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Regulation of Phialide Morphogenesis in *Aspergillus nidulans*

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by

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Filamentous fungi have two distinctive life cycles, vegetative growth and development for sexual or asexual spore formation. The asexual reproduction in development as conidiation in *A. nidulans* is the dominant form of producing spores effectively. A complex conidiophore structure is developed during asexual reproduction process. The conidiophore is formed from hyphal cell and consists of stalk, vesicle, metulae, phialide and conidial spores. Phialides are essential sporogenous cells in the conidiophore structure. The growth pattern is switched from acropetal to basipetal between phialide and spores, which makes phialide a unique cell type in *A. nidulans* and other phialide producing fungi. Study of the phialide morphogenesis would provide significant insight into the morphogenesis variation in fungi and animals. AbaA is the key transcriptional factor that controls phialide formation, but the knowledge to genes which are directly regulated by AbaA and involved in phialide morphogenesis is poorly known. In this study, twelve genes that are up-regulated by AbaA and potentially related with phialide morphogenesis were selected by gene screening with several criteria we set up, including homology search against other ascomycetes and conserved domain search. The RT-PCR result confirmed that the expression of these genes are induced during developmental stage, probably by AbaA as they contain AbaA binding sites. We also characterized the functions of these genes by generating gene deleted mutants. Two genes

have been identified to regulate the proper function of phialide: ndrA (AN11101) and phiB (AN0499). *A. nidulans* Axl2 may regulate the expression of these two genes in development. They may also play roles as the marker for morphogenetic machinery repositioning during conidiation. Other genes also show relationship to phialide morphogenesis since their mutants exhibited defects in conidiophore.

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Table of Contents

LIST OF FIGURES

LIST OF TABLES

Chapter I Conidiophore Morphogenesis in *Aspergillus nidulans*

Overview

Fungi are eukaryotic heterotrophic microorganisms. They play significant roles in the natural ecosystems and in human lives (Newbound *et al*. 2010). Fungi are distributed in the world widely and colonize on a diverse range of habitats. Fungi recycle the nutrients by decomposing dead plant and animal tissues (Barr and Aust, 1994). Some of them are pathogens and are harmful to plants, animals and humans (Monk and Goffeau, 2008). But some fungal species are food sources for animals including human being, such as edible mushrooms. Fungi have been used industrially for fermentation to produce useful biochemicals for a long history (Bennett 1998). Based on the growth form, fungi are classified as multicellular (filamentous), unicellular (yeasts) and dimorphic (it switches between unicellular and multicellular forms) (Osiewacz 2002).

As the name suggests, filamentous fungi are well known for producing elongated, thread shaped growing hyphae. Hyphae are characterized as a tubular structure with a pointed tip called hyphae tip, and apical or lateral branches can develop along the hyphae during growing phase to build complex hyphae cluster named mycelium. The growth of hyphae is achieved by highly polarized extension of the hyphal tip, and the emerging of new hyphal tips as branching (Kaminskyj and Heath, 1996). Most fungal hyphae consist of multiple cells, which are compartmentalized by internal cross-walls termed as septa. The hyphae structure not only facilitates the colonization of fungi in diverse environment and extends fungi to reach for additional nutrient resource, but also establishes the supporting infrastructure of spatial development by cell differentiation during sexual or

asexual reproduction stage. Besides the vegetative growth featured as hyphae elongation, another growth phase is the development characterized by reproduction. The asexual reproduction of Ascomycota, the largest phylum in fungi kingdom, is considered as an important taxonomic characteristic. During the asexual reproductive process, ascomycete fungi produce conidia spores and separate spores from the parent structure; this process is called as conidiogenesis.

For biological research in filamentous fungi, especially in Ascomycota, several fungal model systems have been established. *Aspergillus nidulans* has been recognized by two most fascinating characteristics: its genetic tractability and high manipulation capability, which lead it to be a well-studied model system for fungal morphogenesis. Same as other fungi, *A. nidulans* undergoes two life stages: the vegetative growing state and the distinctive asexual/sexual development stage. *A. nidulans* initiates vegetative growth by spore germination. During germination, spore shifts from a dormant state into a metabolically active state. After the germ tube is released, highly polarized extension is the main process for hyphal growth. In the developmental stage, a sexual or an asexual reproductive process takes place for propagation. The asexual reproduction is the most common way for *Aspergillus* and other conidiogenous fungi to rapidly reproduce abundant spores for dissemination and survival. Understanding of the morphogenesis during fungal asexual development in *A. nidulans* provides insightful information in the following three aspects. Firstly, it could offer meaningful ideas for implementing potential control of related pathogenic fungi, for example, a human pathogen *A. fumigatus,* which imposes increasing health risks due to its mutation based antifungal drug resistance (Chamilos and Kontoyiannis, 2006). Secondly, it would also lead to the

development of manageable means to optimize the growth of beneficial fungal system such as industrial microbial strains. For instance, an important economic Aspergilli species, *A. oryzae*, is used wildly in Asia for soybean fermentation or sake brewing, of which the rapid growth and proliferation is desired. Thirdly and more broadly, the dramatic cell differentiation during asexual development is considered as a comparable model to outline the underlying mechanism of cell differentiation in higher organisms, such as the development of cancer cells in mammals. The progress accomplished to advance the comprehension of morphogenesis during asexual reproductive development stage in *A. nidulans* will be reviewed and summarized in the following sections.

A model organism - *Aspergillus nidulans*

Several conventional fungal systems, such as *Saccharomyces cerevisiae*, *Neurospora crassa,* and *Coprinus cinereus,* are available for research use based on different purposes. (David *et al*. 1997, Davis 2002, Kües 2000). As model systems, they have some common features, such as the ease of experimental manipulation, short growth cycle, and non-specific growing requirements (Karathia *et al*. 2011). *Aspergillus nidulans* (anamorph; teleomorph is known as *Emericella nidulans*) is a well-established ascomycete fungus model system. It belongs to the Eurotiomycetes class under the Ascomycota phylum. In the past decades, this filamentous fungal system has been extensively used to conduct research on a broad range of biological processes, including cell physiology, genetic regulation, pathogenesis, and so on (Morris and Enos 1992). The vast research interests lead to the development of efficient classic genetic experimental techniques on *A. nidulans*, such as genetic crosses with complementary auxotrophic

marker and double mutant selection using conidial color markers (Todd *et al*. 2007). Some experimental methodologies, including target gene replacement by recombination PCR and fluorescent probe attachment, have also been developed to serve functionality analysis of target genes (Chaveroche *et al.* 2000, Osmani *et al*. 2006, Szewczyk *et al*. 2006, Breakspear *et al*. 2007, Virag *et al*. 2007, Suzuki *et al*. 2008). Although the construction of gene disrupted mutants is prevailing for functional analysis purpose, deletion of essential genes in *Aspergillus* strain sometime are problematic, for example, generating false positive phenotype and abnormal expression of regulated downstream genes. Zarrin *et al*. (2005) used a cassette vector carrying a conditional promoter *A. nidulans* alcA and a selectable marker to generate *A. nidulans* transformants, in which expression of the target essential gene is under the control of alcA. This conditional promoter has been widely used as a convenient tool to tightly regulate gene expression in Aspergillus (Waring *et* al., 1989; McGoldrick *et al*., 1995; Romero *et al*., 2003). These lab methods together construct a firm foundation for genetic research on this fungal system. In recent years, with the help of whole genome shotgun sequencing, *A. nidulans* genome was sequenced and published by Galagan *et al*. (on Broad Institute http://www.broadinstitute.org/ 2005). The sequencing of *A. nidulans* genome and its annotation enabled advanced research on understanding Aspergilli evolution and genetic regulation. In the past few years, as the next-generation sequencing technology emerged, the more accurate genome annotation has become available. For instance, *A. nidulans* Genome Database (AspGD http://www.aspergillusgenome.org/) is aimed for updating and re-annotating *A. nidulans* genome (Arnaud *et al*. 2010). Recently, it has been reported to use *A. nidulans* to discover antifungal drug resistance by mutagenesis with

next generation sequencing (He *et al*. 2014). Several mutation sites have been identified to be potentially responsible for the drug resistance.

Overall, *A. nidulans* has been established as a sophisticated and ideal system for fungal genetic research, especially for the fungal morphogenesis study. Similar with other filamentous fungi, *A. nidulans* starts a new life cycle from the process of spore germination by breaking dormancy (Harris 1997, Harris 2006). Then a polarity axis is established for cell surface expansion and germ tube formation. A hypha is now slowly formed. The hyphae are multinucleate cells caused by parasynchronous nuclear divisions (Harris 1997). When the condition is favorable and hyphal cell is large enough, a septum is formed for cell compartment, which process is called septation (Harris 1997). The majority processes in vegetative growth is the extension of hyphae. Later, the fungus enters into development stage that produces the asexual reproductive structure conidiophore, or sexual structure cleistothecium for proliferation. Producing asexual spores by *A. nidulans* and other ascomycetes are the most dominant way to effectively propagate. Understanding of the developmental and morphological processes would provide insight into the morphological mechanism on molecular level.

Conidiophore development and Phialide

Fungal species in the largest phylum Ascomycota are generally called ascomycetes. Conidiation is the asexual reproductive approach in ascomycetes. The asexual reproduction by conidiation has a broad and important impact in agriculture, industry and medicine (Adams *et al*. 1998). The entire asexual fertile multicellular hyphal

system produced by ascomycetes is named as conidiophore. Conidiophores are aerial septate hyphae which branch, differentiate, and produce conidial spores. Conidia are the asexual spores for dissemination and survival (Ebbole 2010). In this dissertation, *A. nidulans* was used as a model system to study morphogenesis during phialide development. The asexual reproductive pathway of *A. nidulans* is well-studied as a model to understand genetic regulation of cell development in filamentous fungi. In *Aspergillus*, the asexual reproductive process is achieved by conidiation, which includes the formation of conidiophore basal structures and uninucleate conidia spores. Conidiophore development in *A. nidulans* starts from the formation of foot cell with thick cell wall. Foot cell is differentiated from vegetative hyphae (Singh 1973). The foot cell rises an aerial branch called stalk, with an apical swelling at the tip of stalk named vesicle (Oliver 1972). A layer of uninucleate cells metulae bud from the vesicle surface, and sequentially another layer of uninucleate sporogenous cells called phialides form on top of the metulae (Etxbeste *et al*. 2010, Figure 1-1 Timberlake 1993). These two layers of cells are called sterigmata. Chains of conidial spores are produced by the repetitive mitosis division by phialide. This form of spore development is called blastic-phialidic sporulation that a series of small spores arise rapidly from the end of the specialized conidiogenous cell phialide (Kendrick 2003, Gupta and Mukerji 2001).

In *Penicillum*, *Aspergillus* and several other ascomycetes, conidiophore branches are terminated by the sporogenous cells named phialides (Harris 2012). Phialides are branches in conidiophores that produce conidia. Phialides are initially uninucleate cells, but the single nucleus divides to two by mitosis and one migrates into the conidial spore during sporulation. The cell division pattern is switched for sporulation in phialides. Until

the formation of phialides, the cell division in hyphae and conidiophore undergoes an acropetal pattern. In this pattern, the new cells emerge by repeatedly budding from the tip of the preceding cells (Sewall *et al*. 1990, Si *et al*. 2012). In contrast, the conidial spores produced from phialide follow a basipetal division pattern, where the youngest conidial spore is closest to the phialide tip. It is different from the acropetal pattern in that the morphogenetic machinery is located at the apex of newly formed spores, and the repositioning of such machinery from the newly matured spores to the top of the phialide is required for a new round of spore generation by phialide (Sewall *et al*. 1990, Si *et al*. 2012). This acropetal to basipetal pattern switch occurred in phialide makes phialide a unique cell type in conidiophore (Cole 1986, Adams *et al*. 1998).

To some extent, phialides in fungi are similar as the stem cells in plant or animal. Stem cells are a unique cell type that can potentially develop into different cell types during growth. When a stem cell divides, it can remain its original function and shape as the mother cell, and the newly divided cell may possess much more specialized functions and differentiated shape such as muscle cell, nerve cell or red blood cell. The cell division by stem cell is mitosis. The phialide cells in fungi show high level of similarity with the properties of stem cells. During sporulation, the phialide cells undergo mitosis to produce conidial spores. The spores and phialides have totally distinctive functions that spores are produced for dissemination and survival, and phialides function as the “stem cell” to divide differentiated spores. In the process of cell division to produce spores, the function and the shape of phialide remain the same. Presumably phialide cells may be considered as the “stem cells” in fungi.

Central developmental pathway in *A. nidulans*

Because of the importance of conidiation in fungal biology, the regulatory genes and the genetic pathway involved in conidiophore development have been well characterized in *Penicillium*, *Neurospora*, and *Aspergillus* (Roncal and Ugalde, 2003, Springer and Yanofsky 1989, Timberlake 1993). In *A. nidulans*, it is commonly agreed that the induction of conidiophore is triggered by chemical, light and air exposure (Harris 2012, Chi and Craven 2013). Based on the poly(A) RNA sequence investigation over the life cycle in *A. nidulans*, 700-1,100 distinctive sequences are discovered that are specifically transcribed during developmental stage (Timberlake, 1980). This result suggests that a set of genetic modules are responsible of regulating development in *A. nidulans* (Timberlake, 1980). Several conidial mutants were generated to determine the regulatory modules. These mutants show no defects in vegetative growth but exhibit faulty conidiophore morphologies (Clutterbuck 1969). Major genes related to the defects in the mutants have been characterized to understand their relationships in the regulatory pathway by epistatic analysis (Clutterbuck 1969, Adams *et al*. 1988). A central developmental pathway (CDP) has been proposed and described, which mainly consists of three transcriptional factors, BrlA, AbaA and WetA. (Boylan *et al*. 1987, Timberlake and Marshall, 1988). The functions of the major transcriptional factors in the central developmental pathway are reviewed here.

BrlA is a regulatory factor in early conidiophore development in *A. nidulans.* Δ*brlA* mutant exhibits a “bristle” phenotype that the aerial stalks extend indeterminately with no formation of vesicles or further cell types (Clutterbuck 1969, Adams *et al*. 1988, Figure 1-2). Overexpression of brlA leads to the termination of vegetative growth, and

causes the formation of conidial spores from hyphae in submerged culture (Adams *et al*. 1998, Figure 1-3). Overexpressed brlA can also activates the expression of abaA and wetA (Adams *et al*. 1998, Adams and Timberlake 1990). brlA gene encodes two transcriptional units, BrlAα and BrlAβ, both of which accumulate to a high level at developmental stage for normal conidiophore development and conidiation (Adams *et al*. 1998). It was reported that the expression of brlA is occurred 5-10 hours after the induction of development, which is related to the timing of vesicle formation (Adams *et al*. 1988, Boylan *et al*. 1987). The BrlA polypeptide is a C2H2 Zinc finger transcriptional factor that may play a role in binding nucleic acid (Adams *et al*. 1998). BrlA binds specifically to the sequence (C/A)(G/A)AGGG(G/A) on promoter region of hypostatic genes to initiate the formation of vesicle and further conidiophore development (Chang and Timberlake 1992). The developmental regulatory genes abaA, wetA, rodA and yA contain multiple BrlA binding sites indicating the role of BrlA as a primary regulator in central developmental pathway (Adams *et al*. 1998).

AbaA has been characterized as an ATTS/TEA transcriptional factor with DNA-binding property (Andrianopoulos and Timberlake 1994). AbaA has been shown to bind to the consensus sequence CATTC(C/T) (Andrianopoulos and Timberlake 1994). This AbaA specific binding site is present in many regulatory genes including brlA, wetA, yA, rodA and also abaA itself (Adams *et al*. 1998). AbaA is activated by BrlA during the middle developmental stage (Adams *et al*. 1998). The transcription of abaA is initiated between 10-15 hours after development induction that matches the timing of phialide formation (Boylan *et al*. 1987). Δ*abaA* mutant represents repeated sterigmata cell development to form abacus structures, and has no conidiation (Sewall *et al*. 1990, Figure 1-4). This

phenotype was described as beads on a string in an “abacus”, where the name of this gene was derived from (Adams *et al*. 1998). The ultra-structure analysis with transmission electron microscopy showed that the extra layer observed in phialide does not appear in repeated sterigmata cells, which implies that the chains of abnormally repetitive cells are metulae-like structures (Sewall *et al*. 1990). In Δ*abaA* mutant, brlA transcript accumulates to a normal level, but transcript levels of many other developmental genes, including wetA, are much lower than the wild type (Adams *et al*. 1998). Overexpression of abaA leads to the interruption of vegetative growth and the accentuation of cellular vacuolization, but not conidiation (Aguirre 1993). Overexpressed abaA can activate the expression of several developmental genes such as wetA and brlA (Adams *et al*. 1998).

WetA gene is activated in late developmental stage that is required for the synthesis of cell wall in conidial spores (Marshall and Timberlake 1991). The loss of WetA function in Δ*wetA* causes that conidial spores undergo autolysis instead of pigmentation, which is described as “wet-white” (Adams *et al*. 1998). There are four layers of cell wall in matured conidial spores, and spores in Δ*wetA* lose the most inner layer, which indicates that WetA is responsible for the maturation of conidial spore (Sewall *et al*. 1990). The transcription of wetA is initiated after 15 hours, which is corresponding to the timing of spore production (Mirabito *et al*. 1989, Boylan *et al*. 1987). In Δ*wetA*, many sporulation specific genes are failed to accumulate to the levels as they do in wild type (Boylan *et al*. 1987). Overexpression of wetA leads to growth inhibition and excessive branching (Marshall and Timberlake 1991). Overexpressed wetA activates the expression of spore-specific genes including spoC1 gene cluster, which is expressed in spores (Marshall and Timberlake

1991). On the other hand, brlA and abaA are not induced by overexpressed wetA (Marshall and Timberlake 1991).

StuA and MedA are developmental modifiers that regulate cell differentiation in conidiophore development. The phenotype of Δ*stuA* mutant was described as "stunted" that aerial stalk are extremely shortened, and conidial spores are produced directly on vesicles or metulae (Adams *et al*. 1998). The morphology of Δ*medA* mutant was described as "medusa" that it produces repeated and branched sterigmata, and occasionally a secondary conidiophores are formed on the mutant conidiophore (Adams *et al*. 1998). Mutants of both genes are able to produce conidial spores, even though they exhibit abnormal conidiophore development (Clutterbuck 1969, Adams *et al*. 1998). It was suggested based on the functional analysis that MedA may involve in proper temporal expression of brlA by stabilizing transcription complex (Miller *et al*. 1985). And StuA is required for the proper spatial expression of brlA (Adams *et al*. 1998).

Upstream regulators that function as activators for the central developmental pathway are called "fluffy" genes in that their mutants exhibit cotton-like fluffy colony morphology (Adams *et al*. 1998). There are six gene in the fluffy family, fluG, flbA, flbB, flbC, flbD and flbE (flb as fluffy low brlA expression, Adams *et al*. 1992). In Δ*fluG* mutants, conidiation is not observed but can be recovered if mutants were grown next to the wild type (Adams *et al.* 1998). Hence fluG is thought to synthesize the signal factors for activating conidiophore development pathway (Adams *et al.* 1998). Overexpression of fluG leads to the activation of conidiophore development in submerged culture by activating the expression of brlA (D'Souza *et al*. 2001). Besides activating brlA, the function of FluG is also required to activate the expression of flbB, flbC, flbD, flbE, to

initiate conidiophore development (Adams *et al*. 1988). flbA is a G protein signaling protein (RGS) (Yu *et al*. 1996). The colony in Δ*flbA* mutant is aconidial that it undergoes autolysis when the colony matures (Wieser *et al*. 1994). Overexpressed flbA leads to misscheduled expression of brlA (Lee and Adams 1994). Hence flbA is required for the activation of brlA (Lee and Adams 1994). The loss of function of other fluffy genes including flbB, flbC, flbD, or flbE delay the development of conidiation (Wieser *et al.* 1994). The proposed pathway for upstream activators are formed in two ways: fluG → flbE → flbD → flbB → brlA and fluG → flbC → brlA (Wieser and Adams 1995). The two genetic pathways independently activate brlA as the double mutant of Δ*flbC* and Δ*flbD* shows defects in conidiation (Wieser and Adams 1995).

Based on the characterization of key regulatory genes in conidiophore development, it was agreed that brlA is first activated by upstream signal produced by the fluffy gene family, and in turn activates abaA, which autogenously regulates abaA itself, activates brlA retroversely, and activates more downstream structural genes including the third regulator gene wetA (Mirabito *et al*. 1989, Marshall and Timberlake 1990, Lee and Adams 1996). Positive regulation of brlA by abaA and autogenous regulation of abaA provide loop feedback to maintain the regulatory pathway in an active state in conidiophore development (Timberlake 1990, Timberlake 1993). While brlA is not found in other filamentous fungi or in yeast (Harris *et al*. 2009), AbaA might play more important role in this regulatory system generally given its autogenous regulation as well as upstream and downstream activation.

During conidiophore development in *A. nidulans*, a precise spatial and temporal control of gene expression is required for cell differentiation (Aguirre 1993). The two

types of uninucleate sterigmata cells, metulae and phialides, are formed based on transformation from hyphal growth of multinucleate stalk cell to a yeast budding-like cell division (Karos and Fischer 1996). Phialides polarly bud from metulae serving as sporogenous structures to produce chains of spore. In the process of spore generation, nuclear is divided during mitosis and migrated from phialide into spore (Queiroz and Azevedo 1998, Ishi *et al*. 2005). The cell division in phialide to generate spores is asymmetric division in that phialide's own character is not changed (Adams *et al*. 1998), which is similar as the bud emergence process in budding yeast. In this developmental process, one of the three transcriptional factors AbaA is required for phialide formation in *A. nidulans*.

AbaA is also required for phialide differentiation in closely related species, such as *Fusarium graminearum* and *Penicillium marneffei* (Son *et al*. 2013, Vanittanakom *et al*. 2006). Deletions of abaA in the mentioned two species lead to abnormal phialide structure and abolished conidia production, which further proves the essential function of abaA in phialide differentiation. Considering that AbaA protein has DNA-binding function as a transcriptional factor, it would regulate the expression of a series downstream genes which play certain roles in conidiophore development, especially phialide morphogenesis. Several genes have been reported to be required for phialide formation and with abaA binding site possession, which suggests the potential role of being epistatically regulated by abaA. phiA gene was shown to be present and important for phialide development (Melin *et al*. 2003). Disruption of phiA causes reduced conidiophore development, absence of conidia chains and more importantly, improper development of phialides. Moreover, PhiA has been shown to localize in phialide and

conidia, and abaA binding motif were identified in phiA upstream promoter region. Based on these evidences, phiA was claimed as an AbaA regulated gene related to phialide development. Another *A. nidulans* gene axl2 was also reported to be phialide related. (Si *et al*. 2012). It was argued that failing to produce long chain of spores by *axl2* null mutant suggests defects in sporogenous structure. The localization of Axl2 is at the phialide-spore junction. Expression of axl2 is up-regulated when abaA is induced, and overexpressed axl2 induces malformed phialide. Therefore, it is demonstrated that axl2 is involved in phialide morphogenesis and regulated by AbaA. Other key gene modules involved in phialide development remain unknown.

In summary, BrlA drives the formation of all conidiophore structures until phialides, and AbaA is required for the proper function of phialide. WetA is specifically required for the formation of spores. (Figure 1-5)

The conserved GTPase protein Septins in *S. cerevisiae* and *A. nidulans*

The budding yeast *S. cerevisiae* has been utilized as a comparable framework to understand biological mechanisms, such as polarized growth and budding emergence (Harris and Momany 2004). To some extent, the process of sporulation in filamentous fungi is an analogous model of bud emergence in yeast, it would be convenient to study the morphogenesis during asexual reproduction in fungi by applying what has been learnt in yeast as a reference.

Septins are conserved GTPase proteins that are essential to ensure proper cytokinesis (Fares *et al*. 1996, Longtine *et al*. 1996). They are first discovered with cell

cycle defective mutants in *S. cerevisiae*. Loss of any one of the five septin members would cause cell cycle arrest and defective cytokinesis because septins play essential roles to ensure proper cytokinesis by forming a barrier structure at the bud neck during asymmetric cell division (Longtine *et al*. 2000). They have also been found in all eukaryotic species including human. There are five septin members expressed in yeast cells: Cdc12, Cdc11, Cdc10, Cdc3, and Shs1/Sep7, which are all localized at the bud neck during bud emergence. Septin assembly at bud neck is reported to function in two essential aspects in cell division: promoting the localization of other important proteins to the bud site (Fladfelter *et al*. 2001), and also providing a barrier structure to restrict certain determinants to particular cortical domains (Faty *et al*. 2002).

Five septin members are found in *A. nidulans*, AspA, AspB, AspC, AspD, and AspE. They are localized in three patterns in *A. nidulans*, including tips or branches, septa, and floating septin filaments (Momany *et al*. 2001). Among these five septins, AspA, AspC and AspE are orthologs of Cdc11, Cdc12 and Cdc10 respectively (Lindsey and Momany 2006, Momany *et al*. 2001). AspB was reported as the mostly expressed septin, and it localizes at the sites of septation, branching and the junction between conidiophore layers (Westfall and Momany 2002, Hernández-Rodríguez *et al*. 2012). During septation, AspB forms a ring at the septation site, and splits into two rings, where one goes into the basal cell, and the other apical one remains through the septation process (Westfall and Momany 2002, Figure 1-6). However, the defective AspB function in temperature sensitive mutant does not lead to failed septum formation but a faint and thin septum (Westfall and Momany 2002). During the development of conidiophore, AspB is first localized at the foot cell in hyphae, and then accumulated at the vesicle as a

cap structure when the stalk tip is swelling to form the vesicle (Westfall and Momany 2002, Hernández-Rodríguez *et al*. 2012, Figure 1-7). AspB is localized as rings in each layer of sterigmata cells after the emergence of first layer uninuclear metulae (Hernández-Rodríguez *et al*. 2012). While the emergence of each layer in *A. nidulans* conidiophore progressing in the same pattern as bud emergence in budding yeast, together with the observed localization pattern of AspB, it implies that septins play essential roles in cell division and mitosis during conidiophore development. Focused on the phialide layer, a yeast bud site selection marker homologue axl2 has been reported to regulate the organization of AspB at the junction of phialide and spores, and also regulate the function of phialide in *A. nidulans* (Si *et al*. 2012).

Axl2 in *S. cerevisiae* and *A. nidulans*

In the spore germination process, the emergence of a gem tube from a swollen spore undergoes the switch from isotrophic growth to polarized growth in *A. nidulans*. The spore first expands and proceeds to nuclear condensation and division. During the isotrophic growth of conidial spore, water uptake is necessary for the swelling process to maintain turgor pressure. Then a polarity axis is established to specify the site for germ tube emergence. This polarity axis also directs the polarized growth via extension of the hyphal tip throughout the vegetative growth in *A. nidulans.* The best paradigm to understand the polarization in fungi is the bud emergence in budding yeast (Harris and Momany 2004). There are two budding patterns in *S. cerevisiae*, the bipolar pattern and axial pattern. The distinctive set of membrane-associated makers specify which pattern to use. The positional signal produced by the makers is then relayed to the GTPase Cdc42

by a Ras-related GTPase Rsr1/Bud1 complex (Wedlich-Soldner and Li 2008). As a result, Cdc42 is activated locally and in turn recruits morphogenetic machinery to the presumptive bud site to initiate bud emergence. In yeast, bud3, bud4 and axl1 are associated protein with axl2 to facilitate axial budding pattern, and bud8, bud9, rax1 and rax2 are landmarks for bipolar budding pattern (Chant 1999, Park and Bi 2007). Disruption of any of the axial budding genes bud3, bud4, axl1 or axl2 causes the switch from axial budding to bipoloar budding (Chant and Herskowitz, 1991, Roemer *et al*. 1996, Sanders and Herskowitz 1996, Cullen and Sprague 2002). Axl1 is expressed in haploid cells and closely associates with axl2 (Fujita *et al*. 1994). Bud3 is a GEP protein, and Bu4 is a GTP-binding protein, both of which are peripheral membrane proteins and localize as a rings at the bud neck in mother cells (Kang *et al*. 2013). Bud3 and Bud4 interact with septins as well (Chant *et al*. 1995, Chant 1999). Axl2 is a cell wall protein that serves as a marker to promote the axial budding in *S. cerevisiae*, which localizes to the incipient bud site and also bud neck (Roemer *et al*. 1996). Bud3 and Bud4 are required for the localization of axl2 to the cortex by a secondary pathway (Roemer *et al*. 1996). Axl2 was first found in yeast to be the suppressor of Δ*spa2* Δ*cdc10* double mutant that axl2 can rescue this lethal mutants (Roemer *et al.* 1996). Cdc10 is one of the septins in yeast, and Spa2 is a scaffold protein that interacts with other bud selection components in yeast (Sheu *et al* 1998, Longtine *et al*. 1996). Axl2 is expressed at peak level in G1 stage at the bud neck site (Roemer *et al*. 1996). For the bipolar budding pattern, Bud8 is distal and Bud9 is proximal pole makers (Chant 1999, Kang *et al.* 2004). Rax1 and Rax2 are membrane proteins that can form complex with Bud8 and bud9 to promote bipolar budding pattern (Kang *et al*. 2004).

Key modules in the yeast budding site regulatory system are absent or poorly conserved in *A. nidulans*. One of the poorly conserved gene axl2 has been studied in detail, and it plays no obvious role in polarity establishment in *A. nidulans* (Si *et al.* 2012). Instead, axl2 is involved in phialide morphogenesis, and septin organization at phialide (Si *et al*. 2012). Axl2 protein sequence contains two tandem cadherin domains at N-terminus (Si *et al.* 2012). Cadherin (calcium dependent adhesion molecules) functions in cell adhesion to ensure cells binding together (Hage *et al*. 2009). It associates with Catenin protein by protein-protein interaction (Nelson and Nusse 2004). During conidiophore development, Axl2 is localized at the junction between phialide and spores (Figure 1-8, Si *et al*. 2012). In the background of Δ*Axl2*, the hyphal morphology shows no difference with the wild type. But it exhibits striking different conidiophore structure than the wild type (Figure 1-9, Si *et al*. 2012). Rather than the long chains of conidial spores produced by wild type conidiophore, the phialides in Δ*Axl2* only bear one or two layers of spores. It is reported that Axl2 may play a potential roles in *A. nidulans* to facilitate the repositioning of the cell division modules. The expression of axl2 is controlled by BrlA and AbaA in *A. nidulans* (Si *et al*. 2012). During conidiophore development, the phialide is the dividing line between acropetal and basipetal growth pattern. Axl2 may serve as the marker at the phialide-spore junction, so the required repositioning of morphogenetic machinery from newly produced spores to phialide tip can be achieved (Si *et al*. 2012). In the absence of Axl2, the machinery could not be directed back to the phialide tip for a new round of sporulation showing as the phenotype in Δ*axl2* mutant. And also Axl2 may involve in the organization of septins in phialide tip

as well (Si *et al.* 2012). Axl2 could function though protein-protein interaction as the interaction between cadherin and catenin.

Gin4, Hsl1, and Kcc4 in *S. cerevisiae*

In eukaryotic cells, the progression of cell cycle is mediated by cyclin dependent kinase (CDK) and cyclins (Nasmyth and Hunt, 1993). Expression of specific cyclins and binding of cyclin regulatory subunit and CDK activates the cell cycle progression (Kaldis 1999). In *S. cerevisiae*, there is only one CDK subunit Cdc28 that drives the progression of cell cycle. Phosphorylation of G1 cyclins Cln1p-Cln3p by Cdc28 initiates cell division, whereas Cdc28's binding to G2 mitotic cyclins Clb1p and Clb2p switches cell growth from the apical growth to isotrophic bud growth (Altman and Kell, 1997). The apical bud growth is restricted at the bud tip whereas the isotrophic growth is not limited but more towards to a rounded growth over the surface of the budding cell (Lew, 2000). The correct cell cycle progression is regulated as the way that the next event depending on the accomplishment of previous event, termed as checkpoints (Hartwell and Weinert 1989). To determine the entry timing of mitosis, it is regulated by the balance of two proteins yet with opposite functions on Cdc28: an inhibitory kinase Swe1 (a Wee1-related kinase) and an activator Mih1 (Okuzaki *et al*. 2003).

The organization of septin assembly is regulated by the function of a signaling network consisting of protein kinases Hsl1, Gin4, Kcc4 and other protein kinases (Barral *et al* 1999, Longtine *et al*. 2000). Hsl1, Gin4 and Kcc4 are homologous protein kinases which are localized at the bud neck in yeast (Okuzaki and Nojima 2001). Hsl1p is

claimed as a Swe1 negative regulator that degrades Swe1p in G2 and M phases in *S. cerevisiae* (McMillan *et al*. 1999). Deletion of hsl1 gene leads to prolonged G2 stage (Ma *et al*. 1996), but mutation of *hsl1* or *kcc4* produces no obvious defects in septin organization in bud neck (Longtine 2000). During bud emergence, Hsl1 is associated with Hsl7 around the bud neck at the daughter cell side (Barral *et al.* 1999). It was suggested that Hsl7 may be the substrate as Swe1 for the Hsl1 kinase (Lew 2000). gin4 in budding yeast on the other hand, is also not essential for cell growth, but it is required to ensure the proper mitotic progression, cytokinesis and septin localization at bud neck (Okuzaki *et al*. 1997, Longtine *et al*. 1998, Wu *et al.* 2010). Gin4 works with mitotic cyclin to promote cell cycle progression through mitosis (Altman and Kellogg 1997). When cell enters mitosis, Gin4 protein becomes phosphorylated under control of mitotic cyclin Clb2. Gin4 kinase activity is activated upon phosphorylation (Altman and Kellogg 1997). Malfunction of Gin4 delays mitosis and causes elongated bud growth. Gin4 also involves in the proper formation of septin assembly at bud neck. Δ*gin4* mutant shows reorganization of septin resulting in defective cell separation (Longtine *et al*. 1998, Gladfelter *et al.* 2002). And during mitosis, Gin4 kinase was found to associate with septin proteins implying that septin is potentially a Gin4 kinase target (Mortensen *et al*. 2002). Moreover, combined deletion mutant of *hsl1*, *gin4* and *kcc4* leads to much worse phenotype of elongated cell shape which is similar to septin deleted mutants (Barral *et al.* 1999). This result suggests that they function together in septin ring formation (Barral *et al.* 1999). The specific function of Kcc4 remains unknown (Longtine *et al*. 1998). Homologues of hsl1, gin4 and kcc4 have been found in multicellular organisms, such as *A. nidulans* and *Candida albicans*. But there is only one copy instead of three individual

ones in *A. nidulans*. Interestingly, it is claimed as Gin4 in *A. nidulans*, but named as Hsl1 in *Candida albicans*. It is tempting to speculate that these genes in filamentous fungi may possess the similar important function in the budding-like process such as sporulation as in budding yeast.

Prospective and aims

Due to its high degree of cell differentiation and the importance to the fungal sporulation, conidiophore development in filamentous fungi has long been studied and are still attracting interest from microbiology researchers. Phialide, as an essential sporogenous part in conidiophore structure and a unique switching system between distinctive growth and division patterns, deserves more attention to reveal the mechanisms underlying the morphogenesis of its development. There are many fascinating myths waiting to be untangled and uncovered, this dissertation focuses on the conidiophore morphogenesis during asexual development, more specifically the regulation of phialide morphogenesis. Although much progress and advancement to understand the biological processes have been made using filamentous fungal models such as *Candida albicans*, *Neurospora crassa*, and *A. nidulans*, there is still much unknown underneath the iceberg, such as polarity growth, cell differentiation, and asymmetric division. Especially for the studying phialide morphogenesis in this dissertation, *A. nidulans* may serve as one of the frontier models with phialidic conidiation in that the regulatory pathway for conidiophore development has been well characterized.

Even though there are some reported genetic modules that involve in regulating phialide function, the broad range of key proteins in this biological process still remains unknown. The aim of this thesis is to develop strategies to study the morphogenesis in phialide development with gene differential expression assessment and phenotype based functionality analysis. These strategies focus on identification of key genetic modules involved in phialide development, which may regulate the morphogenesis of phialide. This includes searching for genes with specific phialidic functions and generation of gene disruptive mutant strains.

Figure 1-1. Morphological development of conidiophore structure in *A. nidulans*.

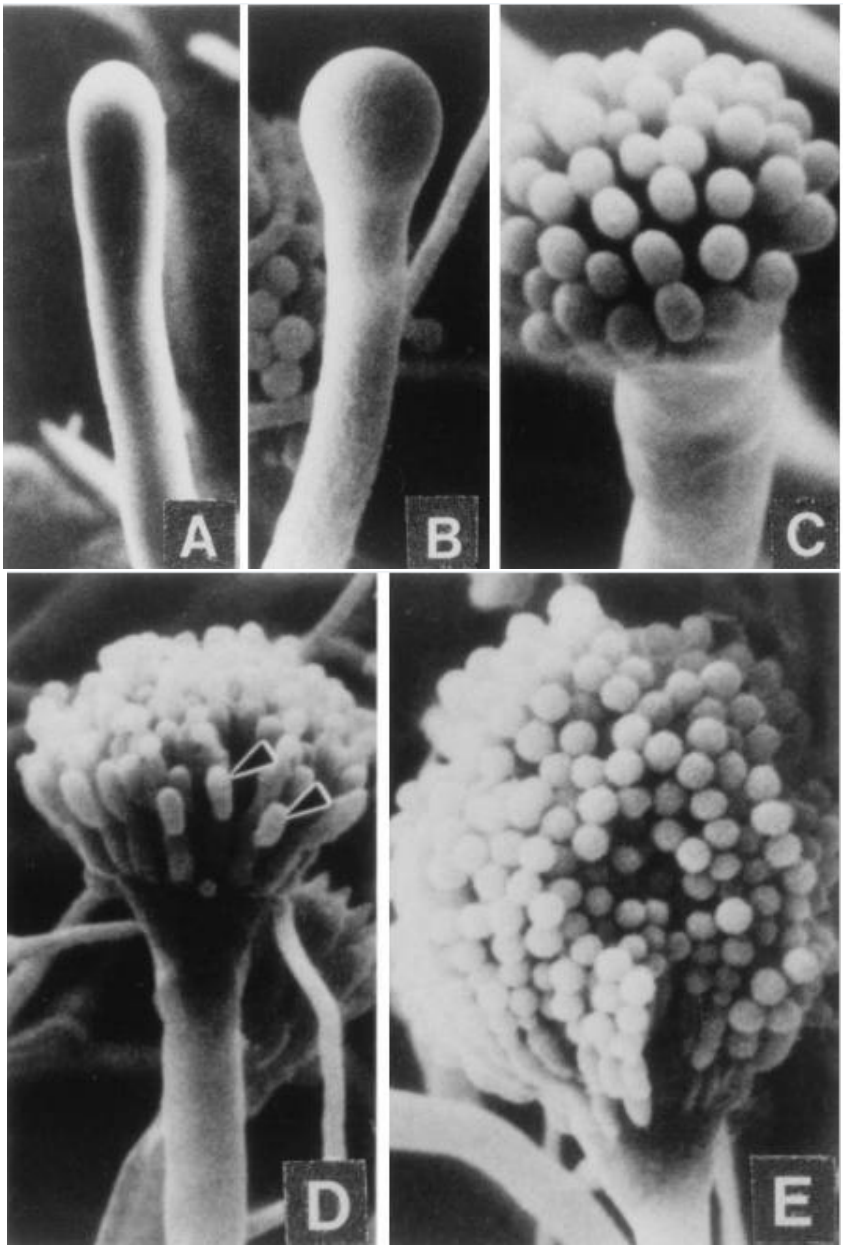

Figure 1-1. Morphological development of conidiophore structure during conidiation in *Aspergillus nidulans*. Panel A: the elongated aerial supporting cell stalk, B: the swelling hyphal tip vesicle, C: uninuclear cell metulae, D: uninuclear cell phialides, E: conidial spore in chains (Timberlake 1993).

Figure 1-2. Conidiophore morphology in *brlA* disruptive mutant

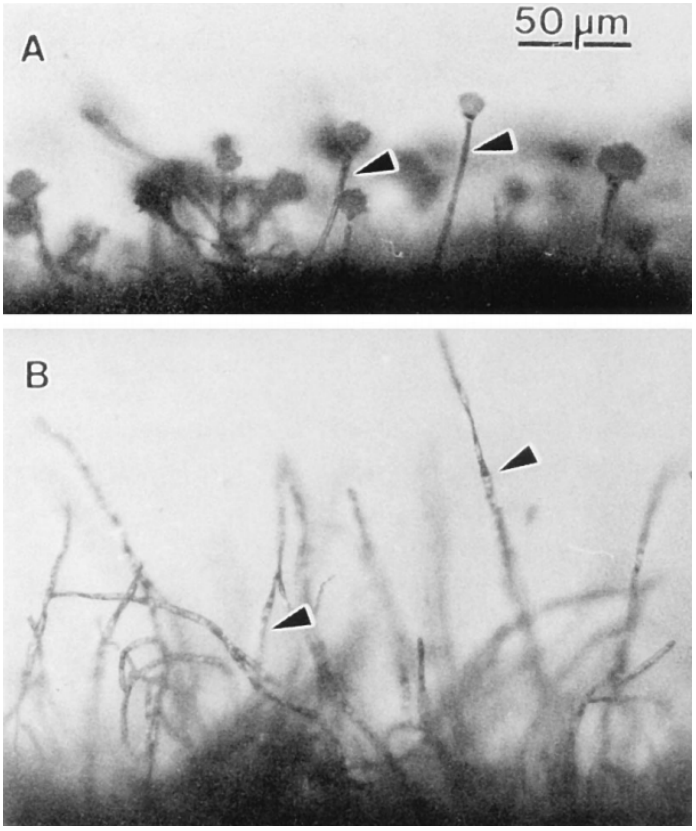

Figure 1-2. Indeterminately extended stalk in Δ*brlA* mutant. A: the wild type conidiophore development. The additional conidiophore structures are formed after stalk elongation. B: in Δ*brlA* mutant, stalk extends indeterminately (marked by arrows) and fails to form vesicle or generates other conidiophore structures (Adams et al. 1988).

Figure 1-3. Over-expression of brlA leads to sporulation in submerged culture.

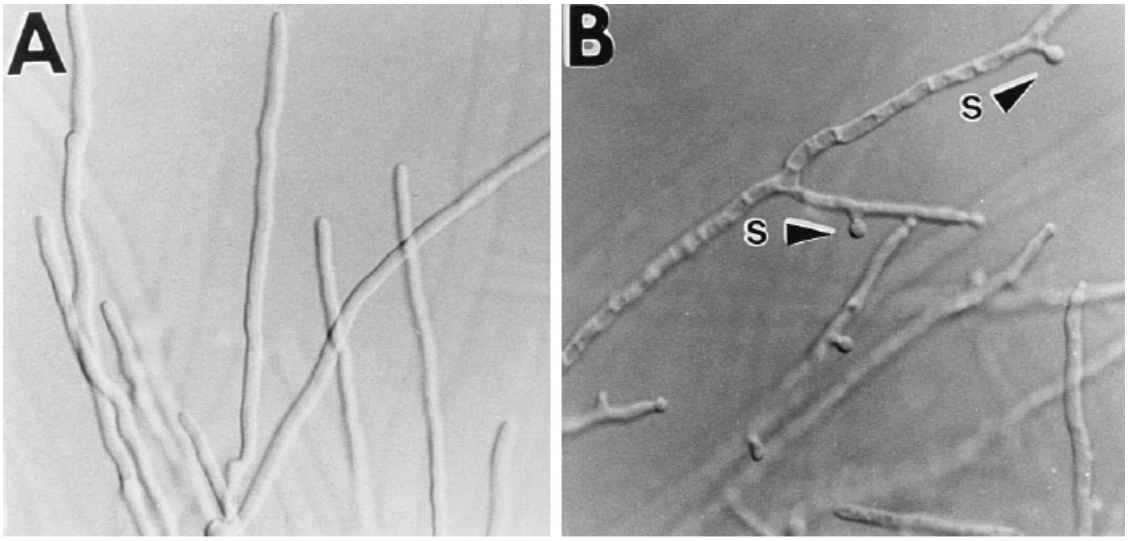

Figure 1-3. The brlA is under the control the alcA-promoter, which is induced by alcohol. Wild-type (A) and *alcA(p)::brlA* (B) strains were grown for 12 hours in liquid minimal medium and then transferred to alcA inducing medium. After 24 hours, wild type exhibits no conidiation, whereas only after 3 hours, overexpressed brlA strain produced conidial spores on hyphae (Adams *et al.* 1998).

Figure 1-4. Micrographs of conidiophores in null *abaA* strain and wild type strain.

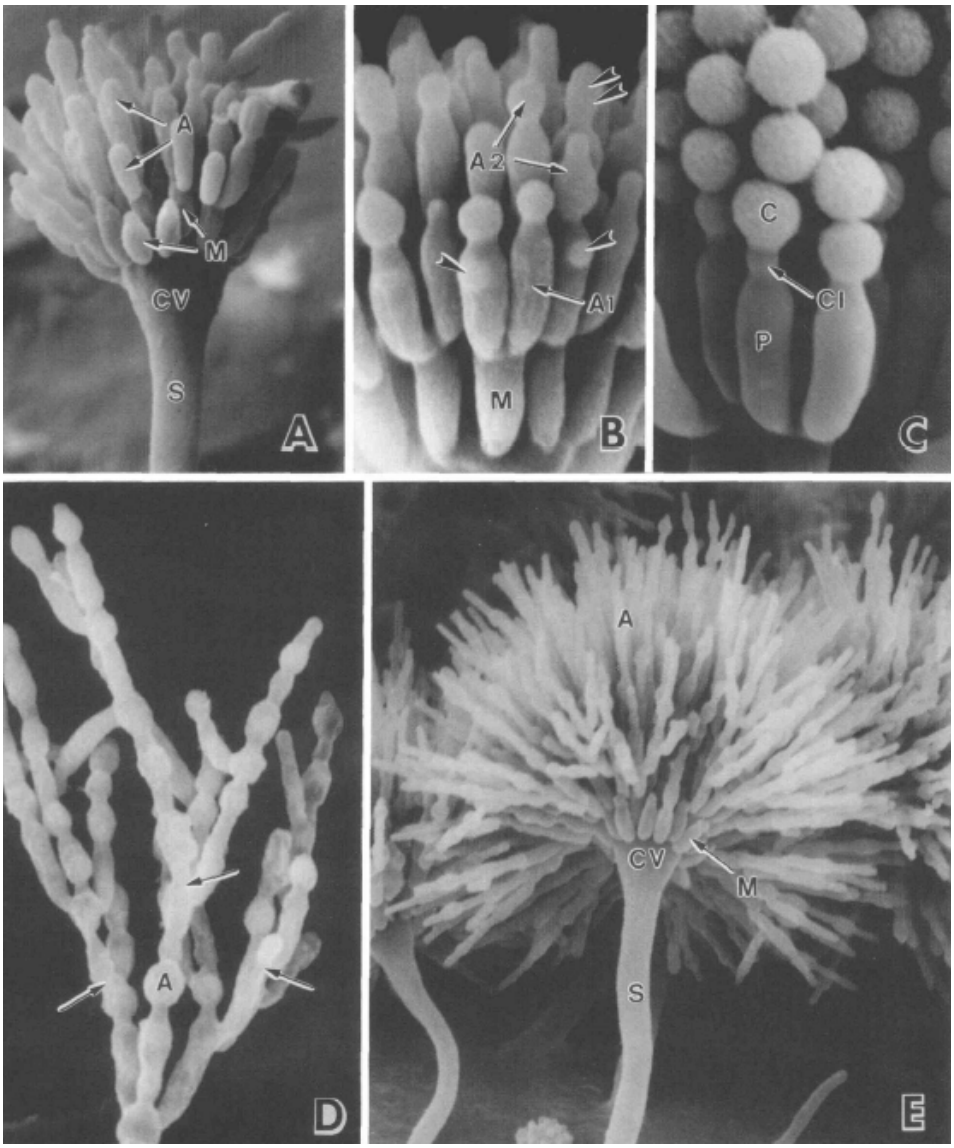

Figure 1-4. Micrographs of conidiophores in null *abaA* strain and wild type strain. Panel A: normal stalk (S), vesicle (CV) and metulae (M) in mutant strain, B: abacus structures (A) formed by budding from metulae (M), C: normal phialide structure (P) with conidia spores (C) in wild type strain, D: apical and lateral abacus structures (A), E: overview of abnormal conidiophore structure (Sewall *et al*. 1990).

Figure 1-5. Regulatory network that regulates the development of conidiophore in *A. nidulans.*

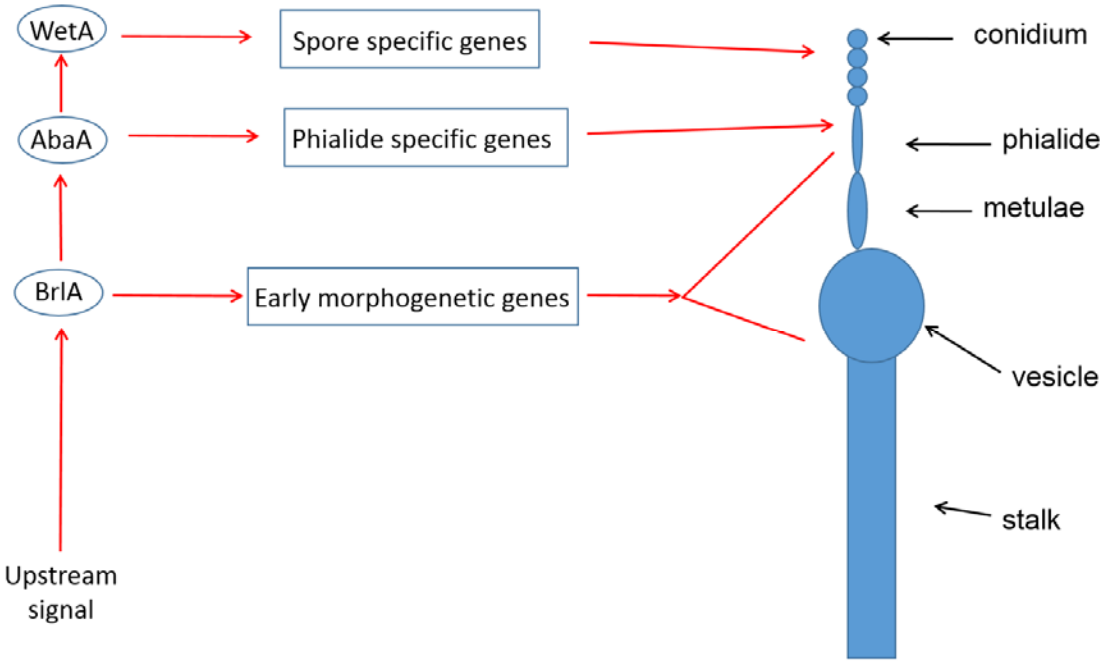

Figure 1-5. Regulatory network that regulates the development of conidiophore in *A. nidulans.* Note that BrlA is required for activating early morphogenetic genes and function through the development until phialide, AbaA is required for phialide-specific genes expression, and WetA is required for spore-specific genes expression (reproduced from Timberlake 1990).

Figure 1-6. The localization of AspB and actin during septation.

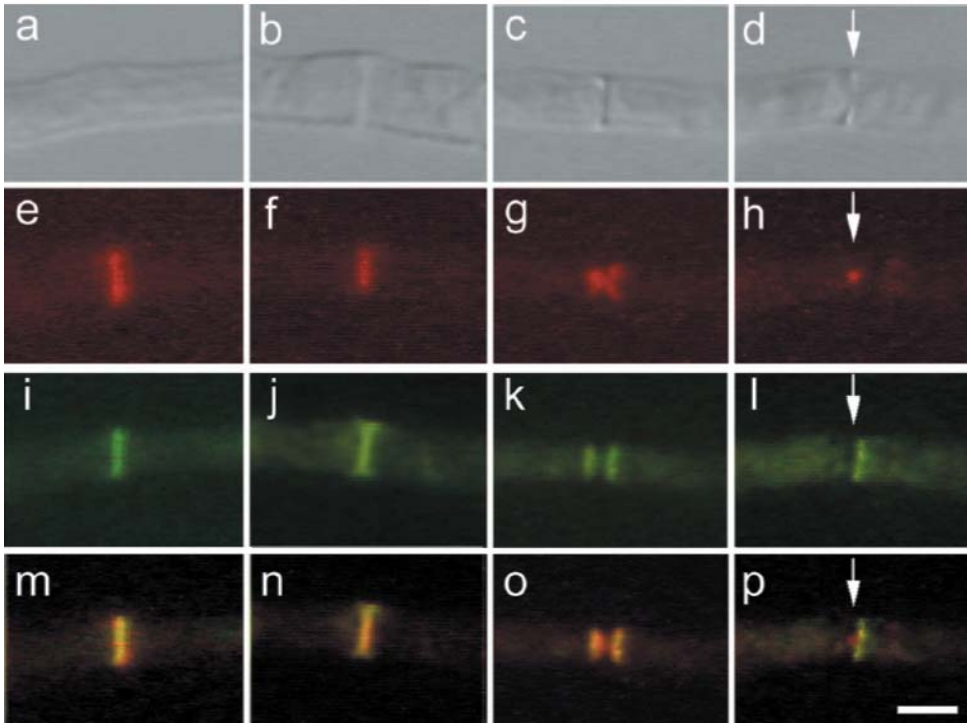

Figure 1-6. The localization of AspB and actin during septation. The first row shows the DIC image of septum. The second row indicates the actin localization. The third row illustrates the localization of AspB. And the fourth row is the combined view of both actin and AspB localization. Notice that the AspB ring splits into two rings in K, and the basal one is disappeared whereas the apical one remains in I. Bar = 5 μm. (Westfall and Momany 2002)

Figure 1-7. The localization of AspB in conidiophore.

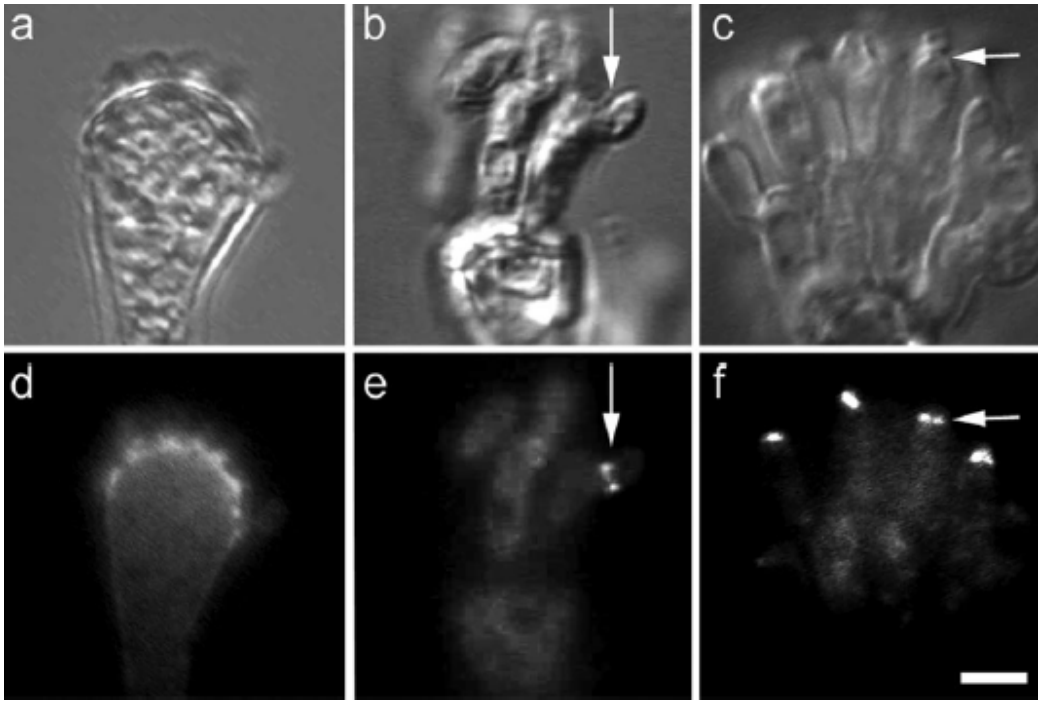

Figure 1-7. The localization of AspB in conidiophore. The first row shows the DIC images, and the second row indicates the AspB localization. Note that AspB localizes at the vesicle/metulae interface in D, metulae/phialide interface in E, and phialide/conidia in F at different stages. Bar = 5 μm. (Westfall and Momany 2002)

Figure 1-8. The localization of Axl2-GFP in conidiophore.

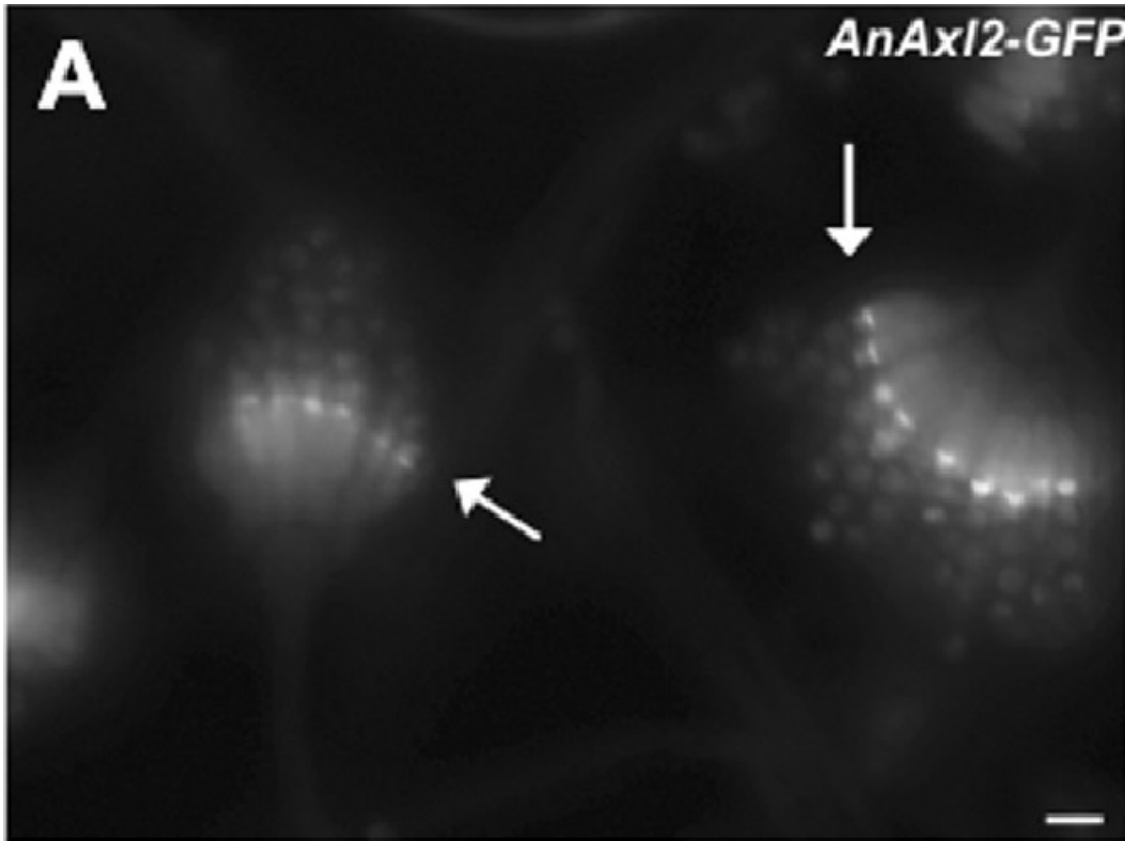

Figure 1-8. The localization of Axl2-GFP in conidiophore. Note that it is localized at the phialide-spore junction. Bar = 10 μm. (Si *et al.* 2012)

Figure 1-9. Phenotype of defective conidiophore in Δ*axl2*.

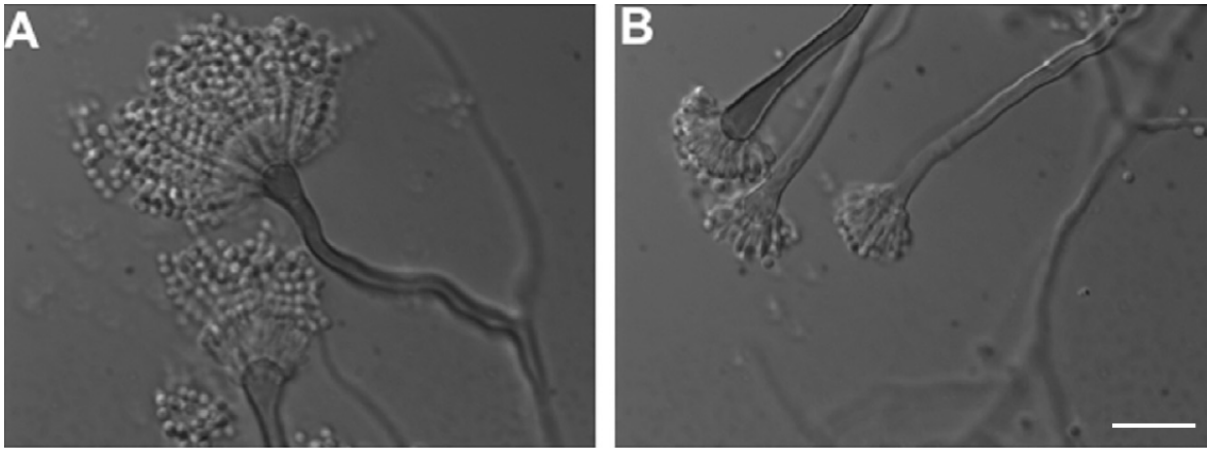

Figure 1-9. Phenotype of defective conidiophore in Δ*axl2*. Left panel is the wild type conidiophore, the right panel shows the defective conidiophore in Δ*axl2*. Bar = 10μm.

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Chapter II Differentially Expressed Genes during Phialide Development in *Aspergillus nidulans*

Abstract

The asexual reproduction in *A. nidulans* is implemented as conidiation. A complex conidiophore structure is developed during asexual reproduction process, and phialides are essential sporogenous cells in the conidiophore structure to generate conidia spores. AbaA is the key transcriptional factor controling the formation of phialide, but the genes which are directly regulated by AbaA and involved in phialide morphogenesis are unknown. In this study, twelve genes that are upregulated by AbaA and potentially involved in phialide morphogenesis were identified by a screening method to select differentially expressed genes. Expression of these genes is elevated when abaA gene is induced. With semi-quantitative RT-PCR, we successfully confirmed that these genes are induced during developmental stage. These genes have homologues in closely related species containing abaA homologues. And AbaA specific binding sites can be found in the promoter regions of 10 out of 12 genes. Our result suggests that most of the 12 genes have potential to be regulated by abaA during transcription. This screening workflow provides a new way to identify potential functional genes in morphogenesis for further functionality analysis.

Introduction

AbaA in conidiophore development

In *A. nidulans*, there are two morphologically distinctive life cycles, the vegetative and the secondary development (Casselton and Zolan 2002). In developmental stage, *A. nidulans* reproduces itself effectively by producing sexual ascospores or asexual conidial spores. The asexual reproduction is the dominant form of propagation. In asexual developmental stage, conidial spores are produced by the specific conidiophore structure. BrlA, AbaA and WetA are three important transcriptional factors that regulate the development of conidiophore during conidiation. brlA is required for the activation of proteins in all developmental steps (Prade and Timberlake 1993). AbaA is induced by brlA and provides positive feedback to brlA during conidiophore development. It is required for phialide differentiation (Sewall *et al*. 1990). WetA is required for the formation of conidial spores (Marshall and Timberlake 1991). In this study, we focus on the phialide formation, which is regulated by AbaA. In *A. nidulans*, a switch of cell division form happens in phialide that makes it so unique. The division pattern until phialides is in an acropetal pattern that new cells bud from the tip of the old cells. On the other hand, spores are produced from phialides by a basipetal pattern. The morphogenetic machinery is required to relocalize from the tip of the new spores to the phialide-spore junction so that the next new spore can be produced. In this study, we focus on the phialide morphogenesis. Axl2 is a marker at the phialide-spore junction to enable this switch and repositioning (Si *et al*. 2012). As axl2 is regulated by AbaA, and involves in phialide development, our hypothesis is that there are broad range of protein regulate the formation of phialide and are regulated by AbaA. In order to identify these genetic

modules, a RNA-seq can be used to screen differentially expressed genes in the abaA induced strain. In the next section, the RNA-seq used to investigate differential gene expression is introduced.

Differential expression of gene, and RNA-seq

The production of proteins in cells is regulated by gene expression through transcription and translation. Transcription refers to the process of messenger RNA (mRNA) being generated by copying DNA, whereas translation is to generate amino acid sequences based on the information carried in the mRNAs. Biological processes in cells are controlled by the activity of proteins, in a way that how much mRNA is transcribed or translated for individual genes. The regulation of gene expression is required since the cellular system needs to prevent unnecessary expression of unneeded genes, or boost expression of needed genes. This regulation is executed in the transcriptional stage as the first throttle. The RNA components called transcriptome in cells reflects a balance of RNA synthesis and decay, which is regulated for expression control. Exploring the composition of transcriptome is significant to advance our knowledge in the subtle genetic regulation of biological processes. Many techniques are available nowadays to help researcher assessing the gene expression level, including serial analysis of gene expression (SAGE), semi quantitative reverse transcription PCR (RT-PCR), microarray, and RNA sequencing (RNA-seq). Among these advanced techniques, microarray and RNA-seq are high throughput methods to investigate large scale gene expression and have been used broadly.

In contrast to forward genetics, which is normally used to identify genotypes of interesting mutagenesis generated strains, reverse genetics is to predict the function of interesting genes by phenotypic analysis. Differentially expressed genes under specific conditions are considered as good candidates for reverse genetic research. When a gene starts to express, the first step happens as transcription where the RNA polymerase enzyme binds to the DNA and copy it into a complimentary sequence of RNA. The regulation of transcription is the key to tightly adjust gene expression in cell. In eukaryotic cells, transcription is restricted by nucleosome, which consists of a segment of DNA wrapped by histone proteins H3, H4, H2A and H2B (Struhl 1999). The activation of transcription requires the release of this complex structure to provide accessibility of DNA to transcriptional enzymes and factors (Bernstein *et al*. 2004). The regulation could also happen during transcript elongation, in which the RNA polymerase travels along and copies the template DNA strand to synthesize pre-mRNA. Pausing of RNA polymerase facilitated by pausing factors NELF (negative elongation factor) and DSIF (DRB sensitivity-inducing factor) complex, and resuming mediumted by P-TEFb (positive elongation factor b) is the main maneuver for elongation control, which limits the rate of expression for many genes (Ghosh *et al*. 2011, Kwak and Lis 2013). RNA processing including capping and polyadenylation is the last step of transcription and controlled by transcriptional regulation (Wang *et al*. 2008, Yang *et al*. 2011). Capping and polyadenylation process adds a modified guaning nucleotide cap to the 5' end of a transcript, and a long tail of adenine nucleotides to the 3' end, which ensures the exportability and stability of mRNA transcript. Since the composition of transcriptome reflects the gene expression, analyzing transcriptome in cells could extend our

understanding in the whole biological system, particularly the expression levels of transcripts. Next generation sequencing (NGS) technology producing high throughput sequences has been widely implemented to identify the mRNA transcripts in a sample (Chu and Corey 2012). The actual sequencing subject is cDNA, which was generated from mRNA by reverse transcription during sequencing library preparation and PCR amplification. There are several NGS platforms available in the market: Illumina, PacBio, SOLiD, Roche454, etc. While they apply different biochemistry steps and processing methods, millions of short sequencing reads are generated from each cDNA fragment. This approach has been utilized by researchers for years to quantitate the expression level for each transcript and gene. Other applications of RNA-seq include discovery of novel gene, single nucleotide polymorphism, and alternative splicing (Griffith *et al*. 2010, Barbazuk *et al*. 2007). The RNA-seq experiment consists sequencing library preparation, sequencing, and RNA-seq data interpretation, which was outline by Wang *et al*. (2010, Figure 2-1). A further application of gene expression analysis by RNA-seq is to explorer differentially expressed genes between samples. To assess differentiated gene expression, transcript abundance is quantified by mapping the short sequence reads to known reference genome, followed by statistical testing of the quantification difference between samples (Oshlack *et al*. 2010). There are many free open source software available for quantification or statistical analysis, most of which are based on R or Pyhon that researcher can comprehend acquired RNA-seq data. Most offered tools for RNA-seq data processing and analysis can be found on http://en.wikipedia.org/wiki/List_of_RNA-Seq_bioinformatics_tools. Even though NGS or RNA-seq produces abundant data from samples, there are complications generated by these technologies too. The sequencing

reads produced by high-throughput sequencing are normally very short compare to the traditional sequencing methods, which leads to the loss of information to assemble the whole structure of transcripts. And short read sequences associated with high throughput inevitably results in the production of huge dataset files, ranging from gigabytes to terabytes. This imposes some obstacles for researchers that both giant data storage and a powerful computing server are required. And since NGS is based on amplification, technical errors and amplification bias are introduced into the process as well to further aggravate this complication. While NGS technology is improving, read length and assembly algorithms have been advanced greatly. Current way to resolve the issues associated with NGS is to invent more efficient algorithms, but more importantly, to develop low quantity RNA-seq without amplification (Ozsolak *et al*. 2010) and direct RNA molecule sequencing (Ozsolak *et al*. 2009, Lipson *et al*. 2009).

The advancement of RNA-seq by sequencing technology has immensely reduced the cost and time required, and increased the yield of sequencing data. In RNA-seq experiments, samples of extracted RNAs are first sheared and reversely transcribed into cDNAs, which is then sequenced on a high-throughput NGS platform, such as Illumina sequencer. The raw sequence reads are mapped to a reference genome (or transcriptome) and counted to link the transcript mRNA level with individual genes to assess the relative gene expression level. As the gene expression is quantified by mapping RNA-seq reads onto annotated reference genome and acquiring the counts for each gene, the Poisson distribution and negative binomial models have been used to model the count data. The R/Bioconductor software package edgeR (Robinson *et al*. 2010) was developed to assess the differential expression of replicated gene count data using an over dispersed Poisson

model. The edgeR tool tests the differential expression by pairwise comparison of counts, and it is also applicable to test count data from other resources as well. The negative binomial parameterization corresponding to over dispersed Poisson model successfully separates biological meaningful variation from technical variation. The significance testing of differentiation for each gene is accommodated by the exact test function (Robinson and Smyth, 2008).

We used RNA-seq to identify AbaA regulated genes. In next section, the method for identification is introduced.

Identify AbaA up-regulated genes

While AbaA is known as the key transcriptional factor that controls the formation of phialide, genes directly regulated by AbaA and involved in phialide morphogenesis are poorly known. In this chapter, we searched for potential phialide related genes by differential gene expression analysis. We compared the gene expression levels in wild type and mutant strain where abaA expression was induced. A gene pool for phenotypic analysis was created, in which genes with elevated expression in induced strains comparing with the wild type strain were selected. The cutoff for elevated expression is set as 10-fold, as axl2 has over 10 fold change when abaA is induced. Also, we used another dataset from Daniel Ebbole lab (unpublished) that RNA from wild type strain was extracted through 0-48 hours to assess the expression level for all *A. nidulans* genes in different growth stages. Genes with increasing expression during developmental stage (16-30 hour) will be selected since they are potentially related to conidiophore

development. A homology search against phialide-plus (*Penicillium, Fusarium, Exophiala*) and phialide-minus (*Ajellomyces*, *Paracoccidioides*) species was used to further select AbaA-induced genes, followed by a conserved domain search to predict hypothetic functions in development.

Here we selected genes in manageable size using RNA-seq screening, homology search, domain search. Genes were selected for RT-PCR verification and AbaA motif search to verify the result of our selection.

Materials and Methods

Strains, media and growth conditions

All *Aspergillus nidulans* strains used in this study are listed in Table 2-1. Stock strains were stored as form of mycelia in 30% (v/v) glycerol solution at -80°C. YGV (yeast extract glucose + vitamins) liquid medium, MN (minimum) medium, MN-threonine medium, and MAG (malt extract + vitamins) medium were made for culturing. MN medium was prepared as described by Kafer (1977). The pH of medium was adjusted to 6.5 with 1.0 N Nacl solution. MNV-threonine medium was made according to Pearson *et al.* (2004). MAG and YGV medium were prepared as described by Harris *et al*. (1994). Uracil/Uridine (UU) and/or Vitamin mix (V) were supplemented in mentioned media as needed to identify strains containing auxotrophic markers. A28 wild type and induced TTA1 (*alcA::abaA*; *abaA*, Mirabito *et al*. 1990, prepared by Haoyu Si) strains were used to extract total RNA for RNA-seq.

RNA extraction from A. nidulans and RNA-seq

Spore suspension of *A. nidulans* strains were cultured in liquid medium for 12-14 hours. Mycelia harvested from liquid culture were filtered through sterile filter paper, and transferred into a precooled mortar. With addition of liquid nitrogen, mycelia cluster was grounded to fine powder using a pestle. Approximately 1.5 gram of dry mycelium powder was mixed with Invitrogen Trizol agent (Invitrogen Inc., Carlsbad, CA) in 1.5 mL Eppendorf tube. Tubes were incubated at room temperature for 5min and then centrifuged for 15min at 12,000 x G. The supernatant was moved into a new tube with addition of 200μL isoamyl chloroform (1:25). Tubes were shaken to mix and then centrifuged for 15min at 12,000 x G. Aqueous phase was transferred to a new tube, and incubated for 10min after adding 500μL 2-propanol. Precipitated RNA was pelleted by centrifuge at 12,000 for 10min, and washed twice with 70% ethanol. RNA pellet was dried at 42°C for 5-10min and re-suspended in 40μL RNA storage solution (5 mM EDTA, 5% SDS) and stored at -20°C. RNA samples were sequenced in the Core Research Facilities in University of Nebraska-Lincoln with an Illumina Genome AnAlyzer II system. One sample of total RNA for each strain was sequenced. Single end reads with 35bp read length were generated. Around 10 million reads were produced for each strain.

Reference genome and building index

The genome sequence of *A. nidulans* was downloaded from Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiDownloads.html). Bowtie/TopHat was used for read mapping. The index of the reference genome was built with the following command line:

> bowtie-build –f nidulans_genome.fa nidulans

Mapping short reads with TopHat

TopHat (Trapnell *et al*. 2009) alignment tool was used to align the short reads to the reference genome. The options we used are: default 2 mismatches allowed, multiple matches not allowed but only unique matches:

>tophat --segment-mismatches 2 --fusion-multireads 1 nidulans <reads_file>

Output of mapped reads was in binary alignment/map format (BAM), a compressed binary version of the sequence alignment/map format (SAM) (Li *et al*. 2009). BAM format file was converted to SAM file with SamTools.

Transcript counts from RNA-seq alignment

To count the reads mapped to each gene, HTSeq (Anders *et al*. 2014) was used. The alignment file in SAM format was fed into HTSeq count tool as input for counting reads. The gene annotation file in GFF format was downloaded from AspGD

(http://www.aspergillusgenome.org/download/gff/). The default option was used to generate output as a table with counts for each gene:

>htseq-count <sam_file> <reference_file>

Gene differential expression analysis

Differential expressed genes were identified based on RNA-seq read counts. RNA-seq count data from *A. nidulans* wild type strain FGSC A28 wild type strain and alcA promoted abaA overexpressed strains were compared. The mRNA abundance comparison between wild type strain and the overexpressed strain was conducted using Fisher's exact test provided by edgeR package in R Bioconductor (Robinson *et al*. 2010).

RPKM calculation

RMKM data of all genes in wild type (Daniel Ebbole, unpublished) through 0-48 hours were used to assess the gene expression change. RPKM, reads per kilobase per million, is used to eliminate the counting bias caused by gene length difference. Briefly, the RPKM is calculated as: $RPKM = \frac{10^9 C}{NL}$, where *C* is the number of mapped reads onto gene exon, *N* is the total number of mapped reads in the experiment, and *L* is the length of the coding regions of each gene (Mortazavi *et al*. 2008).

Homologous analysis and conserved domain serach

Possible homologues of *A. nidulans* genes present in other Ascomycota species were identified using NCBI protein-protein BLAST search tool blastp with E-value threshold = 10^{-5} (Altschul *et al.* 1990, https://blast.ncbi.nlm.nih.gov/). Conserved protein domains were identified using NCBI Conserved Domain Search (Marchler-Bauer, *et al*. 2011, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

AbaA binding site search

Motif identification was performed using the motif finding tool MEME (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Bailey and Elkan, 1994). If the distance from the gene to the upstream neighbor gene exceeds 1000 base pairs, the AbaA binding site motif is searched within 1000 base pair range from this gene.

RT-PCR to verify gene expression level

FGSC A28 strain was grown overnight (12-14 hours) on MAG medium to produce biomass. Mycelia were collected using a vacuum filtering system with sterile filter paper (Whatman Qualitative Filter Papers 4.25 cm diameter, pore size 11µ) and then washed with 1X PBS buffer. Mycelia caught on filter paper was cut into 3 equal sized parts and transferred on MAG medium to induce conidiation. Mycelia samples were collected at 0, 4, 8 hours respectively after conidiation induction. Total RNA was extracted from each sample. RT-PCR was performed using DNase I Amp Grade, Oligo

(dt), SuperScript III Reverse Transcriptase and RNaseOUT (Invitrogen Inc., Carlsbad, CA) according to manufacturer's manuals for complementary DNA (cDNA) synthesis. cDNA was verified with gene-specific primers for semi-quantified gene expression analysis at the transcriptional level.

Results

Selection of twelve potential AbaA-related A. nidulans genes

Axl2 was up-regulated 24.4-fold in the AbaA-induced strain relative to the wild type strain. Here, we used the 10 fold-change as the cutoff to screen AbaA-induced genes. The screening result for analyzing RNA-seq count data revealed more than 200 genes falling into the ">10 fold increased expression" category. In order to reduce the size of this gene pool, we did homology search with closely related Ascomycota fungal species including *Penicillum, Fusarium* and *Exophiala*. These species are reported to have abaA homologues (Son *et al*. 2013, Vanittanakom *et al*. 2006, and Liu *et al.* 2004). AbaA-induced genes are expected to have homologues in these species. Since the early to middle development in *A. nidulans* occurs at 5-15 hours after induction, phialide related genes are presumably to be expressed during this time point. A further investigation was conducted to search for increasingly expressed genes during early development stage, by comparing each gene's RPKM in *A. nidulans* timely extracted RNA-seq data (Daniel Ebbole, unpublished) at 0-48 hours after culturing. A total of 12 genes were selected by filtering methods mentioned above. The log2 fold changes in induced abaA strain for the 12 genes are visualized in Figure 2-2. The RPKM changes over time for the screened 12

genes are shown in Figure 2-3 and Table 2-2 (RPKM values in Figure 2-3 were normalized to range in 0-20 to show the overall PRKM trend of 12 genes).

Homology of 12 genes in other Ascomycota species

The predicted protein sequences of 12 selected genes are available in the annotated *A. nidulans* genome in the Broad Institute database. In order to find out the coexistence pattern of the 12 genes with abaA in other related fungal species, we did protein BLAST search against several Ascomycota genera and species. The homology search result for the selected 12 genes against other Ascomycota species is shown in Table 2-3 (Expect value less than 10E-5). Every gene is shown to have homologues in at least one fungal species with abaA homologue present, while some of the genes are more specifically consistent with abaA distribution than others.

Conserved domain search in 12 genes

The predicted protein domains for each gene are illustrated as in Table 2-4. Within these 12 predicted proteins, AN0499 has a chitin-binding Peritrophin-A domain (Pfam PF01607), which is often associated with a chitinase activity. As chitin is an essential component in the fungal cell wall, it implies that AN0499 is related to cell wall degradation in great diverse biological processes, such as the sporulation process. Gene AN11101 shows ~50% similarity of gin4, hsl1 and kcc4 in the budding yeast. AN11101 also has the serine/threonine protein kinases domain as its homologues in yeast. It is known that the organization of septin ring in bud neck is regulated by gin4 in yeast. The

role of septin (GTP-binding protein) in budding yeast ensures cytokinesis by forming septin rings at the bud neck as diffusion barrier. AspB in *A. nidulans* on the other hand, is the highest expressed member of septin family. It has been reported to localize at the branching site, septum, and also conidiophore structure. In conidiophore structure specifically, this septin protein is localized between vesicle and metulae during metulae formation, and then moved to metulae and phialide interface, finally anchored at the bud neck between phialide and spores during conidiation (Westfall and Momany 2002, Hernández-Rodríguez *et al*. 2012). Similarly to gin4 in yeast, AN11101 may regulate septin proteins organization in *A. nidulans*.

AbaA binding sites

In chapter I of this dissertation, the role of AbaA as a transcriptional factor in *A. nidulans* that regulates conidiophore has been reviewed, especially the phialide development. A binding motif of AbaA, CATTCY, is present in the promoter regions of AbaA-induced genes so that AbaA can regulate their expressions (Andrianopoulos and Timberlake 1993). We examined the sequences between 12 genes and their upstream genes to scan for specific AbaA binding sites. The upstream gene ID, distance to upstream genes in nucleotide and numbers of AbaA binding sites are shown in Table 2-5. Most genes have at least one AbaA binding site within 1000nt upstream. For the two genes (AN5841 and AN6403) in which the AbaA binding site is absent, we can still find the specific BrlA binding site (C/A-G/A-AGGG-G/A, data not shown). Considering the promoter region search result with the fact of the differential expression level in induced abaA strain, as well as their elevated expressions during the development stage, most of

the 12 genes might be regulated by AbaA, and the two exceptional genes are possibly regulated by BrlA.

Semi-quantitative RT-PCR

In order to determine the expression level change at different growing stages and verify the RNA-seq result, a semi-quantitative RT-PCR was conducted. The spores of wild type strain A28 were cultured in YGV liquid medium for overnight, and then mycelia were collected and grown on MAG plates, total RNA was extracted at 0 hour, 4 hour and 8 hour after growing on plates. Gene specific primers were used to assess the cDNA level for each gene after RT-PCR. Since the development is induced when the fungus is transferred from liquid medium to glucose medium, it is expected that genes involved in developmental stage would appear higher expression level at 4 hour and 8 hour time point. As shown in Figure 2-4, except for AN6929, all other genes had increasing expression level over and reached peak level at 8 hours. This fact that expression of 12 genes are induced during early developmental stage confirmed the result of our RNA-seq selection method.

Accordingly, these results suggest the possibility that some of the 12 genes are potentially regulated by AbaA in *A. nidulans*.

Discussion

Screening method for AbaA related genes

As RNA-seq data analysis has been used widely to identify specific target genes, it is also applicable for spotting specific morphogenesis genes. The purpose of this chapter is to identify potential AbaA regulated genes in *A. nidulans* for further genetic study by following criteria including AbaA upregulation, homology search, and conserved domain search and so on. We 12 such genes with our RNA-seq screening method. The main goal of this study is to identify genes involved in phialide morphogenesis. Since AbaA is known as the regulator for phialide development, genes we identified by this screening method are expected to be phialide related. Besides the differential expression level in AbaA-induced strain, the timing of expression of these 12 genes also suggests they are involved in asexual development in *A. nidulans*.

We used semi-quantitative RT-PCR to verify the expression level of the 12 genes at 0, 4 and 8 hour after induction. It is found that all of the 12 genes have minimal or abolished expression at 0 hour, increased expression at 4 hour and reach peak level at 8 hour after induction. Our results confirm the RNA-seq analysis, and implicate that these genes are specifically expressed during secondary developmental stage in *A. nidulans*. In our study, the RNA-seq data without replication was analyzed to select related genes based on differential expression. Hence we used semi-quantitative RT-PCR as a tool to verify our differentiate expression analysis, and from the results of the functionality analysis, our selected genes are related to our research interest. However, it would more applicable if repeats of RNA-seq are conducted in order to derive more confident RNA-seq analysis.

The occurrence of 12 genes in other Ascomycota species

Ascomycota is the largest phylum of fungi. As a diversely colonized habitant on earth, asexual reproduction is the predominant form that Ascomycota fungi undergoes to disperse and spread. The asexual reproduction in Ascomycota is conidiogenesis. While in *Aspergillus* and some other Ascomycota species, conidiophore is developed during conidiation, and phialide is formed as a sporogenous part. Besides *Aspergillus*, *Fusarium, Penicillum, Trichoderma* and *Exophiala* are also known to produce phialide. In our homology search of the 12 genes against other Ascomycota species, homologues of these genes have been found in at least one of phialide produced fungi. At this time, the correlation of these genes with phialide development is promising.

AbaA up-regulation

AbaA is the key transcriptional factor regulating the formation of phialide during conidiophore development. We found multiple AbaA binding sites in the promoter regions of our candidate genes. This result together with the elevated expression during AbaA induction suggests that expression of these genes is regulated by AbaA at the transcriptional level.

Functionality analysis of these 12 genes are necessary to discover their roles in *A. nidulans* developmental growth.

Figure 2-1. The overview of RNA-seq experiment and RNA-seq data analysis

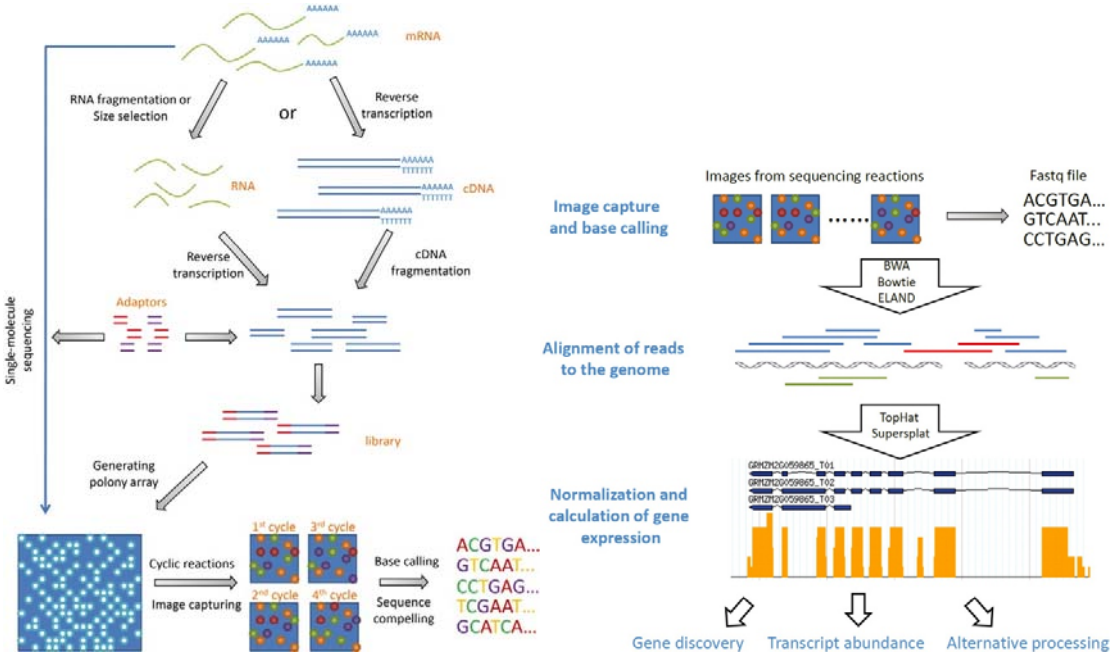

Figure 2-1. The overview of RNA-seq experiment and RNA-seq data analysis.

(Wang *et al*. 2010)

Figure 2-2. Log2 fold change of mRNA level in induced abaA strain and wild type strain of *A. nidulans*

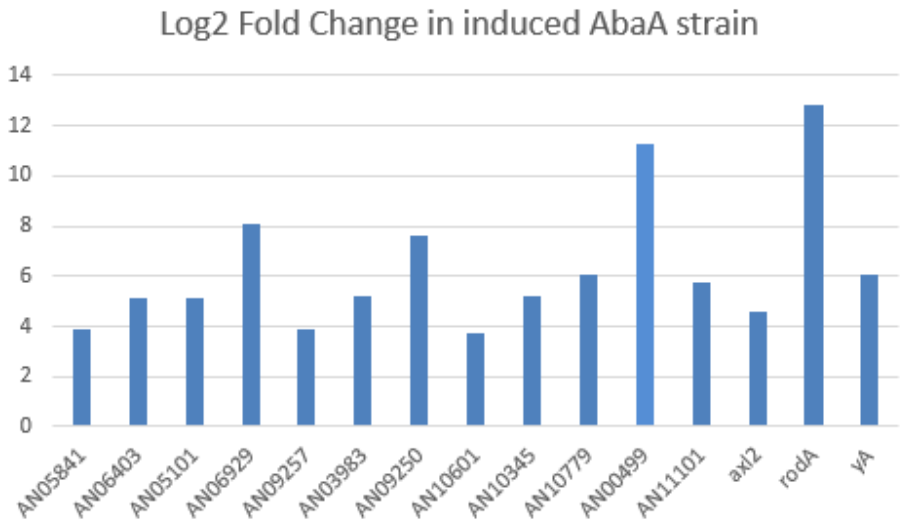

Figure 2-2. Logarithm fold change of mRNA level between induced abaA strain and wild type strain of *A. nidulans*. AbaA regulated genes (axl2, rodA and yA) as references

Figure 2-3. Normalized RPKM (in range 0-20) during 0-48 hours.

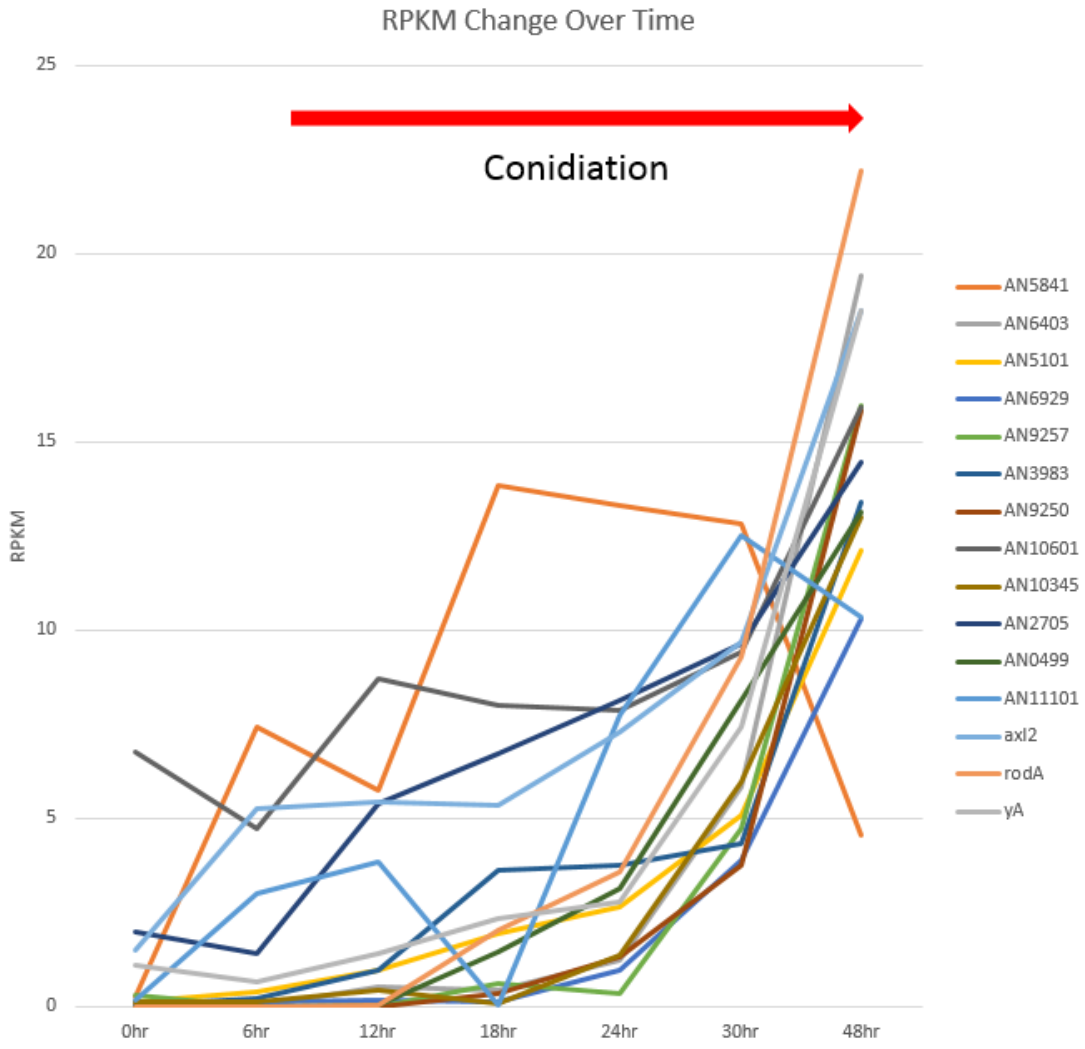

Figure 2-3. Normalized RPKM (in range 0-20) during 0-48 hours to show expression pattern over growing stages. Most genes exhibit the similar expression pattern as axl2, rodA and yA.

Figure 2-4. RT-PCR result.

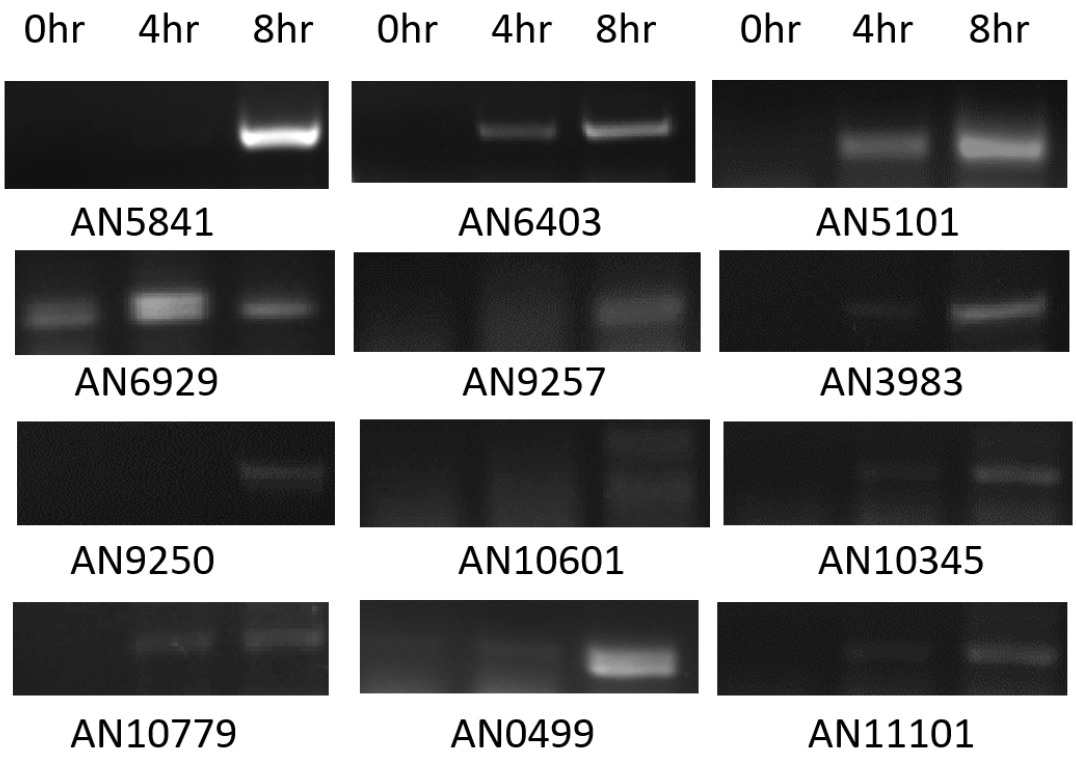

Figure 2-4. RT-PCR result showing the expression level at 0 hour, 4 hour and 8 hour after induction for the twelve genes. Note that every gene, except for AN6929, are induced at 4 hour and 8 hour, which is coincident with the early development stage.

Table 2-1. Strains used in this chapter

Strain	Genotype	Source
A28	*pabaA6 biA1*	FGSC
A1149	*pyrG89; pyroA4; nkuA::argB*	FGSC TNO2A3
TTA1	*alcA::abaA; abaA*	Mirabito *et al*. 1989

Table 2-2. RMKM of 12 genes and references in 0-48 hours after induction

ID	0hr	6hr	12hr	18hr	24hr	30hr	48hr
AN5841	1	22	17	41	40	38	14
AN6403	0	0	0	0	1	6	19
AN5101	1	4	10	19	26	51	121
AN6929	0	0	0	0	1	4	10
AN9257	1	0	0	2	1	19	64
AN3983	21	206	935	3615	3755	4335	13380
AN9250	0	0	0	1	4	11	48
AN10601	14	9	17	16	16	19	32
AN10345	0	0	1	0	3	12	26
AN10779	71	38	39	46	39	46	64
AN0499	0	0	1	29	63	163	263
AN11101	0	1	1	3	6	18	36
axl2	4	13	14	13	18	24	46
rodA	0	0	26	2023	3577	9305	22248
yA	13	8	17	28	33	89	222

Table 2-3. Homology search in other Ascomycota genera using blastp with E-value threshold at 1.00E-5.

abaA	Genus	AN5841	AN6403	AN5101	AN6929	AN9257	AN3983
	Trichophyton		+				
	Coccidioides		+	+			
	Paracoccidioides		+				
	Ajellomyces	+	+				
+	*Fusarium*	+	+	+	+	+	+
+	*Trichoderma*	+	+	+	+		
+	*Exophiala*	+	+	+			
+	*Candida*		+				
+	*Neurospora*	+	+	+	+	+	
	Magnaporthe	+	+	+	+		
+	*Penicillium*	+	+	+	+	+	

Table 2-3. Continued:

abaA	Genus	AN9250	AN10601	AN10345	AN10779	AN0499	AN11101
	Trichophyton		+	+	+		+
	Coccidioides			+	+		+
	Paracoccidioides			+	+		+
	Ajellomyces			+	+		+
+	*Fusarium*	+		+			+
+	*Trichoderma*	+	+	+			+
+	*Exophiala*	+	+	+	+		+
+	*Candida*			+	+		+
+	*Neurospora*	+	+	+			+
	Magnaporthe		+	+			+
+	*Penicillium*	+	+	+	+	+	+

Table 2-4. Conserved domain search in 12 genes.

Gene ID	Length (aa)	Domain	Coverage	Predicted Function
AN5841	240	SDR superfamily	20-230	Short-chain dehydrogenases/reductases
AN6403	483	EEVS	20-380	2-epi-5-epi-valiolone synthase
AN5101	312	ZnMc superfamily	70-300	Zinc-dependent metalloprotease
AN6929	659	Bac-rhamnosid superfamily	400-630	Bacterial alpha-L-rhamnosidase
AN9257	194	No hit		
AN3983	139	No hit		
AN9250	516	Condensation superfamily	100-500	Peptide antibiotics systhesis
AN10601	389	Glyco_transf_25	60-150	Glycosyl transferase
AN10345	462	Glyco_hydro_76	30-400	Glycosyl hydrolase
AN10779	632	Glyco_hydro_16	240-580	Glycosyl hydrolase
AN0499	121	CBM_14	35-80	Chitin binding, cell wall synthesis
AN11101	1234	STKc+BRSK1_2	100-375	Serine/Threonine protein kinases

Table 2-5. AbaA binding site (CATTCY) search in 12 genes.

Gene	Upstream gene	Distance from upstream gene(nt)	Aba binding sites	binding site within 1000nt
AN5841	AN10740	2167	0	0
AN6403	AN11917	659	0	0
AN5101	AN5100	490	2	2
AN6929	AN06928	1380	4	2
AN9257	AN11647	892	2	2
AN3983	AN3982	1480	3	1
AN9250	AN9249	917	1	1
AN10601	AN04843	808	1	1
AN10345	AN11680	874	1	1
AN10779	AN06203	1141	6	4
AN0499	AN0498	2172	1	1
AN11101	AN11109	1090	2	2

Reference:

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Chapter III Characterization of AN11101 in *A. nidulans*

Abstract

The protein kinases Gin4 and Hsl1 have been well studied in the budding yeast *Saccharomyces cerevisiae*. They are required for proper cytokinesis and septin localization during bud emergence and other important life cycle events. Their *A. nidulans* homologue is AN11101, and was named Gin4. We studied the function and localization of AN11101 during hyphal growth and conidiophore development. An AN11101 deleted mutant strain exhibited hyper branching on hyphae. It also has defective phialides which only bear one layer of spores. In comparison, the wild type phialides bear long chains of spores. An AN11101-GFP fusion showed that AN11101 is accumulated at the phialide-spore junction site during the late conidiophore development stage. These results together with its expression being elevated in induced AbaA strain, we hypothesized that AN11101 is regulated by AbaA, and it regulates the phialide morphogenesis. In the double mutant of Δ*AN11101* and septin-GFP fusion strain AspB-GFP, the localization of AspB was observed at the phialide-spore junction site, implying that AN11101 is not required for septin organization at phialide tip. However, this result is contradictory to the prediction of Gin4 function. Based on our observations, we propose to rename AN11101 to ndrA in *A. nidulans* since it is more comparable to the function of Hsl1 in budding yeast.

Introduction

As described in Chapter II, twelve genes were selected to be potential AbaA and phialide related genes. Among these genes, AN11101 has predicted protein kinase function. It was named as gin4 based on the annotation in the *A. nidulans* genome in the Broad Institute database. In *S. cerevisiae*, Gin4 functions as a serine/threonine protein kinase, an enzymes that regulates other proteins by phosphorylation. Gin4 has other two homologues in yeast, Hsl1 and Kcc4. It has been reported that the target proteins of Gin4 in yeast are septins, which are GTP-binding proteins first discovered in budding yeast. Loss of any one of the five septin members causes cell cycle arrest and defective cytokinesis because septins play essential roles to ensure proper cytokinesis by forming a barrier structure at the bud neck during asymmetric cell division (Longtine *et al*. 2000). Hsl1 is a protein kinase localized at the bud neck with the related kinases, Kcc4 and Gin4. Hsl1 protein acts as the negative regulator of Swe1, which inhibits the phosphorylation of clin-dependent kinase, Cdc28. In absence of Hsl1, the Sw1p is highly activated resulting in a prolonged G2 due to the low activity of Cdc28p (Barral *et al* 1999). The activity of Hsl1p requires co-localization of properly assembled septins at the bud neck to inhibit Swe1 activity and facilitate cell to enter mitosis stage (Barral *et al* 1999), but Hsl1 is not required for septin organization at the bud neck (Longtine *et al*. 2000). On the other hand, Gin4 is activated in mitosis to control proper cytokinesis and septin organization. Septin protein bind to Gin4 at the bud neck to activate the kinase activity of Gin4, and Gin4 is consequently required for the localization of septin assembly (Carroll *et al*. 2000, Longtine *et al*. 1998, Wu 2007). The specific function of Kcc4 remains unknown (Longtine *et al*. 1998).

In *A. nidulans*, there are five septins: AspA, AspB, AspC, AspD and AspE, in which AspB has been studied in detail (Cid *et al*. 2001, Momany *et al.* 2001, Westfall and Momany 2002). AspB was reported as the mostly expressed septin, and it localizes at the sites of septation, branching and the junction between conidiophore layers (Westfall and Momany 2002, Hernández-Rodríguez *et al*. 2012). During conidiophore development, AspB is first localized at the foot cell in hyphae, and then accumulated at the vesicle as a cap structure when the stalk tip is swelling to form the vesicle (Hernández-Rodríguez *et al*. 2012). After the emergence of first layer uninuclear metulae, AspB is localized as rings in each layer of sterigmata cells (Hernández-Rodríguez *et al*. 2012). While the emergence of each layer in *A. nidulans* conidiophore progressing in the same pattern as bud emergence in budding yeast, together with the observed localization pattern of AspB, it implies that septins play essential roles in cell division and mitosis during conidiophore development. Focused on the phialide layer, a yeast bud site selection marker homologue axl2 has been reported to regulate the localization of AspB at the junction of phialide and spores and to regulate the development of phialide in *A. nidulans* (Si *et al*. 2012).

Since AN11101 is homologous to Gin4 in yeast, it is possible that AN11101 is another gene that performs a similar regulation function in spore emergence from phialide. To determine the function of AN11101 in *A. nidulans*, we generated a deletion mutant strain and characterized the growth state, hyphal and conidiophore phenotypes. In addition, we also generated a mutant strain in which GFP probe is fused to AN11101 to visualize its localization during vegetative growth and conidiophore development. A

sexual cross between AN11101-deleted mutant and AspB-GFP strain was conducted to investigate the interaction between AN11101 and AspB in *A. nidulans*.

Materials and Methods

Strains, medium and growth conditions

All *Aspergillus nidulans* strains used in this study are listed in Table 3-1. Stock strains were stored as form of mycelia in 30% (v/v) glycerol solution at -80°C. YGV (yeast extract glucose + vitamins) liquid medium, MN (minimum) medium, MN-threonine medium, and MAG (malt extract + vitamins) medium were made for culturing. MN medium was prepared as described by Kafer (1977). The pH of medium was adjusted to 6.5 with 1.0 N Nacl solution. MNV-threonine medium was made according to Pearson *et al.* (2004). MAG and YGV medium were prepared as described by Harris *et al*. (1994). Uracil/Uridine (UU) and/or Vitamin mix (V) were supplemented in mentioned medium as needed to identify strains containing auxotrophic markers.

Construction of gene deletion strain

The ANID_11101 gene was replaced with *A. fumigatus pyroA* auxotrophic marker to generate gene deletion strains. Gene replacement was conducted with gene targeting system designed by Nayak *et* al. (2006) and gene replacement generation method developed by Yang *et al*. (2004). Oligonucleotides for gene replacement were designed using MacVector software (MacVector, Inc. Cary, NC) as shown in Table 3-2.

A. f. pyroA marker fragment was amplified from plasmid pTN1 (Fungal Genetics Stock Center, Kansas City, MO http://www.fgsc.net/). To construct the gene replacement, around 1000 base pairs DNA fragment from upstream and downstream regions were amplified from the *A. nidulans* FGSC A1149 strain genomic DNA (alias TNO2A3 strain, available in Fungal Genetics Stock Center). Genomic DNA was extracted using Mo Bio PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc. Carlsbad, CA). To replace gene, Roche High Fidelity or Long Template PCR System (Roche Diagnostics Corporation, Indianapolis, IN) were used to amplify and fuse upstream fragment, *A.f. pyroA* marker, and downstream fragment together in a Bio-Rad MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). PCR program was configured according to PCR system's recommendations based on the primer Tm temperature and PCR product size. PCR products were verified using electrophoresis in 0.8% agarose gel (Life Technologies, Carlsbad, CA). The QIAquick gel extraction kit was used to gel purify PCR product (QIAGEN Inc., Valencia, CA). The gene-replaced DNA construct was then transformed into *A. nidulans* FGSC A1149 strain, and plated on the supplemented minimal medium with 0.6 M KCl based on transformation protocol described by Osmani *et al*. (2006) Transformation candidates were tested for homologous integration of the gene-replacement construct and the absence of the wild type gene by diagnostic PCR as described by Yang *et al.* (2004)

Construction of GFP-fusion strain

GFP-fusion protein was used to visualize gene localization. Strains with a C-terminal expressed GFP-fusion protein were constructed using a GFP-pyrG fragment,

which was amplified from plasmid pFNO3 (available in Fungal Genetic Stock Center, Kansas City, MO). Gene DNA fragment without stop codon and gene downstream fragment were amplified from FGSC A1149 strain using gene-targeting system protocol (Nayak *et al*. 2006). To amplify and fuse gene fragment and GFP-pyrG fragment, Roche High Fidelity or Long Template PCR System (Roche Diagnostics Corporation, Indianapolis, IN) were used in a Bio-Rad MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The fusion product was gel purified with the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA), and then transformed into FGSC A1149 strain on KCl supplemented minimal medium, to replace wild type gene. Transformants were tested for homologous integration of the gene-GFP fusion by diagnostic PCR (Yang *et al*. 2004).

Hyphal phenotypic analysis and staining

For hyphal growth analysis, conidial spores from corresponding strains were grown on coverslips in liquid YGV medium at 28°C for 12-14 hours. For hyphal morphology microscopy, hyphae that were grown and attached on coverslip were fixed by dipping into 95% ethanol for less than 1 second. For hyphal staining, hyphae attached on coverslip were fixed for 20 min by following a revised standard protocol (Harris *et al.* 1994), then stained with staining solution. Fixation solution was prepared by mixing 3.7% formaldehyde, 25 mM EGTA, 50 mM piperazine-N, N-bis (2-ethanesulfonic acid) (PIPES), and 0.5% dimethyl sulfoxide. Staining solution contained 273 nM fluorescent brightener 28 (Sigma-Aldrich Corporation, St. Louis, MI, Synonym: Calcofluor White M2R) and 160 nM Hoechst 33258, Pentahydrate (bis-Benzimide), (Molecular Probes,

Eugene, OR). Fixed coverslip was then mounted onto microscope slide with mount solution for DIC microscopy (Momany 2001). Mount solution was prepared by mixing 50% glycerol, 10% phosphate buffer, pH 7.0, and 0.1% n-propyl gallate.

Conidiophore phenotypic analysis and staining

Conidiophores were developed for microscopy analysis using sandwich coverslip protocol as described by Lin and Momany (2003). Fungal strains were incubated on MAG plates for 3-5 days to collect conidial spores. Four coverslips were placed on surface of 4% water agar plates, on each coverslip, 1 mL melted MAG medium was pipetted onto each coverslip to build medium dome. Upon solidification of the medium, spore suspension was transferred onto dome top, a second coverslip was then placed on top afterwards. Conidiophores were developed after 3-4 days of incubation and attached to the top coverslip. Coverslip was then taken and dipped into 95-100% ethanol for fixation, and was mounted for DIP microscopy. For Calcofluor/Hoechst staining, the coverslips were stained before mounting on slides.

GFP localization on conidiophores

To localize AN11101-GFP, the spore suspension of AN11101-GFP strain were grown on MAG or MNTVUU medium with the sandwich slide method as described in conidiophore phenotypic analysis. After 2-3 days incubation at 28°C on MAG medium, or 4-5 days on MNTVUU medium, coverslips were observed under the microscope.

Hülle cell counting for evaluating sexual development

100μL 10^4/ml conidial spore suspension was spread onto MN medium and incubated for 7 days at 28°C in dark condition. The agar squares were cut and crushed in a 1.5mL eppendoff tube, mixed with 1mL 0.5% Tween20 and then shaken at medium speed for 1hr to shake off the spores from agar. The spore suspension was diluted 100 fold for counting on a hemocytometer (Waltham, MA) using microscope.

Microscopy

Conidiophore or hyphae mounted on slides were observed using an Olympus BX51 microscope with Sutter Instruments Lambda 10-B optical filter changer system (Novato, CA) and a Photometrics CoolSnap HQ camera (Tucson, AZ). Digital images were taken and processed with MetaMorph for Olympus 7.5.6.0 (Molecular Devices, Inc. Sunnyvale, CA) and Microsoft PowerPoint (Microsoft Corporation, Seattle, Redmond, WA).

Double mutants by sexual cross

In order to determine the interaction between AN11101 and septin genes, *ΔAN11101* x aspB-GFP double mutant was produced by a normal sexual crossing between *ΔAN11101* strain and AspB-GFP strain on minimal medium. Successful crosses

were screened through selective medium (MN). GFP localization in conidiophore was observed using sandwich slide method.

Results

Function of AN11101 in A. nidulans morphogenesis

To characterize the potential function of AN11101, we generated a Δ*AN11101* mutant strain by target gene replacement from the wild type strain TNO2A3 using combinatory PCR. After 7 days of growth, *ΔAN11101* strain appeared to have restricted growth on rich medium and on minimal medium comparing with wild type TNO2A3 strain (Figure 3-1). Notably, *ΔAN11101* strain expressed early sexual reproduction phenotype on both rich and minimal medium shown as yellowish patches on the colony. The yellowish patches are large thick wall cells called hülle cells served as nursing cell to form cleistothecial primordia, and became as the cleistothecium wall (Yager 1992). Hülle cells were counted and repeated for 5 times after 7 days of growth to compare the sexual developmental state between wild type and Δ*AN11101*. The result is shown in Table 3-3. Based on the T-test of the counts of two strains, they have a significant difference in terms of the numbers of sexual cells. Thus AN11101 may suppress the sexual development in *A. nidulans*.

In order to examine the hyphal morphological phenotype in Δ*AN11101* strain, calcofulor and hoechst 33258 staining was used to show septa and nuclei in the wild type and *ΔAN11101* hyphae. Both strains were grown in YGVUU for 14 hours and observed under microscope. There was no striking difference between the wild type and the

AN11101 mutant strains in terms of septa formation and nuclei distribution (Figure 3-2, 3-3). But short branching was noticeable on *ΔAN11101* hyphae (Figure 3-3).

To investigate the conidiophore morphogenesis in Δ*AN11101* strain, coverslip cultures with the previously described sandwich slide method were used for microscopy. The stalk, metulae and phialide structure conidiophore were normal in Δ*AN11101* compared to the wild type strain. The conidiophore in mutant strain only carried one layer of spores on top of phialides, whereas long chains of spores in the wild type strain (Figure 3-4).

AN11101 localizes to the junction of phialide and spores

In order to visualize the localization of AN11101, a C-terminal AN11101-GFP fusion strain was generated using target gene replacement strategy. First, we tried to investigate the localization of AN11101 in hyphae. To do so, the strain was cultured on coverslip in YGV medium for 14 hours. The coverslip carrying hyphae was examined under the microscope. No AN11101-GFP localization on hyphae during vegetative growth was observed (data not shown). Next, we explored the localization of AN11101 on conidiophore. To do so, we inoculate the conidia spore suspension on coverslip bearing 1 mL MAG medium, as described in the sandwich slide method. After two days, the top coverslip carrying hyphae and conidiophores was examined under the microscope. AN11101-GFP was localized at the junction of phialide-spores as rings (Figure 3-5). To further investigate the localization of AN11101, we used the submerged culture to produce reduced conidiophores of AN11101-GFP strain for better view.

AN11101-GFP was only localized at the phialide tip when a spore was emerging (Figure 3-6). These results suggest that AN11101 may function only in the later stage of conidiation and expression of AN11101 is regulated during cytokinesis in mitosis. And the phenotype of hyper branching on hyphae implies that AN11101 is also functioning in vegetative growth but we failed to localize AN11101 on hyphae, which may due to the low concentration of AN11101 in hyphae.

Interaction between septins and AN11101

AN11101 was annotated as gin4 in the *A. nidulans* genome database (Broad Institute). The result of BLAST protein search indicates that AN11101 has 50% similarity with its homologue in budding yeast. In yeast, Gin4 was reported to interact with septins that septins is the target of Gin4 protein kinase. And in *gin4* deleted mutant strain, septin assembly is not properly organized at the bud neck, which implies that Gin4 is required for septin recruitment. On the other hand, AN11101 also has a 47% similarity with Hsl1 in budding yeast. Hsl1p is not required for septin localization at the bud neck, but it is essential for proper cell progression, especially in cell division and mitosis during bud emergence.

We have investigated the role of AN11101 in regulating conidiophore morphogenesis in *A. nidulans.* We want to further determine the interaction between AN11101 and septins in *A. nidulans*. To do so, we made a double mutants through a sexual cross between Δ*AN11101* mutant strain and AspB-GFP strain. The double mutant strain showed a similar phenotype as Δ*AN11101* strain as expected. With microscopy

using the sandwich slide protocol, the localization of AspB-GFP was visualized at the junction site of phialide-spore (Figure 3-7). This result indicates that AN11101 is not required for organization of the septin assembly at the phialide tip.

Discussion

Function of AN11101 in phialide morphogenesis in *A. nidulans*

The purpose of this chapter is to partially characterize AN11101 functions in *A. nidulans*, which is the homologue of protein kinases Gin4 and Hsl1 in budding yeast. The result of our functionality analysis indicates that AN11101 plays a role in hyphal and conidiophore morphogenesis. The actual function of AN11101 in vegetative growth was not clear since we could not localize AN11101 on hyphae. We suspected that AN11101 may relate to the cytokinesis in newly formed branching. Without AN11101, the fungus initialized branching along the hyphae, but a further growth from that point cannot be carried out. It has similar phenotype as the Hsl1 deleted mutant in budding yeast. In absence of Hsl1, the cell is arrested at the G2 stage, and elongated budding structure is formed due to improper cytokinesis. During the development process, it has observed that AN11101 functions only at the late stage of conidiophore development. Before the phialide layer, there was no abnormal phenotype in conidiophore structure. AN11101 seems to regulate the proper function of sporogenous structure phialide.

AN11101 is regulated by AbaA in A. nidulans

As shown in Chapter II, the expression of AN11101 correlates with abaA induction, and two AbaA binding sites were found at the promoter region of AN11101.

AN11101 also has the same localization as axl2, solely localizes at the junction site of phialide-spore. AbaA is the transcriptional regulator that controls the development phialide during conidiation, and it regulates the expression of phialide related genes (Sewall 1990). Combining these results, it is suggested that AN11101 is regulated by AbaA and related to phialide development. It has also observed that Δ*AN11101* mutant strain has an earlier development of sexual reproduction than the wild type strain. AN11101 may play a role in repressing sexual development.

AN11101 may be named as ndrA

Our results indicated that AN11101 is related in morphogenesis regulation in *A. nidulans.* Without this gene, the fungus performs improper hyphal branching and presents malfunctioned phialide structure. The sporulation is the continuous procedure that conidial spores are generated from phialide by bud growth. This sporulation process is considered as a similar biological process of bud emergence in yeast. The function of AN11101 is similar with its protein kinase homologues gin4, hsl1, and kcc4 in budding yeast. But it is not decided to which kinase AN11101 is closer. Moreover, AN11101 is the only homologue in *A. nidulans* of gin4/hsl1/kcc4 NDR kinase family (nuclear dbf2-related kinases). Therefore here we propose to rename AN11101 as ndrA instead of gin4 in *A. nidulans.*

Figure 3-1. Effects of the AN11101 deletion in colony morphology.

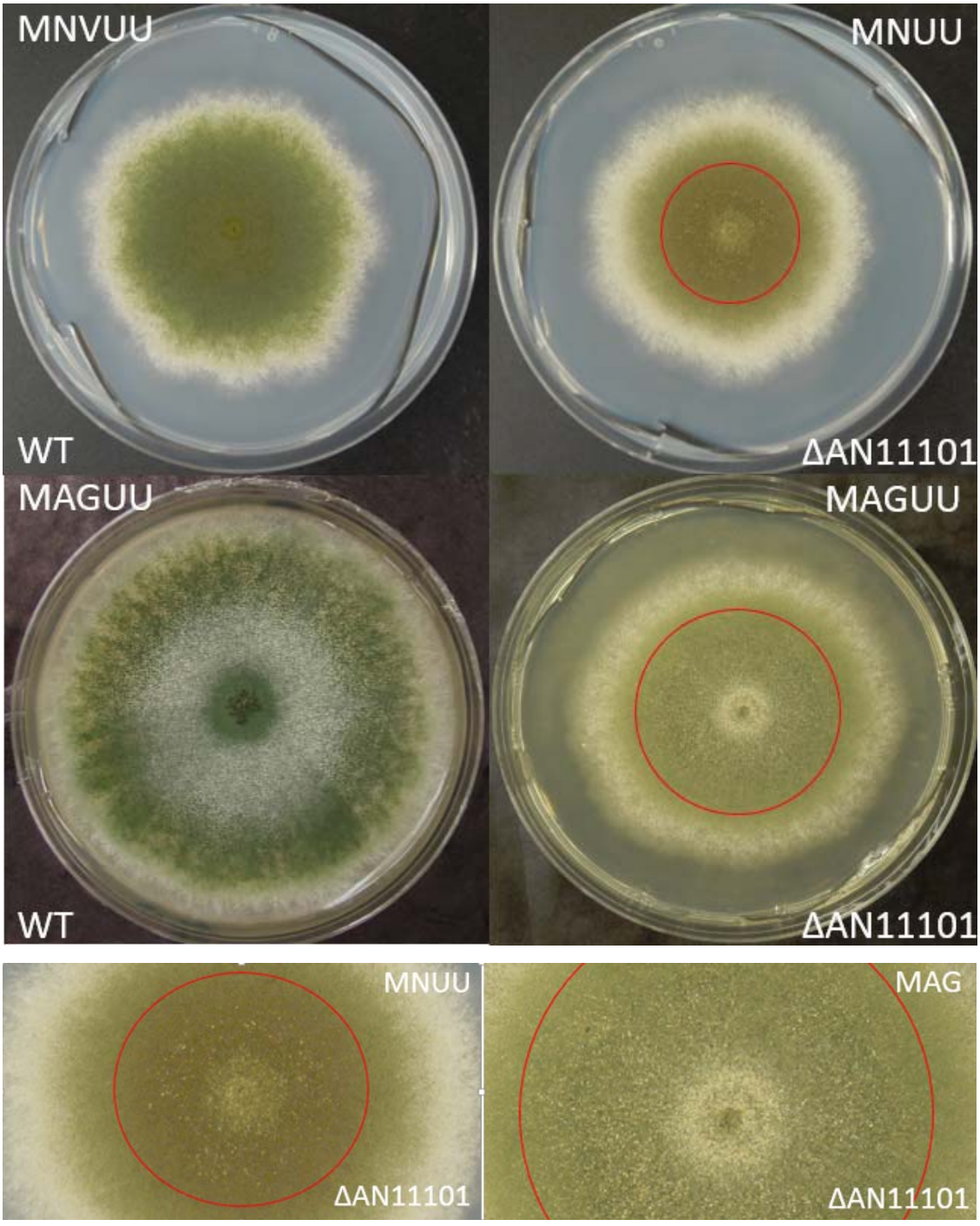

Figure 3-1. Top panel: Wild type strain (WT) and Δ*AN11101* grown on minimal medium (MN) and rich medium (MAG) for 7 days at 28°C. Notice the yellow patches (hülle cells)

on B and D in the region marked by red circles. Bottom panel: magnified image to show yellow patches on colonies.

Figure 3-2. Calcofulor and Hoechst 33258 staining to show septa and nuclei in hyphae.

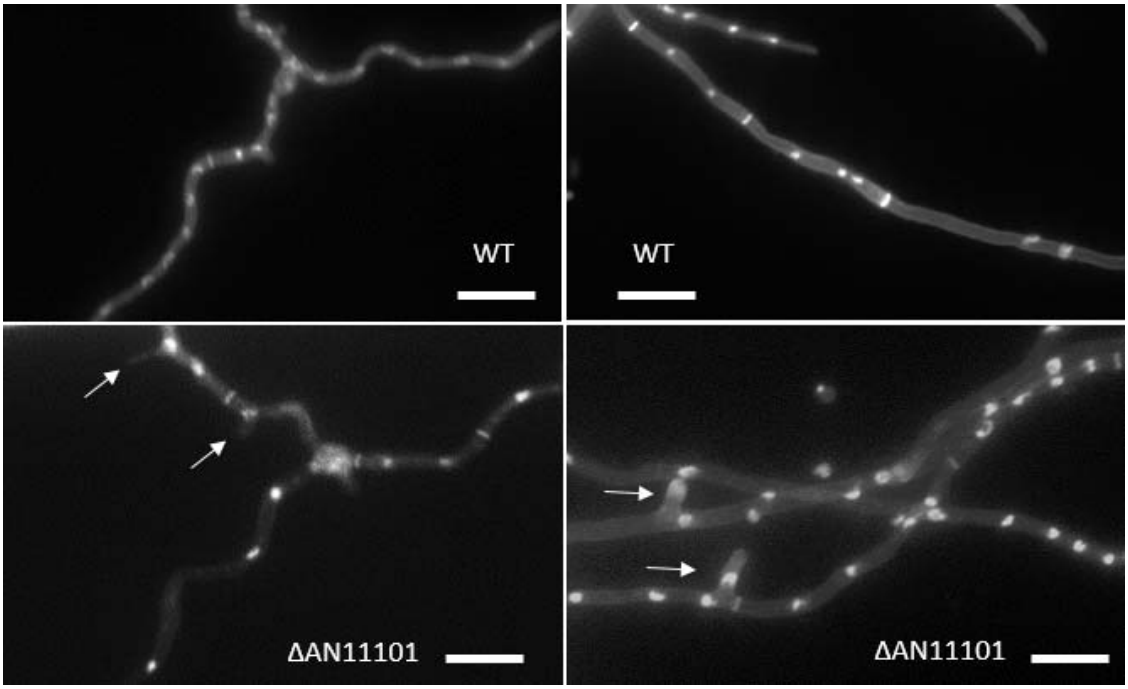

Figure 3-2. Calcofulor and Hoechst 33258 staining to show septa and nuclei in wild type and *ΔAN11101* hyphae. Both strains were grown in YGVUU liquid medium for 14 hours and observed under microscope. There is no striking difference between wild type and the AN11101 mutant strains in terms of septa formation and nuclei distribution. But short branching is noticeable on *ΔAN11101* hyphae (marked by arrows). Bar = 10μm.

Figure 3-3. Coverslip culture to show hyper branching in *ΔAN11101*.

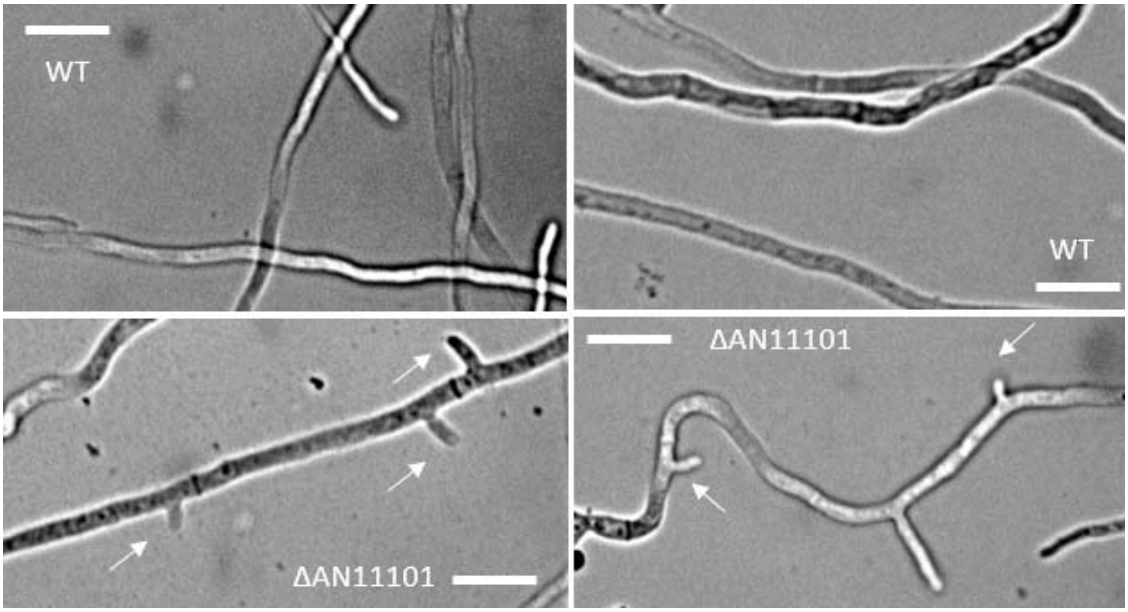

Figure 3-3. Coverslip culture to show hyper branching in *ΔAN11101*. Both strains were grown in YGVUU liquid medium for 14 hours and observed under microscope. Short branching is noticeable on *ΔAN11101* hyphae (marked by arrows). Bar = 10µm.

Figure 3-4. Phenotype of *ΔAN11101* conidiophore morphology.

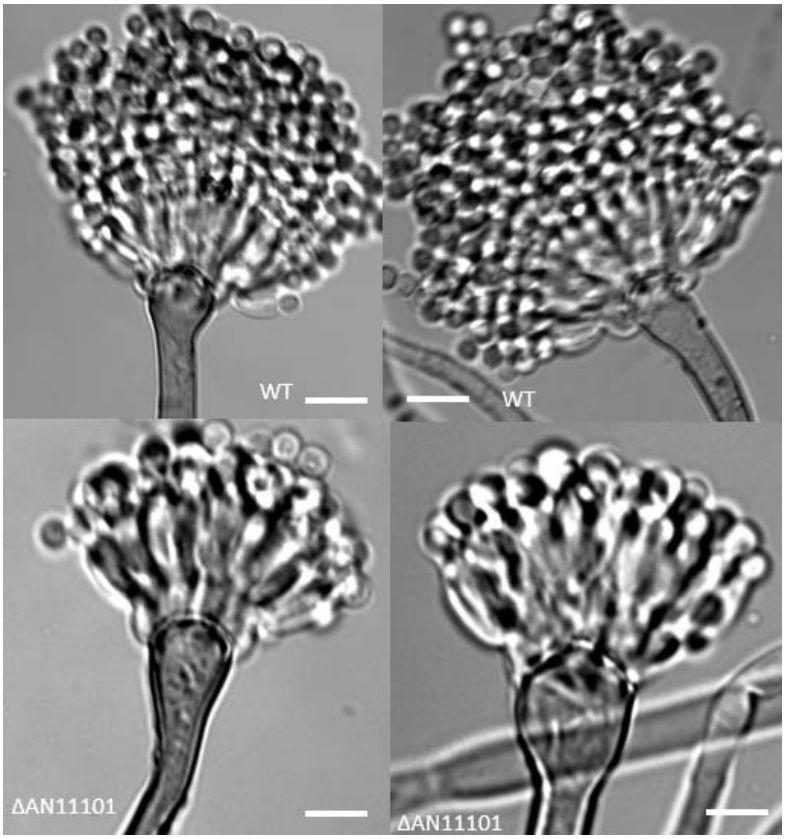

Figure 3-4. Phenotype of *ΔAN11101* conidiophore morphology. Both wild type TNO2A3 and *ΔAN11101* strains were grown on rich medium for 3 days. Notice only one layer of spores from the conidiophore of *ΔAN11101* (same phenotype in *Δaxl2*). Bar = 10μm.

Figure 3-5. Localization of AN11101-GFP.

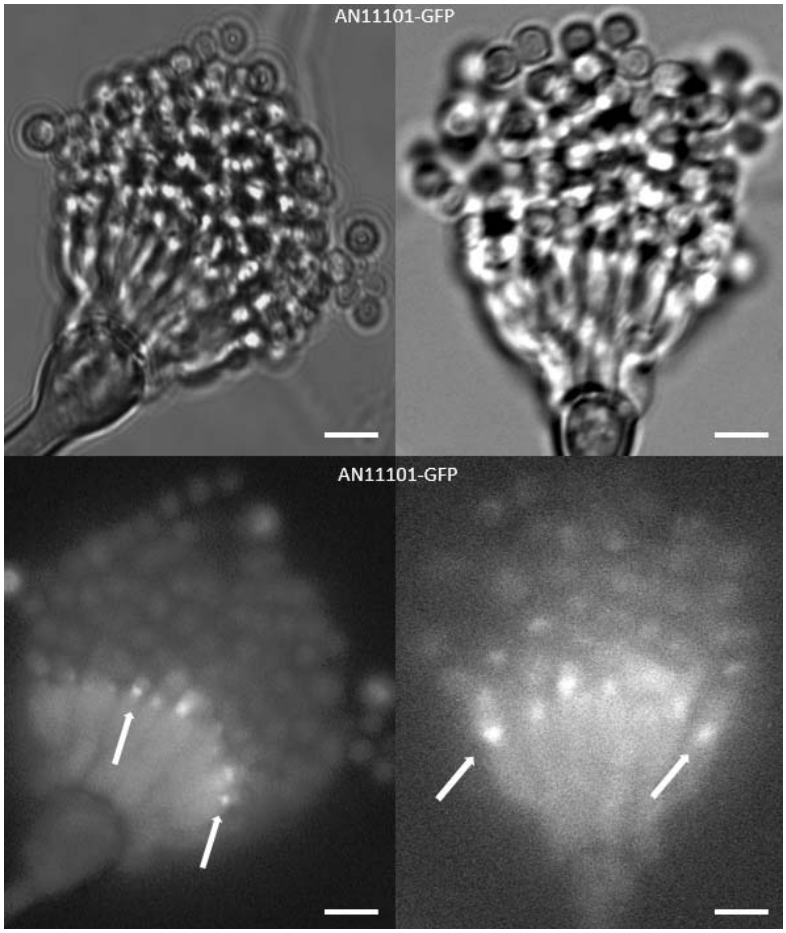

Figure 3-5. Localization of AN11101-GFP. Strain was grown on rich medium for 3 days. Notice AN11101 is localized at the junction of phialide and spores. Arrows indicate AN11101 localization. Bar = 10μm.

Figure 3-6. Submerged culture of AN11101-GFP strain with reduced conidiophore to show localization of AN11101.

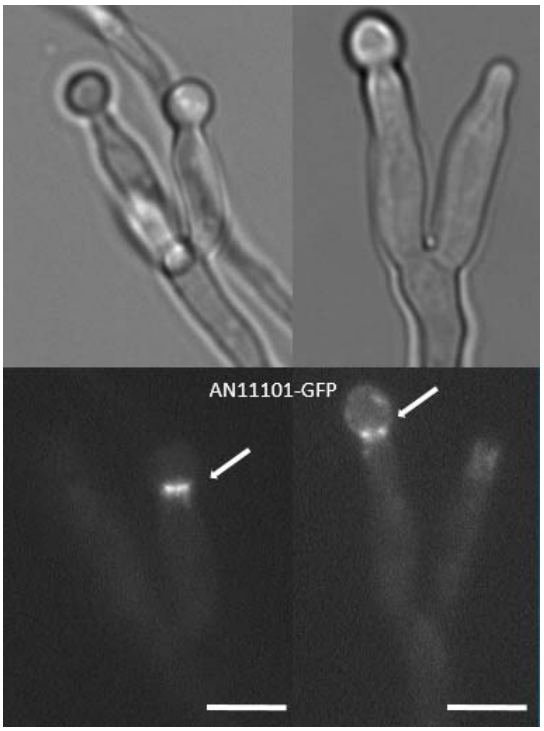

Figure 3-6. Submerged culture of AN11101-GFP strain with reduced conidiophore to show localization of AN11101. Strain was grown in MNV-Thr submerged medium for 5 days. Arrows indicate AN11101 localization. Bar = 10µm.

Figure 3-7. AspB-GFP localization in absent of AN11101.

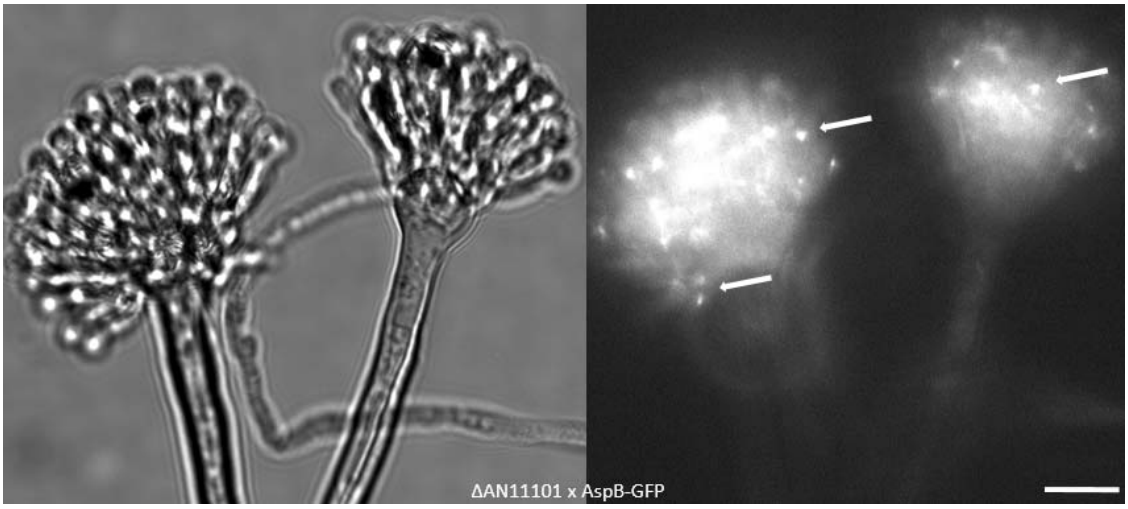

Figure 3-7. AspB-GFP localization in absent of AN11101. Strain was grown in rich medium for 3 days. Arrows indicate AspB localizes in junction of phialide and spores.

Bar = 10μm.

Table 3-1. Strains used in this chapter

Strain	Genotype	Source
A28	*pabaA6 biA1*	FGSC
A1149	*pyrG89; pyroA4; nkuA::argB*	FGSC TNO2A3
AHY2	*pyrG89; argB2; ndrA::pyroA pyroA4 nkuA::argB*	This study
AHY3	*pyrG89; argB2; ndrA::gfp::pyrG pyroA4 nkuA::argB*	This study

Table 3-2. Primers used in this chapter

Primer	Sequence
AN11101::pyroA up F	5′-AGTCAAAATAGCCCAGAACCTGTG-3′
AN11101::pyroA up R	5′-ATTACCTTAGTAATCCAGCATCTGATGTCCGCGACAAAAGTGCTGTAATGCC-3′
AN11101::pyroA down F	5′-AATCCGTCAGTCATCTACTCACCG-3′
AN11101::pyroA down R	5′-GCATTTGTCCTTCATTATGTAGACACTCGCTCAGACAGCCCTGCTATTTCCTC-3′
AN11101-gfp up F	5′-AGATTCCAAGACGATGCGGTC-3′
AN11101-gfp up R	5′–GGCACCGGCTCCAGCGCCTGCACCAGCTCCGGCATTCGGAAACGCGTC-3′
AN11101-gfp down F	5′-GGCATCACGCATCAGTGCCTCCTCTCAGACTCAGACAGCCCTGCTATTTCCTC-3′
AN11101-gfp down R	5′-AATCCGTCAGTCATCTACTCACCG-3′

Table 3-3. Hülle cells counting for TNO2A3 and Δ*AN11101* after 7 days of growth in dark at 28°C. A t-test was used to determine if the difference between two strains is significant.

Strain	1	2	3	4	5	Mean	STDEV	P-value
TNO2A3	47	49	48.5	47	39	47	4.068	< 0.001
Δ*AN11101*	219	191	177	240.5	195	219	25.000	

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Chapter IV Comprehensive characterization of the twelve AbaA related genes in *A. nidulans*

Abstract

Despite the advanced knowledge about the genetic organization of *A. nidulans* genome, there are many genetic modules awaiting for characterization. In this study, we intend to describe the selected 12 genes. To characterize their functions, we generated gene disruptive mutants. The colony growth of mutants are restricted comparing with the wild type. Δ*AN11101* and Δ*AN0499* were much more sensitive to the osmotic or drug stress than wild type and other mutants. The structures of the conidiophore in mutants were well formed as in the wild type. However, some defects were found when we looked closer. Δ*AN0499* exhibited malfunctioned and malformed phialide development, including one layer of conidia on phialide, and random phialide branching from metulae. Here we propose to name AN0499 as phiB as its potential function in phialide morphogenesis. Other mutants exhibited hyper branching on hyphae as Δ*AN11101*, indicating a role in septation. And overall conidiophore in mutants are well developed as in wild type, showing as less conidial spore layers and compact conidiophore head.

Introduction

The filamentous fungi *A. nidulans* has been a successful model system for discovering key genetic modules in regulating biological processes in fungi for over 60 years (Martinelli and Kinghorn 1994). The research on this system covers cell cycle control, secondary metabolite production, cytokinesis, polarized growth and so on (Fischer *et al* 2008, Bayram and Braus 2012, Momany *et al*. 2001, Lee *et al*. 2008, Li *et al*. 2006, Harris *et al.* 1997). The genome sequence of *A. nidulans* and its genome annotation has been done in 2005 (Galagan *et al*.). The knowledge of *A. nidulans's genome* provides insightful information about the genetic composition and organization of *Aspergillus.* This species has been found to consist of ~30 million nucleotide base pairs over eight chromosomes, and total of 9,541 protein-coding genes have been predicted and annotated (Galagan *et al*. 2005). Despite the advancement of massive information regarding the genome annotation, the majority of its genes still needs characterization. There are many interesting genetic modules still awaiting to be revealed, for instance, what we are interested in the phialide development during conidiation. The uniqueness of phialide development is associated with the transition from default thallic fungal growth to blastic conidiogenesis. The understanding of the genetic modules triggering this switch would help to expand our knowledge of the regulation of fungal morphogenesis.

A. nidulans is a spore producing fungus that colonizes in widely diversified habitats. There are two fundamental stages in the life cycle of *A. nidulans*: the growing stage and the developmental stage. The spore production is happened in the developmental stage. Two distinctive forms of developments exist in the developmental

stage, the sexual and asexual sporulation (anamorph and telemorph), of which the anamorph is the dominant one. In the asexual reproduction form, a spatial structure named conidiophore is formed from the basal hyphal cell. Within the complex structure of conidiophore, phialides are the uninucleate cells responsible for sporulation. In order to understand the genetic regulation in asexual reproduction in *Aspergillus* and other conidiation fungi, identification of the genes involved in phialide development is essential. However, despite the importance of phialide morphogenesis, we have limited knowledge about these genes and their regulation. In 1990, AbaA was identified as the functional key factor that directs the phialide differentiation (Sewall *et al*. 1990). AbaA is induced by brlA and then directs cell differentiation from the stem cell metulae to the specific sporogenous cells phialide. During this process, many functional genes are expected to be bound and induced by AbaA and involved in the development. After abaA was discovered in 1990, only a few genes have been reported under regulation of AbaA and characterized as the regulatory modules in phialide development. Besides axl2 described in previous chapters, yA is reported to be regulated by AbaA, and encodes the conidial laccase that is required for the synthesis of green conidial pigment (Aramayo and Timberlake 1993). In the Δ*yA* strain, the phialide morphology and conidial spore chains are different from the wild type - the phialide direction is random and the sporulation is defective (Aramayo and Timberlake 1993). Given that only few of such genes are reported, we focused on the identification of more AbaA and phialide related genes in *A. nidulans* in order to better understand the genetic regulation in phialide morphogenesis.

In Chapter II, we reported twelve genes which are up-regulated by AbaA and contains AbaA or BrlA binding sites. Then, we comprehensively characterized these

genes in terms of stress sensitivity and morphology of hyphae and conidiophore by generating gene disruptive mutants. We report the result in this Chapter. Besides the gene ndrA (AN11101), another gene AN0499 shows striking phenotypes in both hyphae and conidiophore in deleted strain. Because of the abnormal phialide morphology and defective sporulation in Δ*AN0499* strain, we name this gene as phiB.

Materials and Methods

Strains, medium and growth conditions

All *A. nidulans* strains used in this study are listed in Table 4-1. YGV (yeast extract glucose + vitamins) liquid medium, MN (minimum) medium, MN-threonine medium, and MAG (malt extract + vitamins) were made for culturing. MN medium was prepared as described by Kafer (1977). The pH of medium was adjusted to 6.5 with 1.0 M NaCl solution. MNV-threonine medium was made according to Pearson *et al.* (2004). MAG and YGV medium were prepared as described by Harris *et al*. (1994). Uracil/Uridine (UU) and/or Vitamin mix (V) were supplemented in the medium recipe as needed to select strains containing auxotrophic markers. In stress sensitivity tests, strains were grown on MAGUU plates at 28°C, 37°C and 42°C, and on plates supplemented with NaCl (1.5 M), Hydroxyurea (HU, 15 and 25 mM), Menadione (20 and 40 μM), methyl methanesulfonate (MMS, 0.01 and 0.02% v/v) or Congo red (CR 50 μg/mL), respectively. Water agar medium was made with sterile water containing 4% agar for conidiophore development. All fungal strains were stored in 30% glycerol under -80°C.

Construction of gene deletion strains

All premade gene deletion cassettes were ordered through FGSC, which includes both upstream, downstream DNA fragments and *A. fumigatus pyrG* marker fragment for each gene. To replace gene, Roche High Fidelity or Long Template PCR System (Roche Diagnostics Corporation, Indianapolis, IN) were used to amplify and fuse upstream fragment, selective marker, and downstream fragment together in a Bio-Rad MJ Mini Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). PCR program were edited according to PCR system's recommendations based on primer Tm temperature and PCR product size. PCR products were verified using electrophoresis in 0.8% agarose gel (Life Technologies, Carlsbad, CA). If gel extraction was needed, the QIAquick gel extraction kit was used to gel purify PCR product (QIAGEN Inc., Valencia, CA). The gene replaced DNA construct was then transformed into *A. nidulans* FGSC A1149 strain, and plated on supplemented minimal medium with 0.6 M KCl based on transformation protocol described by Osmani *et al*. (2006). Transformation candidates were tested for homologous integration of the gene replacement construct and the absence of the wild type gene by diagnostic PCR as described by Yang *et al.* (2004)

Hyphal phenotypic analysis and staining

For hyphal growth analysis, conidial spores from corresponding strains were grown on coverslips in liquid YGV medium at 28°C for 12-14 hours. For hyphal morphology microscopy, Hyphae grew and attached on coverslip were fixed by dipping into 95% ethanol. For hyphal staining, hyphae attached on coverslip were fixed for 20

min with a revised standard protocol (Harris *et al.* 1994), then stained with staining solution. Fixation solution was prepared by mixing 3.7% formaldehyde, 25 mM EGTA, 50 mM piperazine-N, N-bis (2-ethanesulfonic acid) (PIPES), and 0.5% dimethyl sulfoxide. Staining solution contained 273 nM fluorescent brightener 28 (Sigma-Aldrich Corporation, St. Louis, MI, Synonym: Calcofluor White M2R) and 160 nM Hoechst 33258, Pentahydrate (bis-Benzimide), (Molecular Probes, Eugene, OR). Fixed coverslip was then mounted onto microscope slide with mount solution for DIC microscopy (Momany 2001). Mount solution was prepared by mixing 50% glycerol, 10% phosphate buffer, pH 7.0, and 0.1% n-propyl gallate.

Conidiophore phenotypic analysis and staining

Conidiophores were developed for microscopy analysis using sandwich coverslip protocol as described by Lin and Momany (2003). As of this method, fungal strains were incubated on MAG plates for 3-5 days to collect conidial spores. 4 coverslips were placed on surface of 4% water agar plates, on each coverslip, 1 mL melted MAG medium was pipetted onto each coverslip to build medium dome. Upon solidification of medium, spore suspension was transferred onto dome top, a second coverslip was then placed on top afterwards. Conidiophores were developed after 3-4 days of incubation and attached to the top coverslip. Coverslip was then taken and dipped into 95-100% ethanol for fixation, and was mounted for DIP microscopy. For Calcofluor Hoechst staining, the coverslips were stained before mounting on slides.

Microscopy

Conidiophore or hyphae mounted on slides were observed using an Olympus BX51 microscope with Sutter Instruments Lambda 10-B optical filter changer system (Novato, CA) and a Photometrics CoolSnap HQ camera (Tucson, AZ). Digital images were taken and processed with MetaMorph for Olympus 7.5.6.0 (Molecular Devices, Inc. Sunnyvale, CA) and Microsoft PowerPoint (Microsoft Corporation, Seattle, Redmond, WA).

Results

Gene disruption

We selected twelve genes from the RNA-seq experiment as described in Chapter II, which appear a correlation with AbaA in *A. nidulans*. To characterize the potential functions of these twelve genes, we generated gene deletion mutant strains by target gene replacement from the wild type strain TNO2A3. The gene deletion cassettes were ordered from FGSC. After 7 days of growth on minimal medium, mutant strain colony size was similar to that of the wild type strain. On the rich medium MAGUU, the mutants appeared to have restricted colony size compared to the wild type strain. Some of strains with striking colony phenotypes are shown in Figure 4-1. Besides Δ*AN11101* (ndrA) described in Chapter III, Δ*AN9257* also exhibited early sexual development by forming hülle cells as the yellowish dots on the colony.

To illustrate mutant strains for defects in hyphal morphology, coverslip culture was used and observed under microscope. Even though several mutants display normal

phenotype of hyphal growth as the wild type strain, some mutants display the similar hyper branching features as Δ*AN11101* (Figure 4-2). By Calcofluor Hoechst staining, coverslip cultures were used to determine the nuclei distribution in hyphae. We found there was no difference in the number of nuclei per compartment between mutant and wild type strains.

The potential defects in conidiophore were determined and observed using the standard sandwich slide protocol as describe by Lin and Momany (2003). The overall conidiophore structure of all mutants were normally established as the wild type that they all formed stalk, vesicle, metulae, phialide and conidial spores. However, when we looked closer to compare the conidiophores of some mutants with the wild type strain, some defective phenotypes were noticed. We observed that septa in the conidiophore stalk were much more frequently formed in some mutant strains than the wild type strain (Figure 4-3). Also we noticed that the stalk length in wild type strain were higher than the mutant strain in general. In some mutant strains, the chains of conidiophores formed a compact head than the wild type strain. And more importantly, some mutant strains including Δ*AN11101* only bore few layers of conidial spores on phialide top compared with wild type strain, which had long chains of spores (Figure 4-4).

We counted the septa in stalk, measured the stalk length, compared the head/vesicle ratio, and enumerate the layers of spores, to summarize and quantify the difference in the hyphal and conidiophore phenotypes. We counted the hyper branching on hyphae for mutant and wild type strains. In 200 hyphae for each strain, the short branches (shorter than 40μm) were counted and put into three bins based on the length, 0-10μm, 10-20μm, and 20-40μm. Besides Δ*AN11101*, we also found that Δ*AN5841*,

Δ*AN6403*, Δ*AN6929*, Δ*AN9257*, and Δ*AN9250* had dramatically high number of short branching in hyphae compared to the wild type (Figure 4-5). These result demonstrated that the mentioned genes may play a crucial role in regulating hyphal morphogenesis in *A. nidulans*.

Stalks in 100 conidiophore of each mutant strain and the wild type strain were observed, in order to count the septa frequency in stalk. We found that some mutant strains formed up to 2 septa in the stalk, which is very rare in the wild type strain's stalk. Specifically, Δ*AN5841*, Δ*AN5101*, Δ*AN6929*, Δ*AN9250*, Δ*AN10601*, Δ*AN10345*, and ΔAN10779 have septa in more than 50% percent of stalk, whereas about 8% in the wild type strain (Figure 4-6). These results suggested that these genes may play a role in septation.

We also measured the stalk length of 100 conidiophores in mutants and wild type (Figure 4-7). The mean of stalk length of mutants is generally shorter than the wild type. In order to find out the significance of difference between mutants and wild type, we did a one way ANOVA test. The p-value is less than 0.001, indicating that the difference between stalk lengths is significant. So we further used Tukey's HSD (honest significant difference) test to group all strains. As shown in Figure 4-7, the wild type strain falls into its only group that it has longer stalk than all mutants. Δ*AN3983* has the shortest stalk length in average. We did measurement for the ratio of head to vesicle in conidiophore as well, by dividing the range of phialides to the diameter of vesicle (Figure 4-8 A). And with the same statistical analysis, we found that the mutant strains had more compact conidiophore head than the wild type (Figure 4-8 B), especially in ΔAN3983 and ΔAN10345. It appeared that the mutant strains tend to utilize less surface of the vesicle to

generate metulae cells. These results indicated that these genes are involved in regulating morphogenesis of stalk and vesicle structures in conidiophore.

Finally, we counted the layers of spores on phialide top in conidiophores of mutants and wild type. To do so, we took 1000 conidiophores for each strain and calculated the mean number of layers of spores. Similar as Δ*AN11101*, Δ0499 also had one, at most two layers of spores after two days of growth in sandwich slide, in contrast to about 7 layers in wild type (Figure 4-9). Except for ΔAN5101, phialides of all other mutant strains born less layers of spores than the wild type. This result demonstrated that the phialide function in the mutants, especially in Δ*AN0499* and Δ*AN11101* are adversely affected, hence suggesting that these two genes may play important roles in phialide morphogenesis. The summary of phenotype analysis is shown in Table 4-1.

Random branching of metulae in Δ*AN0499*

In conidiophore phenotypic analysis using sandwich slide method, a noticeable defect in Δ*AN0499* conidiophore was found that random branching of phialide happened laterally on metulae, which is different from the apical phialide branching from metulae in wild type (Figure 4-10). And as discussed above, Δ*AN0499* phialides only born one or two layers of spores. These results implied that AN0499 is required for the normal organization of metulae and phialide during conidiophore development.

Stress sensitivity test

A series sensitivity tests of osmotic and drug stress have been done to score the stress resistance of the mutants. Spores of mutants and wild type strains were inoculated on medium containing different chemical agents and grown for 4 days. Colony of the strains on plates were recorded and compared, some notable differences between mutants and wild were noticed. The growth of 12 mutants and wild type was uniformly inhibited on minimal medium with the presence of 1 or 1.5 M NaCl. However, under osmotic stress of 1.5 M NaCl, Δ*AN11101* was extremely inhibited that the growth was very restricted (Figure 4-11 A). Next we did a test against DNA damaging agent methyl methanesulfonate (MMS) on the strains. MMS is a DNA alkylating agent that methylates DNA, which lethally inhibits DNA synthesis if DNA repair system is defective (Beranek 1990). All mutants and wild type were grown on rich medium supplemented with 0.01% or 0.03% MMS for 4 days. All mutants were grown similarly as the wild type. But unlike the wild type, Δ*AN0499* and Δ*AN11101* showed strong septation that the green area (conidiation) was restricted on the colony (Figure 4-11 B). This strong septation indicates defective developmental growth and conidiation. We also tested the growth of mutants on Hydroxyurea (HU), which is a strong inhibitor of ribonucleotide reductase (Gräslund *et al*. 1982). Mutants and wild type were grown on rich medium containing 15mM or 25mM HU for 4 days. The growth of 12 mutants and wild type was dramatically inhibited, especially for Δ*AN5841*, Δ*AN6403*, Δ*AN0499* and Δ*AN11101* on 25mM HU plates (Figure 4-11 C). The sensitivity to menadione, a strong oxidant, were tested for mutants and wild type at concentration of 15μM and 40μM on rich medium. Δ*AN9257* and Δ*AN11101* exhibited strong sensitivity on 40μM menadione (Wu *et al.* 2010) (Figure

4-11 D). Finally we test the sensitivity of strains against Congo red, which interacts with chitin and interferes with cell wall structure (Ram and Klis 2006). The growth of mutants were similar as the wild type strain after 4 days of culture. But Δ*AN11101* showed strong sepetation, indicating that the conidiation was defective. The results of stress sensitivity tests are summarized in Table 4-2.

Discussion

The purpose of this chapter is to characterize the function of the twelve genes we selected, and to determine if function of any genes is related to phialide morphogenesis. Utilizing gene deletion constructs from FGSC, we generated gene deletion strains foe the twelve genes and we comprehensively characterized the phenotypes of these disruptive mutants. Besides Δ*AN11101*, we successfully identified another mutant Δ*AN0499* to cause severe phenotypic defects in conidiophore development. However, the phenotypes of some mutants were not strikingly different from the wild type strain except for restricted growth. It is expected as these genes may not be essential for growth or development in *A. nidulans*.

Our observations implicate that several genes are involved in proper branching on hyphae. Δ*AN5841*, Δ*AN6403*, Δ*AN6929*, Δ*AN9250* and Δ*AN11101* exhibited hyper branching during vegetative growth. In conidiophore development, several mutants including Δ*AN5841*, Δ*AN0499* and the previously described Δ*AN11101*, showed severe phialide malfunction. And in the stress tests, Δ*AN0499* and Δ*AN11101* displayed strong sensitivity to several stress agents in contrast to the wild type and other mutant strains.

But our considerable interest are in conidiophore development. Besides the ndrA (Δ*AN11101*) gene, here we successfully identified another phialide related gene Δ*AN0499*.

We failed to localize AN0499 by fusing GFP probe to the gene since the fusion PCR did not successfully connect fragments together. But we suspect that AN0499 would also localize at the junction site of phialide and spores. The reasons are: similar as axl2 and ndrA, AN0499 also has multiple AbaA binding sites. AN0499 has a unique chitin biding domain which may involve in the cell wall construction during the formation of new spores. In absence of AN0499, the function of phialide is defective. The phialide in mutant only bears one layer of spores, suggesting that the function modules for normal spore generation are failed to recycle to the phialide tip to proceed another round of sporulation. And the phialide randomly branches from metulae. So here we name this gene as phiB to indicate its function related to phialide morphogenesis.

Figure 4-1. Colony morphology of wild type and selected mutant strains.

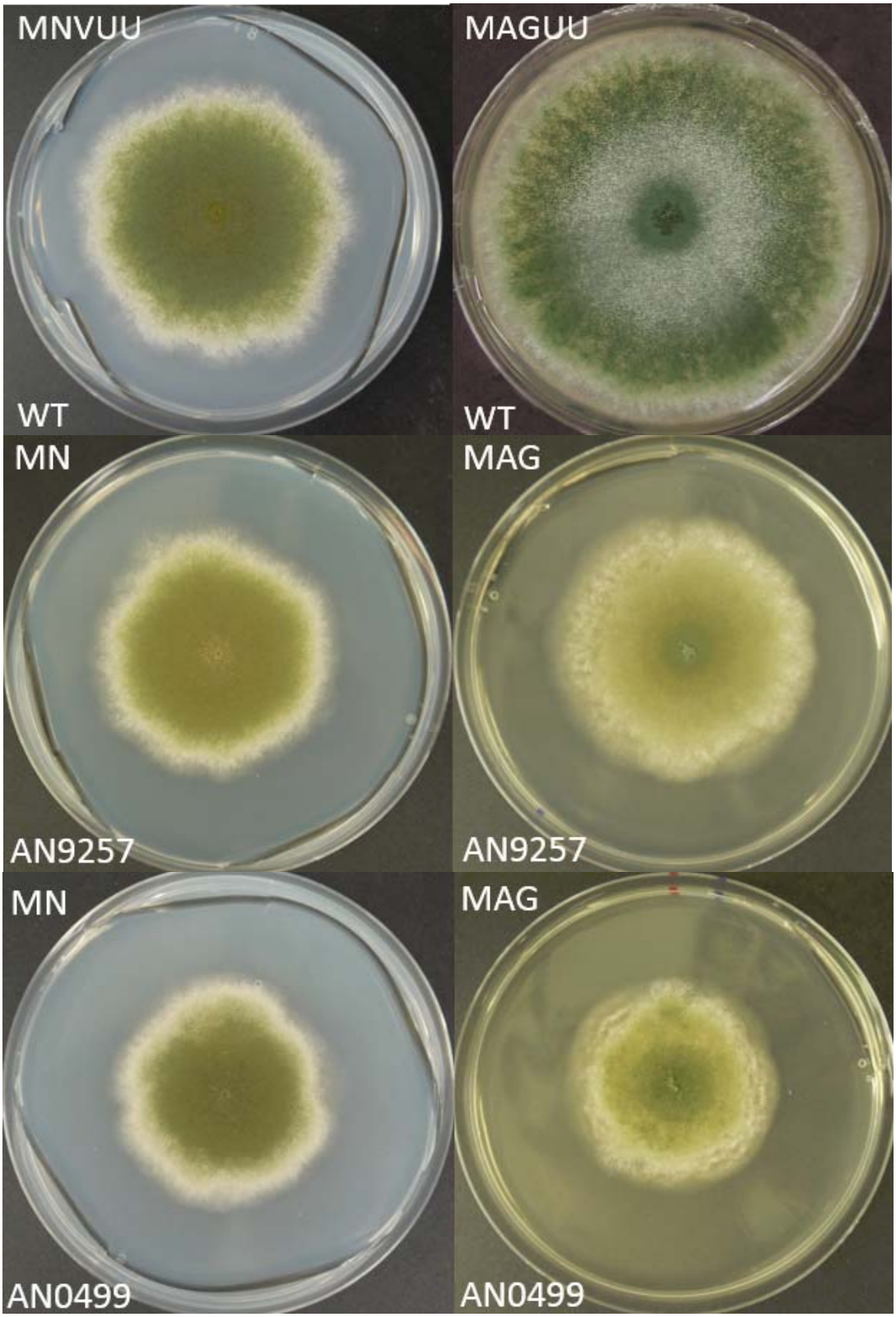


Figure 4-1. Colony morphology of wild type and selected mutant strains. Δ*AN9257* showed early sexual development in minimal medium. Δ*AN0499* had a more restricted growth than other mutants on both minimal and rich medium.

Figure 4-2. Coverslip culture to show hyper branching in mutant strain.

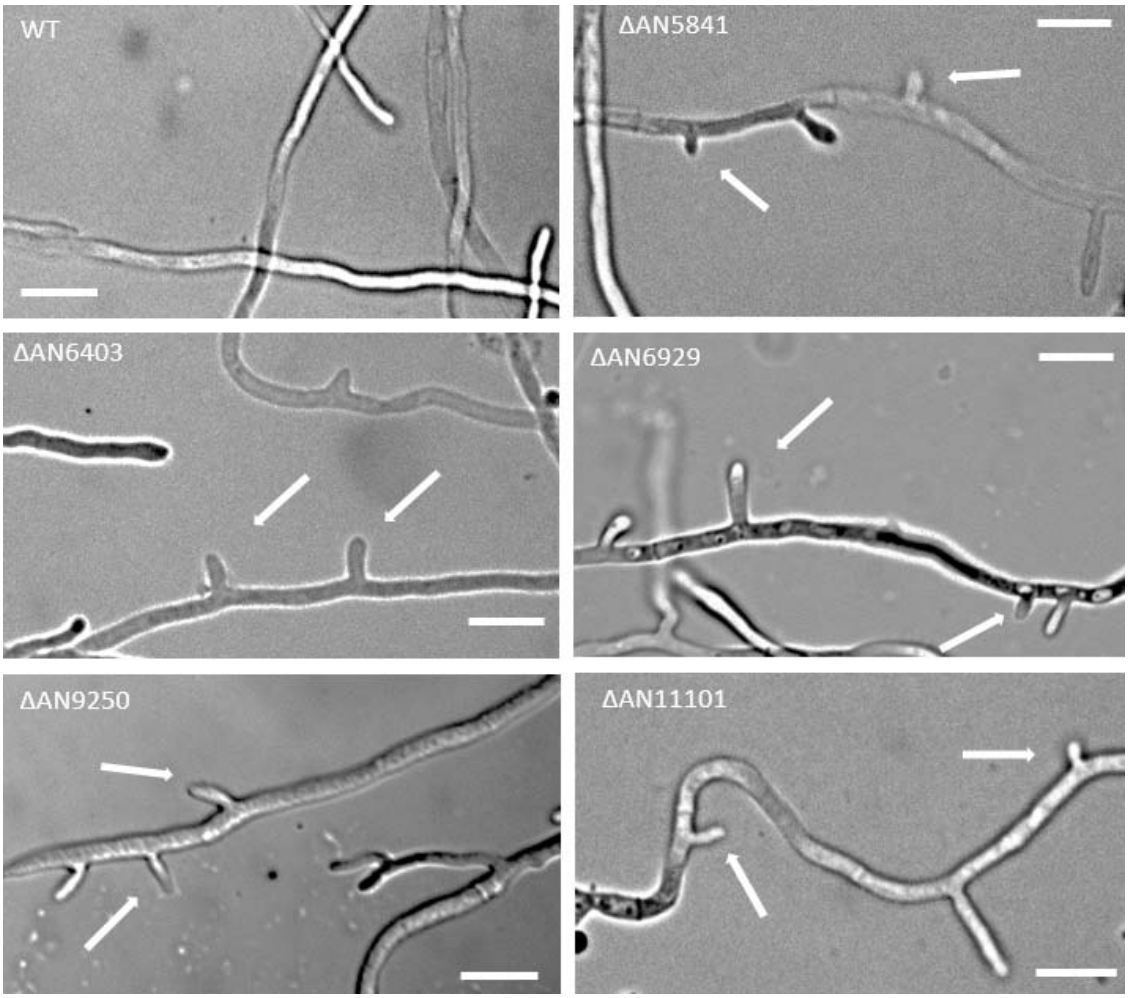


Figure 4-2. Coverslip culture to show hyper branching in mutant strain. All strains were grown in YGVUU for 14 hours and observed under microscope. Short branching is noticeable on hyphae from several mutants (marked by arrows). Bar = 10μm.

Figure 4-3. Conidiophore phenotype showing frequent septa in stalk in mutants.

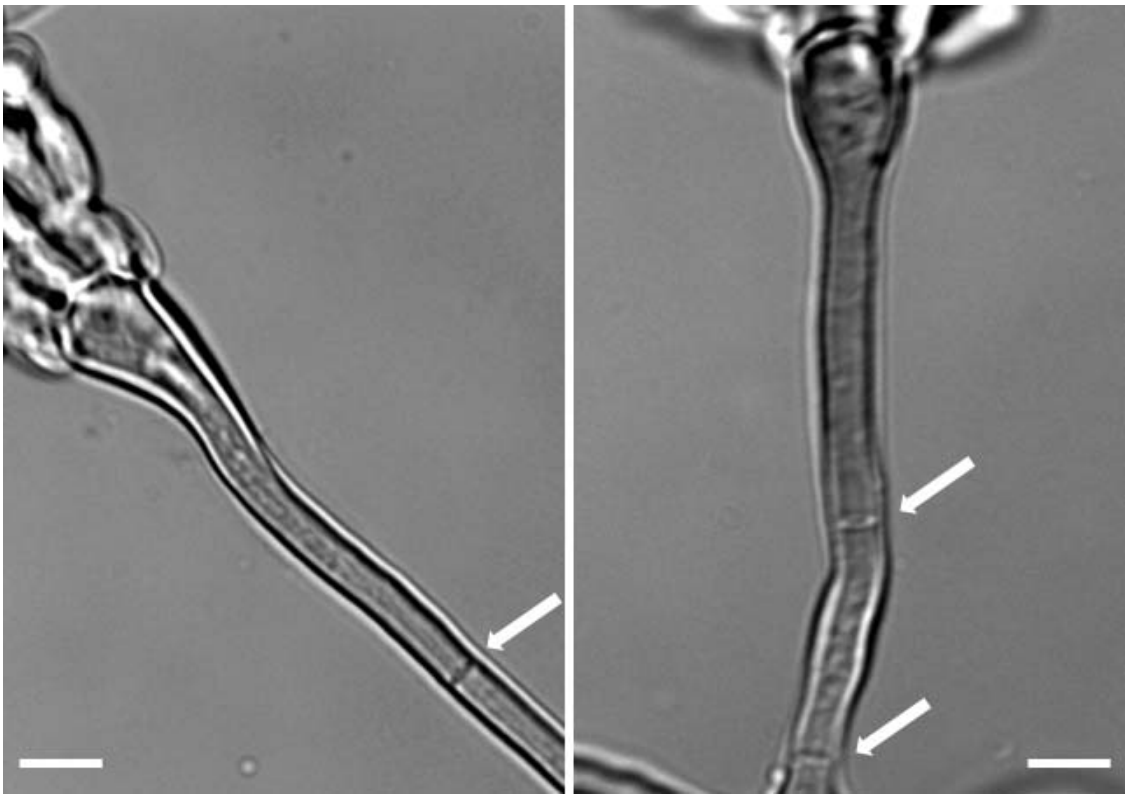


Figure 4-3. Conidiophore phenotype showing frequent septa in stalk in mutants. Strains were grown on rich medium for 3 days. Septa in stalk are marked by arrow. Bar = 10μm.

Figure 4-4. Phenotype of abnormal conidiophore.

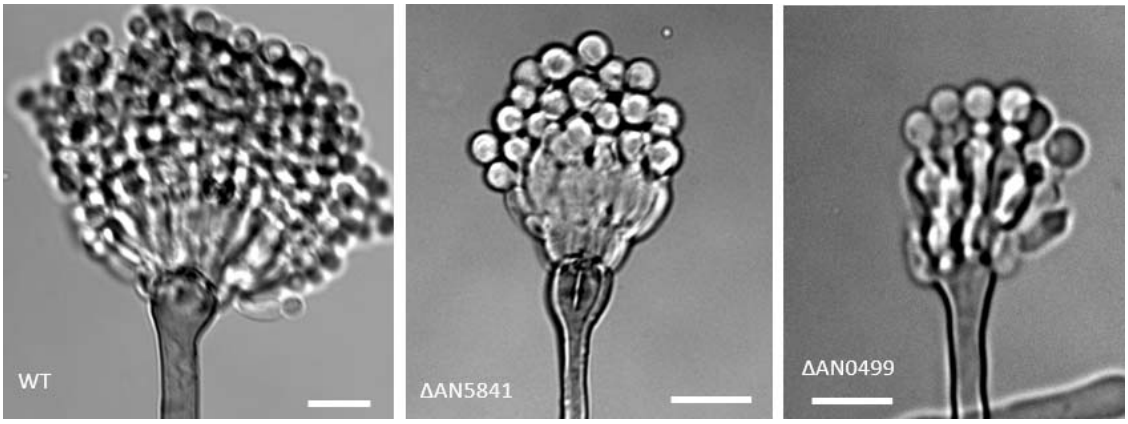


Figure 4-4. Phenotype of abnormal conidiophore. Strains were grown on rich medium for 3 days. Notice only one or two layers of spores from the conidiophore of *ΔAN5841* and *ΔAN0499*. Bar = 10μm.

Figure 4-5. Quantitative analysis of the short hyphal branching.

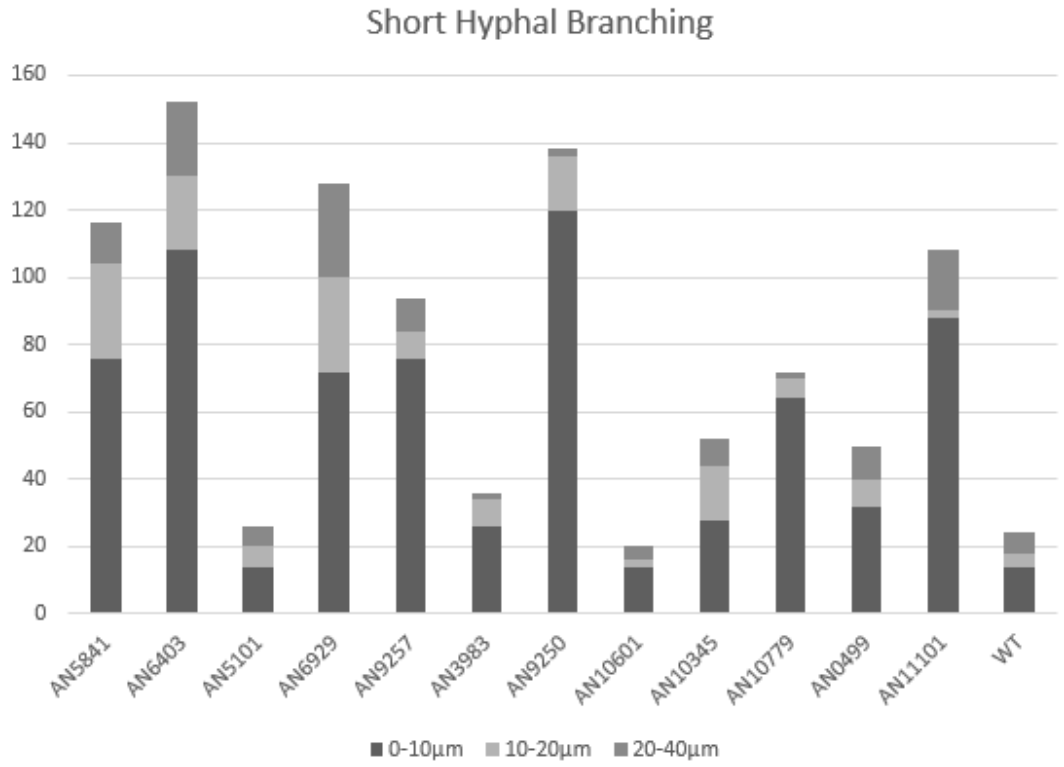


Figure 4-5. Quantitative analysis of the short hyphal branching in mutants and wild type. Strains were grown in liquid medium for overnight. Short branching on 200 hyphae were counted and grouped based on length for each strain. Note that several mutants exhibited hyper branching in contrast to wild type.

Figure 4-6. Quantitative analysis of the frequency of stalk septa.

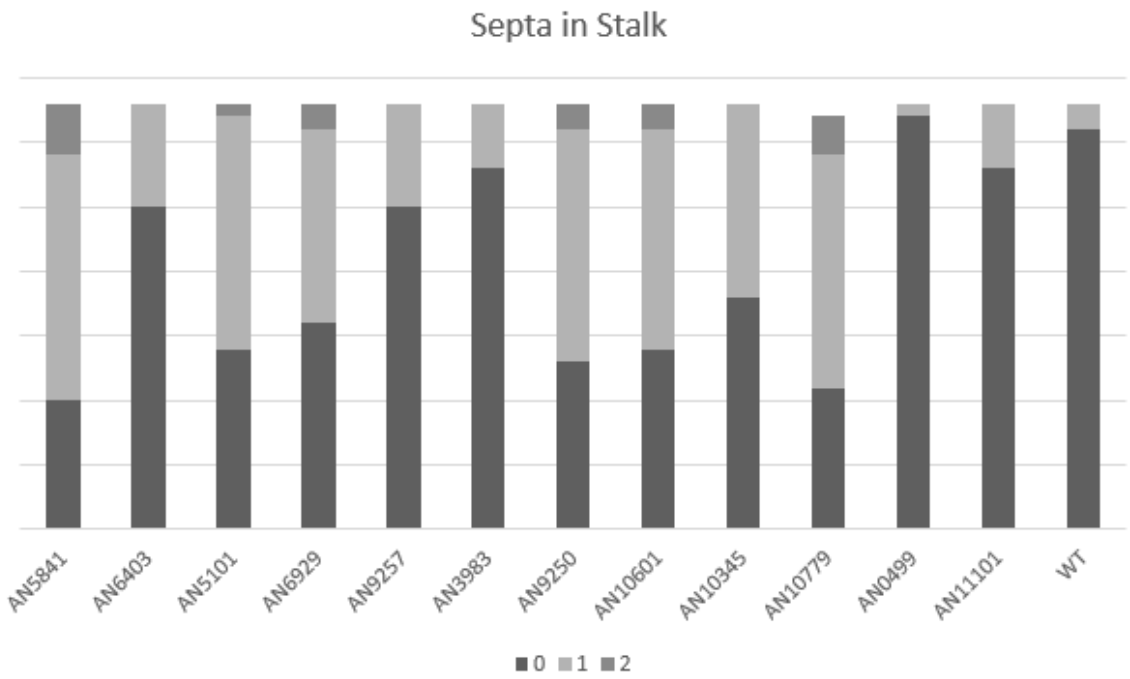


Figure 4-6. Quantitative analysis of the frequency of stalk septa in mutants and wild type. Strains were grown with the sandwich slide method for 3 days. 100 conidiophore were observed for each strain to count the percentage of septa frequency.

Figure 4-7. Quantitative analysis of the stalk length.

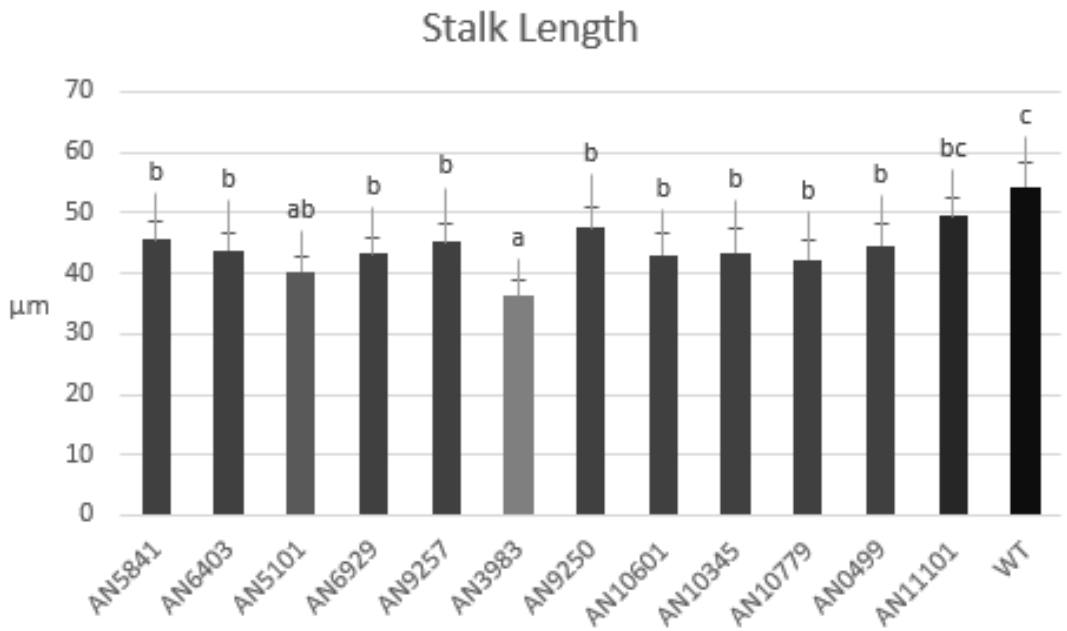
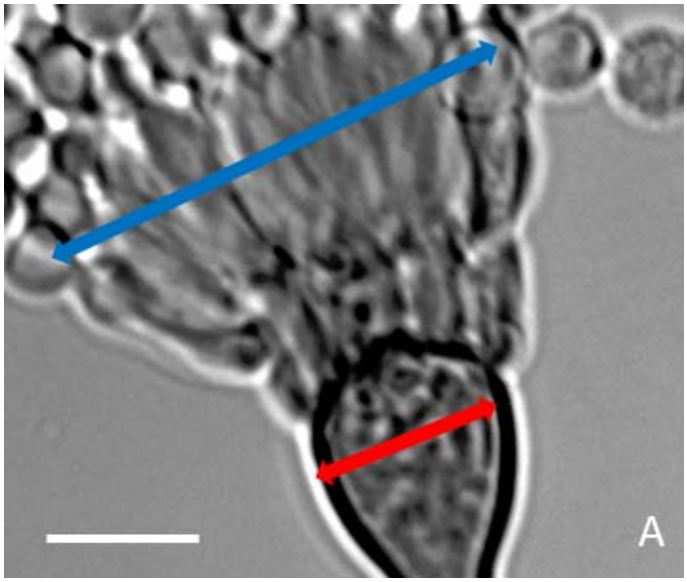


Figure 4-6. Quantitative analysis of the stalk length in mutants and wild type. Strains were grown with the sandwich slide method for 3 days. 100 conidiophore were observed for each strain to measure the average length of stalk. Letters above bars indicate the significant difference between groups. Note that wild type strain has the longest stalk in general.

Figure 4-8. Quantitative analysis of the conidiophore head width.



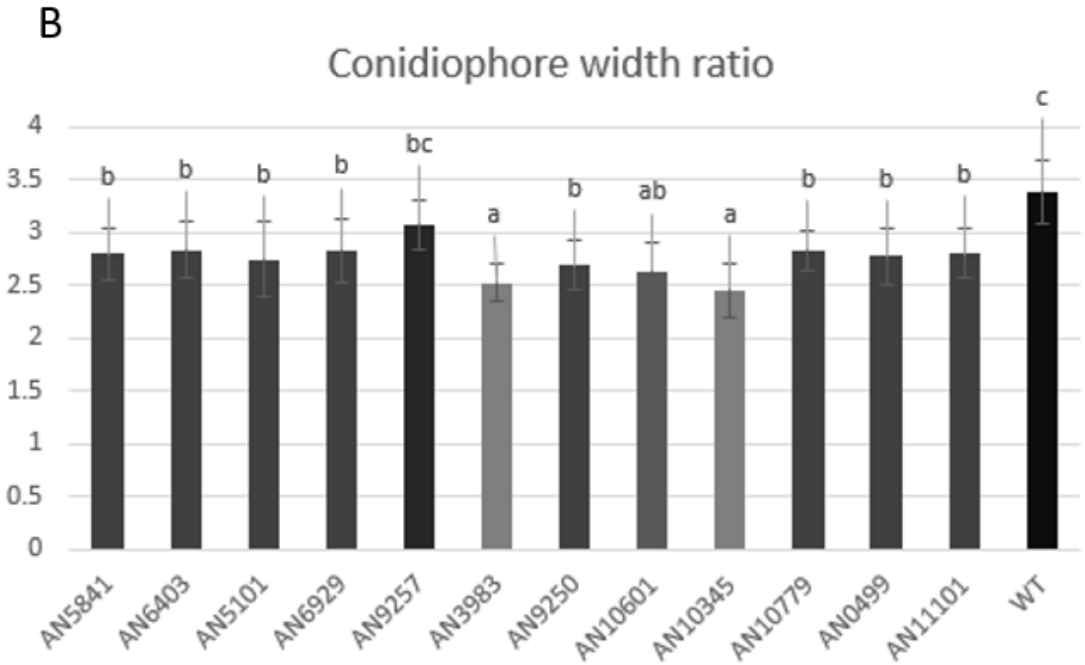


Figure 4-7. Quantitative analysis of the conidiophore head width in mutants and wild type. The ratio was calculated as shown in A, the range of phialide (blue) is divided by the diameter of vesicle (red). Strains were grown with the sandwich slide method for 3 days. B, 100 conidiophore were observed for each strain to measure the average length of stalk. Letters above bars indicate the significant difference between groups. Note that wild type strain has the longest stalk in average.

Figure 4-9. Summary of the difference of conidial spore layers born by phialide in mutants and wild type.

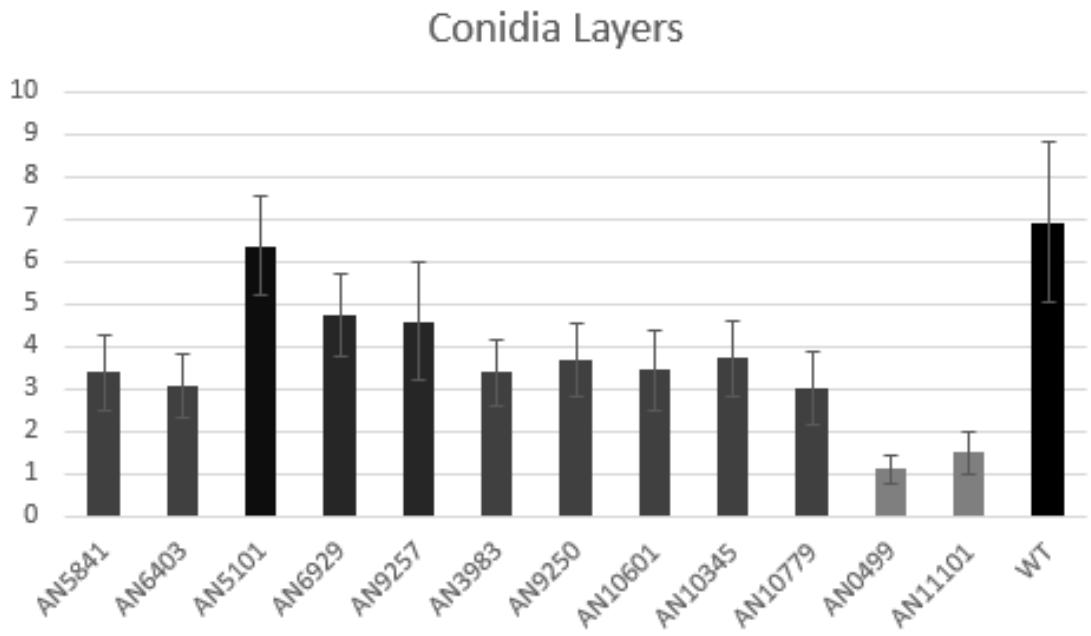


Figure 4-9. Summary of the difference of conidial spore layers born by phialide in mutants and wild type. Strains were grown with the sandwich slide method for 3 days. 100 conidiophore were observed for each strain to count the layers of spores on phialide. Note that Δ*AN0499* and Δ*AN11101* have dramatically reduced conidial layer than the wild type.

Figure 4-10. Random phialide branched from metulae in Δ*AN0499*.

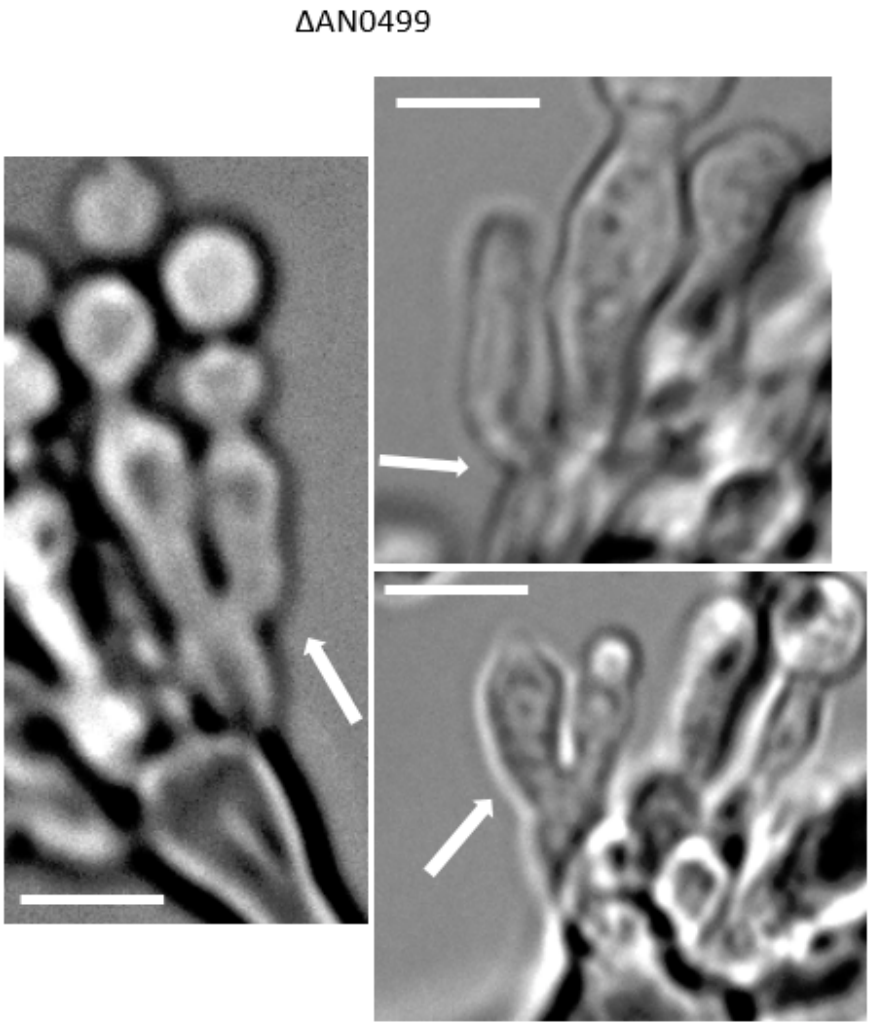


Figure 4-10. Close-up at the phialide in Δ*AN0499* to show the random phialide branched from metulae as marked by arrow. Bar = 10μm.

Figure 4-11. Stress test results.

A.

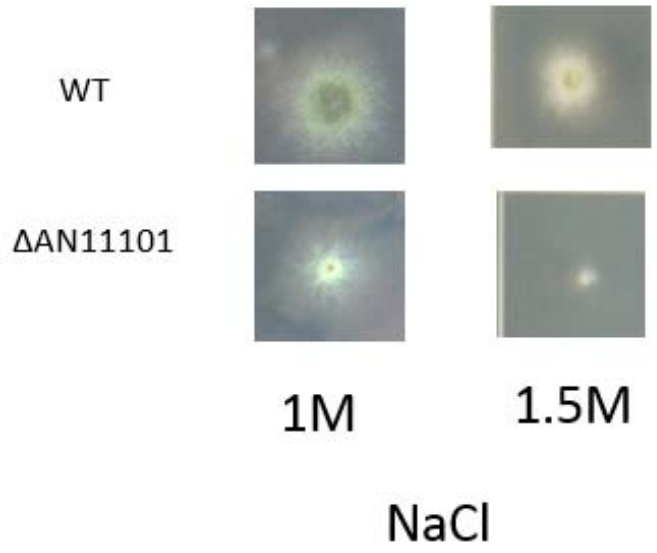


Figure 4-11

B.

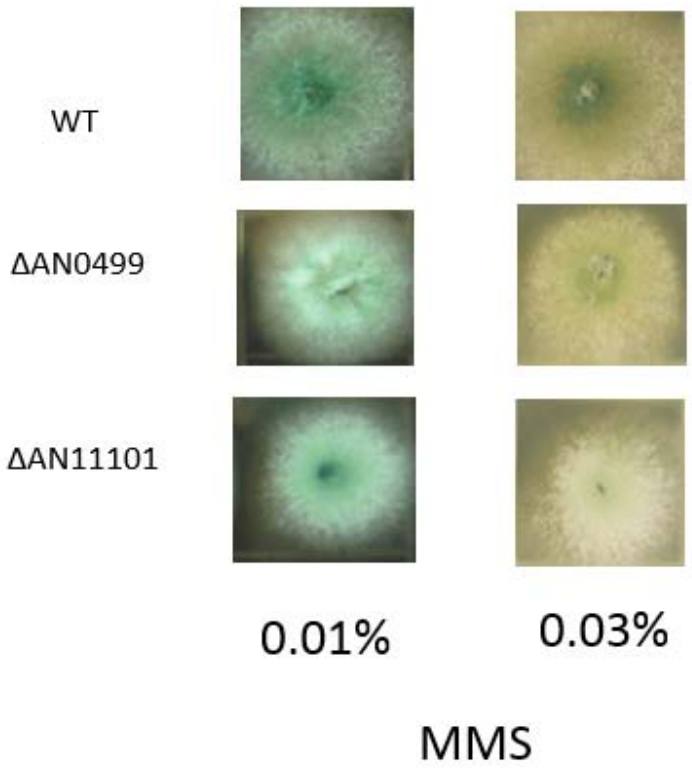


Figure 4-11

C.

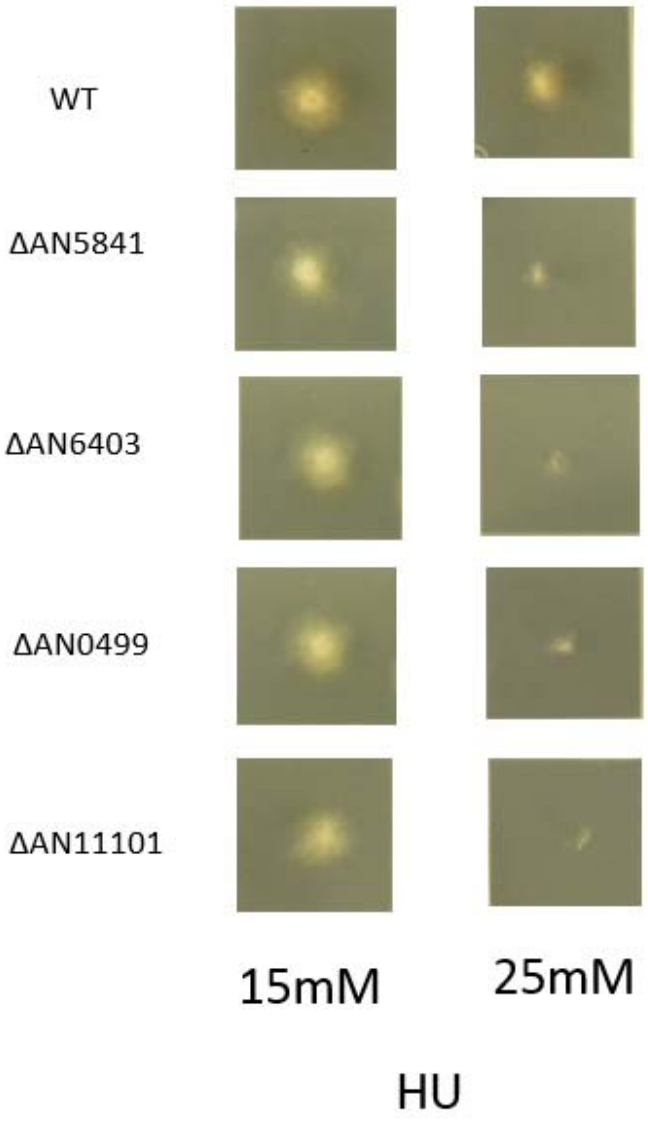


Figure 4-11

D.

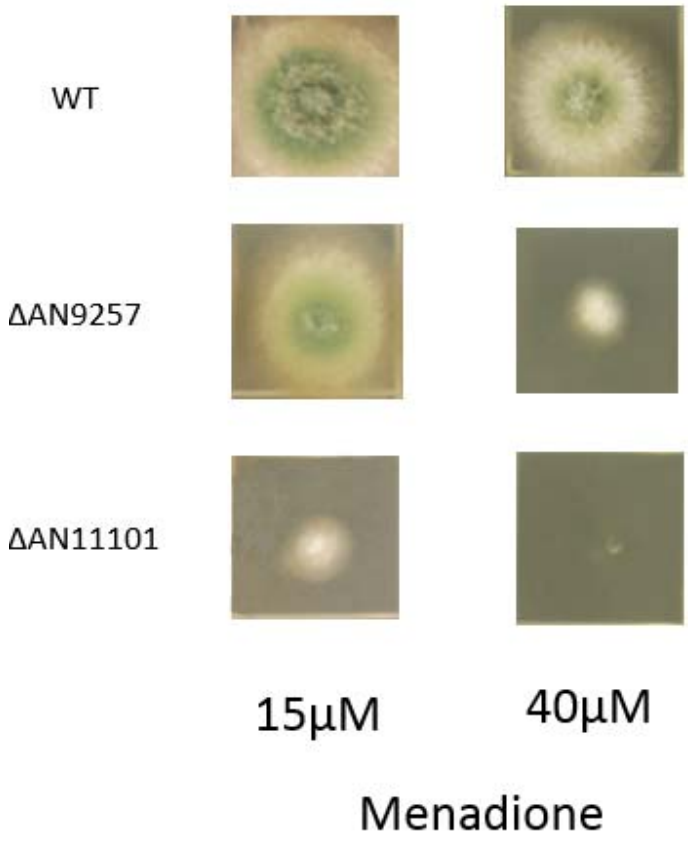


Figure 4-11

E.

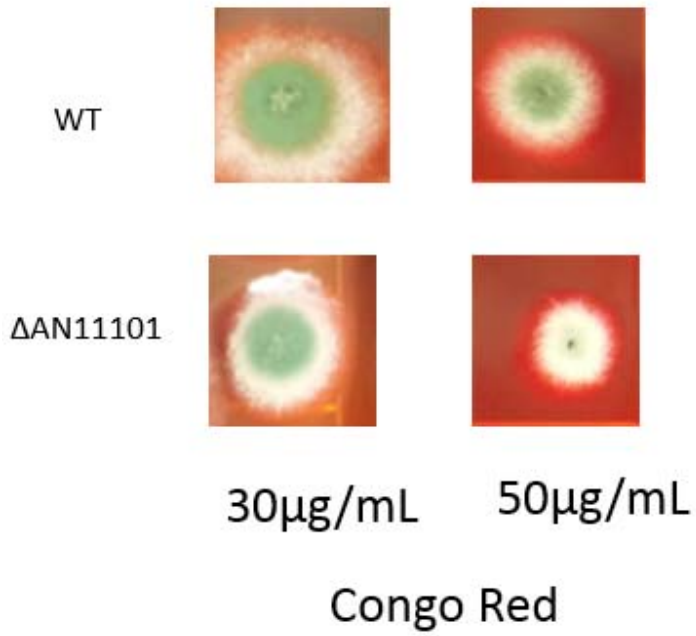


Figure 4-11. Osmotic stress test on minimal medium with NaCl, or drug sensitivity test on rich medium containing HU, MMS, Menadione, or Congo red. In general, AN11101 and AN0499 have higher sensitivity to the stresses than wild type or other mutants.

Table 4-1. Summary of phenotypic analysis, + indicates positive abnormal phenotype, or the value of length/width; - indicates no such phenotype; and +/- shows weak phenotype.

Strain	Hyper branching	Septa in stalk	Stalk length	Conidia layer	Conidia width
ΔAN5841	+	+	++	3	++
ΔAN6403	+	-	++	3	++
ΔAN5101	-	+	+	6	++
ΔAN6929	+	+	++	4	++
ΔAN9257	+	-	++	4	+
ΔAN3983	-	-	+	3	++
ΔAN9250	+	+	++	3	+
ΔAN10601	-	+	++	3	+
ΔAN10345	(+/-)	+	++	3	++
ΔAN10779	(+/-)	+	++	2	++
ΔAN0499	(+/-)	-	++	1	++
ΔAN11101	+	-	++	2	++
WT	-	-	+++	7	+++

Table 4-2 Summary of stress sensitivity tests, + indicates growth, - indicates lethal, and +/- shows strong septation and defective conidiation. Notice that Δ*AN0499* and Δ*AN11101* exhibit higher sensitivity against MMS and Hu than wild type. Δ*AN11101* is sensitive to menadione.

	NaCl		MMS		HU		Menadione		CR	
Strain	1M	1.5M	0.01%	0.03%	15mM	25mM	15μM	40μM	30 μg/mL	50 μg/mL
ΔAN5841	+	+	+	+	+	-	+	+	+	+
ΔAN6403	+	+	+	+	+	-	+	+	+	+
ΔAN5101	+	+	+	+	+	+	+	+	+	+
ΔAN6929	+	+	+	+	+	+	+	+	+	+
ΔAN9257	+	+	+	+	+	+	+	-	+	+
ΔAN3983	+	+	+	+	+	+	+	+	+	+
ΔAN9250	+	+	+	+	+	+	+	+	+	+
ΔAN10601	+	+	+	+	+	+	+	+	+	+
ΔAN10345	+	+	+	+	+	+	+	+	+	+
ΔAN10779	+	+	+	+	+	+	+	+	+	+
ΔAN0499	+	+	(+/-)	(+/-)	+	-	+	+	+	+
ΔAN11101	+	-	(+/-)	(+/-)	+	-	(+/-)	-	+	(+/-)
WT	+	+	+	+	+	+	+	+	+	+

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Chapter V Summary and Prospective

The regulatory system that specifies conidiophore morphogenesis has been known to be the central regulatory pathway in *A. nidulans*. However, the extent to how the downstream genes functioning in phialide development are regulated by the components of this system, especially AbaA still remains unknown. Here we identified several genetic modules and characterized two of them in detail to determine their functions in phialide formation. Notably, ndrA and phiB appear to be specifically required for the proper function of phialide as the sporogenous cell that forms and divides nascent spores. Our observations reveal the new insight into the phialide specific morphogenesis in *A. nidulans* and other filamentous fungi.

Protein kinase ndrA in phialide morphogenesis

We have demonstrated that ndrA in *A. nidulans* is the homologue of yeast Hsl1/Gin4/Kcc4 modules. ndrA plays an essential role in phialide morphogenesis. Conserved domain search revealed that ndrA has serine/threonine protein kinase that is similar as its homologous counterparts in yeast. However, ndrA is not essential for septin organization at the phialide tip. In yeast, Hsl1, Gin4 and Kcc4 are similar to each other functionally and structurally. There is slight difference between them and ndrA is more related to the function of Hsl1. Based on the homology search, ndrA is widely existed in Ascomycota fungi, however, only few higher species produce phialide. In our hypothesis, ndrA in other species may play different roles and may be regulated by other key genes instead of axl2 or abaA. To determine the function of ndrA in other phialide or non-

phialide ascomycetes, gene disruptive mutants can be generated. Also, to further examine the regulation of ndrA by Axl2 in *A. nidulans*, an inducible axl2 by introducing a promoter such as alcA(p) can be used to assess the expression of ndrA under axl2 induction. As we noticed that ndrA blocks sexual development during early development stage, since the Δ*ndrA* mutant exhibits early sexual reproduction, it is necessary to investigate the function of ndrA in this process.

Septins are essential proteins in fungi that ensures proper cytokinesis during septum formation. In Chapter III, we have showed the interaction between AspB and ndrA that ndrA does not function at upstream of AspB, but further studies need to be done to elucidate specific interactions between them and also between ndrA and other septin members in *A. nidulans.* The localization of ndrA-GFP in the septin deleted mutants, especially in AspE and AspD deletions, in that these mutants are less defective in conidiation than other septin mutants. If ndrA is not properly localized in the absence of septin, it would provide a genetic proof for the reversed direction of interaction between septin and ndrA. Similarly, septin-GFP can be used to localize septins in the background of absent ndrA.

phiB in phialide morphogenesis

Even though we failed to localize phiB due to unsuccessful attempt of gene replacement, we believe that phiB may have the similar localization as axl2 and ndrA, at the phialide-spore junction (Figure 5-1), based on the functionality analysis. And phiB may be another module being regulated by axl2, as axl2 serves as the spore site marker to relocalize proteins required for proper sporulation at the phialide tip after each spore

generation. This is implicated by the phenotype of phialide bearing one or two conidia layers in both Δ*axl2* mutant and Δ*phiB* mutant. The specific localization of PhiB needs to be determined. Unfortunately, we could not fuse GFP probe to the N-terminal of the phiB gene. A C-terminal GFP fusion can be designed. A sexual cross between PhiB-GFP and Δ*axl2* could provide insightful information about the relationship between Axl2 and PhiB to test our hypothesis. Moreover, to test our hypothesis of the interaction between Axl2 and PhiB, an Axl2 intracellular part can be cloned and the interaction can be assessed by yeast two-hybrid method (Young 1998) as the β-catenin binds to the cytoplasmic domain cadherins in axl2.

Timing of expression of ndrA and phiB

In addition to the spatial expression of ndrA at phialide-spore junction, right timing of expression may be also needed for proper phialide development. We have indicated the expression level before and during developmental stages after induction using semi-quantitative RT-PCR, and the expressions of ndrA and phiB are only induced at the early development. More specific timing of their expression may be needed to investigate. A northern blot analysis can be used to examine expression levels during different time points. And qPCR is also useful to quantify their expression levels over time.

In order to examine the importance of proper timing expression of ndrA and phiB, an tightly controllable promoter such as alcA(p) can be used to induce their expressions. We can investigate the defective phenotype of mutant caused by wrong timing of expression by inducing ndrA or phiB at 0 hour, 4 hour and 8 hour after overnight

vegetative growth. And also a RNA extraction followed by RT-PCR can be used to verify their induction.

NdrA and PhiB are recruited by Axl2

Axl2 is the *A. nidulans* homologue of the axial bud site selection marker in *S. cerevisiae*. In yeast, axl2 along with Bud3, Bud4 form a regulatory module to specify the axial budding pattern (Chant, 1999). In *A. nidulans,* it is has been reported that during development, Axl2 functions at the late stage of conidiophore development (Si *et al*. 2012). Axl2 is regulated by the transcriptional factor AbaA, and its proper expression is required for proper function of phialide. According to Si's report, Axl2-GFP is solely localized between phialides and nascent spores, but not at other septation site in hyphae or conidiophores (Figure 5-1).

We suspected that NdrA is a genetic module being regulated by AbaA in terms of expression. To perform specific function, Axl2 may involve in recruiting ndrA to a high level at the phialide-spore junction site, which was detected by GFP-fusion. We reasoned this relationship in several aspects. First, same as Δ*axl2* mutant, Δ*ndrA* also fails to generate the long chains of spores as the wild-type phialides, but normally produces only one or two spores on each phialide. Second, NdrA-GFP localizes at the phialide-spore junctions as well, but not at other septation sites (Figure 5-1). Third, expression of ndrA is up-regulated during conidiophore development, or under forced induction of abaA. Fourth, NdrA fails to localize at the phialide-spore junction when axl2 is absent. In addition, NdrA is not localizing in hyphae. Finally, ndrA is also involved in sexual development as the axl2.

Proposed model of interaction between central pathway, axl2, Septin, ndrA, and phiB

BrlA, AbaA and WetA are transcriptional factors in central pathway that regulate the conidiophore development in *A. nidulans*. BrlA is required for the expression of proteins involved in all developmental steps from the formation of vesicle and afterward steps (Prade and Timberlake, 1993), and AbaA is up-regulated by BrlA and serves as the key for activating expression of genes in mid development, and also for phialide differentiation (Chang and Timberlake 1993). As it is already known that axl2 is regulated by AbaA and recruits septins to the phialide-spore junction (Si et al. 2012), which plays important roles in cytokinesis during septum formation. NdrA is another essential module for phialide formation that is recruited by Axl2, and phiB is suspected to function similarly as ndrA. Here we propose the genetic regulation scheme as shown in Figure 5-2 for phialide morphogenesis.

More AbaA related genes to be characterized

In this study, we selected 12 genes based on a single RNA-seq comparison between abaA induced strain and wild type strain. Replicates of RNA-seq can be beneficial to provide more accurate result, but would be expensive. Since our gene selection is mainly for producing a manageable gene pool for functionality analysis, the majority of the AbaA up-regulated genes remain uncharacterized. With our RNA-seq screening method, more genes can be selected and studied. In this study, we also noticed

that the phialides in Δ*AN10779* and other several mutants also bear much less layers of spores than the wild type strain, indicating that these genes are also involved in regulating the phialide function. It would be worthy to characterize these genes in detail.

Figure 5-1. Illustration of septin, axl2, ndrA, and phiB localization.

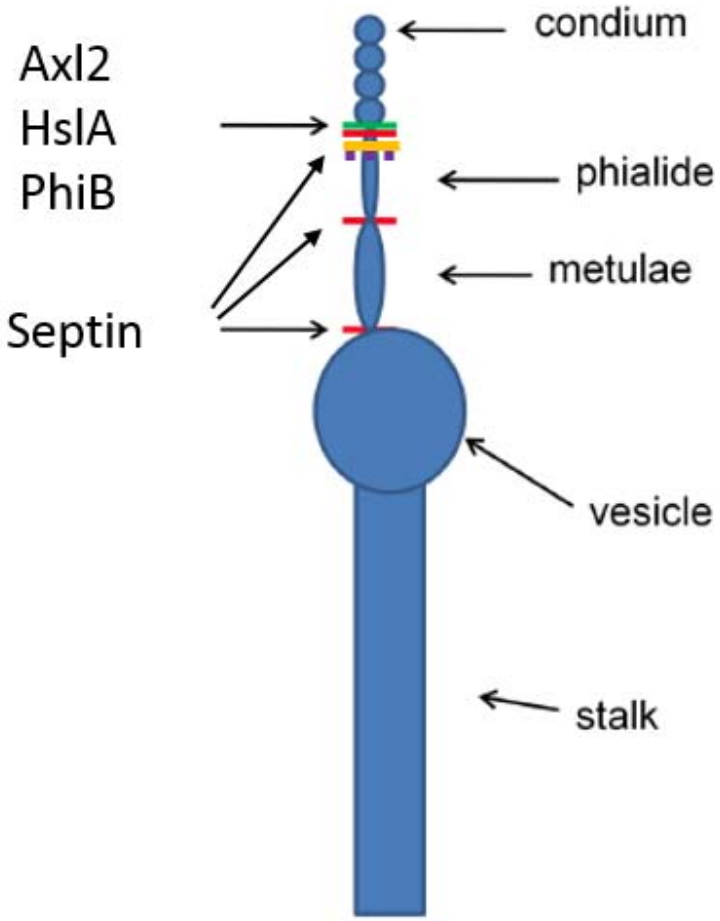

Figure 5-1. Illustration of septin, axl2, ndrA, and phiB localization. Red lines indicate septin, green line indicates axl2, yellow line indicates ndrA, and purple dot line shows the hypothetic localization of phiB.

Figure 5-2. Proposed regulation of conidiophore morphogenesis.

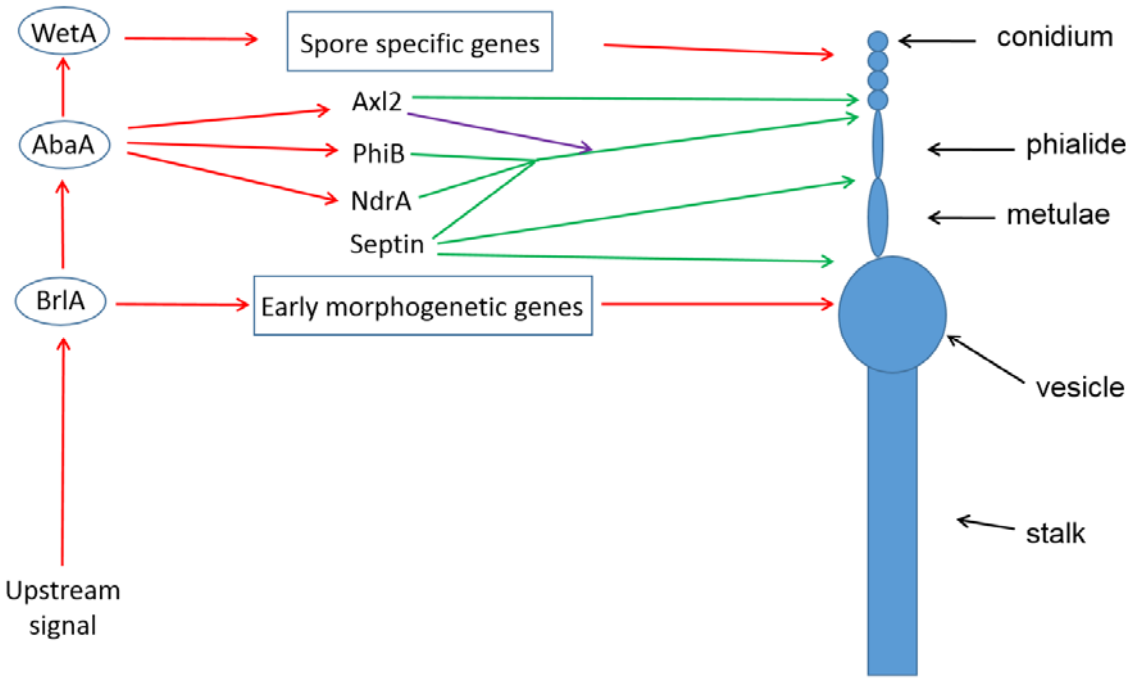

Figure 5-2. Proposed regulation of conidiophore morphogenesis. BrlA is required for the expression of a series of proteins in conidiophore development steps. AbaA is required for phialide differentiation. AbaA regulates the expression of axl2, ndrA, and phiB (red arrows). Axl2 accumulates (green arrows) and recruits (purple arrow) ndrA, phiB as well as septins (green arrows) at the phialide-spore junction. These modules regulate the phialide function. WetA is required for spore production.

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