821). The genome also contains two THN reductases, PKR-1 (NCU09390) and PKR-2 (NCU06905). Melanization of the ascospores is dependent upon PKR-1 while both THN reductases participate in peridium melanization. The genome encodes a single scylatone dehydratase, SCY-1 (NCU07823). Although the enzymes involved in DHN production would be predicted to be located in the cytosol, the DHN pathway enzymes have been shown to be associated with intracellular endosomal vesicles in *A. fumigatus* (Upadhyay et al., 2016d). Our results showed that the *N. crassa* DHN biosynthetic enzymes are associated with intracellular vesicles. We demonstrated that a particular laccase, LACM-1 (NCU02201) is largely responsible for catalyzing the final step in *N. crassa* melanin formation, the oxygen free radical mediated polymerization of DHN into melanin. LACM-1 has an N-terminal signal peptide. We show that the LACM-1 is located in vesicles during early perithecium development but secreted into the cell wall space during perithecium maturation. We present a model for cell wall melanization in which DHN and the LACM-1 laccase are independently delivered to the cell wall space from different intracellular vesicle populations, and melanin polymerization occurs within the cell wall space. In this model, the cell wall chitin, glucans, and glycoproteins become encased within the melanin polymer to provide for the extremely strong cell wall structure characteristic of melanized fungal cell walls.

2. Materials and methods

2.1. Strains, culture conditions, and phenotype characterization

The strains and deletion mutants used in the study were obtained from the Fungal Genetics Stock Center. The gene deletion mutants were generated as part of the Neurospora genome project (Colot et al., 2006). In these deletion mutants, the deleted genes are replaced by a hygromycin resistance gene. The replacement of the deleted genes with the hygromycin resistance cassette was verified via Southern blot analysis. The stocks were routinely maintained on Vogel's sucrose medium slants (Davis and DeSerres, 1970). The NCU02201 mutant, which was found to be a heterokaryon, was rendered homokaryotic by streaking conidia on sorbose medium (Davis and DeSerres, 1970). Mating experiments were carried out on two sterile 8 cm in diameter discs of Whatman 3 MM chromatography paper placed in a Petri dish with 5 ml of sterile synthetic crossing medium (Davis and DeSerres, 1970). The paper serves as a carbon and energy source for the fungus. The strains were allowed to grow in the dark at 22 °C for three days. During this time the fungus begins to produce protoperithecia, the *N. crassa* female mating structure. The developing protoperithecia were fertilized by the addition of a suspension of conidia of the opposite mating type in sterile water. Following fertilization, the perithecia rapidly increased in size and melanin was formed on their outer surfaces. Sterile water was added as needed to maintain the filters in a moist state. The production of melanin was followed visually. Approximately fourteen days post-fertilization, the perithecia eject mature ascospores. The formation of melanin in the ascospores was routinely followed by observing the relative numbers of black and “white” ascospores that were shot onto the lid of the Petri dish fourteen days after fertilization. The ascospores were collected in sterile water and pictures of the ascospores were taken with a Canon Powershot A620 camera fitted with a microscope adaptor.

2.2. Cloning and transformation experiments

The primers in Table 1 were used to PCR amplify and clone the DHN pathway genes into the pMF272 vector to generate GFP-tagged versions of the proteins and into pMF334 vector to generate RFP-tagged proteins (Bowman et al., 2009). The primers for insertion into the pMF272 vector contain sequences to allow for the in-frame insertion of the genes upstream of the GFP coding sequences in pMF272. The In-Fusion HD cloning kit (Clontech Laboratories Inc, San Francisco, CA) was used to insert the sequences into pMF272 that had been cut with the NotI and SmaI restriction enzymes. The primers for insertion into the pMF334 vector contain sequences to allow for the in-frame insertion of the genes upstream of the RFP coding sequences in pMF334 that has been cut with ApaI and EcoRI using the In-Fusion HD cloning kit. The pMF272 and pMF334 vectors contain a portion of the *N. crassa his-3* gene and a region from the neighboring *his-3* gene 3′

Table 1
Primers used for the cloning of GFP-tagged and RFP-tagged versions of the *N. crassa* melanin biosynthetic pathway.

For *pkh-1* NCU01903)	
1903CFr	AAAAGCTGGGTACCGTCGAGCATCCGTCGATCGGAC
1903CRr	CACCATGTTTTGGAATTGCTCTCTGATGGAAGCCATGAC
For *pkh-2*(NCU05821)	
5821CFr	AAAAGCTGGGTACCGTCCAAGTCGCCTTCCTCAGCC
5821CRr	CACCATGTTTTGGAATTGCTTCGTCTTACCCCCCAACAA
For *pkr-1*(NCU09390)	
9390CFg	ACCGCGGTGGCGGCCCTCTGCCGTTGTTGCTTCTGC
9390CRg	CATGTTAATTAACCCCATGCAGGCGCCGCCATCAAC
For *pkr-2* NCU06905)	
6905CFr	AAAAGCTGGGTACCGCAGCTGGAAGAGGACAGAAGC
6905CRr	CACCATGTTTTGGAATTGCTGAGAAGAACCACCAGAGAT
For *scy-1* NCU07823)	
7823CFr	AAAAGCTGGGTACCGTGCGAGTTCACGATCCCATGC
7823CRr	CACCATGTTTTGGAATTGGTTGACTTCGCCCAGTTCCTC
7823CFg	ACCGCGGTGGCGGCCTGCGAGTTCACGATCCCATGC
7823CRg	CATGTTAATTAACCCGTTGACTTCGCCCAGTTCCTC
For *lacm-1*(NCU02201)	
2201CFr	AAAAGCTGGGTACCGAAGGTTGCCAAAGGTCTCGTC
2201CRr	CACCATGTTTTGGAATTGAATCCCCGAATCCTCCTGCGG

The Primers used for PCR amplification of the melanin biosynthetic genes with their upstream regulatory elements and inserting the genes in pMF334 and pMF272 for fluorescent tagging of the proteins are given in Table 1. Primers labeled CFr and CRr are for insertion into pMF334 (RFP) and primers labeled CFg and CRg are for insertion into pMF272 (GFP).

untranslated region (Bowman et al., 2009). The vectors were designed for the transformation of *his-3* mutant strains. When the vectors are inserted by homologous recombination, the *his-3* mutant is transformed from a histidine auxotroph into a prototroph, which facilitates the isolation of transformants with the targeted GFP-tagged or RFP-tagged gene inserted into the *his-3* 3′ untranslated region. All of the plasmids used for the expression of RFP-tagged and GFP-tagged versions of the proteins involved in melanin synthesis were sequenced to make sure that the *N. crassa* sequences were free of mutations.

For complementation experiments, we isolated *his-3*, *rid-1*, DHN pathway gene deletion mutants from mating experiments between a *his-3*, *rid-1* isolate and the deletion gene mutants. These mutant isolates were then transformed with an RFP-tagged or GFP-tagged version of the deleted DHN pathway gene. Transformants were isolated and mated with a *his-3*, *rid-1* deletion mutant isolate to obtain single ascospore *his3*$^{+}$ progeny containing the tagged version of the pathway gene. The ability of these transformants to produce melanized peridia and ascospores was assessed visually. The *rid-1* mutation was included in these experiments to protect the transforming gene from the RIP phenomenon. RIP is a phenomenon in which a DNA sequence found in more than one copy in the haploid genome of a developing *N. crassa* ascospore is subjected to multiple mutational events (Selker, 1999). Since the promoter regions from the transforming genes are found in two copies in the transformants, these sequences would be subject to RIP mutations, which could affect their expression in developing ascospores.

2.3. Expression of the RFP-tagged proteins and GFP-tagged proteins

The expression of RFP-tagged and GFP-tagged proteins in developing ascospores and peridium tissue was observed with a Model EC LUMAM-RPO Working Fluorescence Microscope (Bioscience Tools, San Diego, CA). The expression of RFP-tagged and GFP-tagged DHN pathway genes was clearly evident within the thick multicellular peridium. However, it was difficult to observe in the expression of the fluorescently tagged proteins in individual ascospores.

To determine the intracellular location of the RFP-tagged and GFP-tagged proteins, individual perithecia were manually harvested three days post-fertilization, and “perithecia squashes” were done by placing several perithecia in sterile water on a slide, covering the perithecia with a cover slip, and pressing down on the cover slip with the eraser end of a pencil to break open the peridia. The intracellular locations of RFP-tagged and GFP-tagged proteins in unmelanized areas of the crushed peridia close to the cover slip were observed with a Ziess LSM710 Confocal Laser Scanning microscope. A Plan-Aprochromat 63X/1.40 oil DIC M27 objective lens was used for GFP and RFP imaging. GFP images were collected at 493–598 nm with excitation at 488 nm. RFP images were collected at 600–703 nm with excitation at 580 nm.

3. Results

3.1. Genetic characterization of the N. crassa DHN pathway

A BLAST search of the *N. crassa* genome for the DHN pathway enzymes demonstrated that the genome contained all of the genes in the pathway. Fig. 1 shows the *N. crassa* DHN biosynthetic pathway. The *N. crassa* genome contains one polyketide synthase gene (NCU03584) for the first step in the pathway, two polyketide hydrolase genes (NCU01903 and NCU05821) for the second step of the pathway, two 1,3,6,8 tetrahydroxynaphthalene (THN) reductase genes (NCU06905 and NCU09390) for the third and fifth steps in the pathway, one scytalone dehydratase gene (NCU07823) for the fourth and sixth steps in the pathway, and five laccase genes (NCU02201, NCU03498, NCU00526, NCU09279, and NCU09023) that might represent the final step in melanin formation. To determine whether these encoded proteins functioned in the *N. crassa* melanin biosynthetic pathway, we examined the perithecium and ascospore melanization patterns in deletion mutants lacking these genes (Table 2, Figs. 2 and 3). Since the peridium is totally derived from the female, peridium melanization is a maternally inherited characteristic while melanization of the ascospore has been shown to be an ascospore genome dependent characteristic (Johnson, 1977). A number of *per-1* mutants (mutants in the NCU03584 encoded polyketide synthase) have been described in the research literature (Howe, 1976; Howe and Benson, 1974; Johnson, 1976, 1977; McCluskey et al., 2011), and an examination of their melanization patterns verified that the *per-1* mutants produce unmelanized perithecia when used as the perithecial (female) parent in a mating (Fig. 2). We also verified that *per-1* ascospores were unmelanized (Fig. 3). This was true regardless of whether the *per-1* ascospores were produced from a mating of a *per-1* mutant with a wild type partner or from a mating between two *per-1* partners. The Neurospora single gene deletion library contained a deletion mutant for the single scytalone dehydratase gene (NCU07823) which we will call *scy-1* for scytalone dehydratase 1. An examination of the mutant phenotype showed that when used as the perithecial parent (female) in a mating with a wild type conidial parent (male) or with a second *scy-1* partner, the mutant produced unmelanized peridium (Fig. 2). When used as either the female or male partner in a mating with a wild type isolate, the *scy-1* mutant produced a 1:1 ratio of melanized and unmelanized ascospores. Analysis of these ascospores showed that the unmelanized ascospores were *scy-1* mutants. When two *scy-1* mutants were mated together, all of the ascospores were unmelanized (Fig. 3). Based on these observations, it is clear that the DHN pathway is being used for the melanization of both perithecia and ascospores.

In characterizing the deletion mutants for the two polyketide hydrolase genes (step 2 in the pathway), we found that the library contained the deletion mutant for both of the genes. We found that

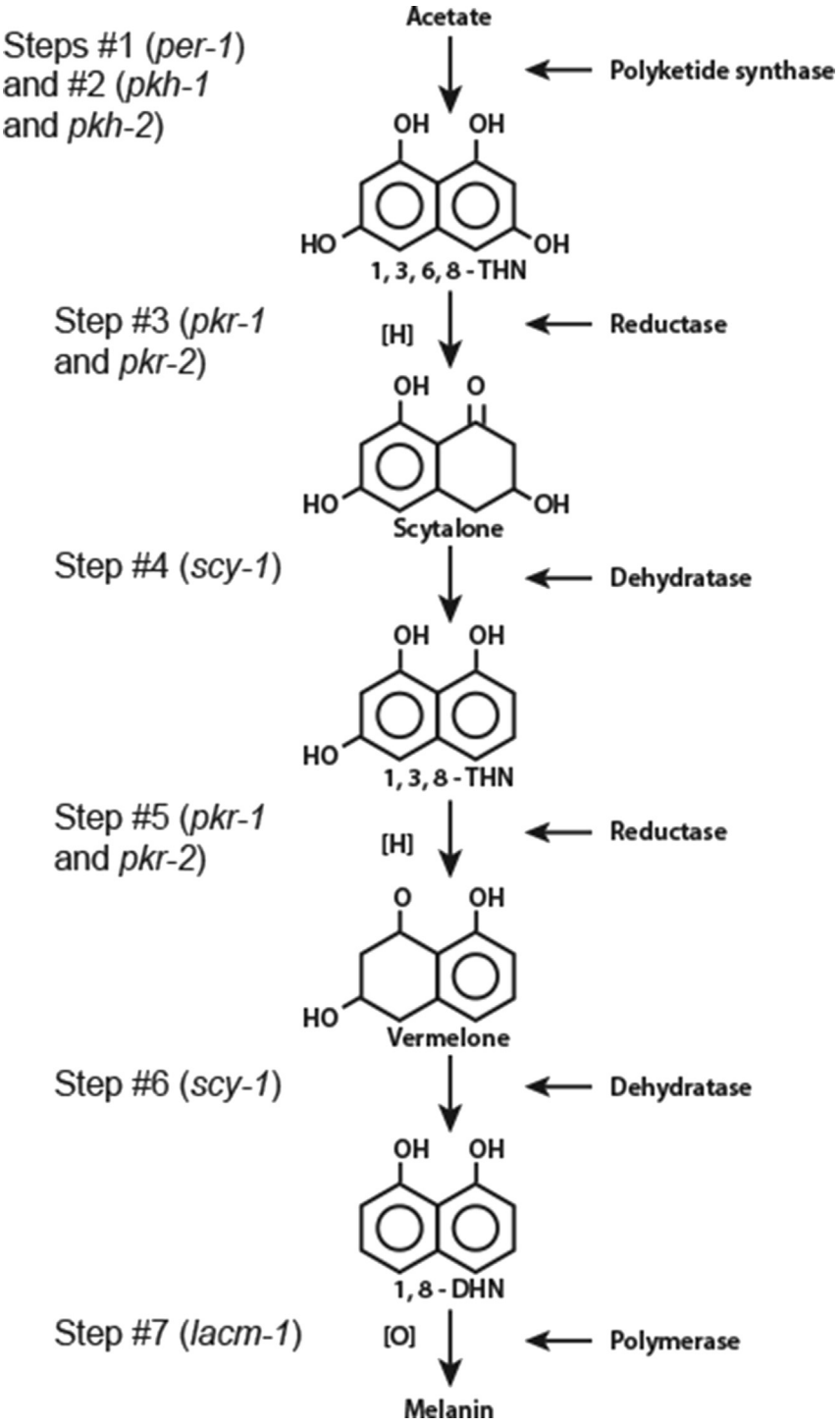

Fig. 1. The *N. crassa* biosynthetic pathway leading to the synthesis of DHN melanin is depicted. The enzymes that catalyze the various reactions are noted.

the *pkh-1*(NCU01903) mutant made a melanized peridium and contained a 1:1 ratio of melanized and unmelanized ascospores when used as a female in a mating with the wild type (Fig. 3). The unmelanized ascospores were found to be *pkh-1* mutant ascospores. This indicates that PKH-1 was required for the synthesis of melanin in the ascospores. The *pkh-2* (NCU05821) deletion mutant produced a melanized peridium and melanized ascospores (Figs. 2 and 3). We conclude that PKH-1 and PKH-2 both contribute to the melanization of the peridium while PKH-1 functions as the sole polyketide hydrolase in the developing ascospores.

We also characterized the melanization patterns of the single mutants for the two 1,3,6,8 THN reductases, which catalyze steps 3 and 5 in the biosynthetic pathway (Fig. 1). We found that the perithecia were melanized in both of the single mutants, indicating that both enzymes were produced in the perithecia and that the enzymes had redundant activity. The *pkr-1* (NCU09390) deletion mutant produced a 1:1 ratio of melanized to unmelanized ascospores when mated with a wild type partner, and the unmelanized ascospores were found to be *pkr-1* mutants. When mating two *pkr-1*mutants together, we found that all of the ascospores were

Table 2
Genes in the DHN melanin biosynthetic pathway in *N. crassa*.

Gene	NCU# (LG#) FGSC#s	Peridium phenotype	Ascospore phenotype	Peridium Expression of GFP-tagged and RFP-tagged proteins
per-1 (polyketide synthase) Step 1	NCU03584 (LG 5) #3309 & #3310 Classical Mutant	White perithecia	White ascospores in self cross & 1:1 ratio of white:black in matings with WT	NA
pkh-1 (polyketide hydrolase) Step 2	NCU01903 (LG 1) #13365	Black perithecia	1:1 ratio of white:black in matings with WT	RFP-tagged PKH-1 shows expression in peridium
pkh-2 (polyketide hydrolase) Step 2	NCU05821 (LG 7) #13696 & 13697	Black perithecia	Black ascospores in self cross & in matings with WT	No expression observed for RFP-tagged PKH-2
pkr-1 (1,3,6,8 THN reductase) Steps 3 & 5	NCU09390 (LG 7) #17987 & 17988	Black perithecia	White ascospores in self cross & 1:1 ratio of white:black in matings with WT	GFP-tagged PKR-1 shows expression in peridium
pkr-2 (1,3,6,8 THN reductase) Step 3 & 5	NCU06905 (LG 7) #14183	Black perithecia	Black ascospores in matings with WT	RFP-tagged PKR-2 shows expression in peridium
scy-1 (Scytalone dehydratase) Steps 4 & 6	NCU07823 (LG 3) #14353	White perithecia	White ascospores in self cross & 1:1 ratio of white:black in matings with WT	RFP-tagged and GFP-tagged SCY-1 show expression in peridium
lacm-1 Laccase Step 7	NCU02201 (LG 7) #16149	White perithecia	White ascospores in self cross & 1:1 ratio of white:black in matings with WT	RFP-tagged LACM-1 shows expression in peridium

Characterization of *N. crassa* genes involved in the melanization of ascospores and peridia. The gene names and the functions of the encoded proteins are given in the first column. The second column indicates the gene number, the linkage group where the gene is located, and the FGSC# of the isolates used in the study. The third and fourth columns indicate the mutant peridium and ascospore phenotypes respectively. The expression of RFP-tagged and GFP-tagged versions of the proteins in the peridium is given in the fifth column.

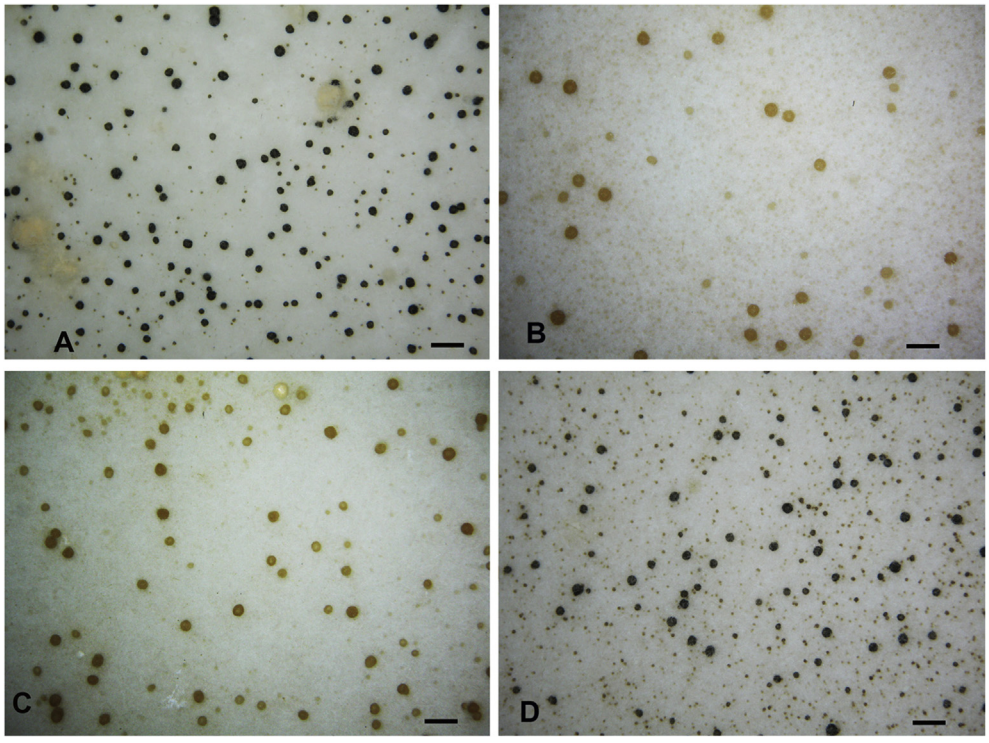

Fig. 2. Peridium melanization in *N. crassa* mutants. Pictures of perithecia growing on Whatman 3 MM paper. Panel A: Melanized perithecia from a wild type isolate. Panel B: Unmelanized perithecia from a mating in which the *per-1* mutant functioned as the female. Panel C: Unmelanized perithecia from a mating in which the *scy-1* mutant functioned as the female. Similar unmelanized perithecia are formed when the *lacm-1* mutant serves as the female. Panel D: Melanized perithecia from a *scy-1* isolate that has been transformed with the GFP-tagged version of the *scy-1* gene. The scale bar shows a 5 mm length.

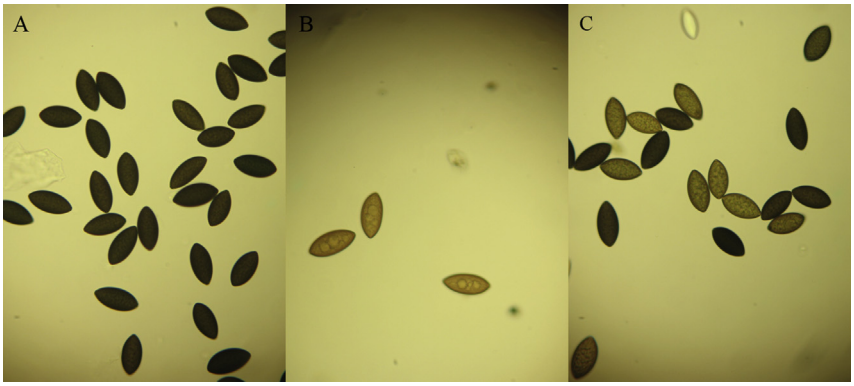

Fig. 3. Ascospores from *N. crassa* mutants. Pictures showing examples of melanized and unmelanized ascospores. Panel A shows melanization ascospores from a *pkh*-2 A x *pkh-2 a* mating. Panel B shows unmelanized ascospores from a *pkr*-1 A *x pkr-1 a* mating. Similar ascospores are produced in *per*-1 A x *per-1 a*, *pkh*-1 A x *pkh-1 a*, *scy*-1 A x *scy-1 a* and *lacm*-1 A x *lacm-1 a* matings. Panel C shows ascospores from a *pkh*-1 A x wild type *a* mating with a 1:1 ratio of unmelanized (*pkh-1*) and melanized (wild type) ascospores.

unmelanized (Fig. 3). The *pkr-2* (NCU06905) deletion mutant was not affected in the melanization pattern of the ascospores. These results demonstrate that a single 1,3,6,8 THN reductase, PKR-1, functions in the developing ascospores, while both reductases function as redundant enzymes within the perithecium.

Single gene deletion mutants were available for four secreted *N. crassa* laccases (NCU02201, NCU03498, NCU00526, and NCU09023). We found that three of these produced normally melanized perithecia and ascospores. However, in mating deletion mutants for the NCU02201 laccase, we found that perithecium melanization was clearly delayed when the NCU02201 deletion mutant was the female partner. In a mating between two NCU02201 deletion mutants, we found that melanization was delayed in the perithecium and for the ascospores, and that the ascospores were poorly melanized. These results suggest that the laccase encoded by NCU02201 is the major laccase used in melanization process in both the perithecium and in the ascospores, but that other laccases may be making minor contributions to the process. We have named the NCU02201 laccase gene *lacm-1* for laccase for melanization-1.

Our genetic results show that *N. crassa* utilizes the DHN pathway to generate melanized peridia and ascospores. We conclude that the developing ascospores use *per-1*, *pkh-1*, *pkr-1*, *scy-1*, and *lacm-1* to generate melanin. The melanin pathway in the developing peridium includes the use of *per-1*, *pkh-1* and *pkh-2*, *pkr-1* and *pkr-2*, *scy-1*, and *lacm-1*.

3.2. Expression patterns for GFP-tagged and RFP-tagged enzymes

Our initial characterization of the DHN pathway deletion mutants demonstrated that the *pkr-1* and *pkh-1* genes were expressed in the ascospores, but that the *pkh-2* and *pkr-2* genes were not. This indicates that some of the DHN pathway enzymes are being expressed in a cell type-specific manner. To further characterize the expression patterns for the enzymes involved in melanin formation, we examined the expression of RFP-tagged versions of *pkh-1*, *pkh-2*, *pkr-2*, *scy-1*, and *lacm-1*. We also examined GFP localization in transformants expressing GFP-tagged versions of *pkr1* and *scy-1*. All of these tagged genes were expressed under the control of their normal promoters. The PKH-1, PKR-2, SCY-1 and LACM-1 RFP-tagged and the GFP-tagged PKR-1 and SCY-1 were able to complement melanin formation in the peridium and/or ascospores of gene deletion mutants, demonstrating that these tagged proteins were functional (Figs. 2 and 3). We used these tagged genes to look at the cell type expression patterns of the DHN pathway genes. In characterizing the expression of the RFP-tagged and GFP-tagged proteins, we found that it was difficult to routinely demonstrate their presence within the developing ascospores. These experiments were done by mating two transformants containing the tagged gene so as to minimize the involvement of the MSUD phenomenon (meiotic silencing of unpaired DNA), which turns off the expression of unpaired DNA sequences (Shiu et al., 2001). Both mating partners contained *rid-1* mutations so that the RIP (repeat induced point mutation) phenomenon would not cause mutations within the tagged genes (Freitag et al., 2002). Our inability to routinely visualize the expression of the tagged proteins in developing ascospores may be indicative of an expression level below our detection limit and/or the presence of melanin within the developing ascospores obscuring the signal.

We were able to see expression of the RFP-tagged and GFP-tagged genes in the female-derived, thick multicellular peridium. The surface of the peridium is rapidly melanized after fertilization, which precludes localizing the proteins in this tissue. This was expected because melanin absorbs light at both the excitation and emission wave lengths. However, we were able to visualize the tagged proteins in the subsurface peridium tissue. Fig. 4 shows the expression of the RFP-tagged PKR-2 (NCU06905) in a 3 d post-fertilization perithecium squash sample. In Fig. 4, the tightly woven peridium tissue has been squashed to expose subsurface regions of tissue, where we are able to detect the RFP fluorescent tag in individual cells with the confocal fluorescence microscope. The results in Fig. 4 demonstrate that PKR-2 is being expressed within the peridium. Similar results were obtained for the peridium expression of all of the other RFP-tagged and GFP-tagged constructs except for the RFP-tagged PKH-2 (Figs. 5 and 6). We were unable to see expression of the RFP-tagged PKH-2 (NCU05821) in the peridium, suggesting that PKH-2 may be expressed at lower levels in the peridium or the tagged protein may be rapidly degraded. Our characterization of the *pkh-1* mutant shows that *pkh-2* contributes to the melanization of the peridium. Our results with GFP-tagged and RFP-tagged proteins showed that PKH-1, PKR-1, PKR-2, SCY-1, and LACM-1 were all expressed in the peridium. These results are in agreement with the melanization patterns we observed in our characterization of melanin production in the deletion mutants. The results of our analyses for the *N. crassa* melanin biosynthetic pathway are summarized in Table 2.

We were particularly interested in knowing the intracellular locations of the RFP-tagged and GFP-tagged proteins. In *A. fumigatus*, the DHN biosynthetic pathway proteins have been shown to be associated with intracellular vesicles (Upadhyay et al., 2016a, 2016d). To examine the location of the proteins, three-day old perithecia were squashed to break them open and expose

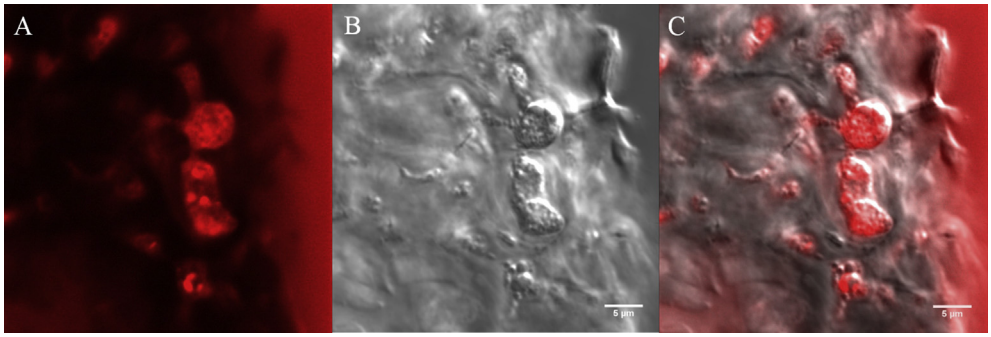

Fig. 4. The expression of the RFP-tagged PKR-2 (NCU06905) in the peridium. Three day post-fertilization perithecium were squashed to break the structures open and the presence of RFP-tagged PKR-2 assessed by confocal fluorescence microscopy. Panel A shows the RFP image. Panel B shows the DIC image. Panel C contains the merged image.

subsurface tissues, which were then examined with a confocal fluorescence microscope. We found that the GFP-tagged versions of SCY-1 were found in a non-uniform, punctate-type intracellular location (Fig. 5), suggesting the protein was associated with intracellular vesicles. The RFP-tagged versions of PKH1 (NCU01903), and PKR-2 (NCU06905) all showed a similar pattern of punctate intracellular location (Fig. 4). We conclude that the DHN biosynthetic pathway proteins are associated with intracellular vesicles, and hypothesize that the DHN pathway enzymes exist as a protein complex.

We also examined the location of the LACM-1 laccase, which generates the free radical oxygen involved in polymerizing the DHN into melanin. LACM-1 has a classical signal peptide sequence at its N terminus, suggesting that the enzyme is transported through the canonical secretory pathway. RFP-tagged LACM-1 was found to be localized in association with the cell wall space in maturing peridia (Fig. 6). This indicates that the LACM-1 is being released into the cell wall space as the peridium undergoes melanization.

4. Discussion

During *N. crassa* female development, there are two cell types that have heavily melanized cell walls. These are the developing ascospores and the peridium layer of the perithecium, an outer layer of cells that are thought to provide protection to the developing ascospores (Figs. 2 and 3). We were interested in knowing how the melanin in these cells was generated. We have previously shown that *N. crassa* makes DOPA melanin and that tyrosinase mutants were unable to form perithecia, the female mating structure (Fuentes et al., 1994). Mutants affected in the *N. crassa per-1* gene were previously known to produce perithecia and ascospores which lack the melanin normally found in these cell types (Howe, 1976; Howe and Benson, 1974). The *per-1* gene encodes the polyketide synthase with close homology to the polyketide synthases at the head of *A. fumigatus* DHN pathway. This suggests that the DHN pathway operates to generate the melanin during peridium and ascospore development and that the block in female development seen with the tyrosinase mutants is not due to a defect in melanization. The role that tyrosinase plays during female development remains an open question. The DHN melanin biosynthetic pathway has been studied in *A. fumigatus* and other fungi and the steps of the pathway have been well-defined (Eisenman and Casadevall, 2012; Langfelder et al., 2003; Nosanchuk et al., 2015). A depiction of the pathway is shown in Fig. 1.

All of the enzymes needed for DHN melanin synthesis are encoded in the *N. crassa* genome. However, unlike *A. fumigatus* and some other fungi in which the genes are found in a gene cluster, the DHN pathway genes are scattered throughout the *N. crassa* genome (Table 2). The first step of the pathway is the synthesis of a polyketide by a polyketide synthase, a large multi-domain protein that functions similar to fatty acid synthases to convert acetyl-CoA and malonyl-CoA to a polyketide. The *N. crassa* polyketide synthase, PER-1, has close homology across its entire length with the *A. fumigatus* Abl1 polyketide synthase, which has been shown to synthesize a heptaketide (Tsai et al., 2001), which suggests that *N. crassa* makes the heptaketide. The *N. crassa* genome contains two genes (*pkh-1* and *pkh-2*) encoding polyketide hydrolases, the second step in the *N. crassa* DHN pathway. Both genes have close homology to the *A. fumigatus* Ayg1 polyketide hydrolase that converts the heptaketide to THN (Tsai et al., 2001) the common precursor of DHN pathways throughout the fungal kingdom (Fig. 1). We showed that PKH-1 was required for the melanization of the developing ascospores, and that PKH-1 and PKH-2 both contributed to the melanization of the peridium. While RFP-tagged PKH-1 was

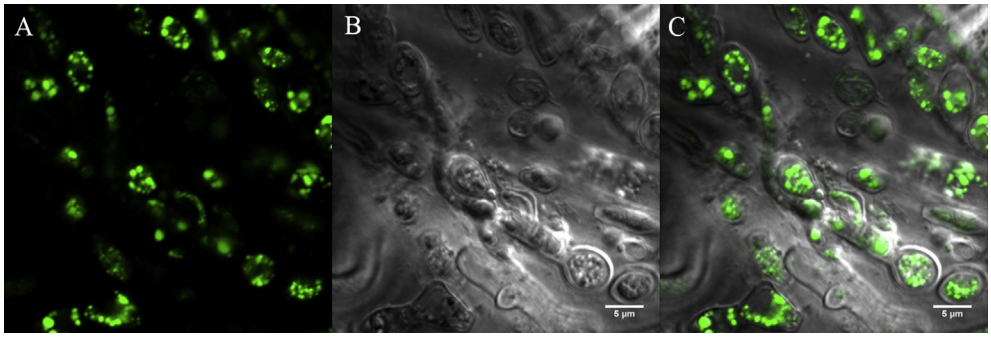

Fig. 5. Intracellular localization of GFP-tagged SCY-1 (NCU07823). Three day post-fertilization perithecia were squashed to break the perithecia open and the presence of fluorescently tagged SCY-1 was assessed with a confocal fluorescence microscope. Panel A shows the fluorescent image of a peridium area containing GFP-tagged SCY-1. Panel B shows the DIC image of the peridium. Panel C contains the merged image.

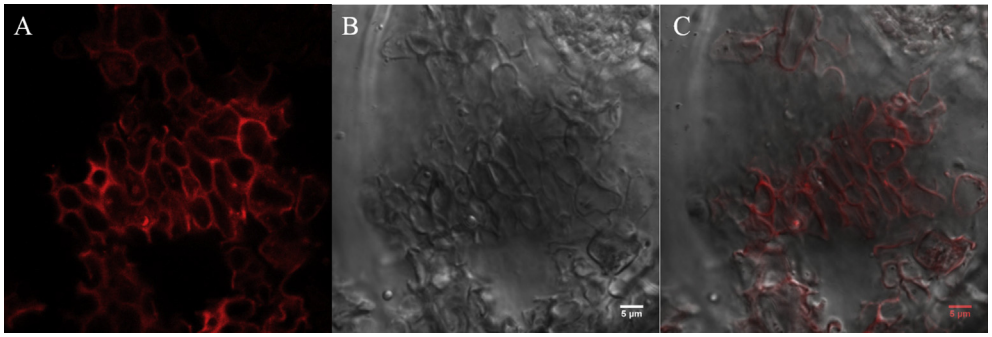

Fig. 6. LACM-1 is localized in the cell wall in maturing peridia. Panel A shows the fluorescent image of LAM-1 in the peridium cell wall space of a maturing (three-day post fertilization) perithecium. Panel B shows the DIC image of the peridium. Panel C contains the merged image.

expressed in the peridium (Fig. 3), we were unable to visualize the expression of RFP-tagged PKH-2. This suggests that PKH-2 might be made at much lower levels than PKH-1 or that the RFP-tagged version of the PKH-2 is rapidly degraded. The third and fifth steps in the DHN pathway are carried out by a THN reductase. Two THN reductase genes (*pkr-1* and *pkr-2*) were found in the *N. crassa* genome. We showed that ascospores rely on the *pkr-1* gene for THN reductase activity (Fig. 3) while both *pkr-1* and *pkr-2* are functioning in a redundant manner in the DHN pathway within the developing peridium. The fourth and sixth steps in the pathway are catalyzed by scytalone dehydratase, and the *N. crassa* genome has a single gene encoding this enzyme (*scy-1*). We show that SCY-1 functions in both the ascospore and peridium DHN pathways (Figs. 2 and 3). A computer analysis of all of the enzymes demonstrated to function downstream of the PER-1 heptaketide synthase (PKH-1, PKH-2, PKR-1, PKR-2, and SCY-1) showed that they lack signal peptides, transmembrane domains, and lipid attachment sites.

We were unable to routinely see the expression of the DHN pathway in developing ascospores. This may be because the rapid formation of melanin in the cells masks the fluorescence signals, or because of a lower level of expression of the enzymes within the ascospores. However, we were able to readily observe the expression of the GTP-tagged and RFP-tagged DHN pathway proteins in the subsurface tissue from developing peridia. Our results demonstrated that PKH-1, PKR-1, PKR-2, and SCY-1 were expressed within the tight woven peridium (Figs. 4 and 5). Using confocal fluorescence microscopy, we were able to show that the proteins were located with a non-uniform punctate distribution, suggesting that they were associated with a population of intracellular vesicles. This suggests the pathway may exist in the form of a multimeric vesicle-associated enzyme complex. In *A. fumigatus*, the DHN pathway enzymes have be shown to be associated with intracellular endosomal vesicles and that the enzymes are palmitoylated (Upadhyay et al., 2016a). The mechanism by which the *N. crassa* DHN pathway enzymes might become associated with an intracellular vesicle and transfer DHN into the lumen of the vesicle remains to be determined. The most likely explanation is that the enzymes form a multi-enzyme complex and that the PER-1 polyketide synthase is being palmitoylated to anchor the entire complex on the membrane. The metabolites could be shuttled from one enzyme to another during DHN synthesis. Presumably, the enzymes could interact with the membrane in a way that allows the DHN to be released into the lumen of the vesicle and eventually secreted.

We identified four laccases that had some homology to the laccases used in generating melanins in other fungi (Sapmak et al., 2015). Characterization of deletion mutants for these laccases demonstrated that the *lacm-1* (NCU02201) deletion mutant was affected in the melanization of both ascospores and peridia, demonstrating that *lacm-1* encodes the major laccase functioning in the oxidative polymerization of *N. crassa* melanin. LACM-1 contains a signal peptide that would direct it into the secretory pathway, but does not have a GPI-anchor signal at the C terminus, suggesting that the laccase travels through the secretory pathway and is released into the cell wall space. Our results with RFP-tagged versions of LACM-1 show that the protein has been secreted into the intracellular space in maturing peridia (Fig. 6). Thus, at the stage of peridium maturation when melanin is being formed, the LACM-1 is localized to the cell wall space while the DHN biosynthetic enzymes are associated with intracellular vesicles

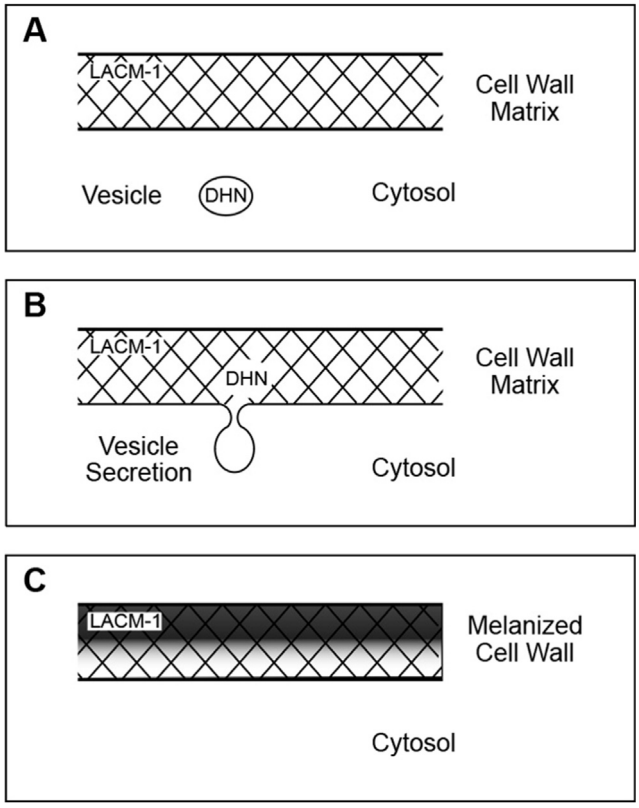

Fig. 7. Model for melanin formation within the *N. crassa* peridium. During peridium maturation, the DHN biosynthetic pathway is localized to intracellular vesicles and DHN is produced within these vesicles. The LACM-1 laccase is located in the cell wall (panel A). As the peridium cells secrete DHN into the cell wall space, the laccase generates oxygen free radicals to effect the polymerization of the DHN into melanin (panel B). The forming melanin encases the cell wall chitin/glucan/glycoprotein matrix of the cell wall (panel C).

(Figs. 4–6). We hypothesize that the DHN is being produced in secretory vesicles and released to the cell wall space, where the cell wall-associated LACM-1 functions to generate the oxygen free radicals involved in polymerizing DHN into melanin.

Based on our findings, we would present a model for melanin formation (Fig. 7). Our model has LACM-1 and the DHN biosynthetic pathway localized to two different vesicles. LACM-1 is released into the cell wall space as the peridia begins maturation. DHN is subsequently released into the cell wall space and converted into melanin during peridium maturation. This model has several appealing aspects. First, the forming melanin would encase the other cell wall components and generate a much stronger cell wall than would be formed if the cell wall chitin, glucans and glycoproteins were not encased within the melanin. The model posits that the cell wall matrix is buried within a melanin structure and would provide the maximum amount of protection to the cell wall from ROS, UV light, environmental stresses, and cell wall hydrolyzing enzymes produced by other microbes or by host cells. The model is compatible with the fact that EM pictures of DHN pathway melanized fungal cells do not routinely show convincing evidence of melanin being localized within intracellular organelles, which would be expected if melanin was formed within secretory vesicles and exported into the cell wall space (Bell and Wheeler, 1986). Our model of having melanin form within the cell wall space is supported by our observation of having LACM-1 located in the cell wall space and the DHN biosynthetic pathway located on intracellular vesicles within the maturing peridia. The model is also supported by the fact that melanin has been shown to be present in the outer layer of other melanized cell walls (Bell and Wheeler, 1986; Langfelder et al., 2003). It is hard to envision how large melanin polymers could be secreted and transported to the outer layer of the cell wall without disrupting the wall and causing cell lysis. In the *N. crassa* peridium, the melanin is known to be preferentially localized to the outer layers of the peridium. We hypothesize that the localization of melanin to the outer portion of the peridium occurs because oxygen, the laccase substrate for the generation of an oxygen free radical needed to initiate the polymerization of DHN melanin, is readily available at the surface of the peridium, but will be less available within the thick, metabolically-active, multicellular interwoven tissue below the surface of the structure. As a result, melanin will be preferentially formed at the peridium outer surface.

Conflicts of interest

The authors declare no conflict of interest. The authors are responsible for the content and writing of the paper alone.

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