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Université Paris Cité

École doctorale Médicament, Toxicologie, Chimie et Imageries, N° 563
Laboratoire Interdisciplinaire des Énergies de demain (LIED UMR 8236)

**Characterization of secondary metabolites in the
filamentous fungus *Podospora anserina*: for a better
understanding of fungal physiology and the isolation of
new bioactive metabolites**

Par Huijuan WANG

Thèse de doctorat de **Microbiologie**

Dirigée par **Florence CHAPELAND-LECLERC** et
Gwenaël RUPRICH-ROBERT

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Titre : Caractérisation des métabolites secondaires dans le champignon filamenteux *Podospora anserina* : pour une meilleure compréhension de la physiologie fongique et l'isolement de nouveaux métabolites bioactifs

Résumé : Les champignons ont une capacité remarquable à produire une gamme variée de métabolites secondaires (MS) avec des bioactivités potentielles. Ces métabolites secondaires ont des fonctions diverses, notamment la protection contre le stress UV, l'influence sur le développement et l'aide à la pénétration et à l'infection de l'hôte. Chez les champignons, les gènes produisant des MS sont généralement organisés en groupes identifiables appelés clusters, bien que beaucoup restent inactifs dans les conditions de laboratoire. Notre recherche se concentre spécifiquement sur la production de MS chez *Podospora anserina*, une espèce connue pour ses MS essentiels de faible poids moléculaire impliqués dans divers processus biologiques. La régulation de la production de MS chez les champignons implique des mécanismes transcriptionnels, épigénétiques et des stimuli environnementaux, avec des régulateurs importants tels que *NsdD*, *LaeA*, *CreA* et *HapX*. *P. anserina* possède 42 clusters putatifs de MS, dont 17 clusters de polykétide synthase (PKS) et 6 clusters de peptide synthétase non ribosomique (NRPS), etc. Malgré cela, peu de recherches ont exploré les rôles des MS tout au long de son cycle de vie. Pour combler cette lacune, notre approche consiste à réaliser la caractérisation fonctionnelle de *NsdD*, *LaeA*, *CreA* et *HapX*. En générant des mutants, nos objectifs étaient d'analyser leurs caractéristiques phénotypiques et d'effectuer l'identification de la structure de certains composés, dans le but de découvrir la signification fonctionnelle des métabolites secondaires chez *P. anserina*. L'étude de régulateurs globaux tels que *PaNsdD* et *PaLaeA* a amélioré notre compréhension de la production de métabolites secondaires. Les études préliminaires de *PaHapX* et de *PaCreA* ont montré des différences phénotypiques intéressantes, notamment dans la réponse au stress et l'utilisation du carbone. Finalement, notre recherche vise à élucider la régulation complexe de la production de SMs fongiques et ses implications dans divers processus biologiques.

Mots clefs : *Podospora anserina*, métabolite secondaire, cluster de gènes biosynthétiques, *NsdD*, *LaeA*, *CreA*, *HapX*

Title: Characterization of secondary metabolites in the filamentous fungus *Podospora anserina*: for a better understanding of fungal physiology and the isolation of new bioactive metabolites

Abstract: Fungi have a remarkable capacity to produce a diverse array of secondary metabolites (SMs) with potential bioactivities. These SMs serve various purposes, including UV stress protection, developmental influence, and facilitation of host penetration and infection. In fungi, SM production genes are typically organized into identifiable clusters, although many of these clusters remain inactive under laboratory conditions. Our research focuses specifically on SM production in *Podospora anserina*, a species known for its essential low molecular weight SMs involved in diverse biological processes. Regulation of SM production in fungi involves transcriptional, epigenetic, and environmental stimuli mechanisms, with prominent regulators such as *NsdD*, *LaeA*, *CreA*, and *HapX*. *P. anserina* possesses 42 putative SM clusters, including 17 polyketide synthase (PKS) and 6 nonribosomal peptide synthetase (NRPS) clusters and so on. Despite this, limited research has explored the roles of SMs throughout its life cycle. To address this knowledge gap, our approach involves the functional characterization of *NsdD*, *LaeA*, *CreA*, and *HapX*. Through generating mutants, our objectives were to analyze their phenotypic characteristics, perform the structure identification of some compounds, aiming to uncover the functional significance of SMs in *P. anserina*. Investigating global regulators like *PaNsdD* and *PaLaeA* improved our understanding of SM production. Preliminary studies about *PaHapX* and *PaCreA* have shown some interesting phenotypic variations, particularly in stress response and carbon utilization. Ultimately, our research aims to elucidate the intricate regulation of fungal SM production and its implications in various biological processes.

Keywords: *Podospora anserina*, secondary metabolite, biosynthetic gene clusters, *NsdD*, *LaeA*, *CreA*, *HapX*

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Abbreviations

ACN	Acetonitrile
AF	Aflatoxin
BGC	Biosynthetic Gene Cluster
Bp	Base Pair
CBC	CCAAT Binding Complex
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CD ₃ OD	MeOH, Methanol
CDCl ₃	Chloroform
CDS	Coding DNA sequence
COSY	Correlated Spectroscopy
CCR	Carbon Catabolite Repression
CFW	Calcofluor White
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DMATs	Dimethylallyl Tryptophan Synthases
DHN	Dihydroxynaphthalene
DOPA	Dihydroxy Phenylalanine
EtOAc	Ethyl acetate
HOG	High-Osmolarity Glycerol
HSTs	Host-Specific Toxins
HMBC	Heteronuclear Multiple-Bond Correlation Spectroscopy
HSQC	Heteronuclear Singular Quantum Correlation
HRMS	High-Resolution Mass-Spectrometry
HPLC	High Performance Liquid Chromatography
MG	Methylglyoxal
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NRPs	Non-Ribosomal Peptide Synthetase
NOESY	Nuclear Overhauser Effect Spectroscopy
ORF	Open Reading Frame

PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PKs	Polyketide Synthase
PKA	Protein Kinase A
PM	Primary Metabolite
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SM	Secondary Metabolite
ST	Sterigmatocystin
TBY	<i>Tert</i> -butyl hydroxyperoxide
TF	Transcription Factor
TSs	Terpene Synthases
TCs	Terpene Cyclases
TCA	Tricarboxylic Acid
TLC	Thin Layer Chromatography
UHPLC	Ultra-High Performance Liquid Chromatography
UV	Ultra Violet
WT	wild-type

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Chapter 1

Introduction

1.1. Fungi and Fungal specialized metabolites

1.1.1. Fungi

In 1969, Robert Whittaker published the five-kingdom classification which separated fungi from the plant kingdom and gave a separate position to fungi in the kingdom classification¹. The classification of the five kingdoms allowed a large number of traits that belonged neither to animals nor plants to be recognized, and then R.H. Whittaker further subdivided the kingdom of fungi into six categories (Basidiomycota, Microsporidiomycota, Deuteromycota, Oomycota, Zygomycota, Ascomycota) based on different phylogenetic evidence and established an independent kingdom of fungi. Fungi are a group of eukaryotic organisms that produce food, organic acids, alcohol, antibiotics, growth-promoting substances, enzymes, and amino acids. They include microorganisms like molds, yeasts, and mushrooms. Fungi play a vital part in our daily lives, both beneficial and bad. On the one hand, they are the source of life-saving and life-enhancing pharmaceuticals, food additives, and scents, but they also have the ability to contaminate our crops and food and cause serious diseases². Fungi are excellent decomposers of organic waste, preferring to target organic complex compounds like cellulose, lignins, gums³. Fungal biologists debated for more than 200 years about which organisms should be counted as Fungi. In less than 5 years, DNA sequencing provided a multitude of new characters for analysis and identified about 10 phyla as members of the monophyletic kingdom Fungi (**Figure 1.1**). It has been reported 97330 species of described fungi at the “numbers of fungi” entry. The addition of 1300 microsporidians bring the total of

all described fungi to about 99000 species⁴. About 80000 to 120000 species of fungi have been described to date, although the total number of species is estimated at around 1.5 million⁵. Because fungi are heterotrophic creatures, they must get nourishment from natural sources such as sugars or amino acids. Fungi are either compelled to degrade dead organic stuff or to interact with live creatures and get nutrients from other species to do this⁶. Fungi are classified into three categories based on their lifestyle: saprophytic, parasitic, and symbiotic⁷. Saprobiotic fungus decompose deceased creatures' organic matter. Parasitic fungi feed on living host species that have been injured by the parasite. Depending on the host, parasitic fungi are classified into several categories. Mycoparasitic fungi, for example, parasitize other fungal species, phytopathogenic fungi that live on plants⁸, and entomopathogenic fungi that live on insects⁹. Symbiotic fungus gets their sustenance from other living species, but they also benefit the host. Endosymbiotic creatures coexist with other living organisms; for example, endophytic fungus coexist with plants¹⁰.

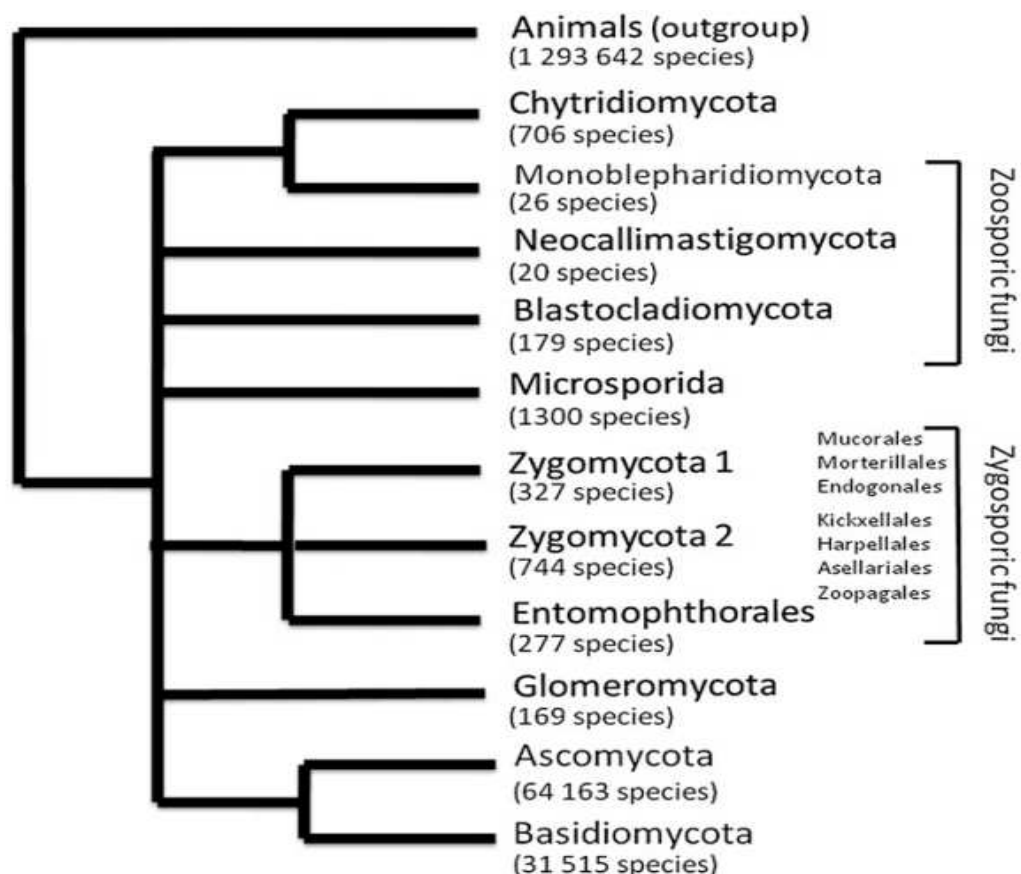


Figure 1.1. Fungal phyla and the estimated number of species in each⁴. Microsporidians are Fungi, according to evidence from gene order conversion and multilocus sequencing¹¹. It is also worth noting that the zoosporic and zygosporic fungal groupings are not accepted as monophyletic¹²⁻¹⁴.

1.1.2. Primary metabolites and specialized metabolites

Fungal organisms can produce a wide range of organic compounds. Specialized metabolites (SMs), also referred to as secondary metabolites, are those that are not generally essential for an organism's primary growth and development, whereas primary metabolites (PMs) are those that are essential^{15,16}. SMs are produced by fungi, bacteria, plants, and some insects. Furthermore, many of these SM compounds, which are also known as natural products and are generally composed of low-molecular-mass compounds with complex molecular structures, offer significant therapeutic effects, among other things¹⁷. When grown in the laboratory conditions, the lack of SMs could result in no visible phenotypic changes in the generating organism. Because some SMs do not have identifiable physiological activities like main metabolites, blocking the secondary metabolic pathway sometimes has little or no effect on fungal growth and reproduction. The roles of SMs, many of which are yet unclear, are being clarified on a regular basis. In contrast, primary metabolites, which include amino acids, nucleotides, polysaccharides, lipids, and vitamins, are found in a wide range of fungus and perform important and frequently observable metabolic processes³.

1.1.3. Chemical classes of Fungal SMs

The heterogeneous SMs produced by fungi can generally be classified into four distinct chemical categories: polyketides (PKs) (e.g. aflatoxins and fumonisins), non-ribosomal peptides (NRPs) (e.g. penicillin, peramine and siderophores such as ferricrocin), terpenes (e.g.

gibberellins, T-2 toxin, deoxynivalenol (DON)) and prenylated tryptophan derivatives (e.g. ergot and indole alkaloids)¹⁸ (**Figure 1.2**). These SMs are classified chemically based on their distinct biosynthetic route, which begins with core enzymes such as polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene synthases (TSs) or cyclases (TCs), and dimethylallyl tryptophan synthases (DMATs). It exists also some hybrid SMs, such as PKS-NRPS hybrids (echinocandin,) and PKS-TC hybrids (fumagilin). The two enzymes may be distinct (valactamide produced by NRPS and PKS) or fused (tenuazonic acid synthesized by NRPS-PKS)¹⁹. In addition, hybrids between these classes have been reported in a number of fungal species (for example, fumagillin and pseurotin)^{20,21}. Most SMs in fungi are formed from either PKS or NRPS. PKS derivatives include, for example, AF, lovastatin, and strobilurin. Penicillin, cephalosporin, and gliotoxin are examples of well-known NRPSs, whereas ergotamine and fumigaclavine are tryptophan derivatives. Indole alkaloids are typically produced from tryptophan and dimethylallyl pyrophosphate, while other amino acids may be utilized as precursors. Ergotamine production in *Claviceps purpurea* and related species is the best understood route. The first committed step is tryptophan prenylation by dimethylallyl tryptophan synthetase (DMATS). Following the methylation of dimethylallyl tryptophan, a succession of oxidation processes leads to lysergic acid via agroclavine. After being triggered by a single-module NRPS, lysergic acid is condensed with a tripeptide generated by a second NRPS and released as ergotamine²². SMs are derived from central metabolic pathways and primary metabolite pools, with acyl-CoAs serving as critical initial building blocks in the synthesis of PKs (for example, aflatoxin) and TSs (for example, carotene), and amino acids serving as the building blocks in the synthesis of NRPs (for example, penicillin)^{23,24}. Certainly, the canonical route mentioned above does not produce a wide range of products. The biosynthesis of norloline, for example, utilizes a pyridoxal-phosphate-containing enzyme and

hence departs from traditional NRPS assembly methods, whereas ustiloxin B is biosynthesized via a ribosomal peptide synthetic (RiPS) pathway²⁵.

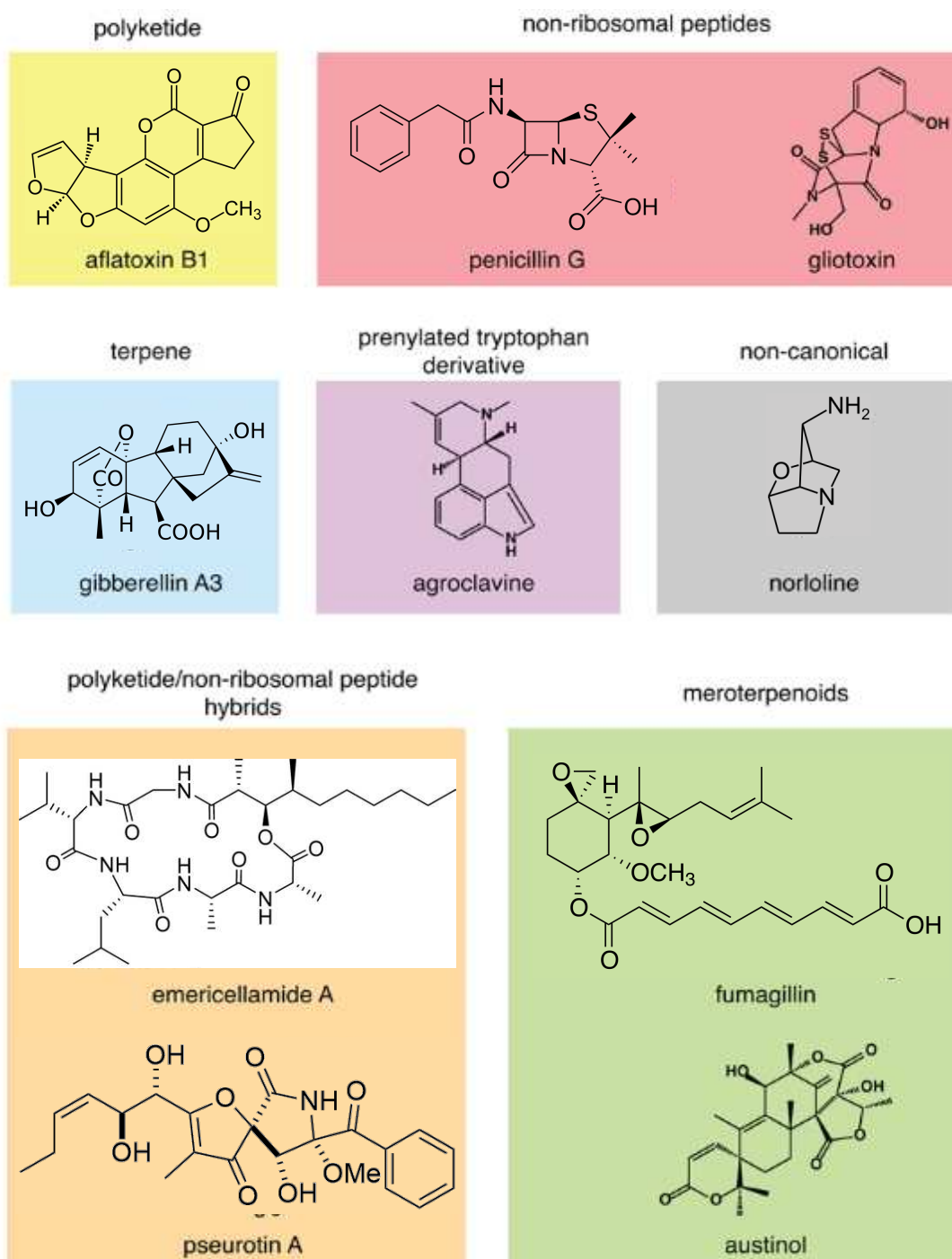


Figure 1.2. Fungal SMs of diverse chemical types generated via distinct biosynthetic pathways¹⁸.

Other SMs also exist, even if their biosynthetic pathways are a little less represented. These include alkaloids, terpenes and RiPPs.

1.1.4. Fungal SM gene clusters

In fungi, in contrast to genes required for the synthesis of a primary metabolite, which are dispersed throughout the fungal genome, genes encoding the enzymatic activities required to produce any SM, are more often arranged in a contiguous fashion as a biosynthetic gene cluster (BGC), hence they have been identified relatively easily from complete genome sequences, such as the aflatoxin BGC^{26–28}. SM biosynthesis genes are often organized in BGCs, which are frequently located at the ends of chromosomes in subtelomeric regions^{29,30}. The typical configuration of BGCs involved in the SMs production includes a key gene encoding enzyme that synthesizes the SM backbone, such as NRPS, PKS, DMATS or TC, and some genes for one or more tailoring enzymes that chemically modify SM skeleton. They may also contain a gene encoding a transporter responsible for exporting the final product and/or a gene encoding a transcription factor (TF) involved in regulating BGC expression³¹. The BGCs of SMs are as diverse in terms of gene content and structure. Some secondary BGCs vary from the typical design and include solely enzymes, such as the endocrocin³² BGCs in *A. fumigatus* and the penicillin³³ BGC in *Penicillium chrysogenum* (Ascomycota, Eurotiomycetes). Some BGCs, however, have several key genes, such as the emericellamide³⁴ BGC in *Aspergillus nidulans* and the cyclosporin³⁵ BGC in *Tolypocladium inflatum* (Ascomycota, Sordariomycetes), which both have a PKS and an NRPS. Other BGCs, lack key genes. In *Aspergillus flavus*, for example, production of the cyclic peptide ustiloxin B is mediated by a RiPS pathway using a precursor peptide²⁵. With the sequencing of the first fungal genomes, one of the major discoveries was that the number of BGCs greatly outnumbered the known SMs, revealing an even larger SM repertoire in these species but also demonstrating that most BGCs are not active or not active enough to produce a detectable amount of SM during standard laboratory cultivation^{24,36,37}.

1.2. The functions of Fungal SMs

SMs have key roles in fungi, some of them are ecological roles and some are bioactivity roles, such as defense and survival in the external environment, as a source of innovative drug formulation, and as antibiotics, antioxidants, anticancer, and antidiabetic drugs, among other things. Because fungi share their habitat with so many different creatures, SMs may provide protection or act as communication intermediaries. SMs are also known as plant and animal pathogen virulence factors. This job most likely developed to protect against amoebae, nematodes, and other invertebrates that feed on fungus. Studies of natural products stimulated development of the separation techniques, spectroscopic approaches to structure elucidation, and synthetic methodologies that now constitute the foundation of contemporary organic chemistry. Interest in natural products was not purely academic but rather was prompted by their great utility as dyes, polymers, fibers, glues, oils, waxes, flavoring agents, perfumes, and drugs. Recognition of the biological properties of myriad natural products has fueled the current focus of this field, namely, the search for new drugs, antibiotics, insecticides, and herbicides. Importantly, this growing appreciation of the highly diverse biological effects produced by natural products has prompted a reevaluation of the possible roles these compounds play in plants, especially in the context of ecological interactions. This section that follows explains the biologically active roles of SMs and the role and function of SMs in the interactions of fungus with microorganisms, plants, and animals.

1.2.1. Bioactivity of fungal SMs

For the past few decades, scientists have tested SMs from over 10,000 fungal species for biological activity. Since bioactive compounds from fungi provide a large pool of unexplored compounds, further investigations into the bioactivity have opened the door to a new era of

antimicrobial agents in higher fungi. In recent decades, fungal SMs are being researched as potential leads in drug development, cosmetics, and crop protection in agriculture.

SMS small molecules possess important role in fungi such as defense and survival in external environment. Natural products SMs are source of novel drug formulation and have vastly enhanced our lives by giving an abundant source of molecules to combat cancer, infections, and cardiovascular illnesses, which has changed medicine³⁸(**Figure 1.3**). Pharmacological research of these bioactive compounds reveals that fungi possess various biological applications such as antibacterial, antiviral, antiprotozoal, anticancer, antidiabetic, antifungal, antihelminthic, and antifungal activities etc. Fungi are prolific producers of a wide range of natural products, and recent advancements in synthetic biology, genetics, bioinformatics, and natural product chemistry have dramatically improved our capacity to mine their genomes for new medications³⁹⁻⁴². After Alexander Fleming's discovery of penicillin in 1928⁴³, antibiotics first came into medical use in the 1950s, now known as bioactive microbial metabolites⁴⁴. Antibiotics have been shown to be effective in the treatment of bacterial, fungal, and protozoal infections, as well as several physiological illnesses (for example, cholesterol reduction). Antibiotics were also utilized in veterinary medicine, as plant protectants (herbicides and insecticides), and as feed additives^{15,45-48}. Many well-known examples of compounds that have an impact on our daily lives are classified as fungal SMs⁴⁹. The 20th century saw the discovery, isolation, and chemical characterization of a wide range of natural compounds derived from filamentous fungi. The discovery of antibiotic action in penicillins from the microfungus *Penicillium notatum* in 1928 had a significant influence on natural product research in many ways^{42,50}. Penicillins and cephalosporins, for example, are β -lactam antibiotics that are the most frequently used antimicrobials in the world, with cephalosporins accounting for 28% and penicillins accounting for 19% of the worldwide antibiotic market⁵¹. Fungal metabolites have a "Janus-faced" spectrum of activities: they can be valuable for

medicinal reasons (e.g., penicillin), but they can also be poisonous and carcinogenic, endangering human, animal, and plant health (e.g., aflatoxins)⁵². *Trichoderma spp.* exhibit antifungal activities against phytopathogenic fungi, in which different groups of SMs, such as terpenes, pyrones, gliotoxin, gliovirin, and peptaibols may be involved^{53–55}. This can be used for the further exploration of new antifungal bioactive metabolites for the management of plant pathogenic fungi.

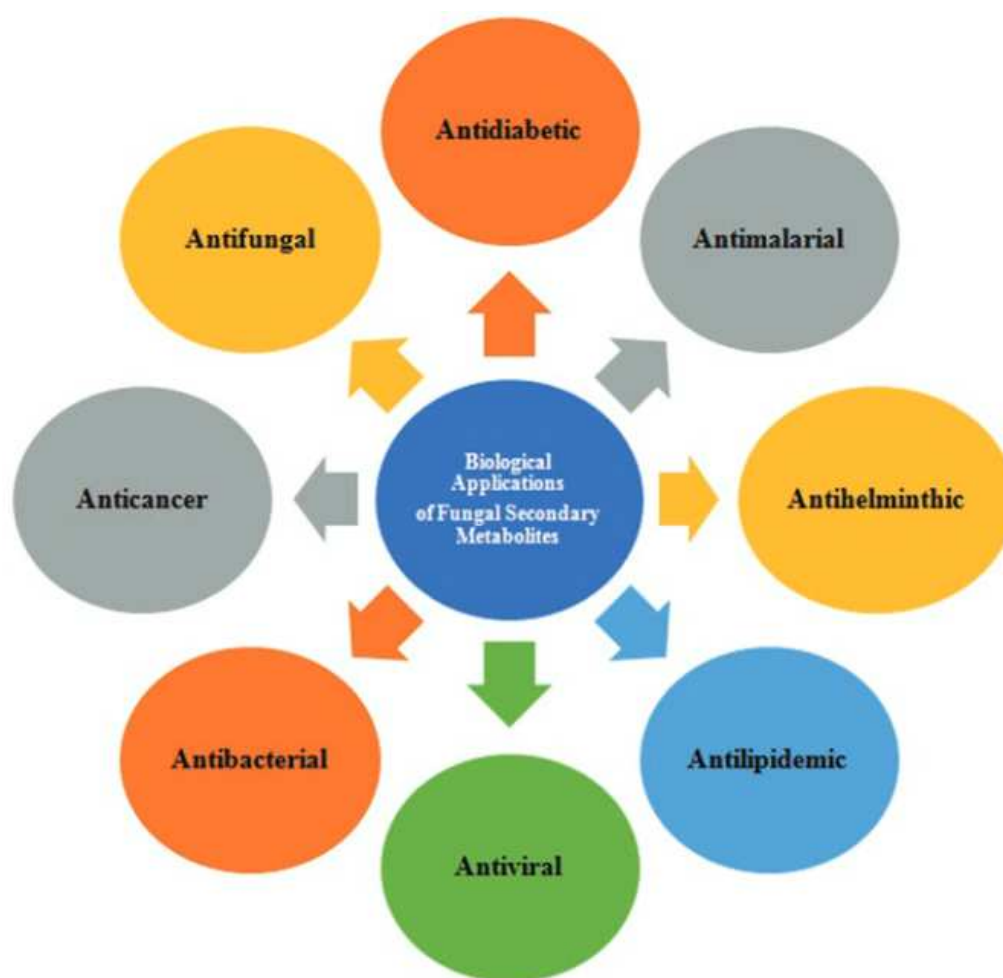


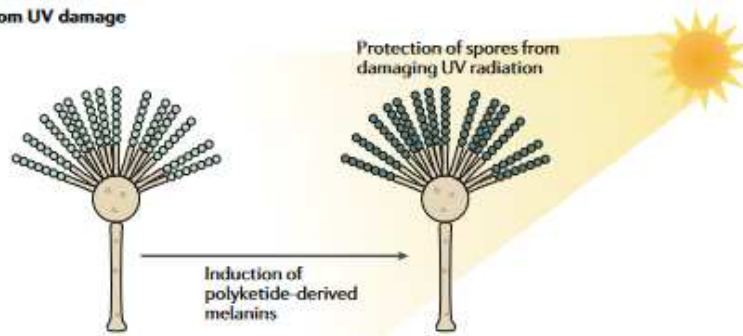
Figure 1.3. Biological applications of fungal SM³.

1.2.2. Ecological roles of fungal SMs

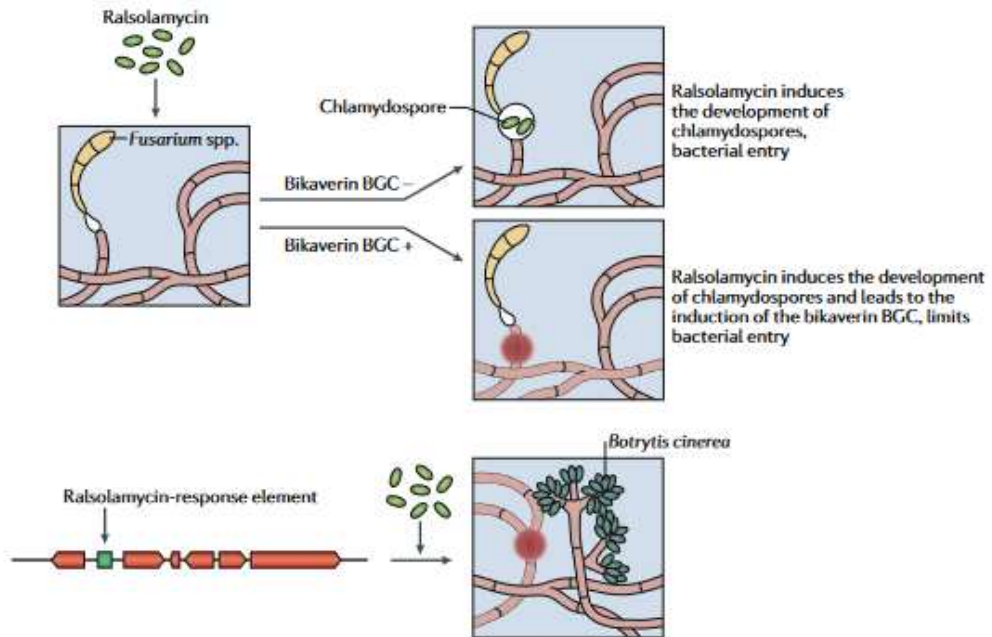
Fungi exist in complex ecosystems where they face a variety of abiotic and biotic challenges, including environmental stress, insect predation, and food restriction induced by

rivals (**Figure 1.4**). Fungi must preserve their niches while contending with competition and predators and ensuring adequate room to develop and absorb nutrients. SMs production coincides with fungal growth, SMs have the potential to alter the course of development, survival, and interaction with other species, both inter- and intra-kingdom. Fungi are a widely diversified group that is well adapted to thrive in practically any environment, and their evolutionary success may be owed, at least in part, to their broad and adaptable SMs repertoire. SMs perform a number of tasks that aid fungal survival in a range of unfavorable situations⁵⁶.

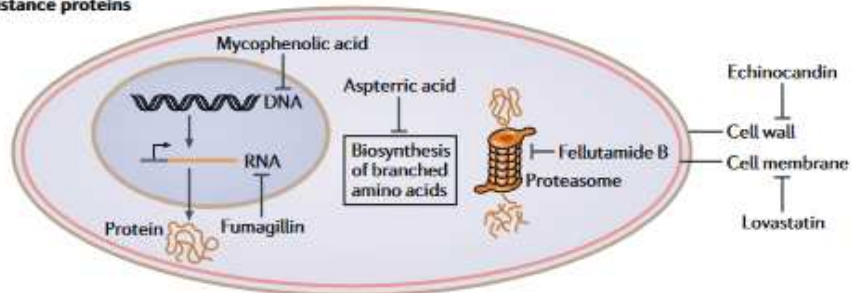
a Protection from UV damage



b Protection from bacterial ingress



c In-cluster resistance proteins



d Fungal development

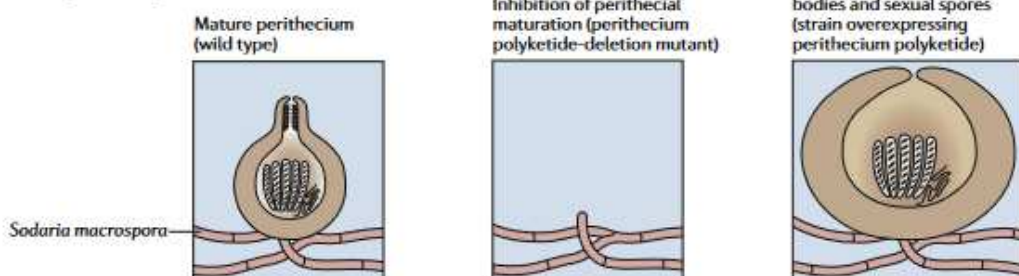


Figure 1.4. The ecological roles of SMs¹⁹. (a) Polyketide-derived melanins, a natural pigment that shields spores from harmful ultraviolet (UV) radiation, are produced by several fungi. (b) The bacteria *Ralstonia solanacearum* secretes the lipopeptide ralsolamycin, which causes fungus to generate chlamydospores and *Fusarium spp.* to express the bikaverin gene cluster. (c) Fungi have developed numerous self-protection methods, including duplicated, resistant copies of target proteins inside a BGC, to shield themselves from their own BGC-encoded antifungal SMs. (d) SMs can affect fungal developmental processes. In the fungus *Sordaria macrospora*, deletion of a PKS suppresses perithecial development, whereas overexpression results in deformed fruiting bodies without the typical perithecial neck.

1.2.2.1. Vegetative, morphogenesis, pigmentation

SMs are not directly necessary for fungal producer short-term growth but are critical for long-term survival. Most SMs are produced after the fungus has finished its first growth phase and mark the start of the development stage, such as the generation of spores⁵⁷. Nonetheless, SMs and fungal morphogenesis have been found to be closely related.

Plants and microbes are the primary suppliers of natural pigments. Plant pigments have many limitations, including seasonal availability and pigment stability and solubility⁵⁸. Bacterial and fungal pigments are an easily accessible source of naturally generated colors (**Figure 1.5**). Bacterial and fungal pigments have several applications and significant advantages over plant pigments, such as simple and quick growth in low-cost media, ease of processing, and weather-independent growth⁵⁹. Aside from color, bacterial and fungal pigments have a variety of biological activities, including antioxidant, antibacterial, and anticancer action⁶⁰. Filamentous fungi are known to produce a wide variety of pigments such as quinones, carotenoids, melanins, phenazines, flavins, monascus, violets, and indigos⁶¹. Fungi fruiting bodies are frequently quite colorful, which piqued the curiosity of chemists early on⁶². These

pigments were discovered to include a wide range of diverse molecules⁶², including alkaloids⁶³, aromatics⁶⁴, polyenes⁶⁵, and even metal complexes⁶⁶.

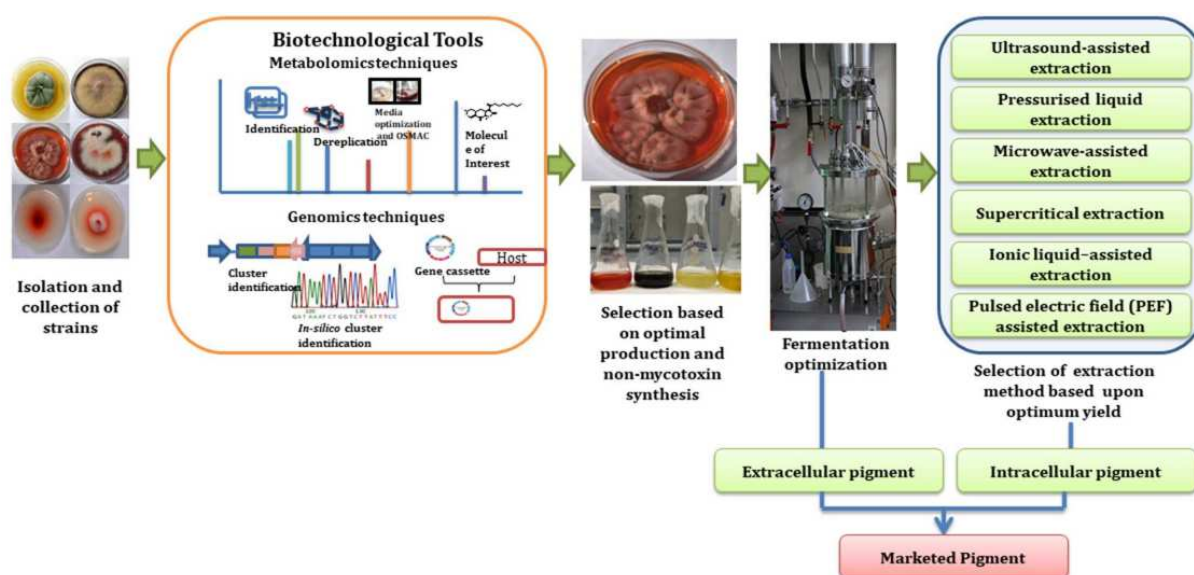


Figure 1.5. Schematic roadmap for a pigment journey from lab scale to industrial scale⁶⁷.

1.2.2.2. Stress protectants

Stress is defined as an environmental shift that causes an internal response in living microorganisms. Fungi have intricate signal transduction pathways that allow them to respond correctly to changes in environmental stimuli at the molecular level. Many fungal SM genes are engaged in stress-related responses, and the varied classes of fungal SMs protect growers against a variety of unfavorable environmental pressures. These stimuli can include biotic and abiotic challenges such as bacterial, viral, and fungal damage, as well as variations in temperature, pH, food availability, oxidative stress, osmotic stress, salt stress, hunger stress, metal ion stress, drought stress, and UV radiation.

Filamentous fungi, actinomycetes, and plants produce a diverse spectrum of SMs, notably throughout the embryonic and aging stages of their life cycle and when challenged with other cells, which frequently correlates to a stress situation⁶⁸. SMs in soilborne fungi have been demonstrated to have a variety of roles, including antibiosis, competitive suppression of other

bacteria, and abiotic stress reduction. Research has shown that the impact of environmental conditions on growth and ochratoxin production, including water activity (a_w :0.85-0.987) and temperature (10-40 °C). Two strains of *Aspergillus carbonarius* obtained from wine grapes from three different European nations and Israel were used in the manufacturing^{69,70}. In another study, it was discovered that single and interacting abiotic environmental stress factors such as water and temperature interactions and the type of solute used to induce water stress have an impact on tolerance mechanisms, molecular ecology, and the relationship with SM production by a group of economically important mycotoxigenic species such as *A. carbonarius*, *A. ochraceus*, *Aspergillus flavus*, *Penicillium nordicum*, and *P. verrucosum*⁷¹. The research of the High-osmolarity glycerol (HOG) pathway and its significance in filamentous fungus growth and secondary metabolism is still in its early stages. *A. nidulans* has been extensively utilized as a model fungus for the study of the molecular genetics of osmotic stress responses, as well as SM and fungal development (**Figure 1.6**). The *A. nidulans* Ssk1 ortholog, SskA, activates the HogA (SakA) pathway in the osmotic and oxidative response. Unlike yeast, deletion of the *A. nidulans* gene expressing SakA, which corresponds to yeast *S. cerevisiae* HogA, resulted in mutant strains with relatively minor susceptibility to high-osmolarity stress⁷². In *A. flavus*, oxidative stress influences sclerotial differentiation and aflatoxin B1 production⁷³. Drought stress is regarded as a key contributor to preharvest aflatoxin contamination induced by *A. flavus*, meaning that aflatoxin infection is worsened by this sort of abiotic stress⁷⁴. Drought-response chemicals, such as reactive oxygen species (ROS), are linked to fungal stress-responsive signaling and SM formation, and can enhance aflatoxin production by *A. flavus* in vitro⁷⁵.

Endophytes are microbes that live in plant tissues without causing significant damage or gaining recompense other than a place to live⁷⁶. Every plant has one or more endophytes that provide nutrients, improve environmental acclimation, and impart biotic and abiotic stress

tolerance in the host plant^{77,78}. During stress, fungal endophytes emit a reduced amount of plant growth-promoting compounds; for example, *Penicillium* sp., which lives on *Suaeda japonica*, secretes the phytohormone gibberellins. Similarly, *Aspergillus* sp., *Cladosporium* sp., *Fusarium* sp., *Penicillium* sp., *Verticillium* sp., and *Ascomycete* sp. that live on *Panax ginseng* release triterpenoid saponins and ginsenosides (which promote root development and biotic and abiotic stress tolerance)^{79,80}.

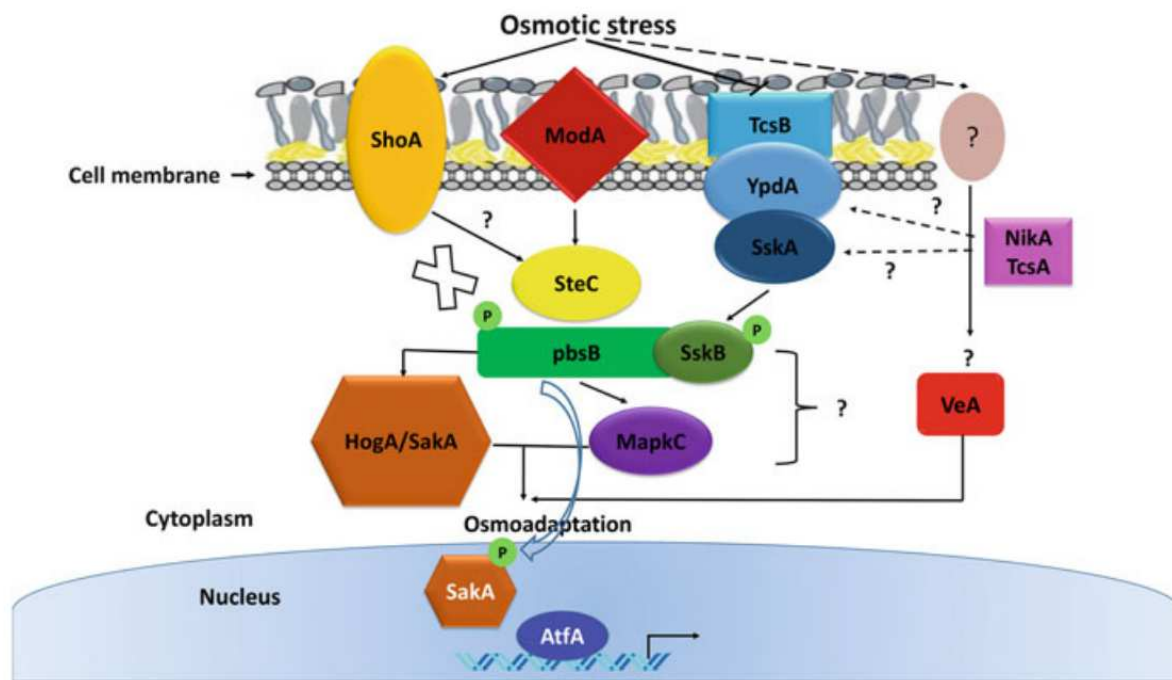


Figure 1.6. The high-osmolarity glycerol (HOG) regulatory mechanism in the model organism *A. nidulans*, as well as the involvement of the osmotic stress response route in fungal development and secondary metabolism⁷⁰.

1.2.2.3. Reproductive development

Many natural compounds are produced by fungi, which are either integrated into developing structures or act as signals to activate developmental programs. Although SMs coordinate main growth, some protective functions are still dependent on SMs, which stimulate fungal development and reproduction. Melanin photoprotects spores, increasing their chances of germination. The production of a specific SM hinders the germination of other spores in the

surrounding environment. *Penicillium sp.* has this outward behavior⁸¹. Several BGCs encode pigments or toxic metabolites that protect fungal sexual structures against environmental extremes or insect predation, in addition to the 1,8-dihydroxynaphthalene (DHN) melanin spore metabolites. Toxins produced by fungus, such as fusarubins⁸² and furocoumarin⁸³, defend sexual structures against predators and fungivores. Similarly, in *A. flavus*, loss of numerous BGC backbone genes causes abnormal sclerotial growth⁸¹. Natural products are also important in spore germination, most commonly in an offensive move in which a fungus or bacterium secretes a SM that inhibits the spore germination of another fungus, such as the polyketide GKK1032A from *Penicillium*, which inhibits the germination of the rice blast pathogen *Magnaporthe oryzae*⁸⁴.

1.2.2.4. Pathogenicity

SMs have critical functions as virulence factors in pathogenic interactions, as evidenced by fungus infecting both animals and plants. The relevance of SMs in fungal biology remains unknown. Pathogenic fungi produce a large number of SMs. An unexplained SM generated by some isolates of the rice blast fungus *Magnaporthe grisea*, for example, is involved in the detection of specific resistant rice cultivars. This metabolite is produced by the ACE1 gene cluster, which includes a hybrid PKS/NRPS and exhibits infection-specific expression⁸⁵. Understanding the precise activities and methods of action of these virulence factors may allow us to tackle fungal diseases more effectively⁸⁶. Mycotoxins contribute to fungal pathogenicity and virulence by being able to subvert or decrease host defenses, facilitating colonization and infection in the hosts⁸⁷. Similarly, certain SMs, referred to as host-specific toxins (HSTs), are peptide effectors that need the presence of a protein in the host plant that is vulnerable to the matching HST in order to induce infection⁸⁸. Plant pathogenic fungi typically generate host-specific toxins that are critical for illness; for example, the HC toxin from *Cochliobolus*

carbonum is required for disease on maize cultivars with the Hm resistance gene and exclusively infects maize cultivars with the HCT-susceptible gene⁸⁹. Melanins are another wonderful example of SMs that are required for fungal pathogenicity⁹⁰. In fungi, two kinds of melanin have been identified as contributing to virulence: dihydroxynaphthalene (DHN) melanin and dihydroxy phenylalanine (DOPA) melanin, and derivatives thereof⁹¹. The human pathogen *A. fumigatus* and the plant pathogen *M. oryzae* are the two most recognized fungal species for which DHN melanin is required for pathogenicity. In *A. fumigatus*, DHN-melanin is mostly synthesized during conidiogenesis and is responsible for the spores' distinctive grey-green color⁹². These spores are common in nature, and it is estimated that each individual inhales hundreds to thousands of conidia every day. This is especially critical for people with compromised immune systems, as *A. fumigatus* is the most common airborne fungal infection in humans.

1.2.2.5. Fungi-host interactions

The involvement of SMs in interactions with other species is of particular interest. They provide a variety of tasks in this context, including facilitating communication within one species or between distinct species, to attack other species by production of toxic compounds or to knock out the hosts defense mechanisms⁹³, nutrition acquisition, and even symbiotic connections⁷. Because fungi share their habitat with so many different creatures, SMs may provide protection or act as communication intermediaries (**Figure 1.7**). SMs are also known as plant and animal pathogen virulence factors. The section that follows explains the role and function of SMs in the interaction of fungus with microorganisms, plants, and animals.

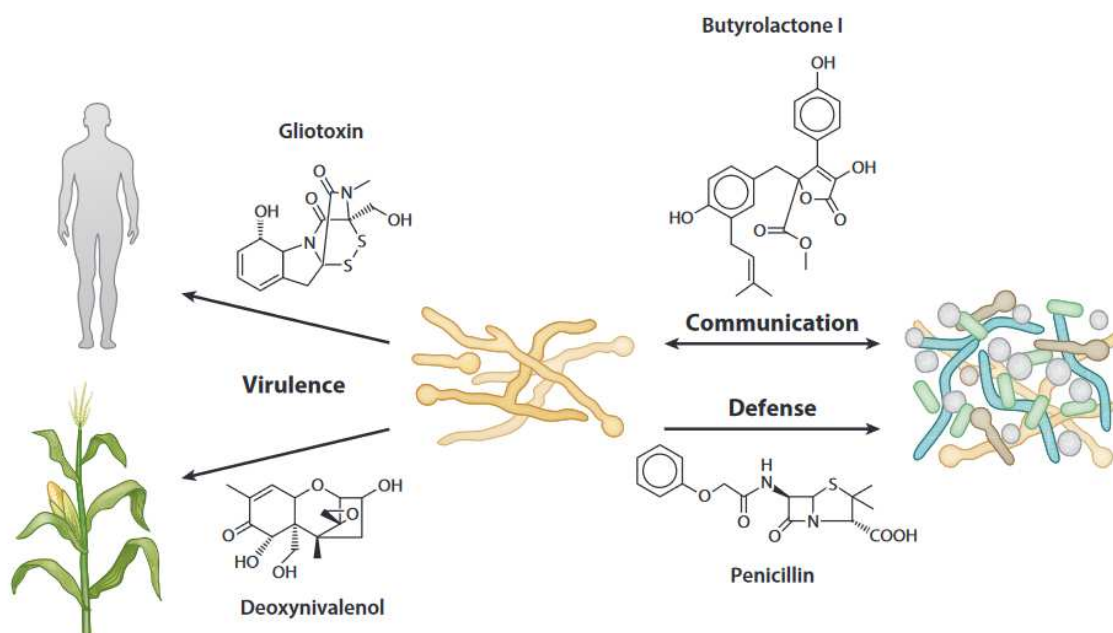


Figure 1.7. The ecological significance of fungal SMs⁹⁴. Fungi generate SMs that act as virulence determinants in plant and animal infections, such as deoxynivalenol and gliotoxin. Furthermore, fungal SM, such as butyrolactone I, can operate as communication molecules or as defense chemicals against other microbes, such as penicillin, so organizing microbial communities.

(1) Interspecies and intraspecies chemical communication

Although Dick and Hutchinson recognized in 1966 that volatile chemicals play a role in fungus-fungus interactions⁹⁵, little is known about fungi's chemical communication with each other and other species such as plants and insects. Chemical communication, for example, is critical in mating, sporulation, and the production of morphological changes⁹⁶. Chemical communication between plants and fungus is also required for mycorrhizal growth⁹⁷. At low concentrations, SMs can operate as signaling molecules, and when accumulated, they can exert antimicrobial action that allows fungus to thrive by impairing or inhibiting the growth and development of other fungi, bacteria, nematodes, insects, or fungivores, or even killing them⁹⁸. For example, in the presence of *Bacillus subtilis*, the endophyte *Fusarium tricinctum* produces

antibiotic depsipeptides from the enniatin family, which are potent growth inhibitors against various bacterium species⁹⁹. Volatiles are evidently very important in the chemical communication of fungus. Many fungi produce (3R)-1-octen-3-ol, which has been shown to activate plant defense in lower concentrations¹⁰⁰ and to demonstrate harmful effects and trigger an oxidative burst on plants in greater quantities¹⁰¹. Furthermore, (3R)-1-octen-3-ol is a fungistatic and fungicidal agent, as well as an insect attractant. In *Penicillium decumbens*, (+)-thujopsene has been identified as a volatile which exhibits autoregulatory properties of the growth of *P. decumbens*. Furthermore, thujopsene inhibited the mycelial growth of other fungus, including *Aspergillus sydowii*¹⁰².

(2) Attack and defense: weapons and armors

Natural compounds that are both protective and weaponized aid fungus in obtaining land rights in highly competitive ecological niches. Competition for food and survival in the open fields is not only interspecies, but also interkingdoms (**Figure 1.8**). To live in the environment, fungi have developed either direct or indirect strategies. Although hundreds of researches have noted the antibiotic effects of fungal metabolites, the majority of these investigations are correlative and include testing extracts against a wide range of bacteria and fungi. Furthermore, extract tests are skewed in terms of concentrations compared to ecological concentrations, and physiologically relevant amounts of a metabolite may behave as a signal rather than a toxin. Penicillin, an antibiotic, was created by *Penicillin notatum* to kill the surrounding bacteria in the culture, such as *Staphylococci*. Penicillin was thereafter recognized as the "miracle drug"⁹⁴. The gradient-dependent effects of *Pseudomonas aeruginosa* phenazine on *Aspergillus* sp. show that a high concentration of phenazine is antifungal, while a moderate concentration causes prolific sporulation in the fungus¹⁰³. Another fungus, *Beauveria bassiana*, has a poisonous metabolite arsenal that may kill insects. The species also infuses the insect with antimicrobial

properties to reduce microbial competition³. In certain circumstances, bacterial-fungal interaction is beneficial for fungi. For example, the endosymbiont relationship between *Burkholderia rhizoxinica* and *Rhizopus* microspores is responsible for rice seedling blight, which worsens after the microbial toxin rhizoxin reaches plant cells¹⁰⁴. To further complicate matters, certain host fungus chemically change rhizoxin to increase phytotoxicity, resulting in a win-win situation for the two microbes^{105,106}. Aflatoxin, another SM generated by *A. flavus*, is poisonous to insects, and a recent research found that aflatoxin gives a fitness benefit to *A. flavus* when it encounters insects^{107,108}. Aflatoxin is a group of carcinogenic substances that increases a fungus's ability to fight and survive in the presence of insects by a factor of 26. Aflatoxigenic strains of the fungus caused higher mortality in *Drosophila melanogaster* and had a measurable fitness advantage over non-toxicogenic strains of the fungus during competition.

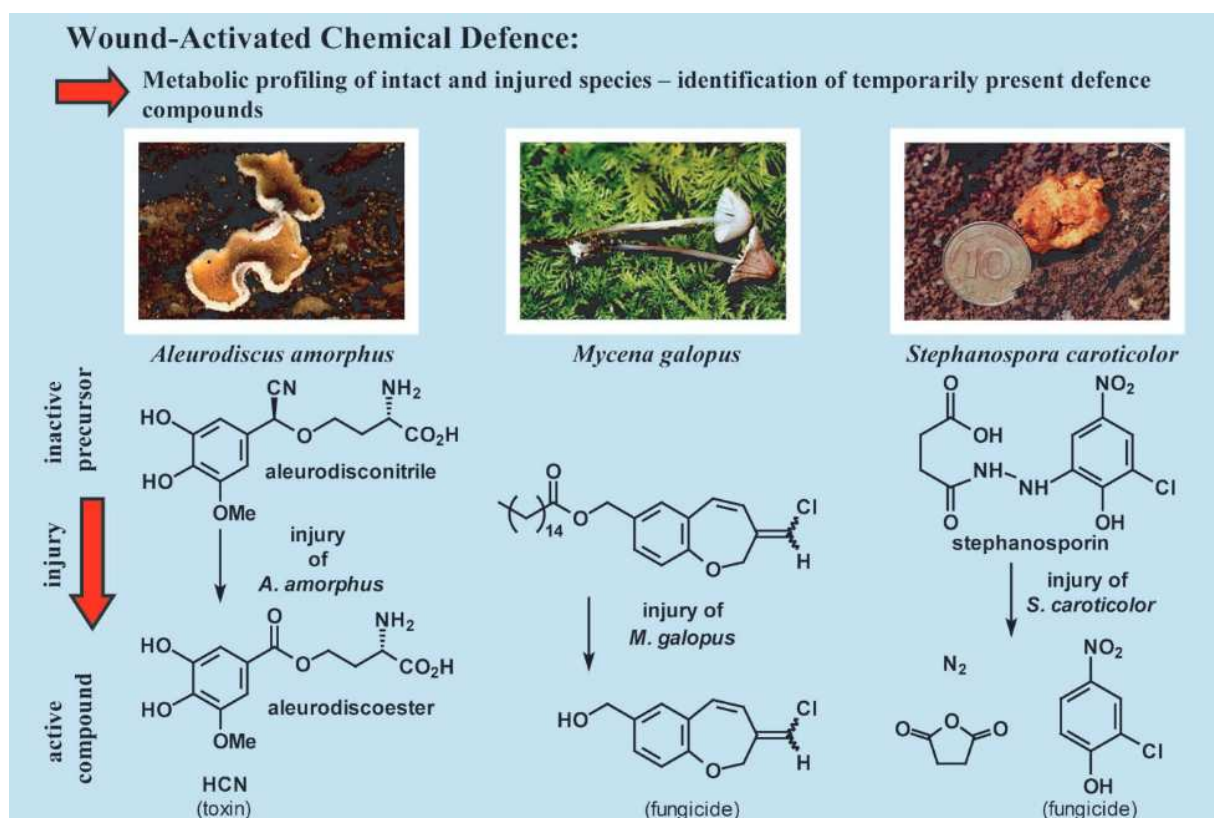


Figure 1.8. The wound-activated chemical defenses of fungi⁹³.

(3) Symbiotic connections

Fungi and plants have evolved a variety of symbiotic associations; for example, endosymbiotic fungus grow within plants¹⁰⁹, mycorrhizal fungi interact symbiotically with plant roots¹¹⁰, and fungi connect with algae in lichens¹¹¹. Furthermore, fungus and insects and fungi and bacteria have recognized symbiotic connections. The endophyte *Neotyphodium coenophialum* can be the producer of ergopeptide alkaloids, such as ergovaline, which is highly toxic to feeding mammals and insects, thus protecting the new grass species tall fescue (*Festuca arundinacea*) from overgrazing^{112,113}. The discovery that endophytic fungus may create powerful bioactive molecules with plant-protective properties accelerated the quest for novel SMs from endophytic fungi. Aside from ergot alkaloids, *Neotyphodium* species generate the indole alkaloid agroclavine, which is harmful to both insects and mammals¹¹⁴, and the pyrrolopyrazine alkaloid peramine, which is toxic to both insects and mammals but not to humans¹¹⁵. *Acremonium coenophialum*, a tall fescue endophyte, may also produce the plant growth hormone indole-3-acetic acid, which can stimulate vigorous plant growth in infected tall fescue¹¹⁶. As a result, fungal endophytes of grasses can protect their hosts from predators and provide them an edge over rivals, making endophyte-infected grasses the dominant species. Endophytes are not just good grass protectors, but also in a variety of other plant species.

The inability of most animals to digest cellulose might be a driving force in the creation of a symbiotic interaction between insects and fungus. For example, the termite *Macrotermes natalensis* cultivates hostile basidiomycete *Termitomyces* cultivar fungus. These fungus feed termites with enzymes like cellulases, which allow them to breakdown plant material. In response, the termites clean their fungus gardens, keeping them free of mycoparasitic fungi like *Trichoderma species*¹¹⁷. Furthermore, termites have a *Bacillus* species that generates the antibiotic bacillaene A, which suppresses hostile *Terminomyces* fungus¹¹⁷.

Fungi are not only nutritional competitors of bacteria but also benefit from SMs of bacteria. Several *Rhizopus* fungi are plant-pathogenic fungi that cause rice seedling blight. The plant disease is caused by the macrocyclic polyketide rhizoxin, which has been discovered in *Rhizopus* species cultures. In rice plants, the phytotoxin stops mitosis. However, it has been demonstrated that the fungus does not create rhizoxin but rather harbors an endosymbiotic bacterium of the species *Burkholderia* that produces WF-1360F, a direct precursor of rhizoxin with an E double bond between C-2 and C-3 rather than the epoxide. The fungus converts WF-1360F to the plant pathogen rhizoxin¹¹⁸. As a result, rhizoxin biosynthesis is a biosynthetic synergism between a bacterium and a fungus.

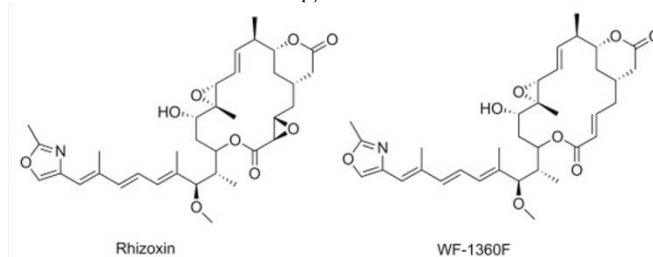


Figure 1.9. Rhizoxin and its precursor WF-1360F¹¹⁸

1.3. Regulation of fungal SMs

Complex regulatory networks govern fungal BGCs. Their regulation is influenced by multilayer mechanisms such as transcriptional regulation, epigenetic control, and environmental signal stimuli. The section that follows goes into depth about these regulatory mechanisms and their influence on SM development.

1.3.1. Transcriptional regulation

In the biosynthesis of microbial natural products, regulatory factors are commonly classified into two types: pathway-specific regulatory factors, which are found in specific BGCs and regulate gene expression in clusters, and global regulators, which are found outside BGCs

and regulates a range of metabolic processes. These TFs help fungal cells respond to environmental cues.

(1) Pathway-specific regulation

Fungal SM gene clusters often contain pathway-specific transcription regulators, such as Zn2Cys6 and Cys2His2 zinc finger proteins^{119,120}. Examples include *AflR* and *LovE* in *Aspergillus terreus*. These regulators control gene expression within specific clusters, impacting SM production. Other regulators like *AreA*, *CreA*, and *PacC* have also been identified^{121,122}. For instance, *VRF1* in *M. oryzae* affects pathogenicity and melanin biosynthesis¹²³. Understanding these regulators enables strategies to optimize SM production by manipulating gene expression patterns. Our understanding of the control of BGCs is rapidly expanding. A cluster-specific TF, most typically a C6-zinc cluster protein that identifies palindromic patterns in cluster gene promoters, is found in up to 50% of fungal BGCs¹⁹. A BGC may contain more than one TF^{124,125}. Originally thought to solely regulate genes within a specific BGC, certain C6 compounds, as hexadecahydroastechrome, are now recognized to influence genes across various BGCs and multiple metabolic pathways^{126,127}. These findings suggest that exploring the intricate networks regulated by cluster-specific TFs could yield unforeseen understandings of the metabolic processes connecting BGC product with the primary metabolism. Metal deficiency, especially copper deficiency, stimulates xanthocillin production, likely via control of the xan BGC C6 TF XanC in *A. fumigatus*¹²⁸. Various 'broad-domain' TFs, including the pH regulator *PacC*, *CreA*, the nitrogen regulator *AreA*, and the CAAX basic leucine zipper protein *HapX*, contribute to both positive and negative control of various BGCs⁹⁴.

(2) Global regulatory proteins

Global regulatory proteins play a vital role in regulating fungal SM by controlling mycelial differentiation, spore formation, and pigment production. They sense changes in nutrient availability and environmental signals, coordinating multiple secondary metabolic reactions, including antibiotic synthesis¹²⁹. Global regulatory proteins are regulated by single proteins, such as *AreA*, *WblA*, *DasR*. These proteins interact with and impact the activity of pathway-specific regulators¹³⁰. In *A. flavus* or *A. parasiticus* strains, for example, the aflatoxin production pathway is controlled by *aflR*, *aflS*, and the global regulator *LaeA*¹³¹. Furthermore, pathway-specific regulators may be influenced by global regulatory proteins. In *Penicillium citrinum*, for example, *VeA* and *LaeA* regulate the output of the polyketide ML-236B by regulating the pathway-specific regulator *mlcR*¹³². The velvet complex (originally reported in *A. nidulans*) is, by far, the most significant transcriptional complex that influences global regulation of SMs across all fungal taxa examined thus far¹³³. The heterotrimeric velvet complex is restricted to fungi and is formed by two transcription factors, VeA and VelB, and the methyltransferase *LaeA*¹³³. In contrast to this complex, *McrA* appears to be a negative regulator of BGCs in *Aspergillus* and *Penicillium* species¹³⁴.

1.3.2. Epigenetic control

Epigenetics involves non-gene sequence changes that affect gene transcription. It includes chemical and molecular genetic modifications such as DNA methylation, histone modifications (methylation, acetylation, phosphorylation, and ubiquitination). Epigenetic modifications have a significant impact on fungal SMs. Efforts to modify the fungal chromosome landscape have been successful in uncovering hidden metabolites from cryptic gene clusters (BGCs) (**Figure 1.9**). Chromatin remodeling governs SM production and controls nuclear activities. DNA accessibility for RNA polymerase II is affected by nucleosome

formation and disassembly. The packed DNA landscape is shaped by factors such as chromatin remodelers, histone variations, and chaperones. Chromatin modifiers affect chromatin shape and function by modifying histones and recruiting remodelers and other proteins^{135,136}. The accessibility of BGCs appears to be influenced by altering transcriptional activity and chromatin structure¹³⁷. Studies have shown that histone acetylation levels correlate with the order of gene expression within BGCs¹³⁸. In 2007, deleting the histone deacetylase gene *hdaA* in *A. nidulans* resulted in increased expression of multiple BGCs. Further research has explored the role of epigenetic regulation in BGCs, identifying compounds through manipulation of histone-modifying enzymes or chemical inhibitors¹³⁹. However, it is likely that epigenetic regulation can also dampen the expression of metabolites, as demonstrated by the downregulation of the *A. nidulans* histone deacetylase *RpdA*¹⁴⁰. In 2010, a study¹⁴¹ in *A. nidulans* found that the sterigmatocystin (ST) BGC is initially silenced during early growth, but its chromatin marks reverse as the fungus enters the SM-promoting phase, facilitated by the global regulator *LaeA*. Another study¹⁴² revealed that *Streptomyces rapamycinicus* bacteria induced expression of the silent orsellinic acid BGC in *A. nidulans* through the activation of the histone acetyltransferase GcnE, part of the SAGA–ADA complex. This complex also induced expression of the ST BGC, suggesting a connection between inter-species communication and the epigenetic machinery¹⁴³. The clustered organization of SM genes in fungus supports co-regulation via chromatin structure. Restructuring nucleosome packing allows for more cost-effective regulation of wider genomic areas. Small inhibitors of lysine acetyltransferase (KAT), lysine deacetylase (KDAC), and DNA methyltransferases have been used in studies to support this concept. Shwab et al. used trichostatin A (TSA), a bacterial-derived KDAC inhibitor, to treat *Alternaria alternata* and *Penicillium expansum*, which resulted in increased synthesis of unidentified SMs. Methylation is another well-studied modification that is likewise renowned for its regulatory intricacy in gene expression¹⁴⁴. One of the earliest fungus types of research looked at the

function of CclA in *A. nidulans*¹⁴⁵. CclA belongs to the COMPASS complex, a conserved multi-subunit complex that both stimulates and represses transcription via histone H3K4 methylation¹⁴⁶. Overall, these studies highlight the role of epigenetic regulation in ST production and the potential for discovering hidden BGCs through manipulation of chromatin structure and regulatory proteins.

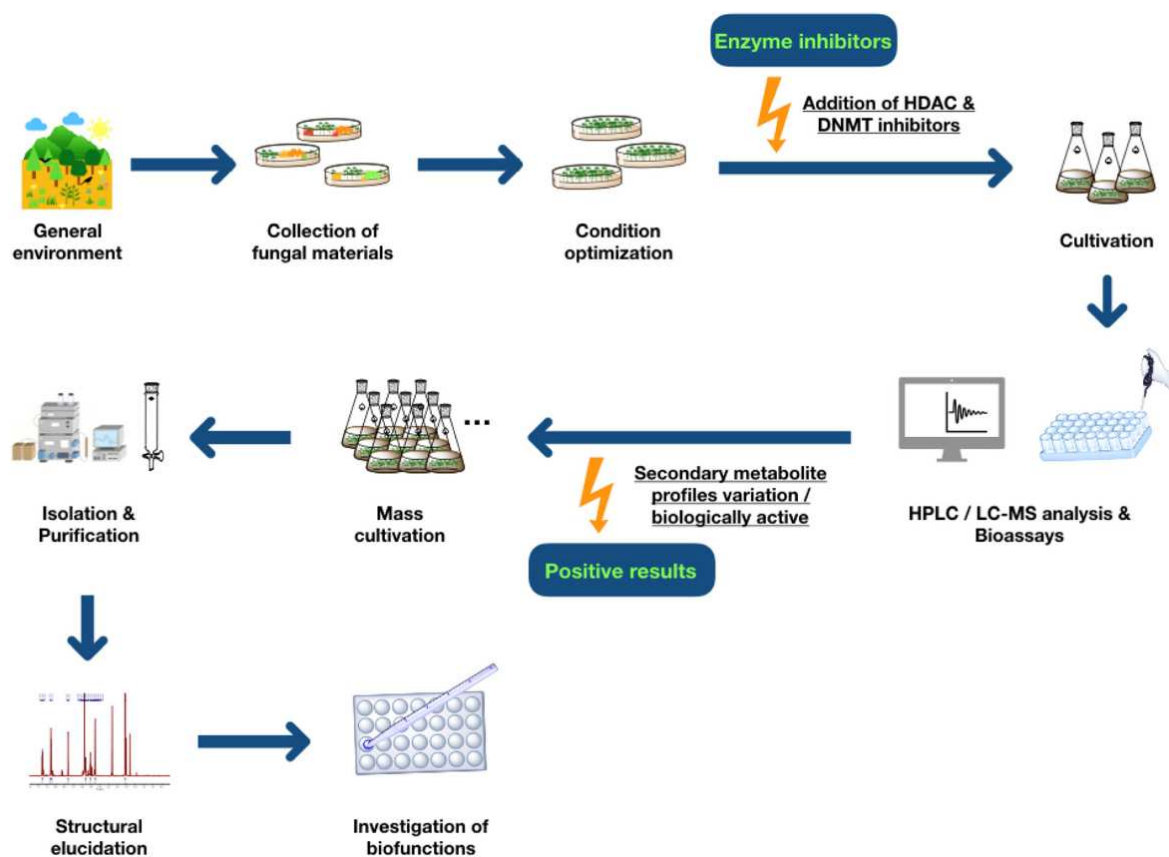


Figure 1.9. The epigenetic approach's general experimental strategy¹⁴⁷.

1.3.3. Environmental signal stimuli

Fungal BGCs are controlled by a complex regulatory network involving multiple proteins and complexes that respond to various environmental stimuli. These stimuli include carbon and nitrogen sources, temperature, light, pH, amino acids in the environment, reactive oxygen species, hypoxic conditions, biofilm formation and iron availability, but also stimuli derived

from other organisms (**Figure 1.10**). However, most of these stimuli are only relevant for certain gene clusters, and this can vary between fungal species. The regulation of BGCs occurs at several levels, some of which are almost entirely specific for the respective pathway, and others of which display a more global regulation of secondary metabolism. As a result, the regulation of secondary metabolism biosynthesis pathways is complex and involves several interconnecting networks.

The activation of BGCs transcriptionally and epigenetically is a result of environmental stimuli and is dependent on the developmental stage of the producing fungus^{148,149}. Environmental signals that impact SM production are obviously interconnected, according to published research. The most advanced aspect of SMs is their ability to function as signaling components, allowing for molecular dialogues with the environment and the establishment of competitive or cooperative relationships between producer fungus and the surrounding living creatures. Fungi have evolved excellent cell-to-cell communication to better response to local environmental changes to ensure the survival of their own colony. SMs initiate virulence factor reproduction, conidiation, or secretion during this communication⁹⁴. For example, the importance of nutritional input has long been recognized, as seen by the one strain-many compounds (OSMAC) method to metabolite mining¹⁵⁰. In *Aspergilli*, for example, fatty acid-derived oxylipins respond to cell density by regulating sexual-asexual reproduction switches, mycotoxin release, and host colonization^{151,152}. Several research groups have found functions for red and blue light photoreceptors and/or their signal transduction pathways during the creation of fungal SMs¹⁵³. Since investigations of aflatoxin generation in *Aspergillus flavus* decades ago, temperature and light have been discovered to stimulate or repress natural product synthesis¹⁵⁴. To regulate the synthesis of natural compounds such as the polyketide dihydrotrichotetronine, photoresponse pathways can cross with known TFs such as *CreA* and/or *CreI* (the carbon catabolite regulator in fungi, named *CreA* in certain species and *CreI* in

others)¹⁵⁵. The transcriptional responses of BGCs to altering environmental stress pathways, notably oxidative stress, lend credence to the idea that SMs (for example, aflatoxin) operate as antioxidants against reactive oxygen species^{156,157}. SMs interact with proteins, nucleic acids, and membranes inside the cell, activating molecular reactions that allow fungus to meet physiological needs¹⁵⁸.

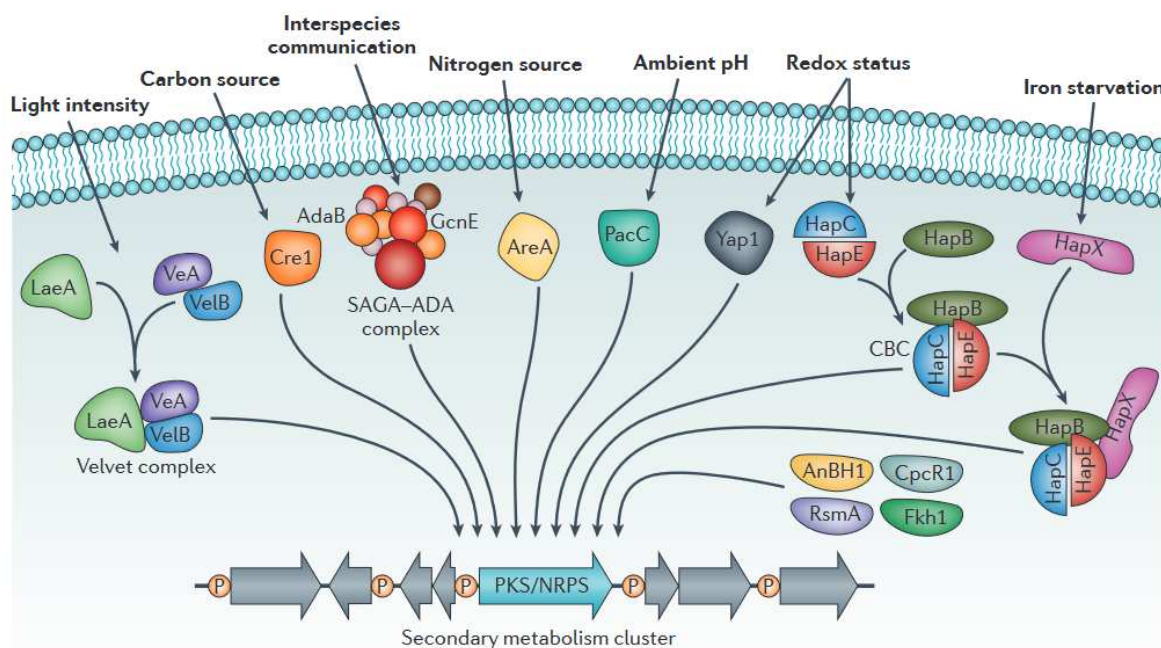


Figure 1.10. Global regulatory proteins are involved in the control of BGCs in several fungi. Environmental signals can impact the regulation of numerous BGCs via regulatory proteins that respond to these cues and modify the clusters' expression⁸⁶.

1.4. Approaches activating transcriptionally silent BGCs

With the sequencing of the first fungal genomes, one of the major discoveries was that the number of BGCs greatly outnumbered the known SMs, thereby revealing an even bigger SM repertoire in these species but also showing that most BGCs are silent or not active enough to produce a detectable amount of SM under the laboratory growth conditions. This indicated that there was a substantial hidden potential of both novel and potentially useful bioactive SMs.

Several approaches have been employed to activate transcriptionally silent BGCs and uncover the production of cryptic or dormant SMs in fungi. In this part, we summarize the approaches that have been used to activate silent BGCs (**Figure 1.11**). We roughly divide all the approaches into two categories for detailed elaboration, one is pleiotropic approaches, including environmental cues, co-cultivation, use constitutive promoters of BGCs, epigenetic mining, manipulation of global regulators, etc., and the other is pathway-specific approaches, including awakening the activator or deleting the suppressors, heterologous expression, etc.

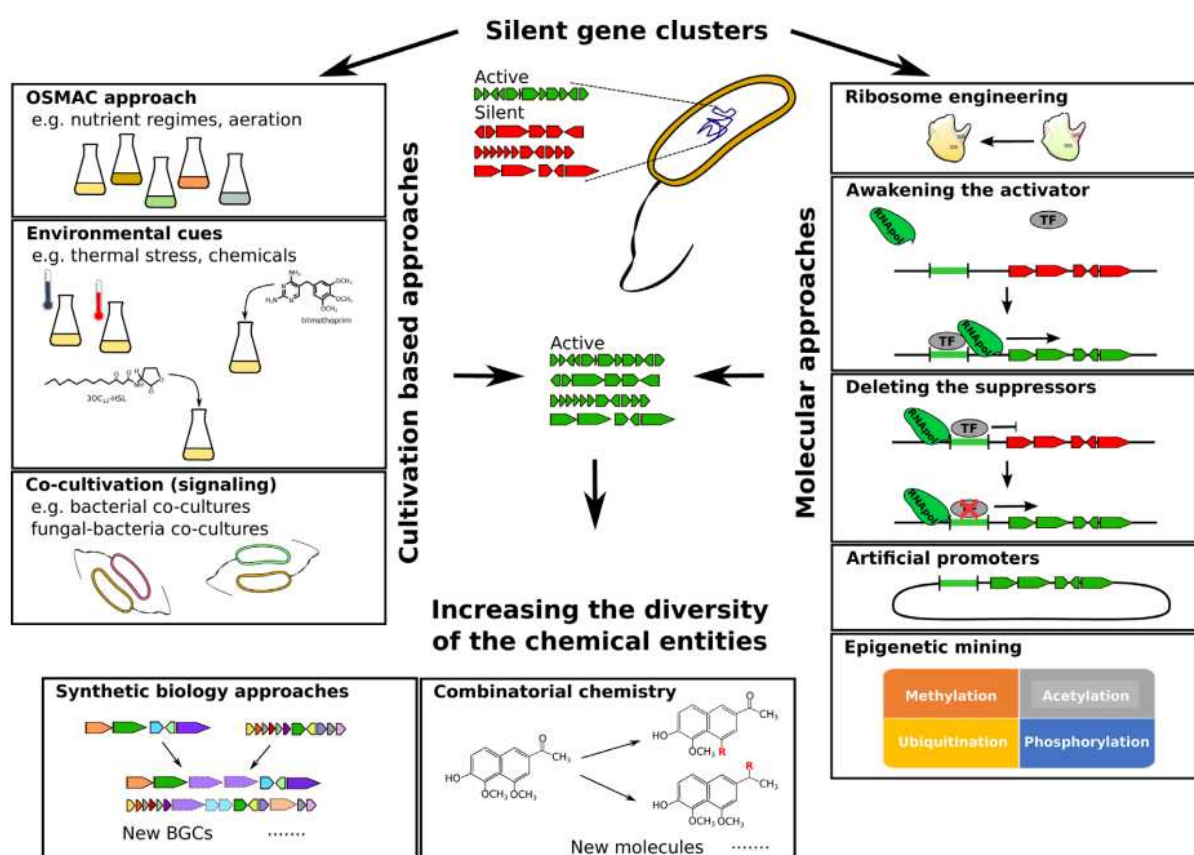


Figure 1.11. The integration of classical culture based and molecular biology-based approaches for the elicitation of natural products from silent BGCs¹⁵⁹.

1.4.1. Pleiotropic approaches

Pleiotropic approaches (**Figure 1.12**), defined by their ability to affect multiple genes and trigger global changes in specialized metabolic pathways, have the potential to yield novel

metabolites even in cases where our understanding of the regulation of specific BGCs is limited. This characteristic makes them advantageous for higher throughput and applicable to diverse organisms. However, due to their empirical nature, predicting the outcomes of pleiotropic approaches can be challenging. The simultaneous induction of multiple BGCs can complicate the identification and isolation of individual metabolites, resulting in the generation of complex mixtures of new compounds. Advancements in understanding BGC regulation will ameliorate these drawbacks, refining pleiotropic approaches for better predictability and targeted metabolite identification.

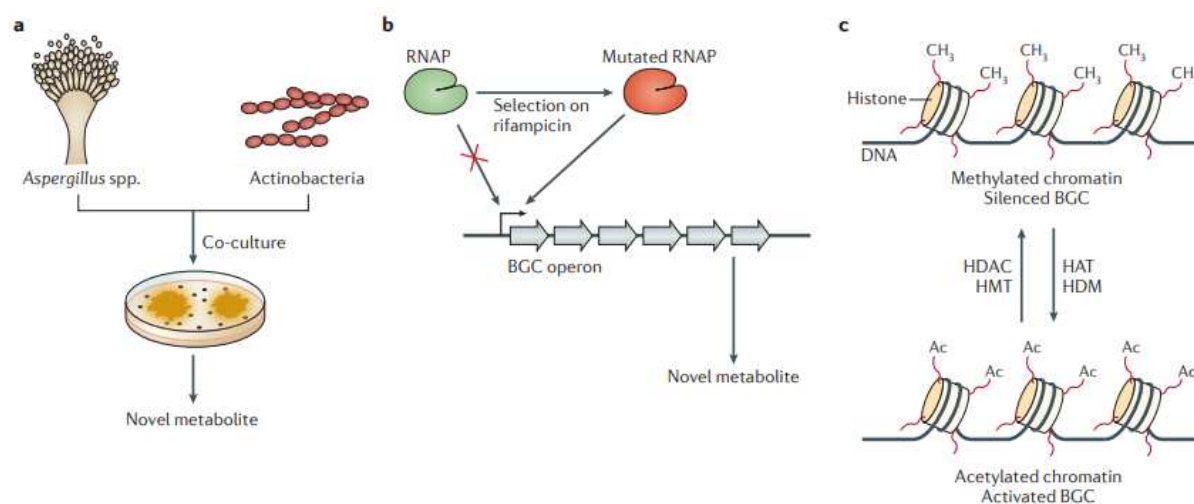


Figure 1.12. Pleiotropic strategies that have been used to activate silent BGCs encoding specialized metabolites¹⁶⁰. (a) One approach to natural product discovery involves the co-culturing of microorganisms to induce crosstalk, which has the potential to activate silent BGCs. (b) Selection for resistance to rifampicin in actinobacteria leads to the H437R mutation (among others) in the RNA polymerase (RNAP) β -subunit, and this increases the affinity of RNAP towards the promoters of BGCs encoding specialized metabolites. (c) Epigenetic perturbation can be used to activate silent BGCs. Histone acetyl transferases (HATs) and histone demethylases (HDMs) can convert methylated histones, which silence BGCs, into acetylated histones, which activate them.

1.4.1.1. Environmental cues

Modifying growth conditions offers a simple and effective means to induce changes in the expression of specialized metabolite BGCs. In natural environments, microorganisms respond to signals from both biotic and abiotic factors. Certain specialized metabolites (SMs) remain dormant in pure laboratory cultures, necessitating the manipulation of growth conditions to unleash their potential. The cultivation environment, media composition, and various conditions significantly influence microbial physiology. By altering growth conditions, such as pH, light, temperature, introducing competing species, chemical elicitors, or additives, the activation of transcriptionally silent SM pathways can be triggered^{18,86}. This approach, often employed as a strain improvement strategy, has been widely utilized for many years. Even subtle changes in growth media can not only result in variations in the quantity of specific compounds but also unlock the production of entirely new molecules.

The 'OSMAC' (One Strain; Many Compounds) strategy is employed to increase the diversity of SMs within a single fungus. By adjusting culture conditions, such as the medium composition or fermentation parameters, the same strain can produce varying quantities and types of SMs. This approach has led to the discovery of numerous novel and bioactive natural products, indicating that modifying culture conditions can activate silent genes and unlock the production of new SMs. The OSMAC method was used to increase the number of compounds identified from six bacterial and fungal species, resulting in the identification of more than 100 compounds from 25 structural classes¹⁶¹. In *A. nidulans*, the application of ionic liquids has demonstrated the ability to stimulate specialized metabolite (SM) production. This stimulation resulted in the up-regulation of approximately 32% of the described backbone genes that are typically silent under normal *in vitro* conditions¹⁶². Numerous studies have investigated the production of aflatoxin/ST in *Aspergillus spp.*, revealing the influence of environmental factors such as nitrate¹⁶³, pH¹⁶⁴ and temperature¹⁶⁵ on their production. Additionally, the biosynthesis

of pseurotin A in *A. fumigatus* has been found to be activated by hypoxia, while the SM in *Fusarium fujikuroi* has been shown to be impacted by nitrogen sources¹⁶⁶.

A panel of more than 45 distinct extracts was obtained by cultivating *A. nidulans* strains under varied growth conditions, resulting to the identification of aspoquinolones A-D¹⁶⁷ and aspernidines A and B¹⁶⁸.

Overall, environmental cues play a crucial role in unlocking the expression of silent BGCs and enabling the biosynthesis of diverse specialized metabolites.

1.4.1.2. Co-cultivation

In nature, microorganisms never exist in isolation but continuously coexist in various environments, actively exchanging and sharing chemical signals, including SMs, among each other. The widespread acceptance of this concept underscores the significance of microorganism interactions as a key driver for SM production. Co-culture, a conventional method for exploring natural products, involves activating silent genes or clusters through interactions between different species. In this mutual exchange of chemical signals, co-cultured strains transmit molecules that influence the compound libraries of both strains, resulting in an increased abundance of compounds. The fungal co-culture strategy effectively activates silent BGCs in fungal strains, leading to the production of cryptic SMs. This strategy typically involves three approaches: fungal-fungal, fungal-bacterial, and fungal-host co-cultures. The co-cultivation of fungal species with bacteria represents another environmental stimulus that has proven successful in activating dormant gene clusters. The discovery of penicillin stands as a significant milestone in the realm of fungal-bacterial co-culture experiments¹⁶⁹. Up until the end of 2022, a total of 49 novel SMs derived from fungal-bacterial co-culture have been documented and classified into two groups: liquid state fermentation (LSF) and solid state fermentation (SSF)¹⁷⁰ (**Figure 1.13**). Another study where they co-cultivated *A. nidulans* with

a collection of 58 actinomycetes isolated from soil and demonstrated that the interaction between the fungi and bacteria resulted in the activation of specific fungal SM genes, including those involved in orsellinic acid biosynthesis¹⁷¹. In fungal-bacterial cocultures, a large macrolactone ibomycin¹⁷², polyketide pestalone¹⁷³, *N*-formyl alkaloids¹⁷⁴, four diterpenoid libertellenones¹⁷⁵, and PKS-NRPS-derived tetramic acid analogs¹⁷⁶ were discovered.

Fungal-bacteria cocultivation also induced natural product formation, exemplified by the discovery of citrifelins¹⁷⁷ and 2-alkenyl-tetrahydropyran class¹⁷⁸ from different cocultures. Such cocultivation implies intricate offensive and counteroffensive interactions between fungi. Co-cultivation of *A. nidulans* with soil-dwelling actinobacteria has been reported to stimulate the expression of specialized fungal metabolite BGCs, resulting in the production of F-9775A and F-9775B¹⁷¹. These compounds, previously isolated from *Paecilomyces carneus*, were not known to be products of *A. nidulans*. In the same way, the co-cultivation of *A. fumigatus* with *Streptomyces rapamycinicus* resulted in the activation of a silent *A. fumigatus* polyketide BGC, leading to the identification of a unique class of polyphenols called the fumicyclines¹⁷⁹. These compounds exhibit moderate inhibitory activity against the growth of *S. rapamycinicus* and are suggested to play a role in fungal defense¹⁷⁹.

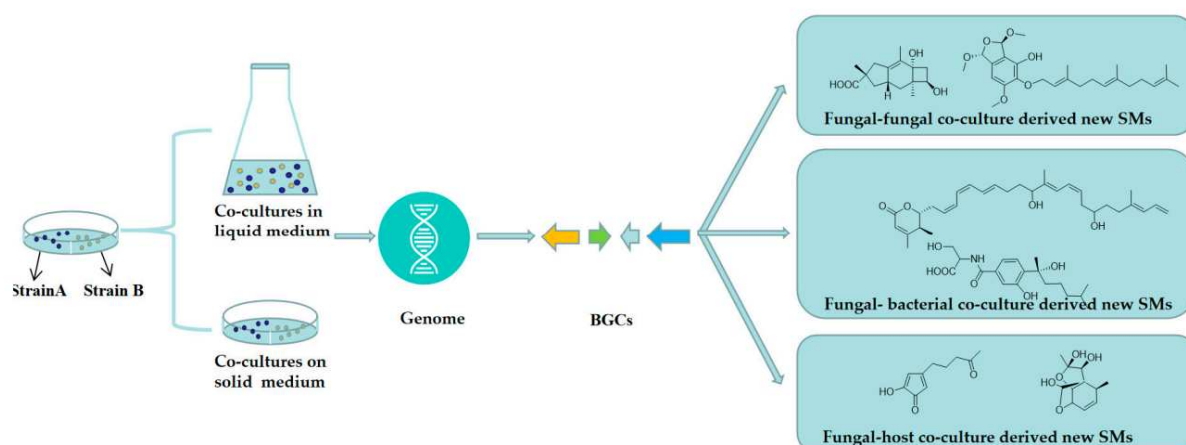


Figure 1.13. Flowchart for a chemical investigation that employs a fungal co-culture method¹⁷⁰.

1.4.1.3. Use constitutive promoters of BGCs

Promoter elements play an indisputably important role in cluster activation, as they are responsible for facilitating efficient transcription, which is the initial step of gene expression¹⁸⁰. Promoter engineering involves utilizing a set of regulatory sequences with known functions to alleviate the native complex regulations and enable subsequent gene expression. Constitutive promoters commonly employed for gene activation include the promoter of the erythromycin resistance gene *ermE* from *S. erythraea*, such as *ermEp1* and its derivatives, the *SF14p* promoter derived from phage I19, and the engineered *kasOp** promoter¹⁸¹. Inducible promoters frequently utilized for gene activation include the thiostrepton-inducible promoter *PtipA*, synthetic resorcinol-inducible and cumate-inducible promoters¹⁸², and the synthetic tetracycline-inducible promoter *tcp830*¹⁸³. DNA-bindings selectively target and bind to upstream activating sequences in the promoter region, regulating filamentous fungal gene expression. Synthetic promoters fuse upstream activating sequences and core promoters or integrate upstream activating sequence elements into native promoters, rewiring gene regulation. Synthetic promoters with inducer-dependent synthetic transcription factor allow precise gene expression tuning. Fusion of *CreA/CreI* DNA-bindings to *Xyr1* enhanced cellulase production in *CreA/CreI* deficient *Trichoderma reesei*^{184,185}. Replacing weak TF's regulatory domain with highly active activation domains activates target SM BGCs. Fusion of *AlnR* DNA-bindings with *AfoA*'s regulatory domain produces asperlins in *A. nidulans*¹⁸⁶. In cases where a cluster-specific transcription factor (TF) is unavailable, an alternative approach involves replacing the promoters of all genes within the BGC. This method was employed by Yeah et al. in their study on the fellutamide B cluster in *A. nidulans*. Specifically, they replaced the promoters of six genes with the regulatable *alcA* promoter¹⁸⁷. A similar strategy was implemented in *A. nidulans* for the cluster responsible for a conidiophore pigment, where the promoters of three genes (*ivoA-C*) were substituted with the *alcA* promoter¹⁸⁸.

1.4.1.4. Epigenetic mining

The function of epigenetic mechanisms in controlling eukaryotic gene expression, which include covalent alteration of DNA and chromatin, is well established^{137,189,190}. Changes in chromatin structure can cause changes in gene expression on a genome-wide scale, affecting a range of cellular functions, including specialized metabolite manufacturing^{137,190}. Epigenetic regulation involves the activation of silent gene clusters in a strain's secondary metabolic pathway through DNA methylation and histone modification, without altering the DNA sequence. This process enables the acquisition of new products by controlling the expression of genes involved in SM^{143,147}. Small-molecule inhibitors of DNA methyltransferases and histone deacetylases have been employed to manipulate the fungal epigenome¹³⁷. When *Cladosporium cladosporioides* cultures were exposed to the histone deacetylase inhibitor suberoylanilide hydroxamic acid, significant alterations occurred in the fungal metabolite profile, leading to the discovery of new cladochrome congeners¹⁹¹. Similarly, exposing *Aspergillus niger* ATCC 1015 to the same histone deacetylase inhibitor unveiled nygerone A, a novel metabolite featuring an unprecedented phenylpyridin-4-one ring system¹⁹². Another histone deacetylase inhibitor, suberoyl bishydroxamic acid, was recently used to inhibit HDAC in *Chaetomium indicum*, resulting in the emergence of three new chaetophenols with unique polycyclic skeletons¹⁹³. Demonstrating the potential of epigenetics, the deletion of *hdaA* (encoding a histone deacetylase) in *A. nidulans* led to significant changes in the fungus's metabolite profile¹³⁹.

1.4.1.5. Manipulation of global regulators

Global regulators are proteins that influence the expression of many BGCs in order to orchestrate a coordinated response to environmental influences. Manipulation of various pleiotropic transcriptional regulators has led to the discovery of novel natural products from

fungi and bacteria by influencing the expression of specialized metabolite BGCs^{86,194}. In fungi, the velvet complex, which consists of at least three proteins (LaeA, VeA, and VelB), plays a crucial role in regulating both specialized metabolism and sexual development^{133,194}. LaeA, first identified in 2004 as a global regulator of specialized metabolism in *A. nidulans* and other *Aspergillus spp.*, plays a key role: deleting *laeA* suppresses the expression of specialized metabolite BGCs, while overexpressing *laeA* promotes their expression¹⁹⁵. The CBC complex, which consist of three proteins (HapB, HapC and HapE), was known to promote the penicillin biosynthesis in *P. chrysogenum*¹⁹⁶. Deletion of GATA-type regulator gene *NsdD* in *Penicillium oxalicum* results in the upregulation of many putative BGCs¹⁹⁷. Various proteins play general regulatory roles in SM. These include AreA^{198,199}, which regulates nitrogen, PacC²⁰⁰, which regulates pH, and CreA²⁰¹, which regulates carbon. Recently, co-expression networks in *A. niger* identified two additional global regulators, *Mjka* and *Mjkb*, involved in SM²⁰². Additionally, specialized regulators such as *AtfA/AtfB* are responsible for aflatoxin synthesis^{203,204}.

1.4.2. Pathway-specific approaches

Among the various methods to activate cryptic BGCs, manipulating pathway-specific TFs has emerged as the fastest, easiest, and most versatile approach (**Figure 1.14**). Unlike pleiotropic approaches, pathway-specific techniques offer enhanced control and predictability. This method allows for precise and controllable activation of targeted BGCs, facilitating clear identification of the resulting metabolic products. These strategies are valuable when predictive bioinformatics analyses reveal structural novelty in BGC products. However, compared to pleiotropic methods, these approaches have lower throughput since specific activation strategies are required for each BGC. Understanding the minimal gene set and regulatory roles of pathway-specific regulators is often necessary.

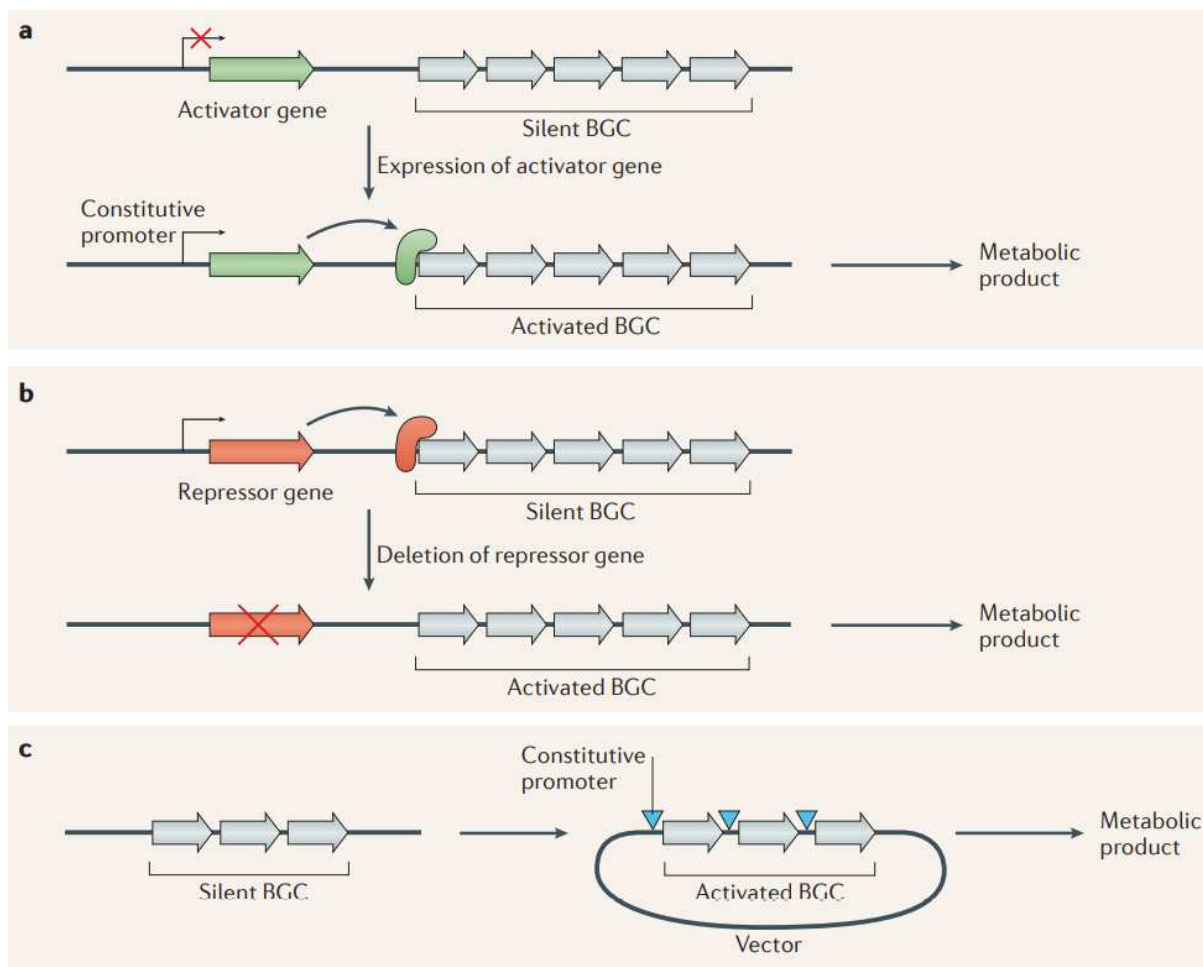


Figure 1.14. Pathway-specific strategies that have been used to induce the expression of silent BGCs encoding specialized metabolites¹⁶⁰. (a) Overexpression of a pathway-specific activator gene has been used to induce expression of the corresponding BGC. (b) Removal of a pathway-specific repressor gene has also been used to induce the expression of silent BGCs. (c) Refactoring, which refers to the replacement of native regulatory elements with constitutive or readily inducible promoters, is another pathway-specific strategy for activating silent BGCs.

1.4.2.1. Awakening the activator or deleting the suppressors

Most pathway-specific TFs function as positive regulators, with only a few acting as negative regulators. Therefore, inducing the expression of pathway-specific activator genes and deleting pathway-specific repressor genes are both promising approaches to activate cryptic

BGCs¹⁶⁰. It is usual to replace the native promoter with either a constitutive promoter or an inducible promoter to increase the expression of positive-acting pathway-specific transcription factors. For instance, activation of the pathway-specific transcription factor gene *apdR*, using the inducible alcohol dehydrogenase promoter *alcAp* from *A. nidulans*, resulted in the expression of the *apd* BGC (*apdA*, *apdB*, *apdC*, *apdD*, *apdE*, and *apdG*) under inducing conditions, leading to the production of aspyridones A and B²⁰⁵. Similarly, overexpression of the pathway-specific transcription factor gene *fsqA* utilizing the *A. nidulans* constitutive promoter *gpdAp* increased expression of the *fsq* BGC and resulted in the creation of many novel isoquinoline alkaloids known as fumisoquins²⁰⁶. Replacing the pathway-specific transcription factor promoter can be highly successful but not always. Reasons include domain function loss, lack of an unknown natural inducer, need for another TF, specific precursor, or post-translational modification²⁰⁷. Synthetic pathway-specific TFs can address these issues in certain cases. The conventional strategy of upregulating the cluster pathway-specific transcription factor could not activate the silent *A. nidulans* BGC encoding a highly reducing PKS gene, AN11191²⁰⁸. One research effectively used a hybrid pathway-specific transcription factor to induce the expression of this cluster¹⁸⁶. Strong expression of the hybrid pathway-specific transcription factor led in the activation of *aln* genes and the generation of (+)-asperlin, the *aln* cluster's final product, under inducing circumstances.

If an active or previously activated BGC is known, its association with a specific SM can be established by deleting or disrupting backbone synthase genes and analyzing the resulting compounds through metabolite profiling. By far the most popular strategy for dissecting gene clusters is gene function elimination. Two commonly used strategies for genetic alterations in the genome are: (1) one-step gene disruptions/deletions, where a selectable marker is inserted into or replaces the gene of interest^{209,210}, and (2) a two-step loop-in/loop-out method using selectable/counter-selectable markers. The latter allows for marker recycling and iterative gene

targeting^{211,212}. Marker systems like *beta-Rec/six* and *Cre/loxP* based on site-specific recombination (SSR) offer similar capabilities¹⁶⁶. It is recommended to restore nutritional markers to wild-type and reinsert deleted genes to confirm phenotypic effects.

The recent application of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology in fungi is anticipated to transform fungal genetic engineering^{213,214}. CRISPR technology utilizes Cas9 nuclease, guided by short RNA, to introduce specific DNA double-stranded breaks in target genes. This enables mutation stimulation or gene targeting through non-homologous end-joining or homologous recombination, respectively. Creating two breaks enhances efficiency and allows for large region deletions. CRISPR offers advantages such as direct use in species without genetic tools, classical gene targeting without selectable markers, and successful implementation in various fungal species. It has been successfully established in diverse fungal species, including *T. reesei*²¹⁵, *Neurospora crassa*²¹⁶, *Candida albicans*²¹⁷, *Pyricularia oryzae*²¹⁸, *Nodulisporium*²¹⁹, *Ustilago maydis*²²⁰, and various *Aspergillus species*²²¹⁻²²³. The technology simplifies gene targeting substrate construction using simple oligonucleotides for gene deletions and point mutations.

1.4.2.2. Heterologous expression

Heterologous expression efficiently unlocks silent or cryptic gene clusters found through genome mining. It offers advantages over native host expression, including the ability to express BGCs from uncultivable or slow-growing hosts, access to mature genetic manipulation tools in the heterologous host, and clearer background information. The process involves three steps: cloning the target BGCs, engineering them, and transforming them into the chosen heterologous host (**Figure 1.15**). If the cluster of interest is found in a species that is challenging or impossible to cultivate in a laboratory or if it is not easily genetically manipulated, heterologous expression strategies can be employed. Moreover, this approach often proves

valuable in providing additional insights that complement those derived from the native host. The initial consideration in heterologous expression is the selection of an appropriate host, which is influenced by various factors. Yeast, bacteria, and filamentous fungus are the three types of hosts. The construct and amount of heterologous expression for all genes in the cluster, including just TF promoter exchange, backbone synthase, or cluster engineering with additional promoters, etc. The cluster and the goal determine the construct to utilize. A more recent study found that the gene encoding the BbBEAS nonribosomal peptide synthetase isolated from *B. bassiana* could be heterologously expressed in *E. coli*, which could subsequently make beauvericin when the precursor D-Hiv was introduced^{224,225}. Heterologous expression in *E. coli* characterized 11 sesquiterpene synthetases from *Omphalotus olearius*²²⁶. AzaH, a FAD-dependent monooxygenase from the azaphilone gene cluster, was heterologously expressed in *E. coli* for in vitro assays and to study its function in azaphilone biosynthesis²²⁷. Tropolone biosynthetic genes from *Talaromyces stipitatus* were expressed and purified in *E. coli*, confirming the expected biosynthetic pathway²²⁸. PKS4 protein from *Gibberella fujikuroi* was also expressed in *E. coli*, producing SMA76a²²⁹. The researchers studied 6-methylsalicylic acid synthesis in both *E. coli* and *S. cerevisiae*²³⁰. In *S. cerevisiae*, they used a heterologous phosphopantetheinyl transferase to activate the *P. patulum* synthase, resulting in double the production compared to the native species and significantly higher than *E. coli*²³⁰. Multiple studies have investigated heterologous expression of BGCs in filamentous fungi. Examples include pyripyropene, citrinin, and Pfma clusters expressed in *A. oryzae* and *A. nidulans*, synthesizing DHN melanin^{231–233}. Heterologous expression in fungi presents challenges, such as altered metabolite profiles and cross-talk with native clusters, hindering the identification of new SMs. One study expressed a polyketide gene cluster in *Fusarium verticillioides*, resulting in the production of fusaric acid, a mycotoxin commonly found in *Fusarium* species²³⁴.

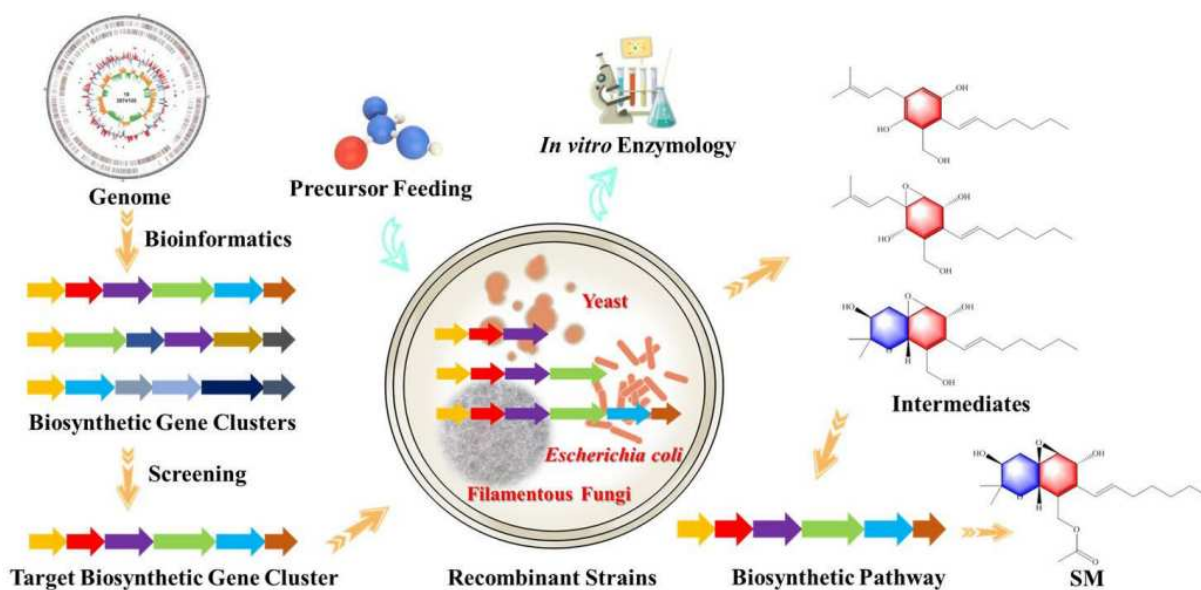


Figure 1.15. The use of a heterologous expression method in the investigation of fungal SM²³⁵. To forecast the biosynthetic route, it mostly consists of screening target BGCs, heterologous expression of recombinant strain, feeding of precursor, *in vitro* enzymatic verification, and identification of intermediate products.

1.5. SMs and *Podospora anserina*

1.5.1. Filamentous ascomycetes fungi

Fungi have potential applications in both applied microbiology and fundamental research. In essence, fungi can be classified into two distinct categories: yeasts and mycelial (filamentous) fungi. Filamentous fungi, such as the ascomycetes *Monascus*, *Fusarium*, *Penicillium*, and *Neurospora*, are being investigated as potential sources of natural pigments with biological usefulness for food, feed, and aesthetic purposes²³⁶. These edible fungi may be utilized in biorefineries to produce ethanol, animal feed, and pigments from waste. Filamentous fungi have long been utilized in the industrial manufacture of economically useful items such as enzymes, antibiotics, feed products, and many more^{61,237}. The notion of biorefinery, or transforming substrates into value-added products, is widely acknowledged in the scientific community. As

a result, research into the diversification of existing and future facilities for the manufacture of a wide range of unique and valuable goods as well as by-products via fermentation is presently a popular topic.

1.5.2. Coprophilous fungi

Animal dung, ecologically rich and composed of easily accessible carbohydrates (hemicellulose, cellulose, and lignin) and significant nitrogen, acts as a thriving substrate for fungal growth. Herbivorous dung can contain up to 4% nitrogen, surpassing that of the original plant material²³⁸. Additionally, it provides essential vitamins, growth factors, minerals, and ample water with a pH level of 6.5. The physical structure of dung, consisting of small fragments in a mucilaginous yet aerated matrix, creates an ideal fungal environment^{239,240}. Fungi play a crucial role in dung decomposition by consuming cellulose and lignin, generating biomass, and conditioning the substrate for invertebrates. Coprophagous fungi, specifically, derive nutrients from fecal matter and contribute to nutrient recycling. With specialized enzymes and adaptations, they break down complex compounds into simpler forms. These fungi thrive in waste-rich environments like pastures, forests, and dung piles.

Coprophilous fungi have been extensively studied, initially as decomposers and later as indicators of past herbivores in sedimentary records²⁴¹. The correlation between spores of coprophilous fungi abundances (*Sporormiella*) and grazing pressure was first observed in the study by Davis et al. at Wildcat Lake²⁴². The pioneering work of B. van Geel and colleagues incorporated fungal remains (non-pollen palynomorphs, NPPs) into traditional palynological studies, expanding our understanding of past ecosystems²⁴³. Categorizing NPPs as proxies required interdisciplinary contributions from zoology, mycology, phycology, and plant anatomy. Some fungal spores can be attributed to modern equivalents, while others are assigned type numbers based on their original identification location^{244,245}. Coprophilous fungi have

endocoprophilous life cycles, with spores consumed and expelled by herbivores (**Figure 1.16**). Most coprophilous fungi spores are obligate, strengthening their use as proxies for past animals²⁴¹. Herbivores consume fungal fruit bodies and spore-laden plant material, with melanised spore walls aiding digestion and preservation²⁴⁶. After passing through the digestive system, spores germinate on the substrate. Fruiting bodies release spores onto herbage, contributing to the sedimentary record. Faeces provide a suitable substrate for coprophilous fungi due to nutrients and favorable conditions. The main coprophilous fungi classes are Mucormycetes, Ascomycetes, and Basidiomycetes, with varying life cycles and fruiting timescales^{247,248}. Understanding fungal succession and spore biology is often overlooked in palaeoecology, highlighting the need for integrating palaeoecological and mycological knowledge.

The order of appearance of fungi on dung follows a taxonomic succession based on their nutritional requirements. Zygomycetes, like *Pilobolus*, *Circinella*, and *Cunninghamella*, are the first to appear due to their ability to metabolize accessible carbon sources. They are followed by Ascomycetes, including *Sordaria*, *Podospora*, *Arthrobotrys*, or *Trichurus*, which can degrade complex carbon sources. Lastly, Basidiomycetes, mainly *Coprinus* and *Coprinellus* species, appear last and persist longer, as they can exploit both cellulose and lignin²⁴⁹. In addition to the nutritional hypothesis, a temporal hypothesis and interspecific competition have been proposed to explain fungal succession on dung^{250–252}. The temporal hypothesis suggests that fungi with simpler structures require less time and nutrients to develop compared to fungi with more complex structures. Interspecific competition involves various interactions such as competition for nutrients, hyphal interference (HI), mycoparasitism, and antibiotic production. HI is a defense mechanism that eliminates competition by causing the death of sensitive partner hyphae²⁵³. Certain fungi can recognize potential competitors through convergent evolution and initiate defense reactions. Initially thought to occur only in late colonizers, HI likely allows

them to overcome early colonizers. Toxic substances play a role in promoting challenger death during HI. Chemical defense, particularly the production of SMs, is another key defense strategy of coprophilous fungi. SMs help them win competition and protect against microbial competitors or fungivorous animals. These bioactive metabolites, such as antibacterial and antifungal compounds, are concentrated in the subphyla Pezizomycotina and Agaricomycotina, especially in orbiliomycetes, eurotiomycetes, dothideomycetes, sordariomycetes, leotiomycetes, and lecanoromycetes⁵⁶. The intense competition among microorganisms inhabiting the dung ecosystem is influenced by niche overlap with other organisms like bacteria, protists, invertebrates, the mammalian digestive system, and fungi.

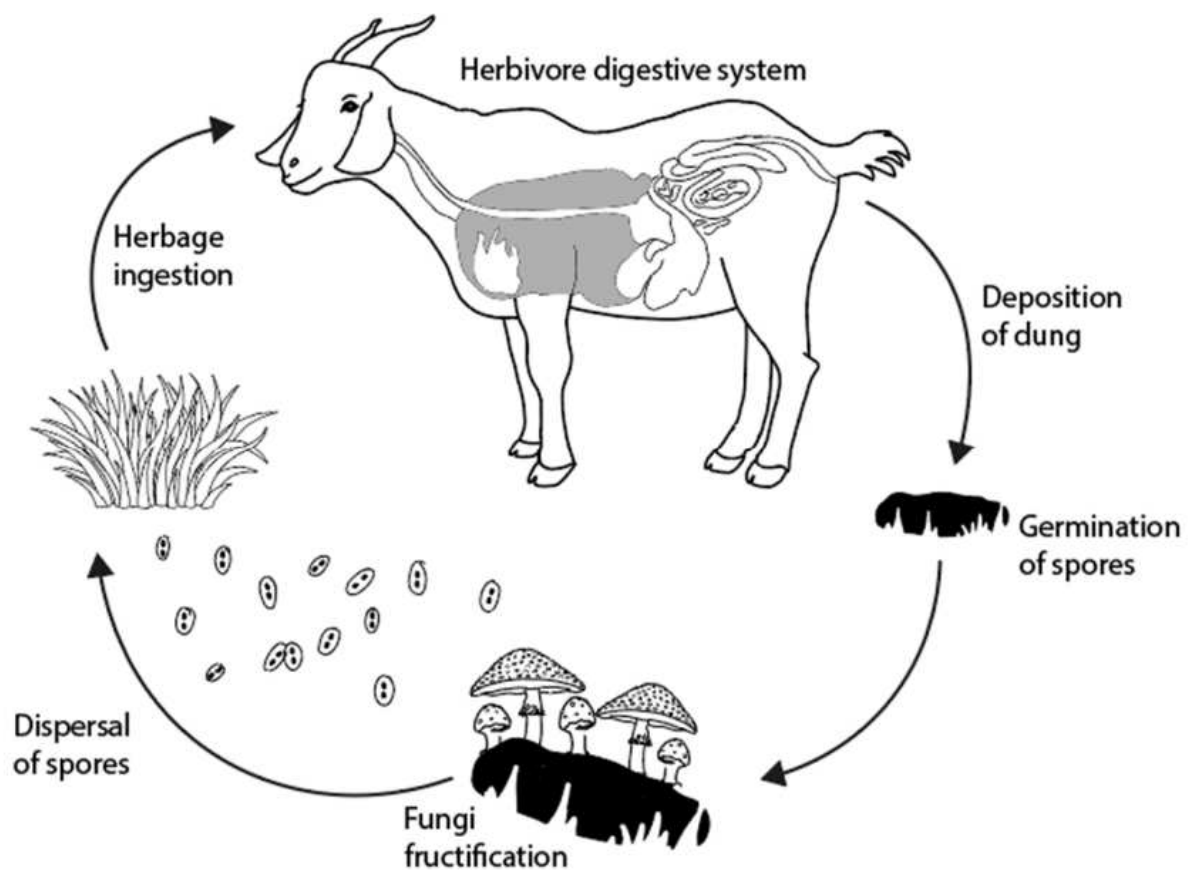


Figure 1.16. The endocoprophilous life cycle of coprophilous fungi²⁵⁴.

1.5.3. *Podospora anserina*

The fungus utilized in this study is *Podospora anserina* (*P. anserina*), an ascomycete filamentous fungus that thrives on dung (coprophilous)²⁵⁵. *P. anserina* germinates and grows from its ascospores, eventually producing fruiting bodies to disperse its offspring. *P. anserina*, has been also used for over a century in laboratories to study various biological processes.

Figure 1.17 illustrates the life cycle of *P. anserina*. This fungus grows rapidly, with a daily expansion rate of 7 mm on standard medium (M2) and completes its life cycle within a week²⁵⁵. *P. anserina* is a heterothallic fungus with two mating types, *mat+* and *mat-*, exclusively relying on sexual reproduction. Within three days of growth, haploid strains of each mating type differentiate into male gametes (spermatia or microconidia) and female gametes (ascogonia)²⁵⁶. Fruiting bodies (perithecia) mature over four days, followed by the ejection of ascospores. Spermatia are easily dislodged by water, while ascogonia remain firmly attached to the mycelium. Fertilization occurs solely between male and female gametes of opposite mating types. Although spermatia can be harvested for fertilizing ascogonia, they cannot disperse through wind and initiate new colonies like conidia. Despite lacking asexual reproduction, hyphal fragments can regenerate new thalli and function as male gametes^{257,258}. After maturation, the majority of ascospores released from perithecia are binucleated and carry distinct versions of the mating type, making them self-fertile. Most asci contain four binucleated ascospores, but around 1% produce two "uni-nucleated" spores instead of a "bi-nucleated" one. The structure of *P. anserina* asci is relatively simple, allowing convenient retrieval of heterokaryotic/homokaryotic ascospores using a needle under a binocular microscope, facilitating microscopic observation and manipulation²⁵⁷.

Rizet and Karl Esser developed essential tools and genetic transformation techniques, leading to molecular genetic studies. Finally, the complete genomic sequence of *P. anserina* was achieved in 2008²⁵⁹. *P. anserina*, like other filamentous fungi, possesses a compact genome

of approximately 35 Mb, which is distributed across seven nuclear chromosomes and one mitochondrial chromosome. Additionally, a robust genetic transformation system has been successfully developed. By inactivating the *mus51* gene responsible for non-homologous recombination, the efficiency of gene deletion through homologous recombination has significantly improved²⁶⁰. This advancement enables the straightforward replacement of a single target gene with a selectable marker such as phleomycin, hygromycin B, nourseothricin, or geneticin. As a result, double and multiple mutants can be generated through genetic crosses.

As a coprophagous fungus, *P. anserina* typically exhibits late fruiting during the succession on dung. *P. anserina* is commonly found on the dung of herbivores worldwide, including sheep, rabbits, and horses. Ascospores attached to vegetation are easily consumed by herbivores and subsequently deposited on dung after passing through the animals' digestive tracts. *P. anserina* possesses an enzymatic repertoire enabling it to break down lignin for access cellulose²⁵⁹. Additionally, it employs a HI defense mechanism, which likely aids in the elimination of competing fungi²⁵³.

P. anserina serves as the initial model system to establish the involvement of mitochondria in the aging process. Research on aging in this organism began in the early 1950s when G. Rizet observed that all cultures of this filamentous ascomycete experience an inevitable cessation of vegetative growth, termed senescence²⁶¹. Due to its easy cultivation, rapid sexual cycle completion within a week, and significantly shorter generation time compared to *A. nidulans* and *N. crassa*, *P. anserina* has become a valuable model fungus in labs. Its exceptional characteristics make it an efficient tool for acquiring mutant strains and advancing genetic research²⁵⁷. It has been extensively utilized across various laboratories to investigate a wide range of biological phenomena. These include fundamental areas such as aging, meiosis, prions, and protein-based inheritance. Additionally, *P. anserina* has contributed significantly to the understanding of fungal-specific processes like sexual reproduction,

heterokaryon formation, and hyphal interference. Moreover, it has been employed in studies related to signal transduction, differentiation, plant biomass degradation, cell fusion, vegetative incompatibility, interactions with other fungi, mitochondrial physiology, translation, secondary metabolism, and more. The diverse research applications of *P. anserina* underscore its significance as a valuable tool in addressing both broad biological questions and specific fungal-related inquiries. Nonetheless, the absence of conidia within *P. anserina* may impose limitations on certain research endeavors. However, technological advancements, including the introduction of equipment like "FastPrep" (Biorad) and the improvement of effective protoplast formation techniques for breaking down mycelia into uninucleated cells, have mitigated the relevance of this previously mentioned limitation.

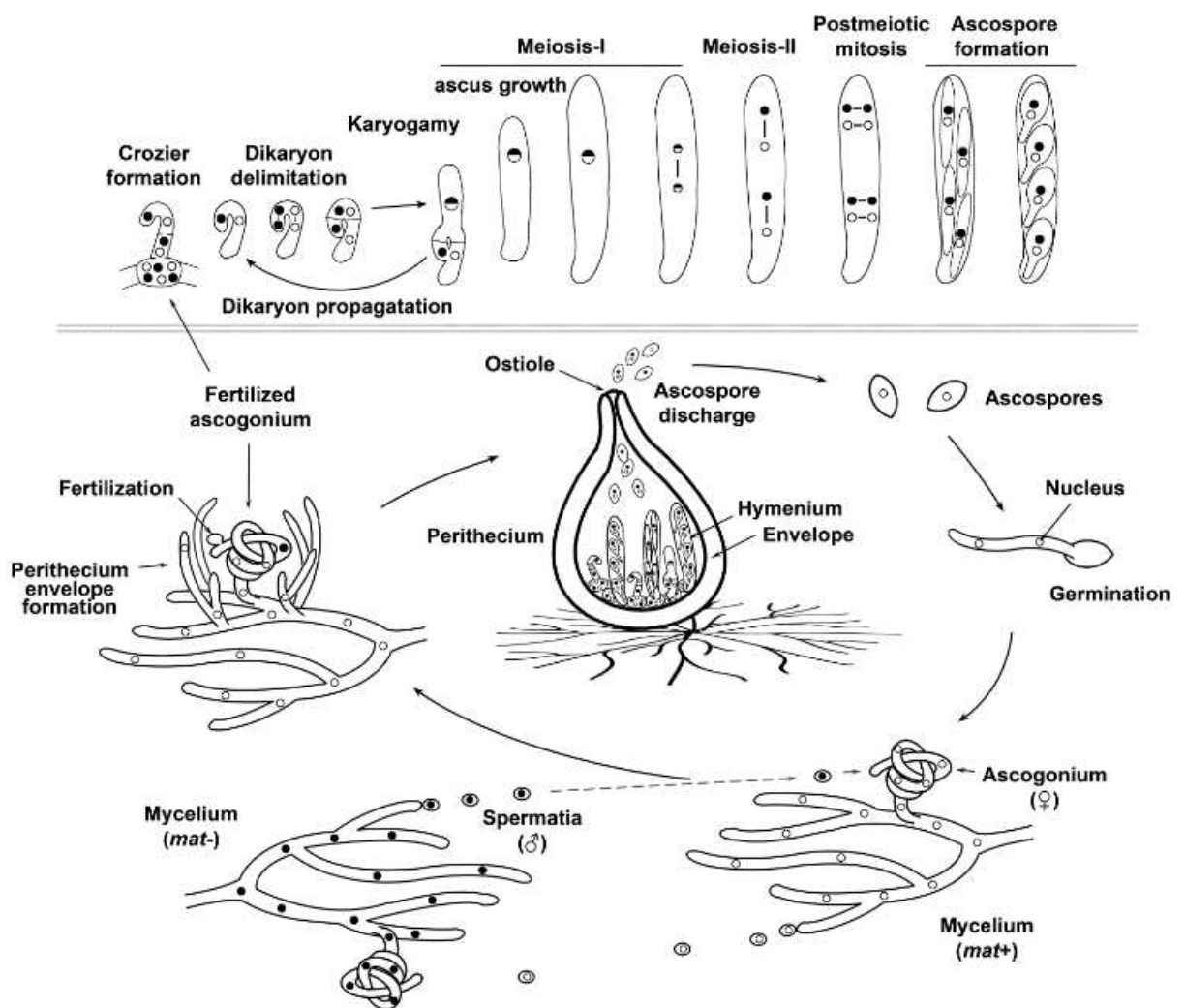


Figure 1.17. The sexual life cycle of *P. anserina*²⁶². Filamentous ascomycetes undergo sexual reproduction involving the formation of ascogonia, surrounded by protective hyphal envelopes. Fertilization occurs through hyphae, asexual spores, or spermatia in heterothallic fungi. The resulting hymenium produces ascospores through karyogamy, meiosis, crozier formation, mitoses, and septa formation. Mature ascospores are enclosed in asci and forcibly ejected for dispersal.

1.5.4. SMs in *P. anserina*

Ascomycetes belonging to the order Sordariales are a well-known reservoir of SMs with potential beneficial applications²⁶³. *P. anserina* belongs to the sordariomycete clade (**Figure 1.18**), which is predicted to produce SMs with high frequency⁵⁶.

It is well understood that SMs serve as chemical defense effectors in order to interact with other species and defend their own producer. To the best of our knowledge, only two benzoquinones (anserinones A and B) have been discovered from liquid cultures of *P. anserina* that were initially isolated from mouse feces²⁶⁴. According to Bills and Gloer's study, ST was ever extracted from an endophytic *Podospora sp.* closely related to *P. anserina* and could be identified in one freshly isolated strain of *P. anserina*^{56,265}. These strains are most likely distinct from the sequenced *P. anserina* reference strain (large S) that we utilized. As a result, few chemical studies on this model fungus have been undertaken to far, hinting that *P. anserina* might be an unexplored source of SMs.

The sequencing of the *P. anserina* genome revealed a higher number of genes encoding SMs than the number of known isolated products. Forty-six potential BGCs are predicted by antiSMASH²⁶⁶ in December 2023 in *P. anserina* (unpublished data, appendix 1) including 18 PKSs. However, two PKS gene involved in melanin biosynthesis and a horizontally transferred BGC from *Aspergillus* have been characterized in *P. anserina*, while the remaining BGCs are

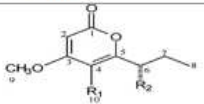
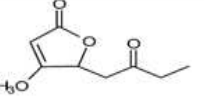
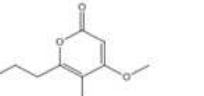
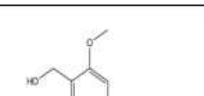
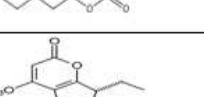
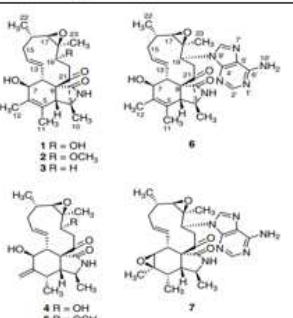
yet to be functionally studied^{267,268}. Furthermore, the expression of several BGCs was found to be up-regulated during *P. anserina*'s antagonistic interactions with *Serratia fonticola* and *Serratia marcescens* bacteria²⁶⁹.

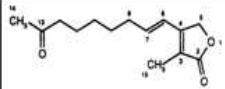
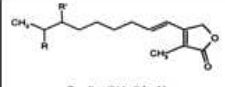
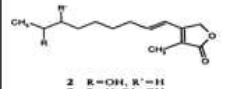
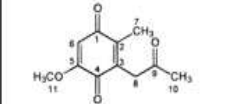
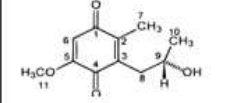
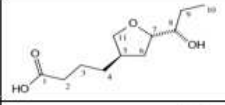
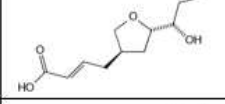
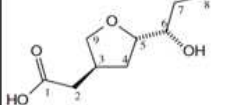
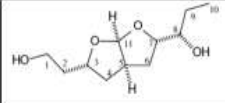
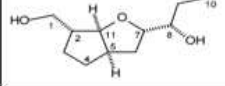
Within our team's existing publications, our primary focus rested on the functional characterization of the *P. anserina* BGC responsible for the biosynthesis of ST, then contributing to a better understanding of ST in fungal physiology²⁷⁰. More recently, it has been shown that the light desensitization protein VIVID was involved in white light-responsive pigmentation, sexual development and ST biosynthesis²⁷⁰.

Simultaneously, we conducted a thorough review of published literature, compiling and summarizing detailed information on natural compounds reported in *P. anserina* and its homologous species (**Table 1.1**).

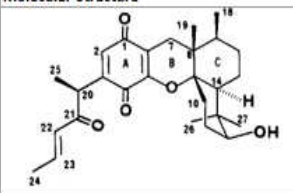
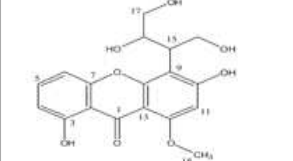
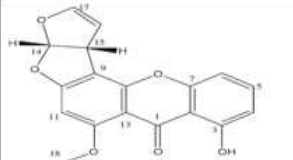
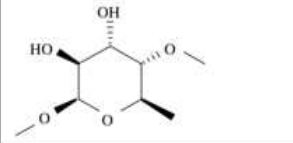
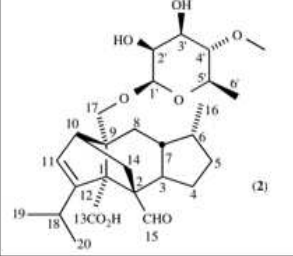
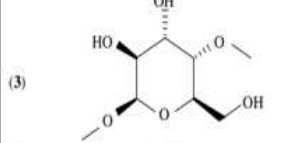

With its well-established molecular manipulation techniques, *P. anserina* holds significant potential for the discovery of novel natural products and the study of the role of SMs in fungal life cycles. In summary, *P. anserina* is a promising candidate for exploring natural products and investigating the functions of SMs.

Table 1.1. A compilation summarizing compounds detailed in the literature, unearthed in *P. anserina* and its homologous species.

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
2	Annularin A	198	199	221	237	419	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C10H14O4	 <p> 1 R₁ = CH₃ R₂ = OH 2 R₁ = CH₂OH R₂ = H 3 R₁ = CH₂OH R₂ = OH 4 R₁ = CH₃ R₂ = H 5 R₁ = H R₂ = H </p>
3	Annularin B	198	199	221	237	419	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C10H14O4	
4	Annularin C	214	215	237	253	451	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C10H14O5	
5	Annularin D	182	183	205	221	387	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C10H14O3	
6	Annularin E	168	169	191	207	359	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C9H12O3	
7	Annularin G	186	187	209	225	395	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C9H14O4	
8	Annularin H	184	185	207	223	391	<i>Annulatascus triseptatus</i>	J. Nat. Prod. 2003, 66, 1302-1306	C9H12O4	
9	Annularin I	196	197	219	235	415			C11H16O3	
10	Annularin J	212	213	235	251	447			C11H16O4	
11	Annularin F	210	211	233	249	443	<i>Podospora anserina</i>		C10H10O5	
12	Alachalasin A	389	390	412	428	801	<i>Podospora vesticola</i>	Bioorg Med Chem-2008-16-p2627-2634	C22H31NO5	 <p> 1 R = OH 2 R = OCH₃ 3 R = H 4 R = OH 5 R = OCH₃ </p>
13	Alachalasin B	403	404	426	442	829	<i>Podospora vesticola</i>	Bioorg Med Chem-2009-17(1)-p428	C22H33NO5	
14	Alachalasin C	373	374	396	412	769	<i>Podospora vesticola</i>	Bioorg Med Chem-2008-16-p2627-2634	C22H31NO4	
15	Alachalasin D	389	390	412	428	801	<i>Podospora vesticola</i>	Bioorg Med Chem-2009-17(1)-p428	C22H31NO5	
16	Alachalasin E	403	404	426	442	829	<i>Podospora vesticola</i>	Bioorg Med Chem-2008-16-p2627-2634	C22H33NO5	
17	Alachalasin F	506	507	529	545	1035	<i>Podospora vesticola</i>	Bioorg Med Chem-2009-17(1)-p428	C27H34N6O4	
18	Alachalasin G	506	507	529	545	1035	<i>Podospora vesticola</i>	Bioorg Med Chem-2008-16-p2627-2634	C27H34N6O4	

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
19	Appenolide A	236	237	259	275	495	<i>Podospira appendiculata</i>	J Nat Prod-1993-56(3)-p341-344	C14H20O3	
20	Appenolide B	238	239	261	277	499	<i>Podospira appendiculata</i>	J Nat Prod-1993-56(3)-p341-344	C14H22O3	 2 R=OH, R'=H 3 R=H, R'=OH
21	Appenolide C	238	239	261	277	499	<i>Podospira appendiculata</i>	J Nat Prod-1993-56(3)-p341-344	C14H22O3	 2 R=OH, R'=H 3 R=H, R'=OH
22	Anserinone A	208	209	231	247	439	<i>Podospira anserina</i>	J. Nat. Prod. 1997, 60, 629-631	C11H12O4	
23	Anserinone B	210	211	233	249	443	<i>Podospira anserina</i>	J. Nat. Prod. 1997, 60, 629-631	C11H14O4	
24	Communiol A	216	217	239	255	455	<i>Podospira communis</i>	Tet Lett-2004-45(37)-p6891-6894	C11H20O4	
25	Communiol B	214	215	237	253	451	<i>Podospira communis</i>	Tet Lett-2004-45(37)-p6891-6894	C11H18O4	
26	Communiol C	188	189	211	227	399	<i>Podospira communis</i>	Tet Lett-2004-45(37)-p6891-6894	C9H16O4	
27	Communiol D	216	217	239	255	455	<i>Podospira communis</i>	Tet Lett-2004-45(37)-p6891-6894	C11H20O4	
28	Communiol E	200	201	223	239	423	<i>Podospira communis</i>	J. Nat. Prod. 2005, 68, 435-438	C11H20O3	

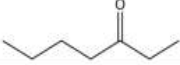
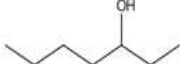

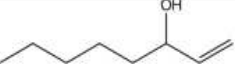
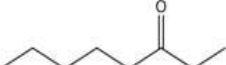
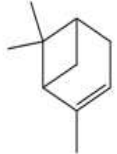
1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
29	Communiol F	198	199	221	237	419	<i>Podospira communis</i>	J. Nat. Prod. 2005, 68, 435-438	C11H18O3	
30	Communiol G	199	200	222	238	421	<i>Podospira communis</i>	J. Nat. Prod. 2005, 68, 435-439	C11H18O3	
31	Communiol H	212	213	235	251	447	<i>Podospira communis</i>	J. Nat. Prod. 2005, 68, 435-438	C11H16O4	
32	Curvicollide A	432	433	455	471	887	<i>Podospira curvicolla</i>	Org Lett-2004-6(8)-p1249-1252	C26H40O5	<p> 1 R=CH₂OH R=H 2 R=CH₃ R=OH 3 R=CH₃ R=H </p>
33	Curvicollide B	432	433	455	471	887	<i>Podospira curvicolla</i>	Org Lett-2004-6(8)-p1249-1252	C26H40O5	
34	Curvicollide C	416	417	439	455	855	<i>Podospira curvicolla</i>	Org Lett-2004-6(8)-p1249-1252	C26H40O4	
35	Decipinin A	600	601	623	639	1223	<i>Podospira decipiens</i>	J. Nat. Prod. 2002, 65, 916-919	C31H36O12	
36	Decipienolide A	378	379	401	417	779	<i>Podospira decipiens</i>	J. Nat. Prod. 2002, 65, 916-919	C21H30O6	
37	Decipienolide B	378	379	401	417	779	<i>Podospira decipiens</i>	J. Nat. Prod. 2002, 65, 916-919	C21H30O6	
38	Deoxyverrucarin E	123	124	146	162	269	<i>Podospira anserina</i>		C7H9NO	
39	13-hydroxyversicolorin B	356	357	379	395	735	<i>Podospira sp.</i>	Parasitol Res-2011-108(3)-p561-566	C18H12O8	

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
40	Podosporin A	440	441	463	479	903	<i>Podospora decipiens</i>	J Org Chem-1988-53(19)-p4567-4569	C27H36O5	
41	secosterigmatocystin	362	363	385	401	747	<i>Podospora sp.</i>	Parasitol Res-2011-108(3)-p561-566	C18H18O8	
42	Stérigmatocystine	324	325	347	363	671	<i>Podospora sp.</i> <i>Podospora anserina</i>	Parasitol Res-2011-108(3)-p561-566	C18H12O6	
43	sordarin	492	493	515	531	1007	<i>Podospora pleiospora</i>	Mycol. Res. 109 (5): 619–626 (May 2005)	C27H40O8	
44	sordarin B	492	493	515	531	1007	<i>Podospora pleiospora</i>	Mycol. Res. 109 (5): 619–626 (May 2005)	C27H40O8	
45	sordaricin	332	333	355	371	687	<i>Podospora pleiospora</i>	Mycol. Res. 109 (5): 619–626 (May 2005)	C20H28O4	(3) 
46	hydroxsordarin	508	509	531	547	1039	<i>Podospora pleiospora</i>	Mycol. Res. 109 (5): 619–626 (May 2005)	C27H40O9	(4) 

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
47	emestrin C	612	613	635	651	1247	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H24N2O10S2	<p>emestrins H–K (1–4), and seven known emestrins: secoemestrin D (5),⁵ emestrin C (6; MPC1001),^{6,7} MPC1001C (7),⁶ emestrin D (8; MPC1001D),^{6,7} emestrin E (9),⁷ MPC1001F (10),⁶ and MPC1001H (11).⁶ Herein, we report on the identification of the producing organisms, their fermentation, the isolation, structure determination, and antibiotic activity of these new and known emestrins and, in particular, their selective antifungal activity toward <i>C. neoformans</i>.</p> <p>1 R₁ = H, R₂ = H 2 R₁ = CH₃, R₂ = H 3 n = 2, R = H 6 n = 4, R = OH</p>
48	emestrin D	644	645	667	683	1311	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H24N2O10S3	
49	secoemestrin D	632	633	655	671	1287	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H24N2O8S4	
50	emestrin E	676	677	699	715	1375	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H24N2O10S4	
51	emestrins xx (MPC1001C)	596	597	619	635	1215	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H24N2O9S2	
52	emestrins xx (MPC1001F)	610	611	633	649	1243	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C29H26N2O11S	
53	emestrins xx (MPC1001H)	546	547	569	585	1115	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C28H22N2O10	
54	emestrin H	418	419	441	457	859	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C20H22N2O4S2	<p>6 n = 2, R = OH 7 n = 2, R = H 8 n = 3, R = OH 9 n = 4, R = OH</p>
55	emestrin I	582	583	605	621	1187	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C29H30N2O7S2	
56	emestrin J	552	553	575	591	1127	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C27H24N2O7S2	
57	emestrin K	566	567	589	605	1155	<i>Podospira australis</i>	J. Nat. Prod. 2016, 79, 2357–2363	C29H30N2O6S2	
58	Podospin A	334	335	357	373	691	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C18H22O6	
59	Podospin B	336	337	359	375	695	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C18H24O6	
60	Podospin C	294	295	317	333	611	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C16H22O5	

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
61	Podospin D	348	349	371	387	719	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O6	
62	Podospin E	348	349	371	387	719	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O6	
63	Podospin F	348	349	371	387	719	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O6	
64	Podospin G	348	349	371	387	719	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O6	
65	Podospin H	350	351	373	389	723	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H26O6	
66	Podospin I	366	367	389	405	755	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H26O7	
67	Podospin J	380	381	403	419	783	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O8	

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
68	Podospin K	364	365	387	403	751	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O7	
69	Podospin L	364	365	387	403	751	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O7	
70	LL-Z1640-1	364	365	387	403	751	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H24O7	
71	(5E)-7-oxozeaenol	362	363	385	401	747	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H22O7	
72	cochliomycin D	362	363	385	401	747	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H22O7	
73	cochliomycin E	362	363	385	401	747	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H22O7	
74	cochliomycin F	362	363	385	401	747	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H22O7	
75	7',8'-dihydrohypothemycin	380	381	403	419	783	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C19H22O7	
76	aigialomycin D	334	335	357	373	691	<i>Podospira sp.</i>	J. Nat. Prod. 2021, 84, 483–494	C18H22O6	

1	Known compounds	[M]	[M+H] ⁺	[M+Na] ⁺	[M+K] ⁺	[2M+Na] ⁺	Species	Bibliographic references	Molecular Formula	Molecular Structure
77	3-Heptanone	114	115	137	153	251	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C7H14O	
78	3-Heptanol	116	117	139	155	255	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C7H16O	
79	Acetic acid pentyl ester	130	131	153	169	283	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C7H14O2	
80	1,3-Octadiene	110	111	133	149	243	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C8H14	
81	1-Octen-3-ol	128	129	151	167	279	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C8H16O	
82	3-Octanone	128	129	151	167	279	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C8H16O	
83	α -Pinene	136	137	159	175	295	<i>Podospora anserina</i>	BBA - General Subjects 1862 (2018) 2174–2182	C10H16	

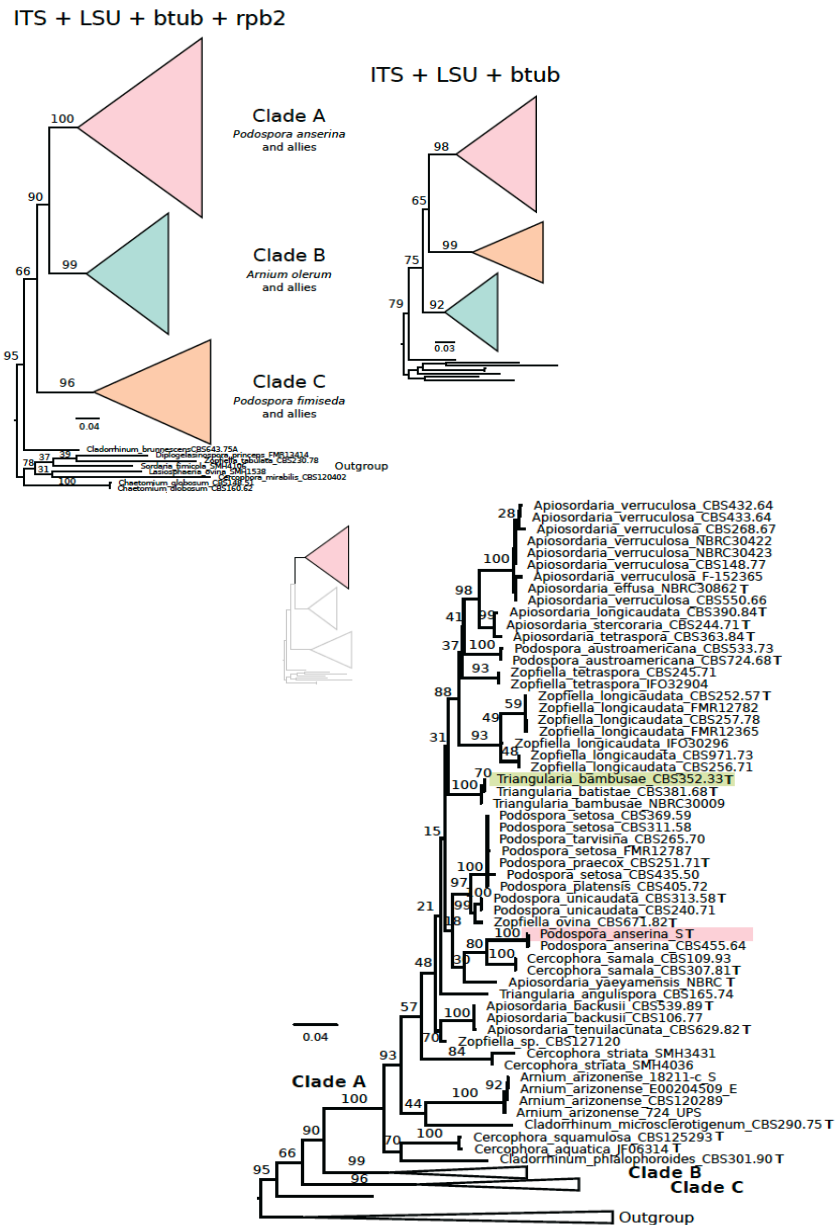


Figure 1.18. Maximum Likelihood phylogeny of the concatenated analysis of ITS, LSU, Brub and rpb2 for the Podosporeae²⁷¹.

1.5.5. Update of BGCs in the genome of *P. anserina*

With genome sequencing advancements, fungal genomes are increasingly identified and reported, allowing for compound prediction. Bioinformatics tools like BLAST and FASTA (sequence similarity searches and homologous gene retrieval from public databases)¹⁶⁶, TOUCAN²⁷² and ARTS²⁷³ (gene mining), antiSMASH^{274–276} and cluster finder^{277,278} (analysis

and prediction of BGCs related to SMs) have been developed, promoting research on fungal SM BGCs. The fungal BGC-specific predictor antiSMASH version 7.0²⁶⁶ has now been released, which enables the research team to take advantage of the continuous improvement of BGC prediction software to conduct a more comprehensive analysis of the *P. anserina* genome and further improve the accuracy of BGC prediction. It has so far permitted the identification of 46 BGCs, including 16 PKS, 10 NRPS or NRPS-like and 5 hybrid NRPS/PKS (appendix 1), (Figure 1.19A). In 23 and 19 BGCs, respectively, specific TFs and potential export proteins were identified (Figure 1.19B).

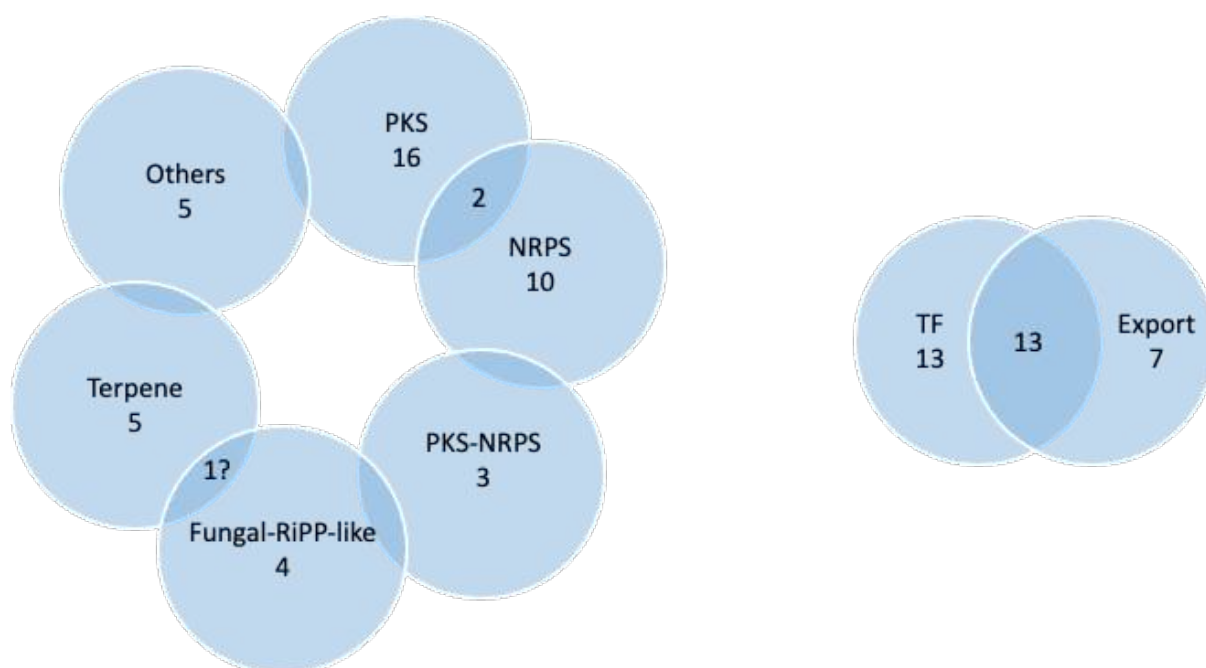


Figure 1.19. Distribution of *P. anserina* BGCs' key backbone enzymes (A) and putative particular TFs and/or putative export protein (B).

1.6. Objectives

Throughout my thesis, I focused on the regulation of SMs production in the filamentous fungus *P. anserina* by inactivating and overexpressing global regulators. This research project is dedicated to acquiring an in-depth understanding of fungal metabolism and its complex

regulatory mechanisms, with the aim of improving our understanding of fungal physiology and isolating new metabolites. This knowledge may provide valuable insights into the role of secondary metabolites in fungal physiology. In addition, this research could reveal new active compounds, meeting a perpetual need in the field of human health. In this context, the main objective is to better understand the global regulation of SMs in *P. anserina* by deleting and overexpressing the global regulators encoded by *NsdD*, *LaeA*, *CreA* and *HapX* genes in this fungus. After this introduction where I provided an overview of fungal SMs, chapter 2 details the experimental methods employed in this study, chapter 3 focuses on my participation in research related to *PaNsdD* topics and presents the published research results, chapter 4 delves into the characterization of SMs in *P. anserina* under the regulation of three selected environmental signal regulators as the most important part of my project and finally, the last chapter involves discussing, analyzing, and summarizing the experimental findings, exploring the ecological role of SMs in *P. anserina*'s natural habitat, and proposing future research directions for my topic.

Chapter 2

Experimental procedures

2.1. Fungal strains and growth conditions

The comprehensive protocols, encompassing standard culture conditions and genetic manipulation procedures for this microorganism, have been detailed by Silar²⁵⁷. Access to these protocols is available at <http://podospora.i2bc.paris-saclay.fr>. The two culture media employed for this study are the widely used M2 medium, which consists of the following components per liter: 0.25 g of KH₂PO₄, 0.3 g of K₂HPO₄, 0.25 g of MgSO₄, 0.5 g of urea, 0.05 mg of thiamine, 0.05 mg of biotin, 5 mg of citric acid, 5 mg of ZnSO₄, 0.25 mg of CuSO₄, 50 mg of MnSO₄, 50 mg of boric acid, 50 mg of sodium molybdate, 1 mg of iron alum, 5.5 g of dextrin, and 10 g of agar, with the pH maintained at 7 using a phosphate buffer. The second medium used is the G (germination) medium, which contains per liter: 25 g of corn flour, 25 g of corn cream, 6 g of ammonium acetate, and 12 g of agar. The optimal temperature for cultivation is 27°C. For resistance selection of transformants, various antibiotics can be added to growth media, the concentration is given as follows: hygromycin B (75 µg/mL), phleomycin (10 µg/mL), geneticin (250 µg/mL) and nourseothricin (40 µg/mL).

Table 2.1 lists the *P. anserina* strains utilized in this study. The strains of *P. anserina* utilized in this study originated from the "S" (big S) wild-type strain, which served as the reference for genome sequencing^{259,279}. The $\Delta mus51$ strain missing the Ku70 member of the protein complex involved in non-homologous end joining was previously identified²⁶⁰. The use of the $\Delta mus51$ strain as a recipient increases the incidence of targeted gene substitution. The

albino *PaPks1-193* mutant has a mutation in the *PaPks1* gene, which encodes the PKS involved in the initial phase of melanin production²⁶⁷.

Table 2.1. *P. anserina* strains used in this study.

Strains	Description
<i>S</i>	Wild type
<i>Δmus51</i>	Deletion of <i>Pa_6_6180</i> Hygro ^R or Phleo ^R or Nour ^R or Genet ^R
<i>PaPks1-193</i>	Single mutation in <i>Pa_2_510</i>
<i>PaNsdD^Δ</i>	Deletion of <i>Pa_2_1880</i> , Phleo ^R
<i>PaNsdD^Δcomp</i>	Complementation of <i>PaNsdD^Δ</i> , Hygro ^R , Phleo ^R
<i>PaNsdD^Δ/PaStcA^Δ</i>	Double deletion, Phleo ^R Genet ^R
<i>PaLaeA^Δ</i>	Full-length deletion of <i>Pa_1_2300</i> , WT background, Hygro ^R
<i>PaLaeA^Δcomp</i>	Complementation of <i>PaLaeA^Δ</i> , Hygro ^R , Genet ^R
<i>OE-PaLaeA</i>	Overexpression of <i>Pa_1_2300</i> , WT background, Hygro ^R
<i>PaLaeA^Δ/PaPks1-193</i>	Deletion of <i>PaLaeA</i> in <i>PaPKS1-193</i> background, Hygro ^R
<i>OE-PaLaeA/PaPks1-193</i>	Overexpression of <i>PaLaeA</i> in <i>PaPKS1-193</i> background, Hygro ^R
<i>OE-PaCreA</i>	Overexpression of <i>Pa_1_22930</i> , WT background, Hygro ^R
<i>PaHapX^Δ</i>	Full-length deletion of <i>Pa_2_6580</i> , WT background, Phleo ^R

2.2. Molecular biology experiments

Next, we will provide a detailed description of the construction process for deletion, complementation, and overexpression strains. As previously described^{270,280}, we conducted targeted gene deletion in *P. anserina* using protoplast-mediated fungal transformation. Briefly, the target-resistance cassette for the target gene was constructed as follows. Two fragments of the target gene flanking sequences were PCR-amplified from genomic DNA using *ad hoc*

primer pairs. Subsequently, each of these fragments was fused to the target cassette, which had been amplified from plasmid pBC- (target resistance) containing the target-resistance selective marker. The resulting fusion PCR products were then used to transform protoplasts of the *Amus51::(target resistance)* strain. Initially, all target-resistance transformants were screened using PCR with primer pairs located within the target-resistance cassette and outside the flanking region. Two candidate transformants were subsequently crossed with the wild-type strain to eliminate potential untransformed nuclei and to segregate the *Amus51* mutation. The most effective strategy, known as the "split marker" method, is elaborated in **Figure 2.1**. Several mat + and mat – strains carrying the target gene deletion were obtained and further confirmed using Southern blot analysis. Appendix (**Table S1, Figure S1**) contains the presented primers, schematics, and Southern blot results.

In order to ascertain that the observed phenotypes in the deletion strain were specifically caused by the inactivation of the relevant gene, the complementation process entailed ectopically inserting the corresponding gene under the control of its native promoter. Using a high-fidelity polymerase (such as Phusion High-Fidelity DNA Polymerase from Thermo Scientific or Q5 High-Fidelity DNA Polymerase from NEB), we amplified a 7.8 kb fragment containing the complete wild-type CDS, its native promoter, and terminator, using the appropriate primer pair. Subsequently, the amplified DNA fragment was cloned into the EcoRV site of the pBC- (target resistance) plasmid, resulting in the creation of a new vector. This plasmid was sequenced and then introduced into the protoplasts of the target gene deletion mutant strain. After sequencing the plasmid, it was introduced into the protoplasts of the target gene deletion mutant strain. Transformants were selected on medium containing target resistance, and the presence of the wild-type allele was confirmed using PCR.

To obtain overexpression mutant strains, it is necessary to construct an overexpression plasmid first. We used the pBC-HA vector²⁸¹ that contains the AS4 promoter²⁸² and the ribP2

terminator cloned into the NotI site of pBC-hygro. A 750 bp fragment carrying the AS4 promoter, a about 2000 bp fragment carrying the complete CDS of target gene and a 216 bp fragment carrying the were amplified using adequate primer pairs. These three amplicons were fused by PCR and then inserted into the EcoRV-digested pBC-hygro plasmid generating a new overexpression plasmid. All the PCR reactions were performed with a high-fidelity polymerase (Phusion High-Fidelity DNA Polymerase from Thermo Scientific or Q5 High-Fidelity DNA Polymerase from NEB). Candidate plasmids were then validated by enzyme digestion and transformations were performed as described previously using protoplasts from the S strain.

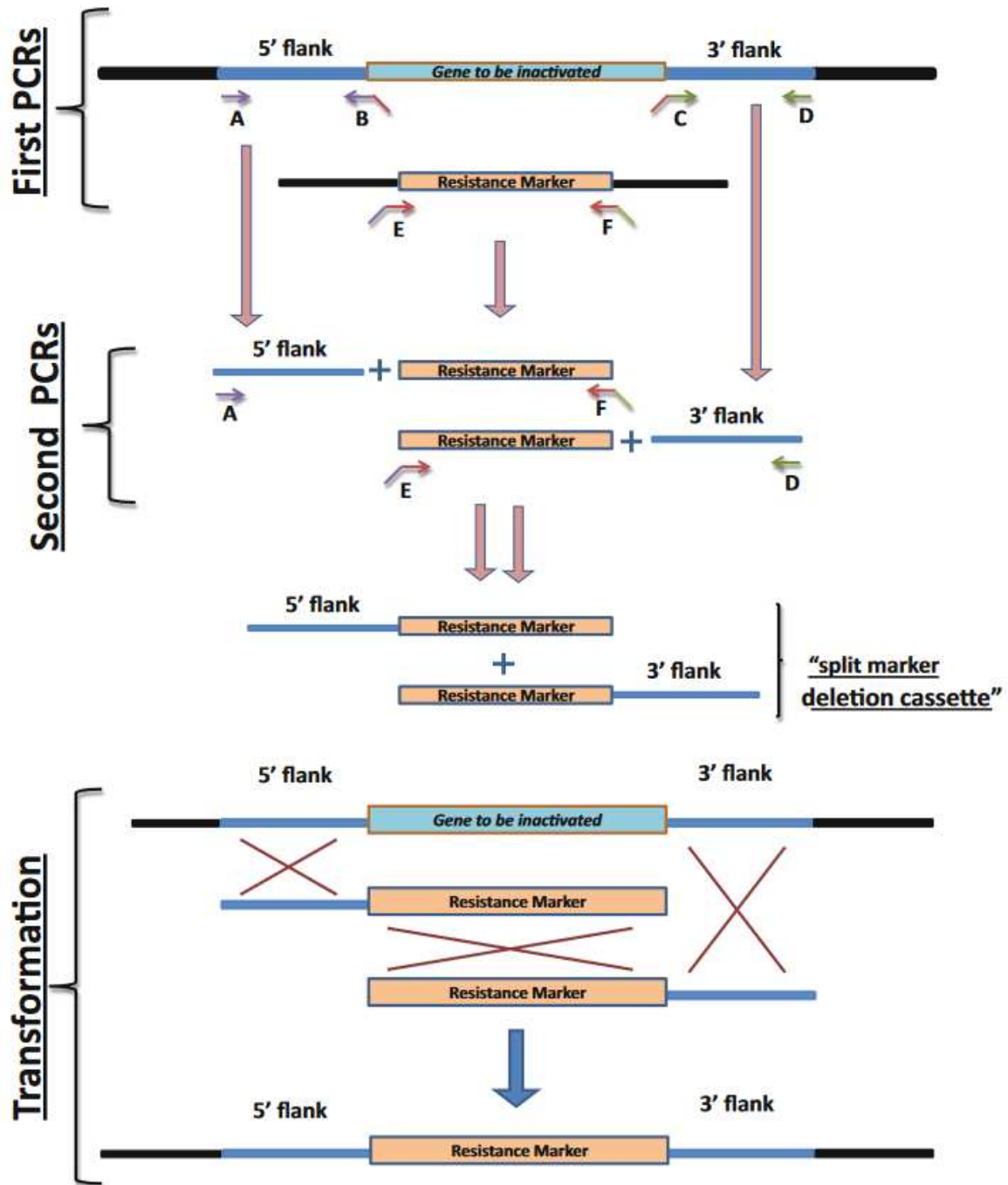


Figure 2.1. Gene inactivation with the “split marker” method²⁵⁷. In the initial step, two PCR reactions amplify the 5' and 3' flanking regions of the target gene for deletion using primer pairs (A, B) and (C, D). Simultaneously, a third PCR reaction amplifies the resistance marker with primers E and F, containing complementary sequences for fusion. In the subsequent step, two separate PCR reactions merge the 5' and 3' flanking fragments with the resistance marker using primer pairs (A, F) and (E, D), yielding a 2-piece deletion cassette, the split marker deletion

cassette. Both fragments are co-transformed into *P. anserina*. Three recombination events (indicated by red crosses) subsequently delete the target gene, replacing it with the resistance marker.

2.3. Phenotypic analyses

2.3.1. Fertility assays and sexual reproduction

After mixing fragmented mycelia from homokaryotic *mat+* and *mat-* strains, the mixture was inoculated at the center of an M2 plate under continuous light exposure. Photos were captured following an 8-day incubation period at 27°C. Fresh mycelia growing on solid M2 medium were observed under a microscope. To quantitatively measure spermatia, the strains were cultured for 3 days, and then spermatia were harvested by flooding the plates with 1.5 mL of 0.05% Tween 20 in sterile water. Subsequently, the resulting suspension was counted using a hemacytometer as part of the microscopy analysis and quantitative measurement of spermatia.

2.3.2. Vegetative growth

To investigate the influence of target genes on the vegetative growth of *P. anserina*, phenotypic analysis was conducted on both mutants and the WT strain. This analysis involved incubating them on solid and liquid M2 media or solid and liquid LB media at 27°C for 4-12 days, representing the optimal growth conditions. The specific steps included taking a small piece of the wild or mutant strains, adding 500 µL of sterile water to each tube, subjecting them to Fast Prep (at a speed of 5000 g for 5 seconds), and finally, adding 10 µL of Mix in the middle of each solid or liquid medium. During their vegetative growth and sexual cycle, observations were made on colony morphology, pigmentation, perithecium formation, ascospore production, ascospore dispersal, and germination. Under identical conditions, M2 liquid and LB liquid

cultures were conducted to assess the fungal pigmentation of the thallus and the coloration of the culture filtrate following the removal of the mycelial suspension. Each treatment was replicated on three plates, and the experiment was repeated once.

2.3.3. Carbon sources

The M0 plates were supplemented with various carbon sources at the following concentrations: 2% Lactose, 2% Glycerol, 5 g/L microcrystalline cellulose (MCC), 5.5 g/L glucose (M3 Medium), and 5g/L Lignin powder. Simultaneously, we prepared MAE (Minimal Agar Ethanol) medium, M0+cellophane (to assess penetration ability, with cellophane placed flat on top of the M0 solid medium), and M0+Whatman (small 1 cm squares of Whatman paper laid flat on the M0 medium). Standardized fragmented mycelium suspensions of WT and mutant strains were inoculated onto the media using 5 μ L spots. The cultures were then incubated at 27°C for 5 days, followed by observations of colony size and morphological characteristics. As controls, these strains were also grown on M0 medium without any supplements. Each treatment was replicated on three plates, and the experiment was repeated once. When the strains are cultivated on a medium with an altered carbon source, they produce hyphae that are lighter in color and less dense. This makes it challenging to capture growth differences in direct sunlight. Consequently, we employed two methods for photography: some images were captured in natural sunlight, while others were taken under the ultraviolet light of an electrophoresis imager.

2.3.4. Oxidative stress response

The M2 plates were supplemented with oxidative stress-inducing agents at the following concentrations: H₂O₂ 500 μ M, Menadione 25 μ M, *Tert*-butyl hydroperoxide (TBH) 50 μ M, and methylglyoxal (MG) 5 mM. Standardized fragmented mycelium suspensions of WT and

mutant strains were inoculated onto the media using 5 μ L spots. The cultures were then incubated at 27°C for 5 days, followed by observations of colony size and morphological characteristics. As controls, these strains were also grown on M2 medium without any supplements. Each treatment was replicated on three plates, and the experiment was repeated once.

2.3.5. Osmotic stress

To assess osmotic stress sensitivity, individual incubations of WT and mutant strains were performed on M2 medium supplemented with osmotic stress reagents, including 0.5 M of NaCl, 0.5 M of KCl. Standardized fragmented mycelium suspensions of WT and mutant strains were inoculated onto the media using 5 μ L spots. As controls, these strains were also grown on M2 medium without any supplements. Each treatment was replicated on three plates, and the experiment was repeated once.

2.3.6. Cell wall-binding compounds

To assess cell wall-binding compounds stress sensitivity, individual incubations of WT and mutant strains were performed on M2 medium supplemented with osmotic stress reagents, including 1 M of sorbitol, and 10 μ M of calcofluor white (CFW) and 100 μ M of Congo red. Standardized fragmented mycelium suspensions of WT and mutant strains were inoculated onto the media using 5 μ L spots. As controls, these strains were also grown on M2 medium without any supplements. Each treatment was replicated on three plates, and the experiment was repeated once.

2.3.7. Confrontation assays against fungi

Plants and animals exhibit defense responses when encountering external invaders. A shared mechanism triggered in both groups involves the release of reactive oxygen species (ROS). In mammals, NADPH oxidase serves as the main generator of this oxidative burst, while in plants, a NADPH oxidase has been identified in *Arabidopsis thaliana*, producing peroxide^{283–285}. Similarly, NADPH oxidases (Nox1 and Nox2) have been found in filamentous ascomycetes and basidiomycetes, with Nox1 present in *Aspergillus fumigatus* and *A. nidulans*^{286,287}. Nox1 and Nox2 enzymes in the filamentous fungus *P. anserina* are implicated in developmental processes and regulated ROS production during interactions with other organisms²⁸⁸. The fungi accumulate peroxide when challenged, resembling an oxidative burst observed in plants and animals, suggesting a recognition mechanism at the contact zone with other microorganisms. The peroxide accumulation and associated death events in *P. anserina* require *PaNox1* and are controlled by the PaASK1 MAP kinase signal transduction cascade²⁸¹, independent of functional respiration, peroxisomes, or the intact *PaNox2* gene.

To gain a deeper insight into whether mutants might experience changes in their capacity to outcompete other fungal adversaries, we assessed two established indicators of resistance: cell death and peroxide production. Diaminobenzidine (DAB) staining is a conventional method used for peroxide detection, leading to the formation of a red precipitate. To detect cell death in the contact zone, we employed the Trypan blue exclusion method as described by Silar²⁸⁹. The peroxide detection and cell death assays were carried out following the methods previously described²⁸⁹. In the confrontation experiments, we used several strains to co-cultivate with *P. anserina*. All the selected confrontation strains (*Mucor circinelloides*, *A. nidulans*, *P. chrysogenum*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Coprinus cinereus*, and *Trametes versicolor*) originate from our laboratory.

Given variations in the growth rates of each strain, we employed two distinct approaches for the confrontation experiments to monitor both cell death and peroxide production. Among

the other strains chosen for confrontation experiments, *M. circinelloides*, *A. nidulans*, *P. chrysogenum*, *A. fumigatus*, and *P. anserina* exhibited nearly identical growth rates. In contrast, the growth rates of *B. cinerea*, *C. cinereus*, and *T. versicolor* were significantly lower than that of *P. anserina*. The *B. cinerea*, *C. cinereus*, and *T. versicolor* strains were initially cultured at the center of M2 plates under optimal temperature conditions for 4 days. Subsequently, the *P. anserina* WT and mutant strains were placed around them. Simultaneously, the *M. circinelloides*, *A. nidulans*, *P. chrysogenum*, *A. fumigatus* strains were inoculated around the *P. anserina* WT and mutant strains on M2 plates and incubated at the optimal temperature for 3 days. Peroxide detection and cell death assays were conducted after an additional 3 days of incubation to allow the fungal thalli to reach the appropriate growth stage, ensuring contact between the central fungus and other fungal thalli.

2.3.7.1. Cell death assay

Cell death in the contact zone was evaluated using the Trypan blue exclusion method, wherein a 0.1% Trypan blue solution in distilled water (5 mL) was evenly spread across the confrontation plates using a glass rod to ensure uniform dye access to all cells²⁸⁹. The plates were incubated at room temperature for approximately 10 minutes and then thoroughly rinsed with distilled water before conducting observations and capturing images.

2.3.7.2. Peroxide detection

Thalli were incubated, and the plates were flooded with either 2.5 mM Nitro Blue Tetrazolium (NBT) in 5 mM (N-morpholino) propane sulfonate-NaOH at pH 7.6 to detect superoxide or 2.5 mM diaminobenzidine and 5 purpurogallin units ml⁻¹ of horseradish peroxidase in potassium phosphate buffer at pH 6.9 to detect peroxide. The solution was spread to ensure contact with all the hyphae. After 30 minutes, the supernatant was removed, and the

plates were incubated for several hours to overnight²⁸⁹. Review the experimental results the following day and promptly proceed with the photography.

2.4. Metabolomics approach

The approach outlined here utilized readily available and cost-effective spectroscopic identification techniques, including UV spectra, low-resolution mass spectrometry, and ¹H NMR data. Strains were cultivated in specifically chosen media to ensure the production of a substantial quantity of material suitable for isolating fractions enriched with secondary metabolites or pure compounds. The acquired data provided ample information for identifying the majority of the isolated metabolites as pure chemical entities.

2.4.1. Fungal growth conditions

A small piece of wild or mutant strains was collected in a 2 mL Eppendorf tube containing 500 µL of sterile water. Tubes were then placed in a Fast Prep (speed 5000 g, 5 seconds) to crush the mycelium. Each liquid fungal culture was prepared by mixing 1200 µL of crushed mycelium (from the wild type or the mutant strains) to 1200 mL of M2 liquid medium in a Roux flask. Typically, 12 Roux flasks were prepared for each experiment. Subsequently, the Roux flasks were carefully laid flat in the dark incubator for 12 days static cultivation at 27°C.

2.4.2. Fungal culture extraction

Initially, the culture medium and mycelia were collected by vigorously shaking the Roux flasks. Subsequently, the complete fungal cultures, comprising mycelia and media, were transferred to a blender, and crushed using Fastprep for 2 minutes. Subsequently, an equal volume of ethyl acetate (EtOAc) was added, and the aqueous phase was extracted under

ultrasonic irradiations for 30 minutes. This extraction step was repeated 3 times for the aqueous phase.

The organic phases were subsequently dried with MgSO_4 and filtered through Whatman paper. The resulting filtrate was evaporated until dry, the obtained compounds were weighed, recorded, and stored in the refrigerator for subsequent experiments.

2.4.3. TLC experiment

The SMs compounds concentrations in each strain were estimated by comparisons to standard spots on thin-layer chromatography (TLC) plates by dilution spotting. The dried extracts were resuspended in an adequate amount of methanol and separate in liquid on TLC plate (TLC Silica Gel 60 F254) using a sufficient volume of cyclohexane: EtOAc (2:1). Pictures of the TLC plate was taken under visible light, UV 254 nm, UV 366 nm and sulfuric vanillin respectively. All experiments were performed in triplicate.

2.4.4. HPLC-UV-MS analyses

The resulting residue was dissolved in 2 mL of methanol, filtered through a 0.2 μm filter, and subjected to reversed-phase high-performance liquid chromatography coupled with UV detection (HPLC-UV) analysis. The HPLC-UV analysis was carried out using Dionex UltiMate 3000 HPLC systems, which were equipped with an X-bridge C18 column (4.6 \times 250 mm, 5 μm , Waters, Ireland) and operated in positive-ion mode with an ESI source. The flow rate was set at 0.6 ml/min. Fresh extracts from all strains were subjected to analysis using a linear gradient ranging from 0% to 100% ACN in H_2O on an Accucore C18 column (100 \times 3 mm, 2.6 μm particle size) with deionized water (A) and acetonitrile (B) as the solvent system. The optimized gradient elution program was as follows: 0–5 min, 0% B; 5-35 min, 0-100% B; 35-

45 min, 100-100% B; 45-46 min, 100-0% B; 46-66 min, 0-0% B. The injection volume was 5 μ L and the flow rate was 0.6 mL/min. The wavelength was set at 200-800 nm.

2.4.5. NMR analyses

NMR spectra were recorded on a Bruker Avance 300-MHz, 400-MHz, and 600-MHz spectrometer at room temperature (Bruker Corporation, Karlsruhe, Germany). All spectra were processed with MestReNova 12.0 (Metrelab). Chemical shifts are referenced to those of the solvent signals.

2.4.6. HRMS

Modern high-resolution mass spectrometry (HRMS) instruments, operating in full-scan mode, capture mass-to-charge ratios (m/z) within predefined mass ranges using specified scanning frequencies and/or resolving power (RP) as per the instrument's specifications. HRMS, renowned for its superior mass resolution and precise mass measurement, greatly enhances the ability to unambiguously determine the elemental composition of known and novel components, often achieving accuracy within 5 ppm. This level of precision enables analysts to effectively distinguish target analytes from co-eluting isobaric compounds. To improve the precision of mass measurements, TOF and Q-TOF instruments frequently utilize a lock mass correction approach, which entails the continuous infusion of a user-selected reference compound (potentially a well-characterized polyphenol) and subsequent adjustment of experimental m/z values based on the reference compound's m/z value.

2.4.7. Preparative HPLC

The purification of the products was performed using a preparative HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-40 delivery system, Rheodyne manual injection valve, FRC-40 fraction collector and SPD-M40 detector connected to a SCL-40 control unit. Product was dissolved in 2mL of MeOH and purified on a Nucleodur C18 Htec (250 mm x 32 mm, 5 μ m) column (Macherey Nagel) with a gradient mobile phase 5 to 100% acetonitrile in water at a flow rate of 35 mL/min in 30 min. Fractions containing the products were pooled and the mobile phase was evaporated under reduced pressure. The refined gradient elution protocol proceeded as follows: 0–5 min, 5% acetonitrile; 5–35 min, 5–100% acetonitrile; 35–45 min, 100% acetonitrile.

Chapter 3

Global regulator PaNsdD

3.1. The publication about PaNsdD

The *NsdD* gene (never undergo sexual development) encodes a conserved fungal GATA-type zinc finger transcription factor critical for regulating sexual development in fungi. This DNA-binding domain is highly conserved among filamentous fungi but not in plants or animals²⁹⁰. *NsdD* was first recognized as a central promoter of sexual reproduction in *A. nidulans* (Eurotiales, Ascomycota) through the observation of complete infertility in the corresponding mutant²⁹²⁻²⁹⁴. *NsdD* governs genes associated with sexual structures and processes, initiating the shift from asexual to sexual development in response to environmental cues, with functions and targets differing among fungal species²⁹³⁻²⁹⁵. In this chapter, we investigate the role of *NsdD* in the model fungus *P. anserina*, which does not engage in asexual reproduction. This research findings were published in Applied and Environmental Microbiology and can be accessed at <https://journals.asm.org/doi/full/10.1128/aem.02378-21>. In this chapter, we conducted a functional investigation of the global regulator *PaNsdD* in *P. anserina*. I contributed with Ling Shen to the mutant construction and the phenotypical characterization presented in this paper.



Functional Characterization of the GATA-Type Transcription Factor PaNsdD in the Filamentous Fungus *Podospira anserina* and Its Interplay with the Sterigmatocystin Pathway

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Gwenaél Ruprich-Robert and Florence Chapeland-Leclerc contributed equally to this article. Author order was determined in order of decreasing seniority.

ABSTRACT The model ascomycete *Podospira anserina*, distinguished by its strict sexual development, is a prolific but yet unexploited reservoir of natural products. The GATA-type transcription factor NsdD has been characterized by the role in balancing asexual and sexual reproduction and governing secondary metabolism in filamentous fungi. In the present study, we functionally investigated the NsdD ortholog PaNsdD in *P. anserina*. Compared to the wild-type strain, vegetative growth, ageing processes, sexual reproduction, stress tolerance, and interspecific confrontations in the mutant were drastically impaired, owing to the loss of function of PaNsdD. In addition, the production of 3-acetyl-4-methylpyrrole, a new metabolite identified in *P. anserina* in this study, was significantly inhibited in the $\Delta PaNsdD$ mutant. We also demonstrated the interplay of PaNsdD with the sterigmatocystin biosynthetic gene pathway, especially as the deletion of *PaNsdD* triggered the enhanced red-pink pigment biosynthesis that occurs only in the presence of the core polyketide synthase-encoding gene *PaStcA* of the sterigmatocystin pathway. Taken together, these results contribute to a better understanding of the global regulation mediated by PaNsdD in *P. anserina*, especially with regard to its unexpected involvement in the fungal ageing process and its interplay with the sterigmatocystin pathway.

IMPORTANCE Fungal transcription factors play an essential role in coordinating multiple physiological processes. However, little is known about the functional characterization of transcription factors in the filamentous fungus *Podospira anserina*. In this study, a GATA-type regulator PaNsdD was investigated in *P. anserina*. The results showed that PaNsdD was a key factor that can control the fungal ageing process, vegetative growth, pigmentation, stress response, and interspecific confrontations and positively regulate the production of 3-acetyl-4-methylpyrrole. Meanwhile, a molecular interaction was implied between PaNsdD and the sterigmatocystin pathway. Overall, loss of function of PaNsdD seems to be highly disadvantageous for *P. anserina*, which relies on pure sexual reproduction in a limited life span. Therefore, PaNsdD is clearly indispensable for the survival and propagation of *P. anserina* in its complex ecological niches.

KEYWORDS transcription factor PaNsdD, sterigmatocystin, sexual development, 3-acetyl-4-methylpyrrole, *Podospira anserina*

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The model fungus *Podospora anserina*, recently described as *Triangularia anserina* (Sordariales, Ascomycota) (1), has long been used in the laboratory to study various biological processes, such as senescence, meiosis, prions, sexual development, signal transduction, cell fusion, hyphal interference, vegetative incompatibility, mitochondrial physiology, and plant biomass degradation (2–5). *P. anserina* is frequently recovered from herbivore dung and is therefore regarded as a typically coprophilous fungus (4, 6). Dung-inhabiting fungi are predominately known to produce various secondary metabolites that act as chemical weapons to enhance competitiveness and ecological fitness (7, 8). These bioactive products are of intense interest to humankind because of their potential pharmaceutical properties (9, 10). Notably, mining of the *P. anserina* genome revealed a large number of putative biosynthetic gene clusters (BGCs) for secondary metabolites (11). However, to date, only very few products that exhibited antibacterial, antifungal, and larvicidal activities have been characterized in this species during past decades (12–14), probably because most BGCs are silent under standard culture conditions (10, 15). Genomics-driven BGC detection and preliminary chemical investigations so far imply that *P. anserina* is a prolific, as-yet unexploited natural product reservoir.

In fungi, secondary metabolism is often governed by intricate regulatory mechanisms that also control other processes (e.g., asexual and sexual development) (16, 17). For example, the global transcription factor LaeA (loss of *glbR* expression) simultaneously regulates numerous metabolic BGCs and fungal morphogenesis in *Aspergillus* spp., as well as in *Fusarium verticillioides* (Hypocreales, Ascomycota), *Monascus pilosus* (Eurotiales, Ascomycota), and *Penicillium dipodomyis* (Eurotiales, Ascomycota) (18–22). As core regulatory elements, transcription factors indeed play an essential role in multilevel gene expression coordination (23). Our previous work characterized the mycotoxin sterigmatocystin (ST) pathway-specific transcription factor PaAfr, which mediates biological processes, including metabolite production, sexual development, pigmentation, and interspecific competition in *P. anserina* (14). Nevertheless, plenty of transcription factors in *P. anserina* still remain to be elucidated (24).

The *NsdD* gene (never undergo sexual development) encodes a GATA-type zinc finger transcription factor, with highly conserved DNA-binding domain in many filamentous fungi but not in plants or animals (25). *NsdD* was initially identified as a key activator of sexual reproduction in *Aspergillus nidulans* (Eurotiales, Ascomycota) due to the complete loss of fertility in the corresponding mutant (25). From then on, *NsdD* and its orthologs (SUB-1/Pro44/Lt1/Csm1/Nsd1) were characterized according to their involvements in biological processes in filamentous fungi. The roles of *NsdD* and its orthologs are summarized in Table 1. For example, *NsdD* affected morphogenesis and aflatoxin (AF) production in *Aspergillus flavus* (Eurotiales, Ascomycota) (26) and was identified as a repressor of asexual development and negatively affected ST production in *A. nidulans* (27). Deletion of *Pro44* caused developmental defects and sterility in *Sordaria macrospora* (Sordariales, Ascomycota) (28). Deletion of *Csm1* resulted in enhanced microconidia formation, reduced stress tolerance, and deregulated expression of BGCs in *Fusarium fujikurii* (Hypocreales, Ascomycota) (29). Loss of *SsNsd1* function affected morphological transition, appressoria formation, and pathogenicity in the plant pathogen *Sclerotinia sclerotiorum* (Helotiales, Ascomycota) (30). However, the role of *NsdD* in the model fungus *P. anserina*, which lacks asexual reproduction, has not been studied.

In the present study, we functionally investigated the global regulator PaNsdD in *P. anserina*. Our results revealed that PaNsdD was required for vegetative growth, sexual reproduction, stress tolerance, interspecific confrontation, and production of 3-acetyl-4-methylpyrrole, a new secondary metabolite identified in *P. anserina* in this study. We also demonstrated the interplay of PaNsdD with the ST pathway. Indeed, the deletion of *PaNsdD* triggers the enhanced red-pink pigment biosynthesis in the presence of the core polyketide synthase (PKS)-encoding gene *PaStcA* of the ST pathway.

RESULTS

Identification of PaNsdD and generation of mutants. In *P. anserina*, a total of 507 transcription factors classified into 41 families can be retrieved from the Fungal Transcription Factor Database (24). Among them, the *P. anserina* genome contains a putative *NsdD* ortholog, namely *PaNsdD* (*Pa_2_1880*). The complete CDS of *PaNsdD*

TABLE 1 Role of NsdD and orthologs in some filamentous fungi

Fungi	Protein	Role	Reference
<i>Aspergillus nidulans</i>	NsdD	Fertility and sterigmatocystin production	25, 27
<i>Neurospora crassa</i>	SUB-1	Perithecia production	56
<i>Aspergillus flavus</i>	NsdD	Morphogenesis and production of aflatoxin	26
<i>Sordaria macrospora</i>	Pro44	Developmental cycle and fertility	28
<i>Trichoderma reesei</i>	SUB1	Regulation in a nutrient- and light-dependent manner of female fertility, pheromone system, and secondary metabolism	36
<i>Botrytis cinerea</i>	BcLtf1	Regulation of light-dependent stress responses and expression of secondary metabolism-related genes	45
<i>Fusarium fujikuroi</i>	Csm1	Microconidia formation, stress tolerance, and expression of biosynthetic gene clusters	29
<i>Sclerotinia sclerotium</i>	SsNsd1	Morphological transition, appressoria formation, and pathogenicity	30
<i>Penicillium oxalicum</i>	PaxNsdD	Regulation of major genes involved in plant biomass degradation, conidiation, and pigment biosynthesis	37
<i>Metarhizium rileyi</i>	MtNsdD	Dimorphic transition and microsclerotia formation	46
<i>Podospira anserina</i>	PaNsdD	Vegetative growth, sexual reproduction, stress tolerance, interspecific confrontation, and production of 3-acetyl-4-methylpyrrole	This study

consists of 1,659 bp and potentially encodes a GATA-type transcription factor, namely PaNsdD, of 411 amino acids. PaNsdD shares 99.32, 57.43, and 56.57% identities with NsdD orthologs from *Podospira cornata*, *Neurospora crassa*, and *S. macrospora*, respectively. In order to study the biological functions of PaNsdD in *P. anserina*, a deletion mutant lacking *PaNsdD* (Δ PaNsdD) and complemented strain (Δ PaNsdD^C) were constructed, verified (Fig. S1), and subjected to further analysis.

Impact of PaNsdD on vegetative growth and colony morphology. To evaluate the impact of PaNsdD on vegetative growth and colony morphogenesis, wild-type and mutant strains were cultured on M2 solid medium for 12 days. In the first stage, the Δ PaNsdD mutant displayed a reduction in growth rate compared to the wild-type strain (Fig. 1). Moreover, the Δ PaNsdD strain formed a flat colony due to sparse aerial

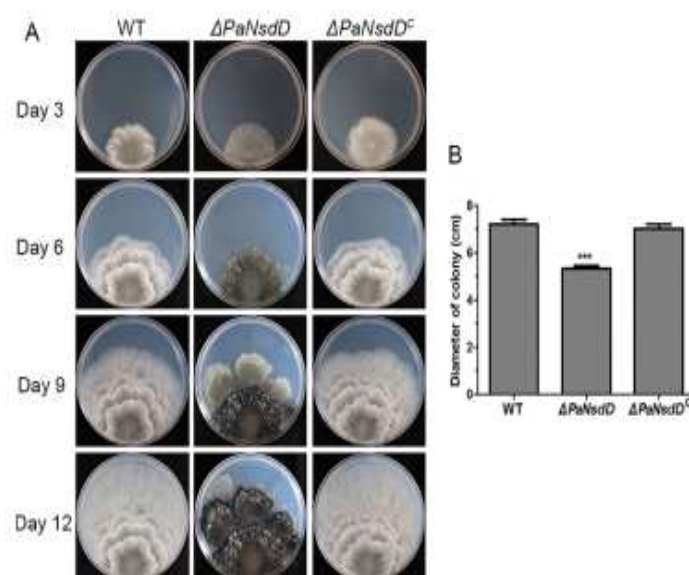


FIG 1 Colony morphology of *P. anserina* strains. (A) The wild-type (WT), Δ PaNsdD, and Δ PaNsdD^C strains were grown on M2 solid medium at 27°C under dark conditions. The pictures were taken after several days of incubation as indicated. Inoculum was positioned on the edge of the petri dish in order to observe the growth of the strains on a large surface, necessary for 12 days of growth. (B) Colony diameter of tested strains after 5 days of growth. As the inoculation point was made on the edge of the plate, the radius of the colony was measured, the diameter is then deduced by multiplying this length by two. The data represent the means \pm standard deviation (SD) from three independent experiments. Asterisks indicate significant differences ($P < 0.01$) of each strain relative to the WT, followed by Student's *t* test.

hyphae formation. Intriguingly, in the second stage from the 6th day to the 9th day, growth of $\Delta PaNs dD$ appeared to suddenly cease since culture began to slow down, and hyphal prolongation at the edge of the colony was clearly blocked to a large extent. Meanwhile, accelerated and excessive accumulation of pigment started to appear on the surface of colony in a short period, usually less than 3 days (Fig. 1). Nevertheless, the remaining hyphae that were not arrested in partial section could escape the front of growth and persist with outward extension. In the third stage from the 9th to the 12th day, active hyphae that went beyond the colony margin restarted a series of processes, including vegetative proliferation, cessation, and melanization (Fig. 1). It should be noted that more and more mycelia were gradually arrested during this periodic process; only some hyphae can avoid the melanized fate and continue their weak growth until final growth cessation. As shown in Fig. 1, deletion of *PaNs dD* eventually led to the irregular peripheral growth, accompanied by hyphal staling and enhanced pigmentation. In contrast, the $\Delta PaNs dD^c$ strain showed wild-type-like growth characteristics, such as normal growth rate, fluffy colony with extensive aerial hyphae, and normal pigmentation. These results indicated that *PaNs dD* was crucially required for the correct growth and morphogenesis in *P. anserina*; otherwise, a cycled stop-start growth pattern might be triggered, leading to an affected ageing process.

Role of *PaNs dD* in sexual reproduction. To study the role of *PaNs dD* in sexual development, we first examined the formation of reproductive organs through microscopic observation. Both opposite mating type strains (*mat+* and *mat-*) of *P. anserina* were able to differentiate male gametes (spermatia) and female gametes (ascogonia). Obviously, the $\Delta PaNs dD$ mutant exhibited enhanced production of spermatia on M2 solid medium following 3 days of cultivation (Fig. 2A). Subsequently, quantification analysis demonstrated that spermatia yield of $\Delta PaNs dD$ was significantly increased (4.5-fold, $P < 0.01$), compared with wild-type strain (Fig. 2B). The complemented strain restored normal spermatia production. Therefore, these data suggested *PaNs dD* functions as a negative regulator of spermatia differentiation in *P. anserina*.

In view of the aberrant formation of spermatia caused by *PaNs dD* deletion, we further determined the fertility of wild-type and mutant strains. As expected, the wild-type strain produced a ring of perithecia after a fertilization event. In contrast, loss of *PaNs dD* led to the radial growth, but no perithecia were produced even if it was cultured for up to 1 month. Genetically complemented strain regained the wild-type-like fertility (Fig. 2C). This result indicated that the lack of *PaNs dD* completely abolished sexual reproductive ability. Due to the sterile characteristic, we then dissected the potential defects that could be present in the process of gamete differentiation, fertilization, or perithecial maturation. We showed that the $\Delta PaNs dD$ mutant acts as a maternal partner that failed to be fertilized by the spermatia of the wild-type strain, which acts as paternal partner (Fig. 2D). It implies that the absence of *PaNs dD* triggered potential defects in ascogonia. We finally confirmed that the female sterile phenotype in $\Delta PaNs dD$ was not dependent on mating type by reciprocal crosses. Taken together, largely consistent with the initial nomenclature, the data above revealed that the *PaNs dD* mutant indeed never undergo sexual development, which may be mainly due to the overproduction of spermatia and the dysfunction of ascogonia.

Function of *PaNs dD* in multiple stress responses. To clarify the function of *PaNs dD* in response to various stressors, we determined the fungal growth on M2 plates supplemented with stress-inducing agents. Strikingly, compared to the wild-type strain, the $\Delta PaNs dD$ mutant showed significantly reduced tolerance to different oxidative stressors, as H_2O_2 (500 μM), menadione (Mena, 25 μM), and *t*-butyl hydroxylperoxide (TBY, 50 μM) (Fig. 3). Especially, the growth of $\Delta PaNs dD$ was almost completely inhibited in the presence of methylglyoxal (MG) at 5 mM, which can induce reactive oxygen species (ROS) production by depleting intracellular glutathione (31). Moreover, growth of $\Delta PaNs dD$ was highly affected in the presence of sorbitol at 1.5 M. Additionally, to a different extent, growth of mutants was also inhibited under cell wall stress conditions triggered by exposure to Congo red (CR, 100 μM) and Calcofluor white (CFW 10 μM), which impair assembly of β -1,4-glucans and chitin in the cell wall,

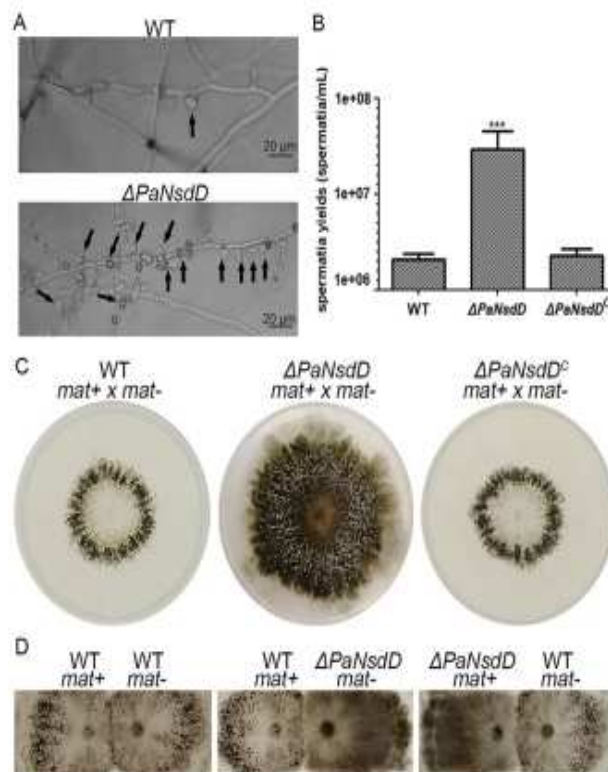


FIG 2 Sexual development of *P. anserina* strains. (A) Microscopic observation of spermatia formation. The pictures were taken after 3 days of growth on M2 plate. The arrows indicate typical spermatia. (B) Quantification analysis of spermatia. The tested strains were cultured on M2 medium for 3 days and then used for the counting and observation of spermatia. The data represent the means \pm SD from three independent experiments. Asterisks indicate significant differences ($P < 0.01$) of each strain relative to the WT, followed by Student's *t* test. (C) Fertility assays. Fragmented mycelia from opposite mating type strains were mixed and then inoculated in the center of plate. The plates were grown at 27°C under constant illumination. The photos were taken after 8 days of incubation. (D) Spreading experiments. Crosses were made by inoculating both strains 1.5 cm apart. After 3 days of incubation, 1.5 mL of water was added and spread all over the plate. Mature perithecia were visible as small dots when the fertilization event was completed.

respectively (Fig. 3). Taken together, increased sensitivity of $\Delta PaNsdD$ to various stressors indicated that PaNsdD was required for the maintenance of stress tolerance and cell wall integrity in *P. anserina*.

Contribution of PaNsdD in interspecific confrontations. To elucidate the contribution of PaNsdD in interspecific interactions, the $\Delta PaNsdD$ mutant and the wild-type strain were coconfronted with three other fungal challengers, as previously described (14, 32). In the cell death assay, we found that *Penicillium chrysogenum* (Eurotiales, Ascomycota) and *Botrytis cinerea* that have been described to be usually killed by *P. anserina* displayed a lower level of cell death in the contact zone when confronted to $\Delta PaNsdD$ compared to wild type (Fig. 4A). In contrast, *Trametes versicolor* (Polyporales, Basidiomycota), which usually can kill *P. anserina* (14, 32), aggravated cell death specifically on the thallus of $\Delta PaNsdD$, compared to confrontation with wild type. These results implied that loss of PaNsdD triggered the declining competitiveness of *P. anserina* during interspecific antagonistic processes. Then, we detected the level of peroxide, which is usually restricted to the contact zone, and superoxide, that is accumulated more or less uniformly on fungal thallus, without any necessary contact with the competitor (32). No obvious difference in peroxide accumulation is observed between wild type and $\Delta PaNsdD$ at the confrontation with contestant strains (Fig. 4B). Moreover,

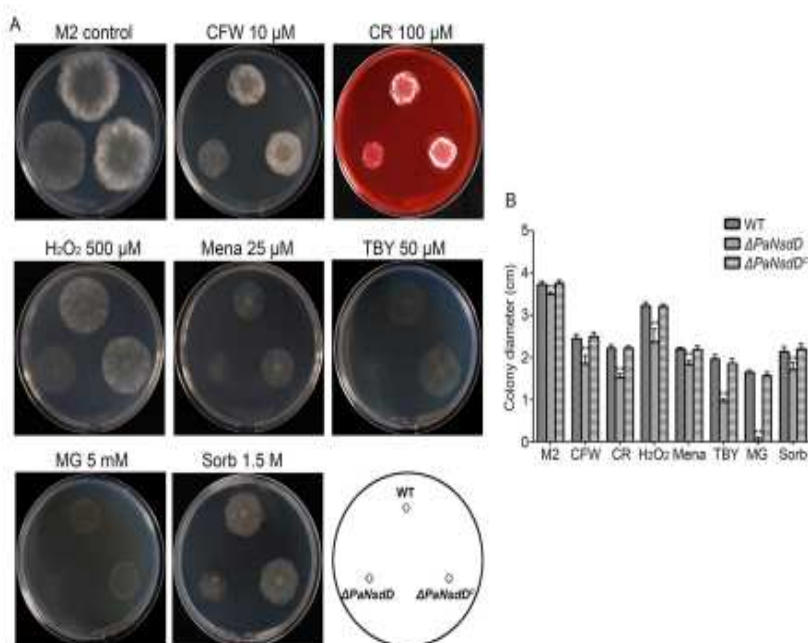


FIG 3 Growth of *P. anserina* strains on M2 plates exposed to abiotic stressors. The tested strains were grown on M2 medium supplemented with different stress agents under certain concentrations as indicated. (A) Colony morphology in the presence of stress-inducing agents. The pictures were taken after 3 days of incubation at 27°C. (B) Colony diameter (cm) of tested strains after 3 days of incubation at 27°C. The corresponding strains that grown on M2 medium without supplements were used for normalization. CFW, Calcofluor white; CR, Congo red; Mena, menadione; TBY, t-butyl hydroxyperoxide; MG, methylglyoxal; Sorb, sorbitol.

peroxide accumulation was not detected on wild-type and mutant thalli, in the absence of competitor (Fig. S2). Finally, nitroblue tetrazolium (NBT) detection assay revealed that $\Delta PaNsdD$ produced a lower level of superoxide over the thallus than that of the wild-type strain (Fig. 4C), indicating that PaNsdD was probably involved in the production of ROS, mainly superoxide. Combined with the decreased tolerance of $\Delta PaNsdD$ to abiotic stresses, we suggested that PaNsdD might contribute to a positive role in protecting *P. anserina* from hostile environment by mediating ROS production.

Effects on fungal pigmentation and interplay of PaNsdD with the ST pathway.

To investigate the impact of PaNsdD on fungal pigmentation, all of the strains were cultured on M2 and LB media, liquid and solid in both cases. As shown in Fig. 5, *P. anserina* wild-type strain was normally greenish on M2 medium, due to accumulation of melanin, and presented a slight red-pink pigmentation in M2 liquid medium and on LB plate. Nevertheless, we first observed that the coloration is more intense under all conditions with $\Delta PaNsdD$ compared to wild type, suggesting an excessive accumulation of pigments in this mutant. Then, one might expect that PaNsdD could downregulate the pigment production or affect its accumulation and could be involved in the melanin biosynthesis in particular. It should be noted here that when strains were grown on G medium and G + yeast extract medium (culture media usually used to promote ascospore germination), the mycelium remained everywhere very poorly pigmented, and the culture medium remained unstained (Fig. S3). Taken together, all these results are in accordance with the fact that fungal coloration highly depends on the medium composition (5).

Nevertheless, in a previous work, we observed (i) that the absence of the red-pink pigmentation when the $\Delta PaStcA$ mutant lacking the ST-core polyketide synthase PaStcA was cultured on M2 and (ii) the accumulation of red-pink pigments in the ST-overproducing strain OE-PaAfr (14), which seem to be identical to those produced by

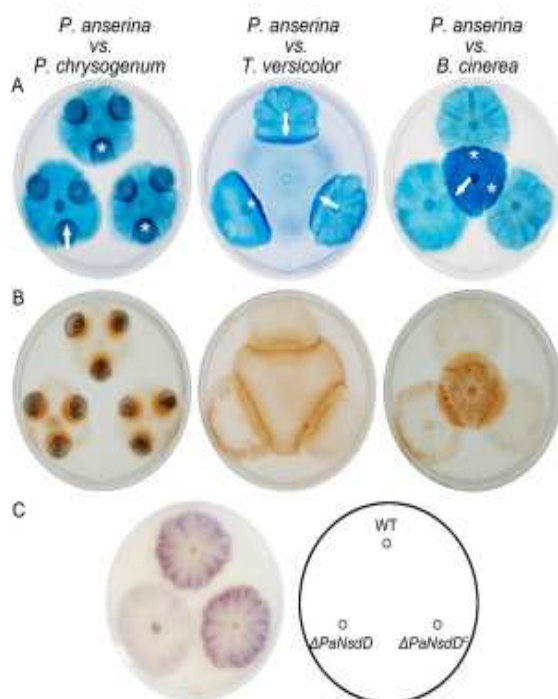


FIG 4 Confrontation of *P. anserina* strains against other fungal species. Cell death assay (A), peroxide detection (B), and superoxide detection (C) were conducted as previously described (14, 32). (A) In the contact zone, the intensity difference of blue color indicates the Trypan blue-stained dead cells triggered by the contestant strains. Asterisks indicate more serious cell death than that indicated by arrows. (B) Peroxide detection of *P. anserina* strains when contestants were involved, (C) Superoxide detection in WT, $\Delta PaNsdD$, and $\Delta PaNsdD^2$ in the absence of challengers.

the $\Delta PaNsdD$ strain. Similar results were obtained here on M2 but also on LB medium (Fig. 5B to D). In order to better understand the putative interplay of PaNsdD with the ST pathway and the occurrence of a similar red-pink pigmentation phenotype, the double mutant $\Delta PaNsdD\Delta PaStcA$ was constructed.

This mutant exhibited a colorless colony phenotype identical to $\Delta PaStcA$ on M2 liquid and LB media (Fig. 5B to D). Therefore, these results suggested that the presence of PaStcA was a prerequisite for the biosynthesis of red-pink pigments and that this gene interplays with pigment production. Our findings here are consistent with those previously described; i.e., that such a red-pink pigmentation requires the presence of functional ST pathway (14). It is worth noting that cultures of $\Delta PaStcA$ were always colorless even when they were subjected to 1 month of incubation under the same conditions (data not shown).

Involvement of PaNsdD in the biosynthesis of a new metabolite in *P. anserina*.

To examine secondary metabolites regulated by the global transcription factor PaNsdD in *P. anserina*, we first conducted a qualitative and semiquantitative analysis by high-performance liquid chromatography UV (HPLC-UV) analysis in all extracts after 14-day cultivation, as previously described (14). We found that the secondary metabolite profile was significantly altered owing to the PaNsdD mutation. First, under M2 liquid condition, the profile of wild-type strain evidenced two major peaks at 16.05 and 27.3 min, respectively (Fig. 6). The second peak at 27.3 min could unambiguously be correlated with ST, as previously seen (14). We may note that the HPLC-UV profiling on LB culture extracts showed only one peak, clearly correlated with ST, in wild-type and $\Delta PaNsdD$ strains, meaning that the second compound is absent under these culture conditions (Fig. S4).

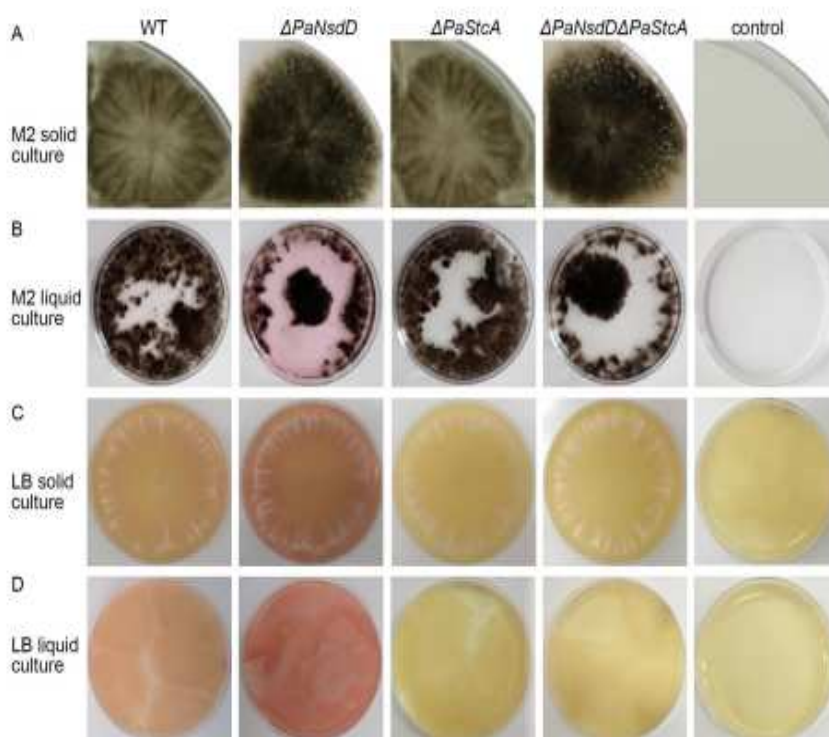


FIG 5 Pigmentation of *P. anserina* on various media. Tested strains were cultured on M2 solid medium (A), M2 liquid medium (B), LB solid medium (C) and LB liquid medium (D), respectively. After 14 days of growth at 27°C, altered pigmentation were clearly observed, and photos were therefore taken.

In order to identify the compound corresponding to the peak at 16.05 min, it was necessary to proceed to its isolation by silica chromatography and preparative HPLC successively. Characterization of this isolated product was conducted by liquid chromatography-mass spectrometry (LC-MS) in electron spray ionization (ESI)-positive and -negative modes. Our results showed that the corresponding signals of this product are two ions at m/z 124 for $[M+H]^+$ and m/z 122 for $[M-H]^-$, respectively (Fig. S5), indicating that the molecular formula of this compound could be C_7H_8NO . 1H and ^{13}C nuclear magnetic resonance (NMR) along with 2D spectra (Fig. S6) allowed identification of 3-acetyl-4-methylpyrrole for the compound at 16.05 min. All of these data are in accordance with the literature (33–35). Thus, deletion of *PaNsdD* in both wild-type and $\Delta PaStcA$ background almost completely blocked the production of 3-acetyl-4-methylpyrrole (at 16.05 min), implying that *PaNsdD* positively regulates its biosynthesis. In contrast, the amount of ST, determined by the UV signal intensity (mAU), was similar in both wild type and $\Delta PaNsdD$, indicating that the production of ST was not affected by the deletion of *PaNsdD* (Fig. 6).

DISCUSSION

***PaNsdD* is an activator of sexual reproduction and a repressor of male gamete production in *P. anserina*.** Given the critical role of *NsdD* in balancing sexual and asexual development in several fungi (29, 30, 36–38), we wondered about the function of *PaNsdD* in *P. anserina*, which has only a pure sexual life cycle without any asexual phase (4). Our data suggested that *PaNsdD* is a positive regulator of sexual reproduction and a repressor of male gametes production in *P. anserina*. Unlike other asexual spores, spermatia produced by *P. anserina* were only used to fertilize ascogonia and had almost no germination (38). Here, we show that the erected sterile hyphae of

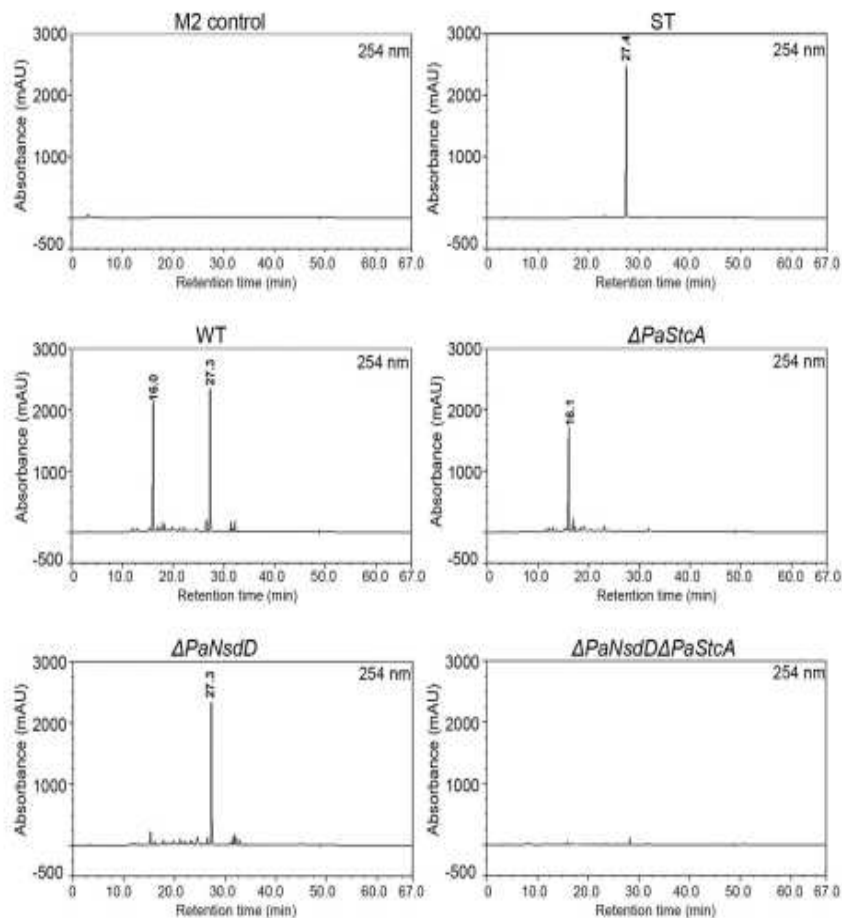


FIG 6 High-performance liquid chromatography UV (HPLC-UV) detection of culture extracts of *P. anserina*. The HPLC-UV profiling displayed the peak intensity (mAU) according to the retention time (rt). Sterigmatocystin (ST) presents a rt of 27.3 min (14). The other major peak presented a rt of 16.05 min. The x axis (min) shows the retention time. The y axis (mAU) shows the signal intensity.

$\Delta PaNsdD$ mutant was able to form anamorphic-like structures containing dozens of spermatia, which strikingly resemble those of *Cladorrhinum/Bahupaathra* species (5).

PaNsdD is involved in the vegetative growth pattern of *P. anserina*. Remarkably, $\Delta PaNsdD$ mutant was distinguished by an unusual growth characteristic that involved an alternation of irregular periods of growth and nongrowth on M2 solid medium. In this study, we presented the infrequent alternative growth pattern in *P. anserina*, however, that resembled the ones observed in *Neurospora* and *Aspergillus* species. For instance, unlike the continuous outward extension of hyphae in the wild-type strain, the "stopper" mutants of *N. crassa* had an aberrant phenotype in which growth of the mycelium started, stopped, and then started again (39). The "vegetative death" mutants of *Aspergillus glaucus* (Eurotiales, Ascomycota) showed many features of senescence similar to those described for *P. anserina*. However, a partial section of *A. glaucus* mutant culture sometimes can escape the dying front of mycelia and continue the patchy extension until ultimate growth cessation (40). Taken together, compared to the wild-type strain of *P. anserina*, the $\Delta PaNsdD$ mutant showed persistent cycles of stopping and impaired starting, with a concomitant excessive accumulation of pigment, leading to a clear disturbance in the ageing process of the thallus. As we know, the ageing model *P. anserina* clearly did not propagate indefinitely and underwent

senescence during its limited vegetative growth period (4, 41). In *P. anserina*, strain-specific senescent phenotypes were characterized by some common features, such as darker pigmentation, growth stop at the hyphal tips, and formation of "barrier of senescence" (5). Noticeably, visible morphological changes in $\Delta PaNsdD$ mutant, such as sparse aerial hyphae, overproduction of dark pigment, accelerated cessation of hyphal growth, rapid transition from juvenile phase to senescent phase, and ragged elongation of partial peripheral hyphae, indicated the premature senescence syndrome caused by *PaNsdD* mutation.

PaNsdD is required for fungal pigmentation and stress tolerance. Pigmentation is generally considered the output of secondary protective mechanisms that protect the producer from adverse environmental stresses, including oxidative stress, light, UV radiation, and temperature variation (42–44). Moreover, given the essential role of NsdD and its orthologs in maintaining the redox homeostasis in other fungi (29, 45), we hypothesized that the overpigmented $\Delta PaNsdD$ mutant would respond to multiple stressors. However, we found that $\Delta PaNsdD$ mutant was hypersensitive in the presence of various oxidant and osmotic stressors, suggesting that here, enhanced accumulation of pigment did not confer a protective function onto *P. anserina*. Moreover, a likely scenario could be that defects in cell morphogenesis might lead to internal stress signals caused by aberrant cell wall formation, which in turn could be the reason for the observed sensitivity toward several stresses or the enhanced melanization. Our findings were in accordance with the functions of BcLtf1 in *B. cinerea* and MrNsdD in *Metarhizium reileyi* in mediating ROS homeostasis and stress response (45, 46).

PaNsdD affects secondary metabolism in *P. anserina*. Apart from the altered growth pattern and differentiation affected by the loss of function of PaNsdD, pigment variation of mutant cultures in both solid and liquid medium indicated that PaNsdD played a vital role in governing some secondary metabolism pathways in *P. anserina*. Indeed, previous studies revealed that NsdD and its orthologs function as global regulators in secondary metabolism processes in several fungi, and in particular, NsdD influenced ST biosynthesis in *A. nidulans* (27). In this study, we have highlighted a dark green pigment and a red-pink pigment that are overproduced in $\Delta PaNsdD$. As the DHN-melanin has been described to be the major pigment that contributes to the greenish colony morphology of *P. anserina* in standard conditions (47), we hypothesize here that the dark green pigment is linked to the biosynthesis of melanin, but further experiments are necessary to clarify this point. Intriguingly, deletion of *PaNsdD* in both wild-type and $\Delta PaStcA$ backgrounds triggered a very dark pigmentation of the whole thalli, which is probably also linked to an excessive accumulation of melanin. We also found that the single mutant $\Delta PaStcA$ and the double mutant $\Delta PaNsdD\Delta PaStcA$ lost their abilities to produce the red-pink pigment, which is visible in both wild-type and $\Delta PaNsdD$ strains on M2 liquid medium and on LB plates, implying that the $\Delta PaStcA$ phenotype predominated in the double mutant under these conditions. In contrast, it seems that growth of *P. anserina* on LB medium was not able to induce fungal melanin production but led to the formation of a red-pink pigment, indicating that at least two pigment biosynthetic pathways exist in *P. anserina*. We do not know yet which chemical compound is responsible for the pigmentation, but we have demonstrated here that the presence of PaStcA was a prerequisite for the biosynthesis of this red-pink pigment and that PaNsdD negatively regulated the two pigmentation pathways.

Finally, secondary metabolites may serve as defense mechanisms during fungal colonization and the adaptation processes (48, 49). The interspecific competitiveness of $\Delta PaNsdD$ was suppressed when confronted with other fungal antagonists. Accordingly, in addition to the reduction of ROS production, the role of the new compound, identified here as 3-acetyl-4-methylpyrrole, needs to be further investigated. To this day, this compound was identified only in *Eupenicillium hirayamae* (Eurotiales, Ascomycota) under the name of desoxyverrucarin E (35) and in *Aspergillus terreus* (Eurotiales, Ascomycota) (34), but its involvement in fungal physiology has not yet been investigated.

To conclude, in this study, we demonstrated that deletion of *PaNsdD* led to a huge change in vegetative growth, pigmentation, stress response, and secondary metabolism

regulation and that a molecular interaction was observed between PaNsdD and PaStcA. Overall, the loss of PaNsdD leads to premature ageing, sterility, and sensitivity to environmental stresses. Such features seemed to be highly disadvantageous for this species whose propagation before senescence and death exclusively relies on sexual reproduction. The presence of PaNsdD is clearly indispensable for the survival and propagation of *P. anserina* in its complex ecological niches.

MATERIALS AND METHODS

Strains and culture conditions. The *P. anserina* strains used in this study were derived from the “S” (big S) wild-type strain, which was used for the sequencing of the *P. anserina* genome (2, 11, 50). The two culture media used for this study are the most commonly used M2 medium (0.25 g/L KH_2PO_4 , 0.3 g/L K_2HPO_4 , 0.25 g/L MgSO_4 , 0.5 g/L urea, 0.05 mg/L thiamine, 0.05 $\mu\text{g/L}$ biotine, 5 mg/L citric acid, 5 mg/L ZnSO_4 , 0.25 mg/L CuSO_4 , 50 $\mu\text{g/L}$ MnSO_4 , 50 $\mu\text{g/L}$ boric acid, 50 $\mu\text{g/L}$ sodium molybdate, 1 mg/L iron alum, 5.5 g/L dextrin, and 10 g/L agar) with pH maintenance at 7 by a phosphate buffer and the G (germination) medium (25 g/L corn flour, 25 g/L corn cream, 6 g/L ammonium acetate, and 12g/L agar).

All the protocols, including standard culture conditions and genetic manipulation for this microorganism, are described by Silar (4) and can be accessed at <http://podospora.i2bc.paris-saclay.fr>. The $\Delta\text{mus51}::\text{nouR}$ strain lacking a *mus51* gene was used for deletion mutant construction, which resulted in an increased frequency of targeted gene replacement (51). The $\Delta\text{PaStcA}::\text{genetR}$ strain harboring a mutation in the PKS-encoding gene *PaStcA*, which acts at the first step of ST biosynthesis, was previously constructed by Shen et al. (14).

Deletion and complementation of PaNsdD. The *PaNsdD* (*Pa_2_1880*) gene sequence was retrieved from the *P. anserina* genome database by BLAST, using the *N. crassa* protein SUB-1 (NCU01154) as query (11). To investigate the function of PaNsdD, targeted gene deletion was carried out according to the “split marker” method, as previously described (4, 52). Briefly, a phleomycin resistance cassette was used as selective marker. The flanking regions (approximately 800 bp) of the open reading frame of *PaNsdD* were amplified and then were fused with the resistance cassette, respectively. The fusion products were then used to transform protoplasts of the $\Delta\text{mus51}::\text{nouR}$ strain. All transformants were screened on minimal medium containing phleomycin at 10 $\mu\text{g/mL}$. Putative transformants were then diagnosed by PCR. The primers used in this study are listed in Table 2. To eliminate the untransformed nuclei and to segregate the Δmus51 mutation, two primary transformants $\Delta\text{PaNsdD}\Delta\text{mus51}$ (PhleoR HygroR) were genetically purified by crossing with the wild-type strain to generate both *mat+* and *mat-* homokaryotic mutant strains ΔPaNsdD (PhleoR HygroS) that contain only the *PaNsdD* deletion but lack Δmus51 . Correct gene replacement was further confirmed by Southern blotting (Fig. S1). The ΔPaNsdD mutant was genetically crossed with ΔPaStcA to generate the double mutant $\Delta\text{PaNsdD}\Delta\text{PaStcA}$, which was resistant to both phleomycin and Geneticin.

Complemented strains were generated by ectopically introducing a wild-type allele of *PaNsdD* into corresponding mutant, as described by Shen et al. (14). To obtain the plasmid pBC-hygro-PaNsd-Comp, a wild-type *PaNsdD* gene containing native promoter and terminator was amplified with primers PaNsdD_cF and PaNsdD_cR (Table S1). Fragment was inserted into EcoRV-linearized pBC-hygro plasmid. The reconstructed vector was validated by sequencing (Genewiz, Germany) and then was transformed into the ΔPaNsdD mutant strain. All hygromycin-resistant transformants were verified by PCR and phenotypic analysis; three out of putative transformants that restored the wild-type-like phenotype were genetically purified, resulting in the complemented strain ΔPaNsdD^c , which was selected for detailed phenotypic characterization.

Phenotypic analysis. To determine the role of PaNsdD in fungal physiology, wild-type and mutant strains were cultured on M2 medium at 27°C. Aliquots (5 μL) of standardized fragmented mycelial suspension were spotted on the plates. For vegetative growth observation, colony morphology, size, and pigmentation were documented each day. For fertility assessment, perithecia formation, ascospore production, and dispersal were examined during their sexual cycle. In order to observe the fungal pigmentation of thallus and the coloration of culture filtrate, M2 and LB liquid cultivation were also similarly conducted. All experiments were performed in triplicate.

Microscopy analysis and quantitative measurement of spermatia. Microscopic observation was made on fresh mycelia growing on solid M2 medium. Quantitative measurement of spermatia was conducted as described previously (53). Briefly, the strains were cultured for 3 days, and then the spermatia were harvested by flooding the plates with 1.5 mL of 0.05% Tween 20 in sterile water. The suspension was counted through a hemacytometer.

Sensitivity to various abiotic stresses. To assess the sensitivity of fungal cells to environmental stresses, fungal growth was determined on M2 medium supplemented with various chemical stressors at a certain concentration: (i) H_2O_2 (500 μM), menadione (Mena, 25 μM), *t*-butyl hydroperoxide (TBY, 50 μM), or methylglyoxal (MG, 5 mM) for oxidative stresses; (ii) Sorbitol (1.5 M) for osmotic stress, and (iii) Congo red (CR, 100 μM) or Calcofluor white (CFW, 10 μM) for cell wall-perturbing stresses. Aliquots (5 μL) of standardized fragmented mycelial suspension were spotted on the plates. Colony diameters were measured daily from three replicates. Quantification of relative growth inhibition of strains exposed to different chemicals was calculated as D_t/D_0 , (where D_t is diameter of control colony without treatment, and D_0 is the diameter of colony treated with stressors).

Interspecific confrontation assay. Detection of cell death and oxidative burst were conducted as previously described (14, 32). Briefly, *P. anserina* wild-type and mutant strains were inoculated on M2 plates with three neighboring *P. chrysogenum* strains and then incubated for 3 days. *T. versicolor* and *B. cinerea* strains were centrally precultured on M2 plates for 4 days, prior to the inoculation of *P. anserina* wild-type and mutant strains. Cocultivation can be ceased until the central fungus was completely in

TABLE 2 Primers used in this study

Primers	Sequence (5' to 3')	Application
PaNsdD_1F	tccagattggcaggacagtg	Deletion of <i>Pa_2_1880</i>
PaNsdD_2R	ctatttaacgacctgcctgaacgggactgggaccgtttttgg	
PaNsdD_2F	ccaaaaacgggtcccaagtcctgggtcagggcagggtcgttaaatag	
PaNsdD_3R	ggtaactactagctccggctccatcgaactggatctcaacagcggtaag	
PaNsdD_3F	cttaccgctgttgagatccagttcgatgggacggagctagattgacc	Junction verification for <i>Pa_2_1880</i> deletion
PaNsdD_4R	actaaagggtcccaacttgc	
PaNsdD_verify_F	cgaattgttccgccttttcg	
Valid5'	tgagaagcacacgggtcac	
Valid3'	tcggggcgaaaactctc	Complementation construct of <i>Pa_2_1880</i>
PaNsdD_verify_R	tgaatccgagcctctgttttg	
PaNsdD_cF	attggcaggacagtgacatc	
PaNsdD_cR	gtctttcaggcgagaacagc	
PaNsdD_verify_cF	ccagtgccctcaacaatgcc	Verification for <i>Pa_2_1880</i> complementation
PaNsdD_verify_cR	gctgttttctcaccatacg	

contact with competing fungal thalli (14). Trypan blue staining was used to reveal the cell death in the contact zone (32). Diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining were used to detect peroxide and superoxide, respectively (54, 55).

Metabolite extraction and analysis. To monitor the production of putative compounds regulated by PaNsdD, *P. anserina* wild-type and mutant strains were stationary cultured on M2 and LB liquid medium at 27°C for 14 days. Secondary metabolite extraction was conducted as described previously (14). The extracts were dissolved in 2 mL of methanol, filtered over a 0.2- μ m filter, and subjected to reversed-phase high performance liquid chromatography coupled to a diode-array detector (DAD) analysis. HPLC analysis was performed on Dionex UltiMate 3000 HPLC Systems using a column (X-bridge C18, 4.6 \times 250 mm, 5 μ m, Waters, Ireland) with a flow rate of 1 mL/min. Fresh extracts of all strains were detected with a gradient of acetonitrile in H₂O: 0% for 5 min, 0 to 100% in 35 min, and 100% for 10 min.

In order to identify peaks detected on HPLC-UV chromatogram, ST standard previously isolated was used (14). Concerning the peak at 16.05 min, which disappeared in the Δ PaNsdD strain, its purification was conducted as follows. The extract (125 mg) was purified by chromatography on silica (20 to 45 μ m) with a gradient of ethyl acetate in cyclohexane (0 to 100%) to give a fraction of 4.5 mg containing the unknown compound. A subsequent purification was made on a preparative HPLC system (Shimadzu, Kyoto, Japan) consisting of an LC-40 delivery system, Rheodyne manual injection valve, FRC-40 fraction collector, and SPD-M40 detector connected to a SCL-40 control unit. The fraction was dissolved in 0.5 mL of MeOH and purified on a Nucleodur C18 Htec (250 mm \times 32 mm, 5 μ m) column (Macherey-Nagel) with a gradient of acetonitrile in water (10 to 80% for 30 min) at a flow rate of 45 mL/min. Fractions containing the products were pooled, and the mobile phase was evaporated under reduced pressure to furnish 1.9 mg of the desired pure compound. NMR and MS data of this metabolite were in accordance with the literature (33, 34) and were permitted to identify the product as 3-acetyl-4-methylpyrrole.

Data availability. All of the data generated in this study are included in the main text and its supplementary information files.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.3 MB.

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We declare no conflict of interest.

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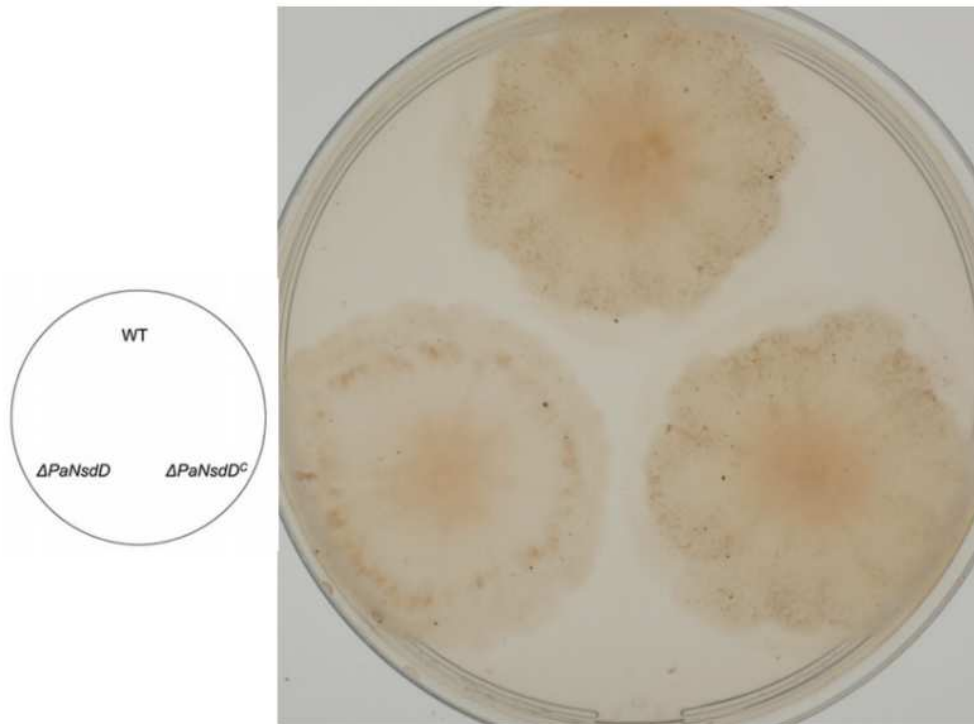
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3.2. Supplementary data

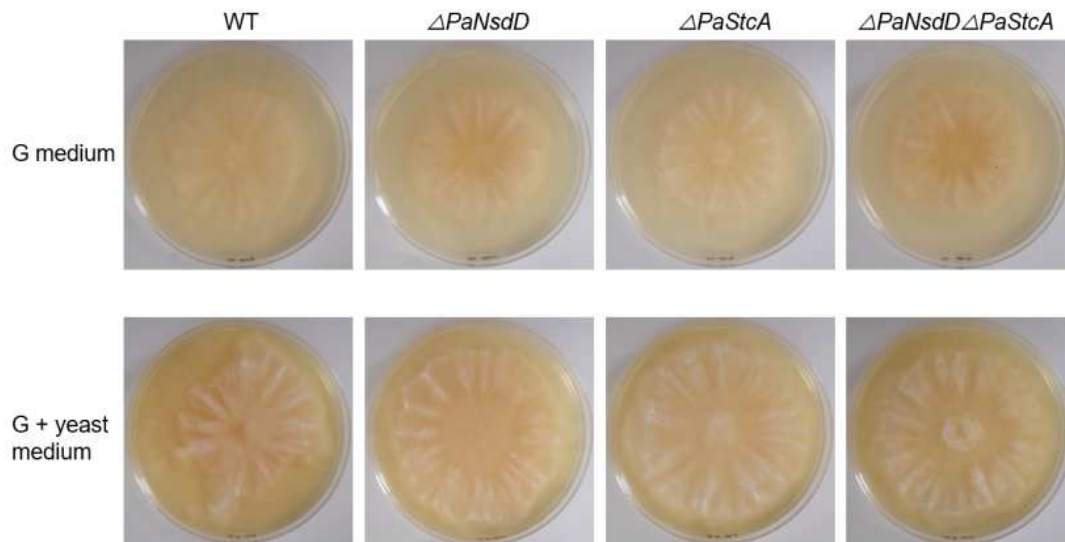
3.2.1. Peroxide detection of *P. anserina* strains when contestants are absents

Detection of oxidative burst were conducted as described in experimental procedures. *P. anserina* WT and mutant strains were inoculated on M2 plates and then incubated for 3 days. Diaminobenzidine (DAB) staining was used to detect peroxide respectively.



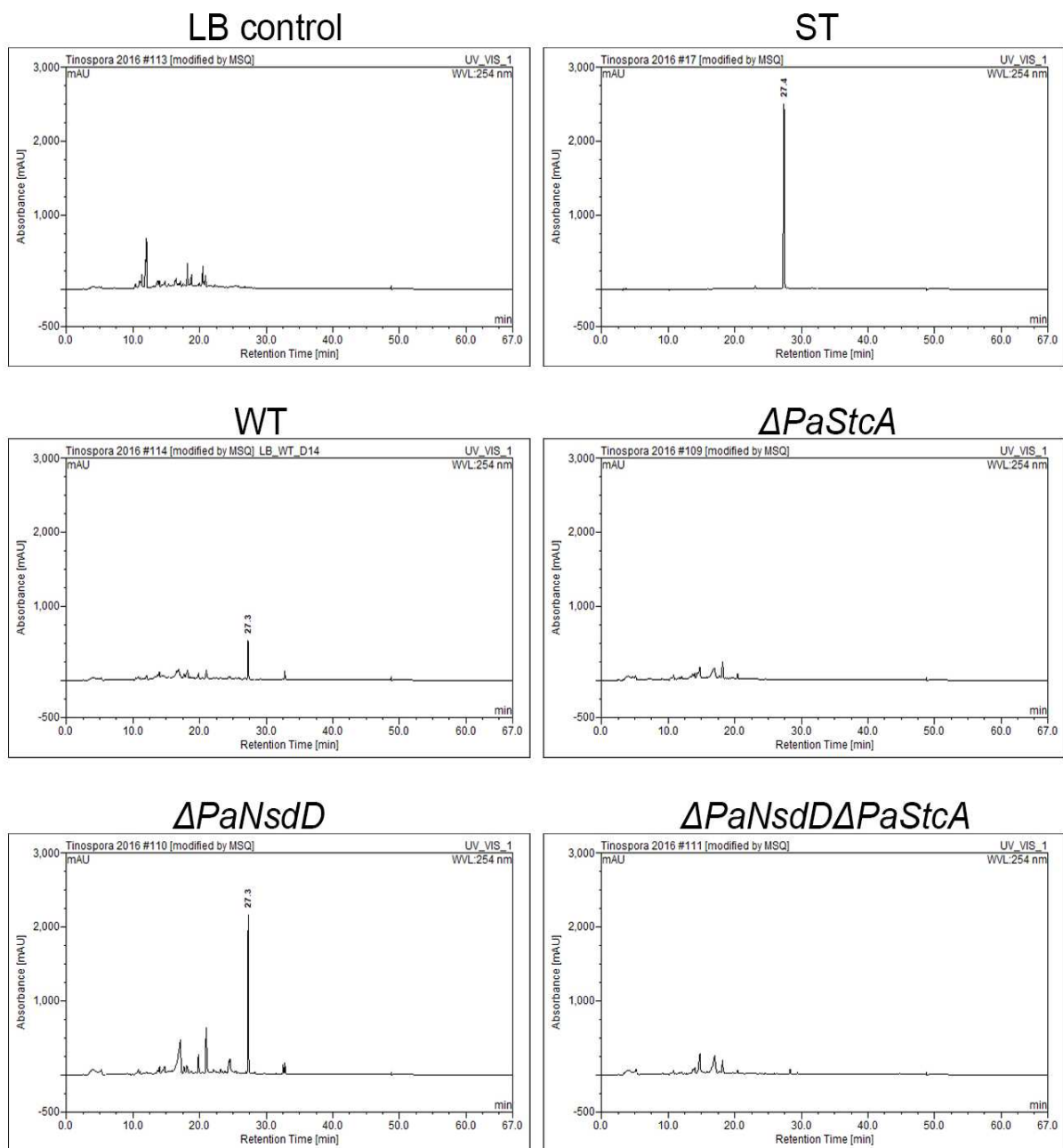
3.2.2. Growth of *P. anserina* strains on G medium and G+yeast medium

The WT, $\Delta PaNsdD$, $\Delta PaStcA$ and $\Delta PaNsdD/\Delta PaStcA$ strains were grown on G and G+yeast solid medium at 27 °C under dark condition. Pictures were taken after eight days of incubation as indicated. WT: wild-type strain.



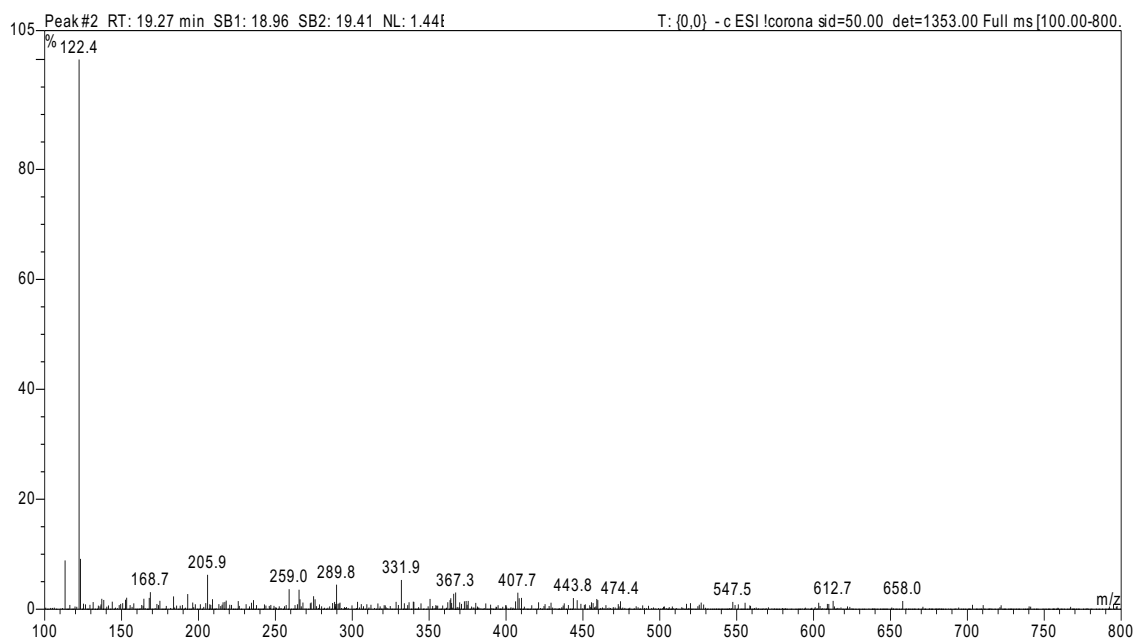
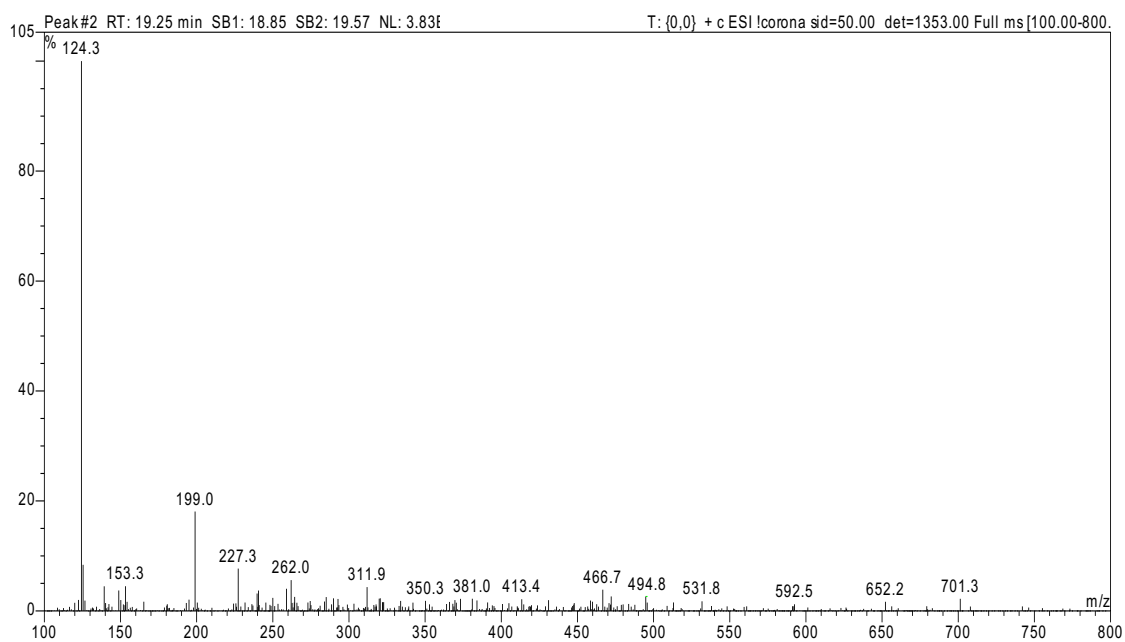
3.2.3. HPLC-UV detection of LB culture extracts of *P. anserina*

The HPLC-UV profiling displayed the peak intensity (mAU) according to the retention time (rt). ST presented a rt of 27.3 min. WT: wild type strain. X-axis (min): retention time. Y-axis (mAU): signal intensity.



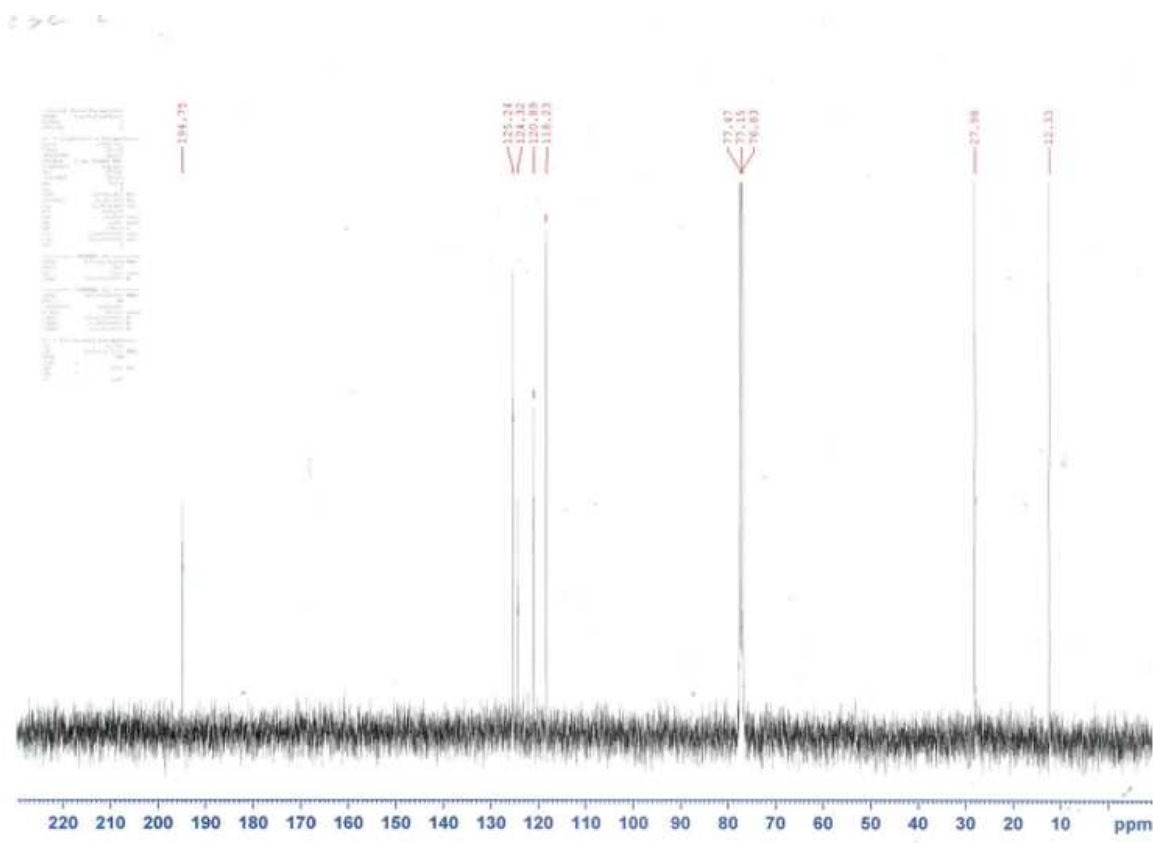
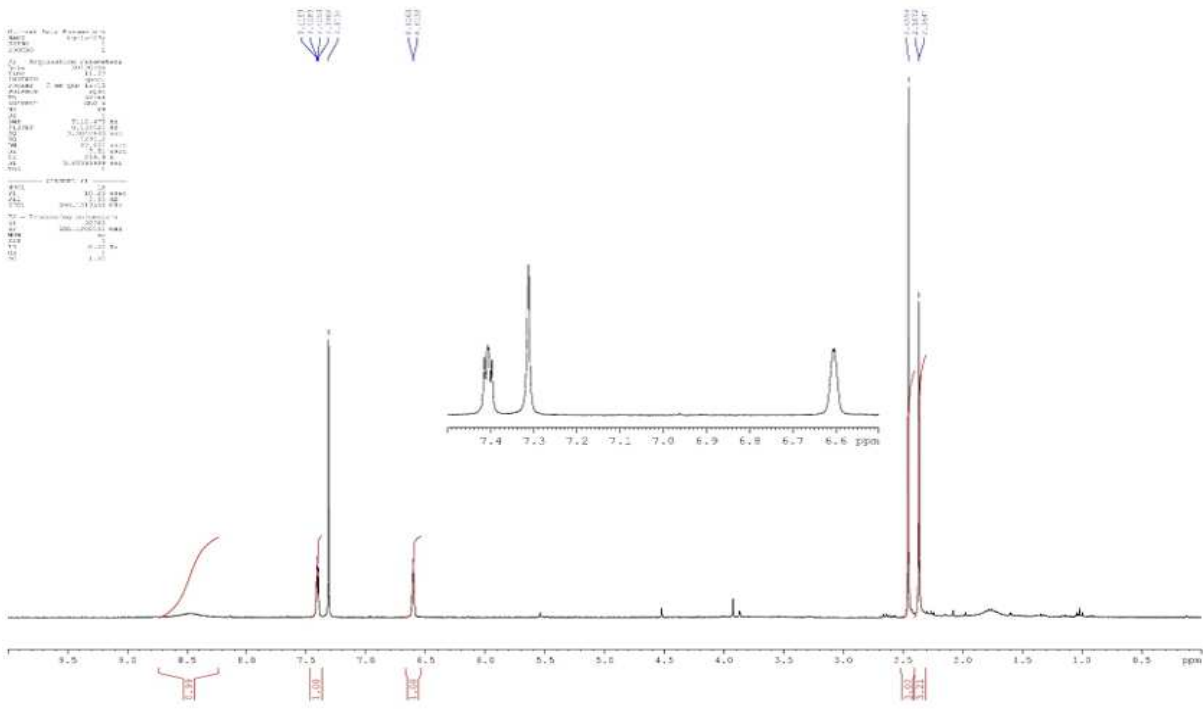
3.2.4. LC/MS analysis

The isolated product corresponding to the peak at 16.05 min was conducted by LC-MS in positive ESI and negative ESI mode, respectively.



3.2.5. NMR analysis

^1H and ^{13}C NMR spectra of the isolated product corresponding to the peak at 16.05 min (solvent CDCl_3).



3.3. Conclusion

Fungal transcription factors are crucial for orchestrating various physiological processes. *P. anserina*, a model ascomycete distinguished by its strict sexual development, represents a rich yet unexplored source of natural products. However, there is limited knowledge regarding the functional characterization of transcription factors in this filamentous fungus. In this study, we investigated the GATA-type regulator *PaNsdD* in *P. anserina*. Our findings reveal that *PaNsdD* plays a pivotal role in controlling fungal aging, vegetative growth, pigmentation, stress responses, interspecific interactions, and positively influences the production of 3-acetyl-4-methylpyrrole. Moreover, we uncovered a molecular interaction between *PaNsdD* and the sterigmatocystin pathway. Specifically, the deletion of *PaNsdD* leads to enhanced red-pink pigment biosynthesis, which occurs exclusively in the presence of the core polyketide synthase-encoding gene *PaStcA* of the sterigmatocystin pathway. In summary, the loss of *PaNsdD* function appears to be highly detrimental to *P. anserina*, which relies solely on sexual reproduction within a limited lifespan. Hence, *PaNsdD* proves to be indispensable for the survival and proliferation of *P. anserina* in its intricate ecological habitats.

Chapter 4

Environmental signals

4.1. Environmental signal PaLaeA

4.1.1. Introduction

Filamentous fungal *Podospora* species rely on genetically programmed biological processes for growth, development, and SM. These processes demand precise coordination among various signaling elements, TFs, upstream and downstream regulators, and biosynthetic genes.

The *LaeA/Lae1* (loss of aflR expression) (**Figure 4.1**) gene was initially discovered in *A. nidulans* and has since been recognized to play a significant role in the biosynthesis of numerous SM in various filamentous fungi, such as *Aspergillus spp* etc¹⁹⁵.

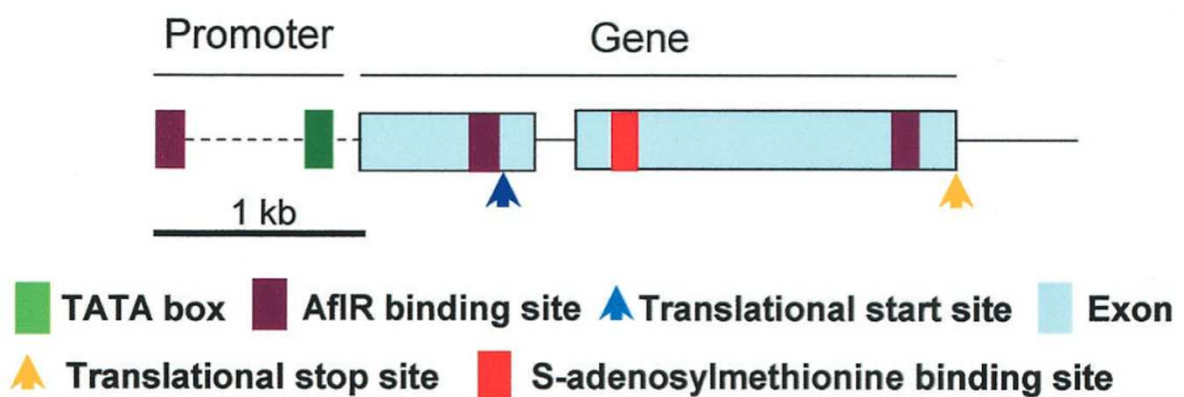


Figure 4.1. The *LaeA* gene is depicted schematically¹⁹⁵.

As mentioned in the introduction, LaeA belongs to the velvet complex, which plays a very important role in the regulation of gene expression by light. It has been shown that, like growth and development, the intensity and wavelength of light can affect the expression of a wide range of secondary metabolites²⁹⁶. This regulation is at least partly mediated by the velvet complex and in particular LaeA, which has been shown to be a global regulator of SM production. LaeA orthologs are widely conserved, having been identified in numerous filamentous ascomycetes^{26,297-299}. LaeA has been demonstrated to be a genuine methyltransferase, methylating itself on a methionine residue at position 207³⁰⁰. The impact of LaeA extends to about 50% of the SM gene clusters in fungi, and its absence leads to decreased SM production. LaeA might control accessibility of binding factors to chromatin regions of SM clusters because the S-adenosyl methionine binding site of LaeA prevents heterochromatin maintenance of some clusters³⁰¹. Loss of LaeA silenced ST and penicillin synthesis in *A. nidulans*, lovastatin production in *A. terreus*, and gliotoxin production in *A. fumigatus*, leading to the notion that LaeA was involved in global control of SM gene clusters³⁰¹.

In *T. reesei*, LaeA regulates expression of cellulases and polysaccharide hydrolases as well as a variety of SM clusters, while in *A. nidulans*, LaeA forms a complex with VeA and VelB that affects the production of SMs and participates in light-responsive developmental regulation. LaeA has also been implicated in regulation of central carbon metabolism and citric acid production in *A. carbonarius*, *A. niger*, and *Aspergillus luchuensis*. LaeA is required not only for the biosynthesis of a diverse spectrum of SM in *A. nidulans*, but also for the proper expression of the relevant SM BGCs. The *LaeA* null mutant shows significantly reduced mRNA expression of *aflR* and *stcU* genes, critical for ST synthesis. Deletion of *LaeA* leads to decreased formation of ST, penicillin (PN), lovastatin (LOV), and mycelial pigments, defining the Δ *LaeA* phenotype^{195,302}. In *A. flavus*, the Δ *LaeA* mutant loses the ability to produce various SMs (aflatoxin B1 and B2, cyclopiazonic acid), while *OE::LaeA* enhances certain SM production

not observed in the WT. *OE::LaeA* also leads to the exclusive production of sclerotia-specific metabolites (paspaline/paspalinine, aflatrem, and aflavinines). Along with its influence on sclerotia development, LaeA has an effect on seed colonization and lipase activity, which is strongly connected to *A. flavus* pathogenicity¹³¹. The deletion of the *LaeA* gene in the *A. fumigatus* strain led to decreased virulence in the mouse pulmonary model, accompanied by a reduced ability to kill neutrophil cells, providing strong evidence supporting the role of LaeA-mediated toxin production in the development of invasive aspergillosis by *A. fumigatus*^{303,304}.

LaeA exerts control not only over secondary metabolism but also plays essential roles in development. In filamentous fungi, fungal development, SM, and Hülle cell formation are linked through velvet family regulatory proteins and LaeA. LaeA, as a crucial part of the velvet complex, plays a pivotal role in coordinating development and SM formation in response to light levels. This heterotrimeric velvet complex, consisting of VelB, VeA, and the nuclear methyltransferase-domain protein LaeA, is responsible for orchestrating responses to light in filamentous fungi¹³³. The velvet family of proteins, exclusively found in fungi, plays vital roles in diverse fungal species, including human pathogens, endophytic fungi, and plant pathogenic fungi, contributing significantly to sporulation, pathogenicity, and the production of SMs^{305,306}. These family regulatory proteins form diverse complexes, with VosA-VelB inhibiting conidial germination but promoting trehalose synthesis and β -glucan biogenesis. The VelB-VeA-LaeA complex controls SM and Hülle cell formation, crucial for nascent sexual fruiting bodies^{133,302,307} (**Figure 4.2**). The *LaeA* null mutant in *A. nidulans* exhibits constitutive sexual differentiation, indicating that LaeA plays a crucial role in inhibiting sexual development in response to light. Furthermore, the absence of LaeA leads to the formation of notably smaller fruiting bodies, attributed to the lack of a specific globose cell type (Hülle cells) that normally nurtures the young fruiting body during its development³⁰⁸.

Deletion of the *LaeA* gene causes an impairment in conidiation in *P. chrysogenum*. Because *LaeA* has sequence similarity to histone and arginine methyltransferases, it may act as a global regulator by influencing chromatin structure. However, other target substrates of *LaeA* are currently not known. Further research is needed to explore additional functions and interactions of this intriguing protein.

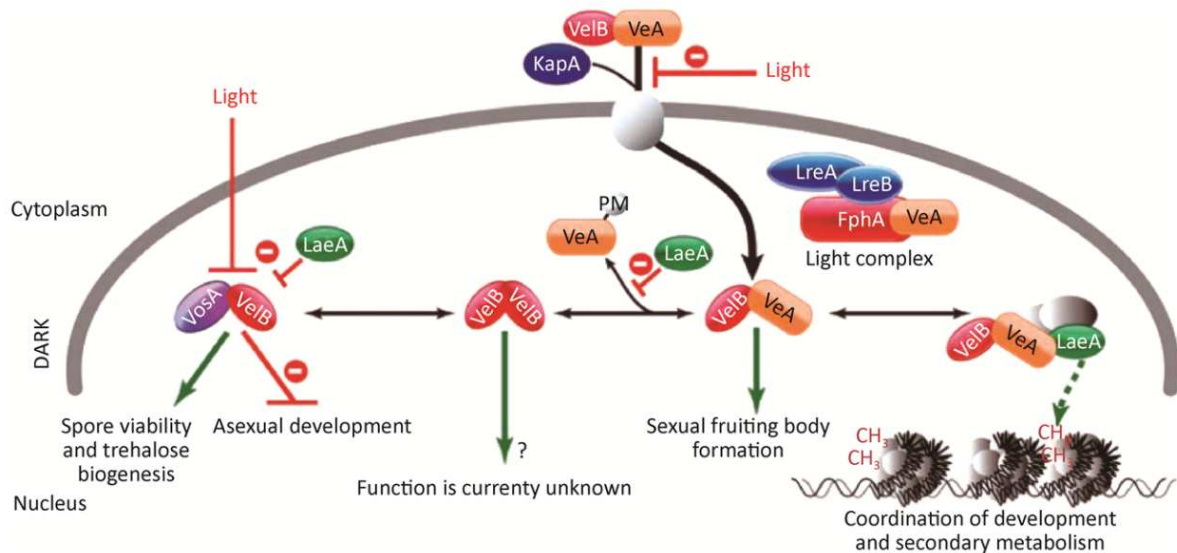


Figure 4.2. The global control network diagram involving *LaeA*²⁹⁷.

In order to characterize the role of *LaeA* in *P. anserina*, the deletion, complementation and overexpression of the gene were conducted. The main results are presented in this chapter and an article is in preparation.

4.1.2. Results

4.1.2.1. Generation of deletion, overexpression, and complementation mutants

In *P. anserina*, the fungal transcription factor database contains a total of 507 TFs grouped into 41 families. The $\Delta PaLaeA$ mutant strain and the complemented strain (*PaLaeA^{comp}*) were previously generated by Pierre Grognet (I2BC, Université Paris Saclay) but remained

uncharacterized. To further investigate the biological functions of PaLaeA in *P. anserina*, we also constructed the overexpressed strain (*OE-PaLaeA*) and verified it. The $\Delta PaLaeA$, *PaLaeA^{comp}*, and *OE-PaLaeA* strains were fully characterized.

4.1.2.2. Impacts of PaLaeA on vegetative growth and colony morphology

To assess the influence of PaLaeA on vegetative growth, we conducted a phenotypic analysis of both WT and mutant strains. This analysis involved incubating the strains on solid and liquid M2 media, as well as solid and liquid LB media, all at the optimal growth temperature of 27°C. After 8 and 12 days of incubation, we conducted comparisons and analyzed distinctions in colony size, morphology, and hyphal pigmentation among the $\Delta PaLaeA$, *PaLaeA^{comp}*, *OE-PaLaeA*, and WT strains, respectively. As depicted in Figure 4.3, it is apparent from both M2 liquid and solid media that the $\Delta PaLaeA$ and *OE-PaLaeA* strains produce fewer hyphae in comparison to the WT strain, with the $\Delta PaLaeA$ strain exhibiting a more significant reduction. In our initial observations, we noted that compared to the wild type, the $\Delta PaLaeA$ strain exhibited lighter staining on both solid and liquid M2 media, indicating that this mutant likely downregulated melanin production or affected its accumulation. On the M2 solid medium, it's noticeable that the hyphae of the $\Delta PaLaeA$ strain aggregate to form a distinct ring, a characteristic that sets it apart from the WT strain and other mutants.

After culturing for over 12 days, it became evident that the $\Delta PaLaeA$ strain exhibited a pronounced deep pink pigment production on both the solid and liquid LB media. This pink pigment was also notably present in the filtrate of the M2 liquid medium.

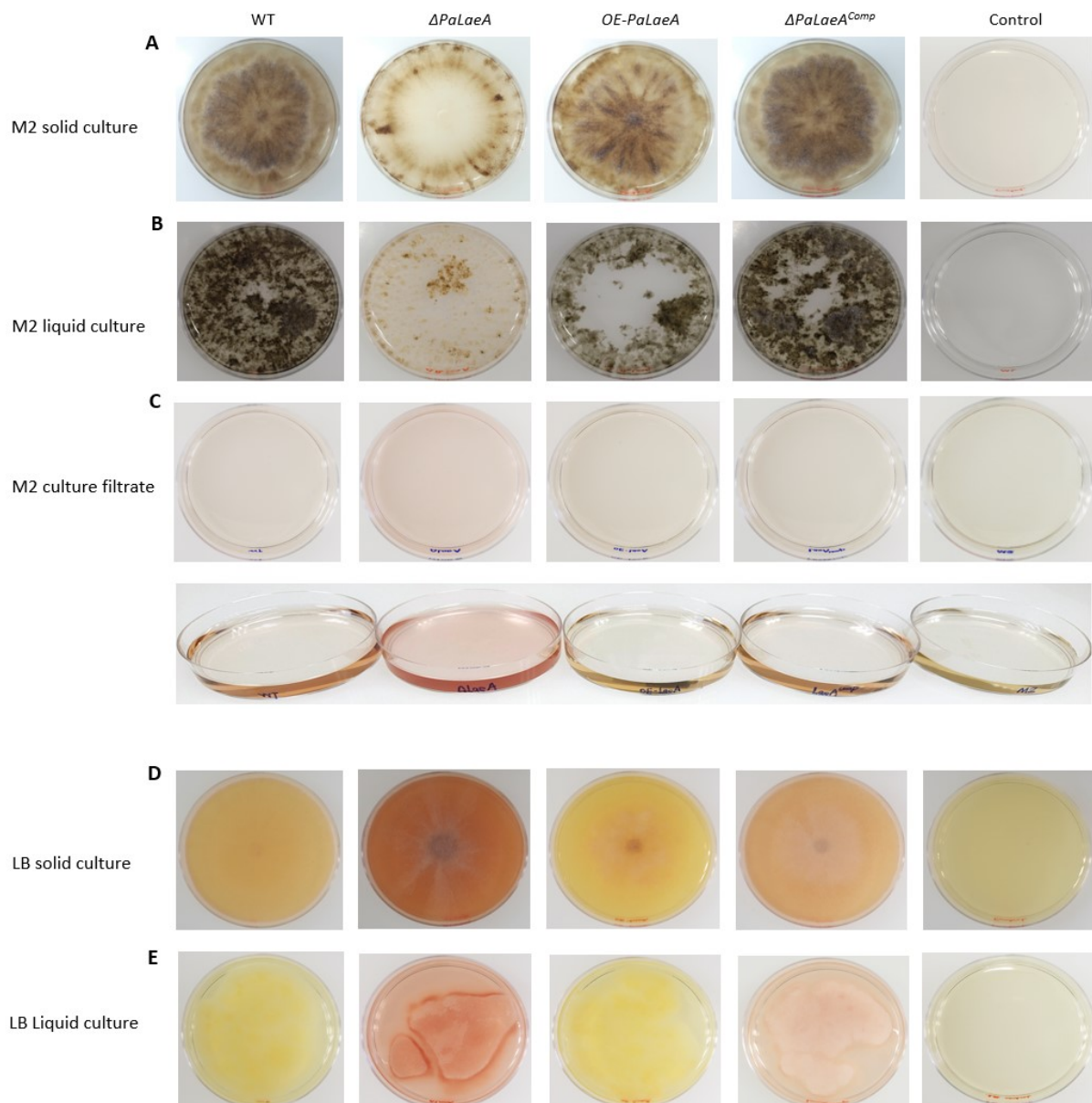


Figure 4.3. Vegetative growth of wild type and mutants. Growth of *P. anserina* strains on M2 solid medium (A), M2 liquid medium (B). Culture filtrate without mycelium (C). Growth of *P. anserina* strains on LB solid medium (D), LB liquid medium (E). Photograph the flat panel from a different angle to contrasting color differences. Photographs of the M2 solid culture plates were captured following 8 days of incubation at 27°C. For the M2 liquid culture plates and LB solid and liquid culture plates, images were taken after 12 days of incubation at the same temperature. WT: wild-type strain.

4.1.2.3. Effects of PaLaeA on mechanisms involved in pink pigment production

To investigate the mechanism behind pink pigment production in the $\Delta PaLaeA$ strain, we generated double mutants $\Delta PaLaeA/193$ and $OE-PaLaeA/193$. The $PaPks1-193$ mutant lacks polyketide synthase activity, leading to the incapacity to produce dark green pigment due to the lack of (DHN)-melanin production²⁶⁷. As shown in Figure 4.4, the $PaPks1-193$ mutant is unable to produce dark green pigment. However, the $\Delta PaLaeA/193$ mutant still exhibited pink pigment accumulation, leading us to conclude that the signaling pathway responsible for regulating this pink pigment production is distinct from the pathway involved in (DHN)-melanin pigment production.

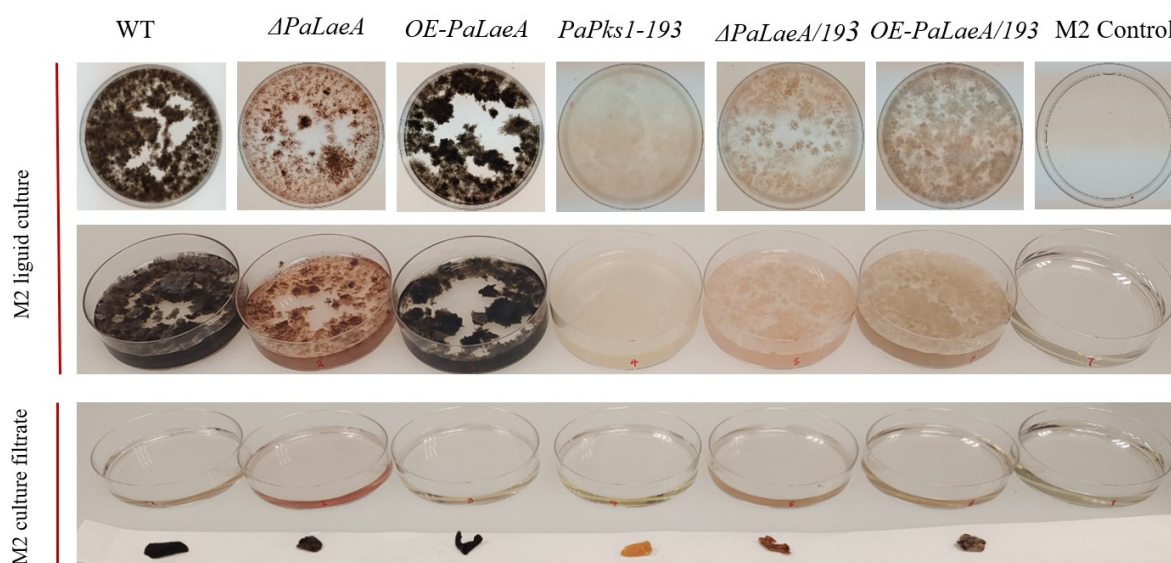


Figure 4.4. Growth of *P. anserina* strains on liquid M2 medium, culture filtrate without mycelium and dry and weight the mycelium. Pictures were taken after 12-days incubation 27°C.

Another mutant, $\Delta PaNsdD$, also presents a pink pigment production as described previously in chapter 2 and in Shen et al. 2022³⁰⁹. The two strains were cultivated on media allowing pigment accumulation as shown on Figure 4.5 for the day 16. To compare and analyze the pink pigments produced by $\Delta PaLaeA$ and $\Delta PaNsdD$ mutants, the cultures were observed

each day. The $\Delta PaLaeA$ strain initiated pink pigment production after 8 days of cultivation, with clear pigment production visible after 12 days. In contrast, the $\Delta PaNsdD$ strain required more than 14 days of culture before pink pigment production was observed. On LB medium, $\Delta PaNsdD$ produces a greater amount of pink pigment on LB solid medium, while $\Delta PaLaeA$ exhibits a higher production of pink pigment on LB liquid medium.

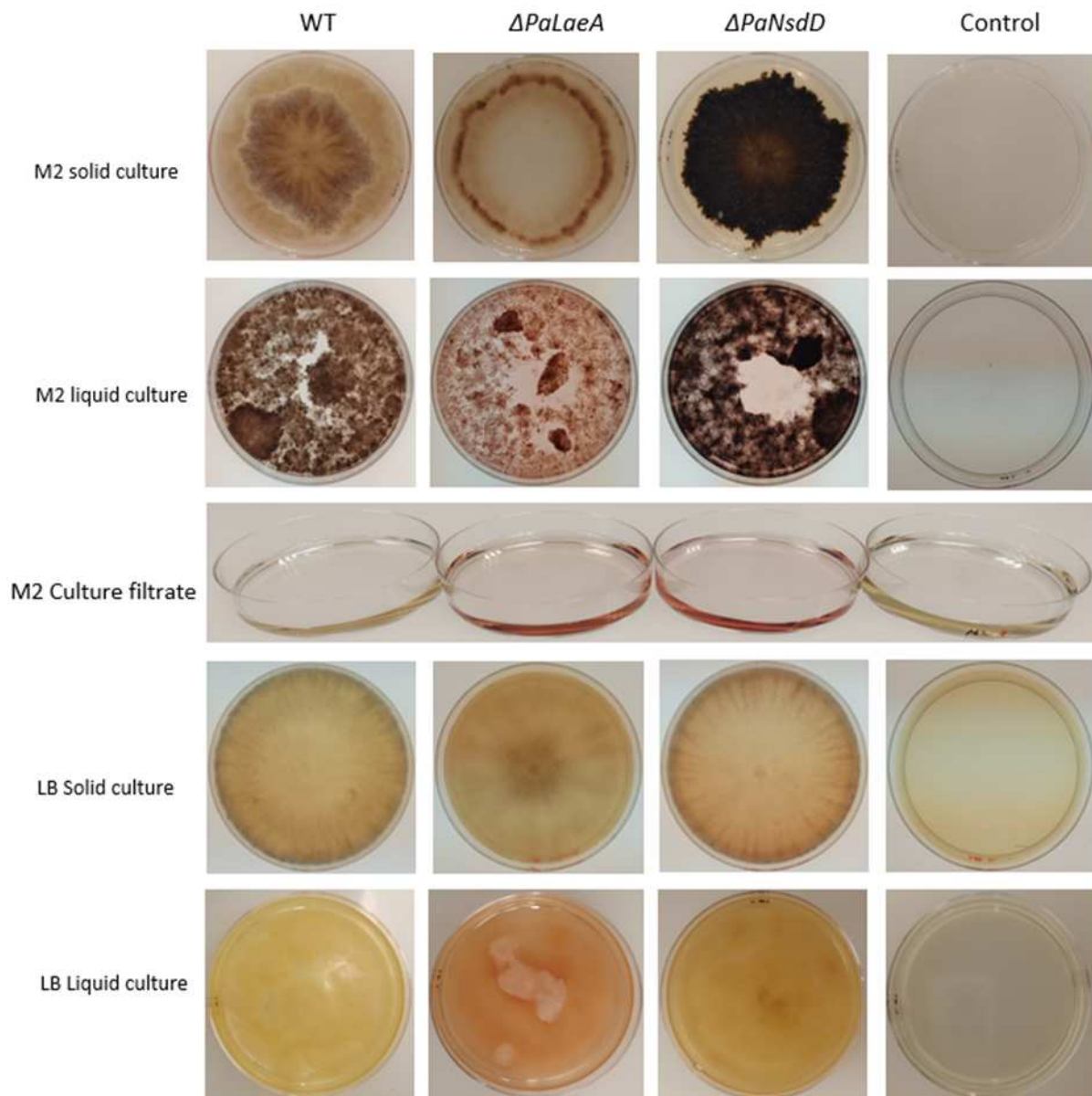


Figure 4.5. Comparative of the pink pigment production from $\Delta PaLaeA$ and $\Delta PaNsdD$ mutants on both M2 and LB solid and liquid media, respectively. Pictures were taken after 16-days incubation 27°C.

To see if the pink pigment produced by $\Delta PaLaeA$ and the pink pigment produced by $\Delta PaNsdD$ share a common origin, additional TLC experimental analyses were conducted. Various concentration gradients were used to determine the optimal eluent ratio, 15% MeOH+85% Chloroform, ensuring the distinct separation of substance bands in the TLC experiment. The pink pigment, easily visible to the naked eye, was researched. To analyze it in the TLC experiment, initially the separated bands under visible light were observed and compared. As shown in Figure 4.6, the TLC bands of $\Delta PaLaeA$ and $\Delta PaNsdD$ exhibited variations as the pink pigment migrated. These differences were further scrutinized under UV 254nm, UV 366nm, and sulfuric vanillin, reaffirming our conclusion. We might hypothesis that at least one compound is similar between the two strains $\Delta PaLaeA$ and $\Delta PaNsdD$ (frontal ratio = 0,3).

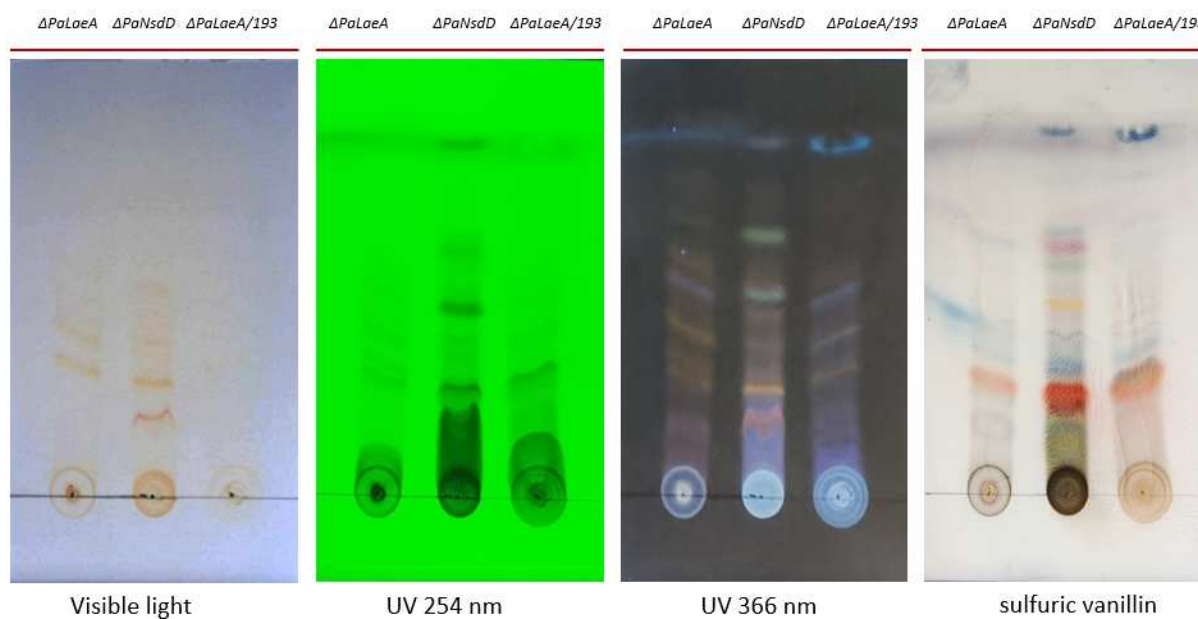


Figure 4.6. TLC eluent: 15% MeOH+85% Chloroform (0.75mL MeOH+4.25mL CHCl₃)

4.1.2.4. Role of PaLaeA in sexual reproduction

To investigate the potential impact of LaeA on the sexual development of *P. anserina*, fertility assays were conducted by genetic heterokaryotic crosses (*mat+* x *mat-*) with both wild-type and mutant strains. In homozygous crosses involving homokaryotic strains of WT, *OE-PaLaeA*, and $\Delta PaLaeA^{comp}$, we observed a ring of numerous perithecia. However, in the $\Delta PaLaeA$ crosses, even after extended incubation (up to 1 month), no perithecia were observed (**Figure 4.7(A)**). These assays allowed us to assess whether PaLaeA plays an essential role in the sexual reproduction of *P. anserina*. These results strongly indicate that the absence of PaLaeA leads to a sexual reproduction defect. To investigate the factors contributing to the sterile phenotype observed in $\Delta PaLaeA$, we conducted additional experiments to assess the impact of PaLaeA on both male and female fertility. This was achieved by crossing the mutant with the wild type (WT *mat+* x $\Delta PaLaeA$ *mat-*, $\Delta PaLaeA$ *mat+* x WT *mat-*) in spreading crosses experiments. In both reciprocal crosses, no perithecia or ascospores were observed on the mutant thallus. This suggest that ascogonia from $\Delta PaLaeA$ were unable to be fertilized by spermatia from the WT, whereas ascogonia from the WT were still capable of normal fertilization by spermatia from $\Delta PaLaeA$ (**Figure 4.7(B)**). These results indicate that the deletion of *PaLaeA* results in a defect in female fertility but does not affect male fertility. Furthermore, it's worth noting that this female sterile phenotype is not dependent on mating type. Interestingly, the mutant thallus has a powdery appearance often correlated with high sperm production. I therefore counted them and remarkably, the $\Delta PaLaeA$ mutant exhibited a significant increase in spermatia production when cultivated on M2 solid medium for 3 days (**Figure 4.7(C)**). Subsequent quantitative analysis confirmed a substantial elevation ($P < 0.001$) in the yield of spermatia in the $\Delta PaLaeA$ strain compared to the WT strain (**Figure 4.7(D)**). The $\Delta PaLaeA^{comp}$ strain restored normal spermatia production and the overexpression has no effect. These observations collectively indicate that PaLaeA functions as a negative regulator of

spermatia differentiation in *P. anserina*. In summary, the data presented above provide compelling evidence that the *PaLaeA* mutant fails to undergo sexual development and produce an excessive amount of spermatia.

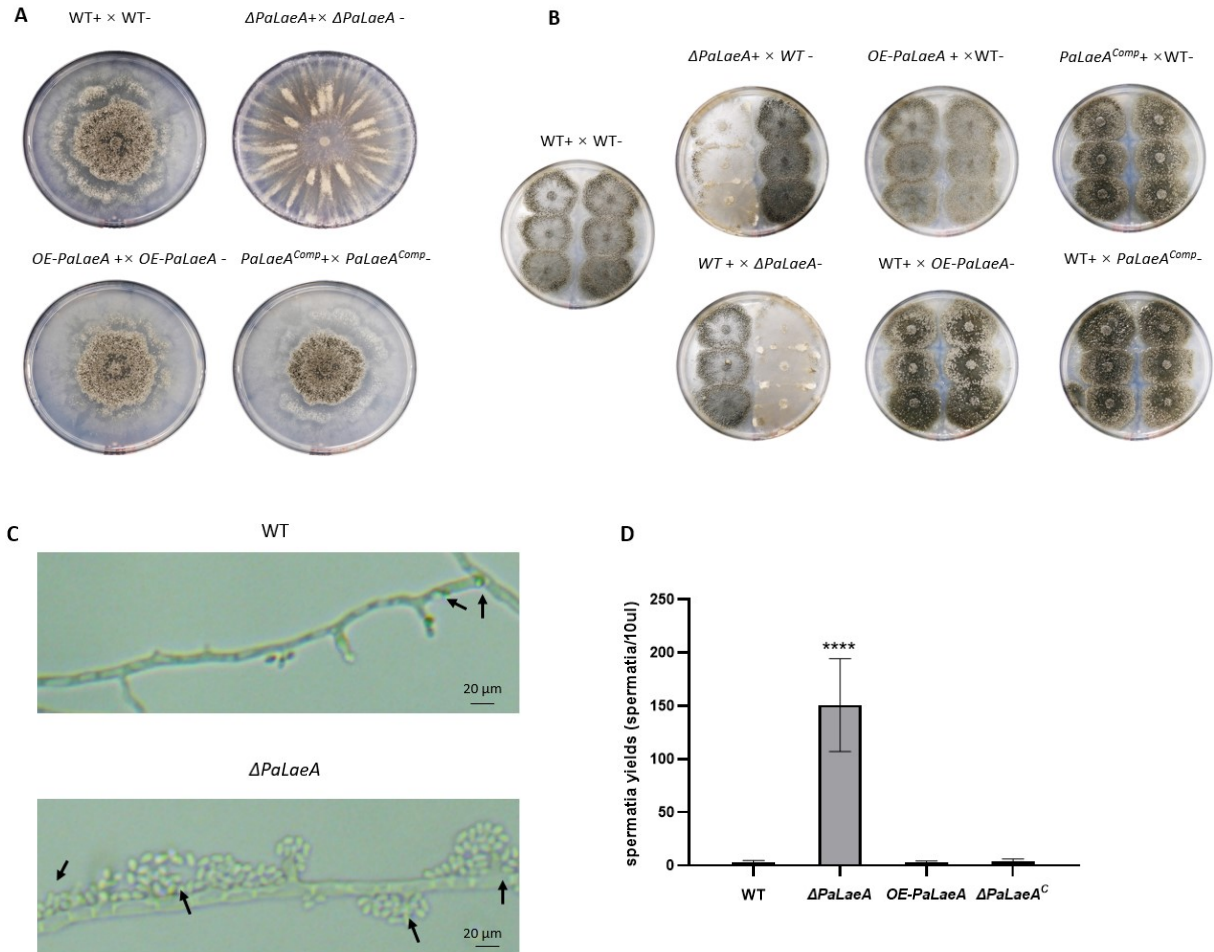


Figure 4.7. Sexual development of *P. anserina* strains. (A) Heterokaryotic crosses assays. Fragmentated mycelia from *mat+* and *mat-* strains were mixed and then inoculated in the center of plate under constant light illumination. Photographs were taken after 8 days of incubation. (B) Watered spreading confrontation crosses assays. Crosses were made by inoculating the strains 1.5 cm apart. After 3 days of growth, 1.5 ml of water was added and spread all over the plate. The pictures were taken 5 days after fertilization, at which time fully mature perithecia expel ascospores. Mature perithecia became apparent as small dots upon the completion of fertilization. (C) Microscopic examination of spermatia formation, with images captured after 3 days of growth on M2 plates. Typical spermatia are indicated by arrows. (D) Quantitative analysis of spermatia. The strains under investigation were cultivated on M2 medium for 3 days,

followed by spermatia counting and observation. Asterisks denote significant differences ($P < 0.001$) in each strain compared to the WT, as determined by a Student's t-test.

4.1.2.5. Effect of different carbon sources

It is well-documented that alterations in growth medium composition can activate previously dormant SM pathways. Therefore, we utilized the basic M0 medium (equivalent to M2 medium but lacking dextrin) and supplemented it with various substances as carbon sources to culture strains. This approach was employed to investigate, among other factors, the influence of different carbon sources on the growth and development of both *P. anserina* mutant and WT strains. Pictures shown in Figure 4.8 show that the *OE-PaLaeA* strain grow slowly than WT on M3, MAE medium, M0 containing Glycerol and M0 containing lignin media. Furthermore, on medium with lignin powder the *OE-PaLaeA* strain shows a deep brown color and exhibits a ring-shaped growth. Additionally, I observed that when the *OE-PaLaeA* strain is cultured on M0 medium containing MCC, it produces dark green substances on the medium. This phenotype is entirely distinct from the characteristics exhibited by the $\Delta PaLaeA$ and WT strains. When I carefully examined various strains and compared them under a microscope at the same size and magnification in identical positions, I observed that the hyphae produced by the *OE-PaLaeA* strain appeared denser, and dark green pigments formed concentrated clusters within the culture medium (**Figure 4.9**). Therefore, I can suggest that the carbon source absorption pathway of the *OE-PaLaeA* strain could be affected.

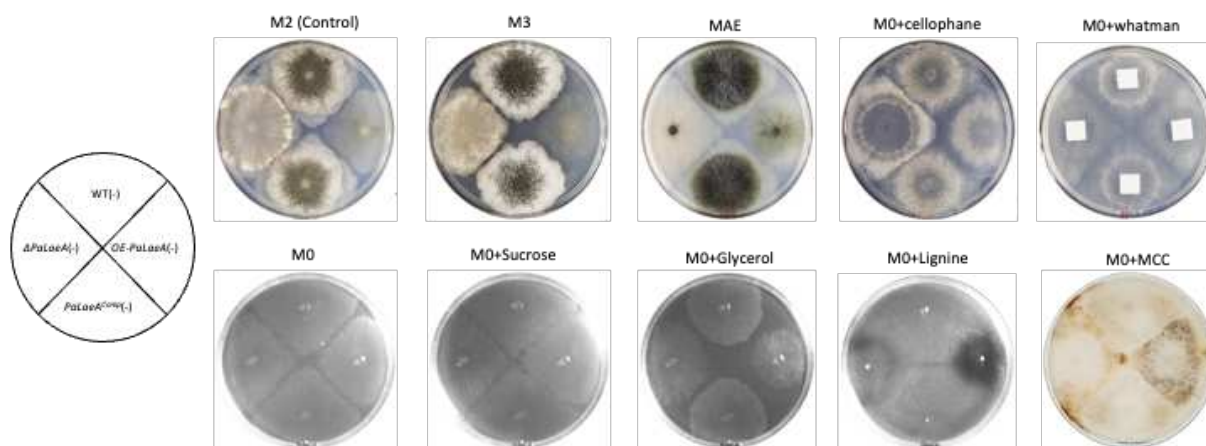


Figure 4.8. Colony morphology WT and various mutants. Some pictures were taken with a camera, and others were taken under gel imaging instrument. M0 is the base medium; M2 Medium=M0 medium + dextrin; M3 Medium=M0 medium + 5.5g/L glucose, MCC: microcrystalline cellulose, MAE: minimal agar ethanol. Pictures were taken after 4-days incubation at 27°C.

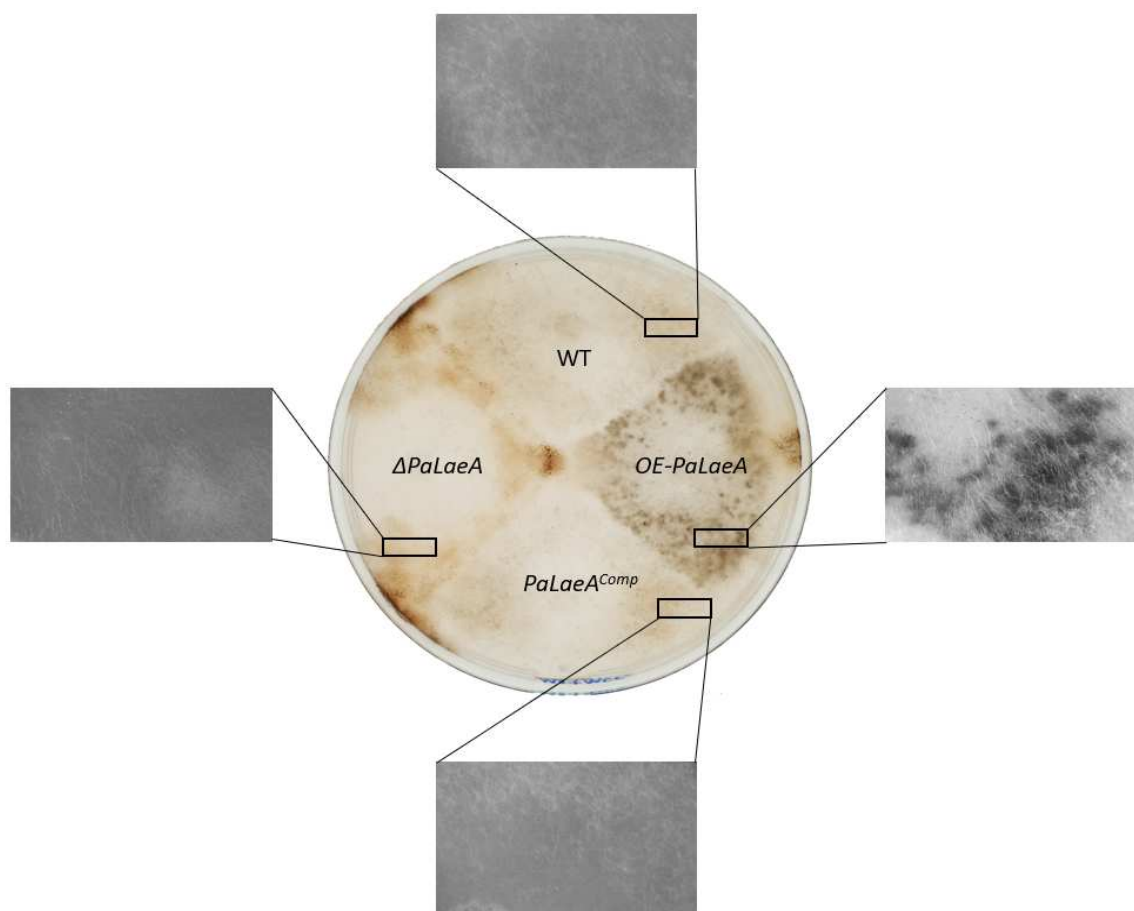


Figure 4.9. The mutants and WT strains of *P. anserina* were cultured on M0 medium containing MCC.

4.1.2.6. Function of PaLaeA in multiple stress responses

To investigate the possible involvement of PaLaeA in regulating various stress responses in *P. anserina*, I conducted experiments to assess fungal growth on M2 plates supplemented with different stressors, including oxidative stressors (H_2O_2 , MG, Mena, TBY), osmotic stressors (NaCl, KCl, Sorbitol), and cell wall-binding compounds (CFW, Congo Red). Clearly, under oxidative stress conditions, the $\Delta PaLaeA$ strain exhibited decreased resistance to certain stressors, particularly MG and TBY, making it more sensitive than the WT to oxidative stress triggered by these compounds (**Figure 4.10**). When all strains were subjected to osmotic stress by adding sorbitol, the WT and *OE-PaLaeA* strains exhibited a circular growth pattern with smooth and regular edges, although their growth rate was inhibited and slowed. In contrast, the $\Delta PaLaeA$ strain was minimally affected by this stress. When the cell wall-binding compound CongoRed was introduced into the culture medium as a stress inducer, the growth of the $\Delta PaLaeA$ strain became sluggish and it failed to produce black deposits, unlike the WT and *OE-PaLaeA* strains. The $\Delta PaLaeA^{comp}$ strain, on the other hand, restored its tolerance to a level comparable to that of the WT strain. This suggests that the loss of PaLaeA function is responsible for this phenotype.

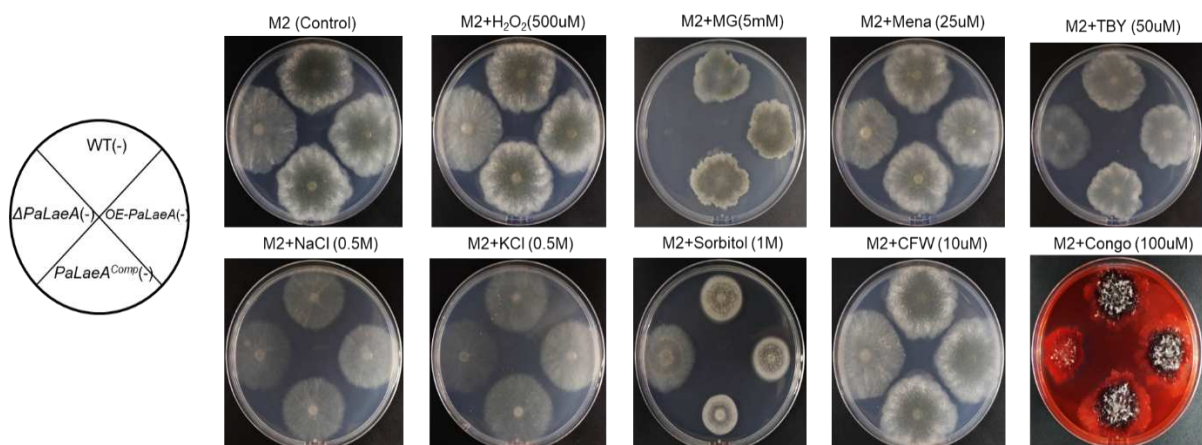


Figure 4.10. Growth of *P. anserina* strains on M2 plates exposed to oxidative stress (H_2O_2 , MG, Mena, TBY), osmotic stress (NaCl, KCl, Sorbitol) and cell wall-binding compounds

(CFW, CongoRed). MG: methylglyoxal, CFW: calcofluor white, TBY: *Tert*-butyl hydroxyperoxide, Mena: menadione. Pictures were taken after 4-days incubation at 27°C.

4.1.2.7. Contribution of PaLaeA in interspecific confrontations

The fungal species used in this experiment as challengers were selected according to their different biological characteristics. The basidiomycete *T. versicolor* is a common saprophytic polypore generally found on fallen trees. The leotiomycete *B. cinerea* and the eurotiomycete *P. chrysogenum* belongs to the ascomycete lineage. *B. cinerea* is a necrotrophic fungus that is at one stage of its life cycle plant pathogen. *P. chrysogenum* is described as a saprophytic fungus, commonly found in soil and decaying matter²⁸⁹. *C. cinereus* is one of two model organisms commonly used to study developmental processes in the homobasidiomycetous fungi. *Coprinopsis cinerea* was defined as saprophytic mushrooms, it is a heterothallic basidiomycete and one of two model organisms commonly used to study developmental processes in the homobasidiomycetous fungi. *Aspergillus* species are filamentous fungi, the majority of *Aspergillus spp.* live as saprophytes and are frequently isolated from soil and decaying vegetation, where they play an important part in the recycling of nutrients such as carbon and nitrogen. *A. nidulans* is a saprophytic filamentous fungus that is found virtually everywhere and is a well-established model organism for genetics and cell biology studies. *A. fumigatus* is commonly found in nature, in decaying organic matter as a saprotrophic filamentous fungus or in compost as a carbon- and nitrogen-recycling organism. Apart from this, because it is an opportunistic aerial fungal pathogen, *A. fumigatus* continuously releases conidia into the environment and can cause allergic and chronic lung diseases, including invasive aspergillosis.

It was shown that interspecific confrontations may be associated with peroxides production by one or both competitors²⁸⁸. We investigated the ability of the *P. anserina* wild-type strain and mutants to produce peroxides. When *P. anserina* is challenged with competitors, we noticed that high levels of peroxides were specifically accumulated at the confrontation zone

as previously described. For each challenger species tested, the level of peroxide accumulation in the confrontation zone was consistent, across all the *P. anserina* strains.

The data depicted in Figure 4.11 reveals that when *OE-PaLaeA* strain was exposed to competitors such as *A. nidulans*, *P. chrysogenum*, *C. cinerea*, and *T. versicolor*, there was a significant increase in peroxide accumulation within the mycelium of the ST-overexpressed strains, as well as at the confrontation zone. In the peroxide detection experiments, we observed that $\Delta PaLaeA$ mutant produced lower levels of peroxide compared to the WT, while *OE-PaLaeA* strain produced higher levels of peroxide than the WT. This suggests that PaLaeA may play a role in orchestrating a global response to competitors. Nevertheless, when we employed the trypan blue method to assess cell death, we observed no significant differences between all the mutant and the WT strains during their confrontations with each antagonistic strain.

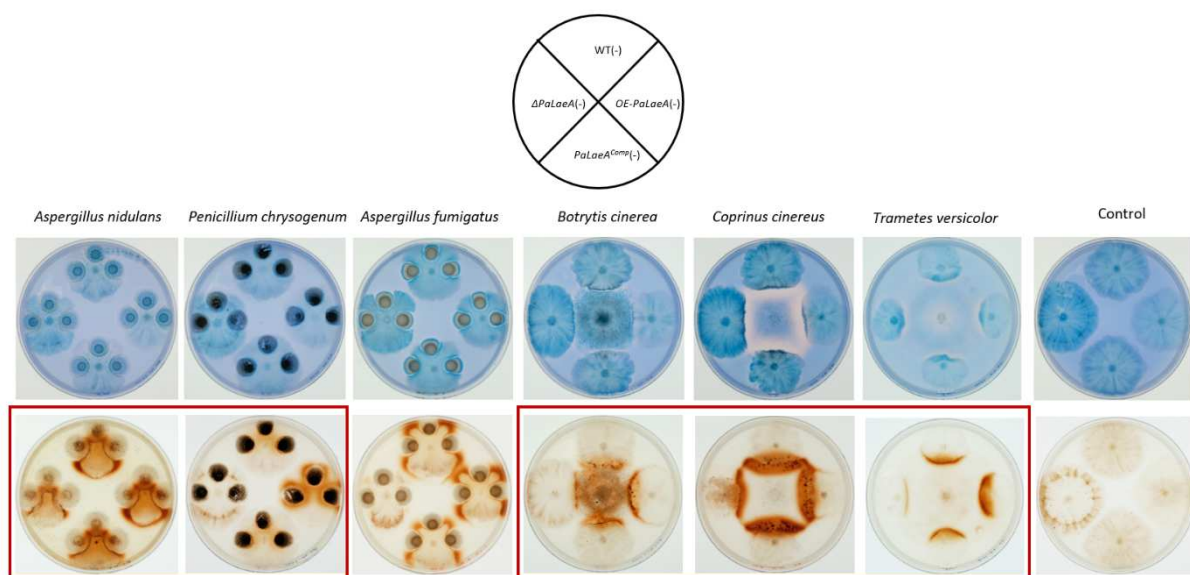


Figure 4.11. Confrontation of *P. anserina* strains other fungal species. Cell death assay and peroxide detection with Diaminobenzidine (DAB) were conducted as previously described.

4.1.2.8. Isolate, and analyze metabolites exhibiting significant production and expression disparities between *PaLaeA* mutants and WT

In the initial metabolite extraction, we individually cultured four bottles of each strain and sequentially extracted the metabolites after 4, 8, and 12 days. We then recorded the weight of the obtained products (**Table 4.1**). During the process of dissolving the extracted metabolites in methanol for HPLC experiments, we encountered an issue where certain metabolites did not fully dissolve in methanol. As a result, I measured and documented both the mass of metabolites that successfully dissolved in methanol and the mass of those metabolites that remained insoluble for each strain (**Table 4.1**). By generating histograms, it is easier to visually compare differences in the total amount of metabolites obtained for each strain (**Figure 4.12**). HPLC-UV-MS (**Figure 4.13**) experimental results show that compared with the WT strain, the $\Delta PaLaeA$ strain has significantly higher metabolite production at the three main peaks at 21.2, 23.9 and 30.8 minutes, and can be obtained on the 12th day of culture. There are more metabolites. Therefore, it is of great significance to identify the main compounds in the $\Delta PaLaeA$ strain, and in subsequent metabolite analysis, the strain was cultured for 12 days each time before extraction and analysis. The solid cannot dissolve in Methanol was then filtered before injection in HPLC and was analyzed by TLC followed by NMR (**Figure 4.14**). It was identified as sterigmatocystin (ST). It indicates that $\Delta PaLaeA$ strain of *P. anserina* produces such a large amount of ST that it precipitates out during sample preparation.

Table 4.1. Weigh and record the mass of extracted metabolites for each strain.

		WT		<i>ΔPaLaeA</i>		<i>PaLaeA^{comp}</i>		<i>OE-PaLaeA</i>	
D4	Weight of residual solid (mg)	13.8	0	14.1	0	12.1	0	13.9	0
	Weight of MeOH soluble extract (mg)		13.8		14.1		12.1		13.9
D8	Weight of residual solid (mg)	19.7	2.6	15.7	0	22.8	4.7	13.9	0
	Weight of MeOH soluble extract (mg)		17.1		15.7		18.1		13.9
D12	Weight of residual solid (mg)	18.1	8.5	17.9	0	20.1	4.6	23.3	0
	Weight of MeOH soluble extract (mg)		9.6		17.9		15.5		23.3

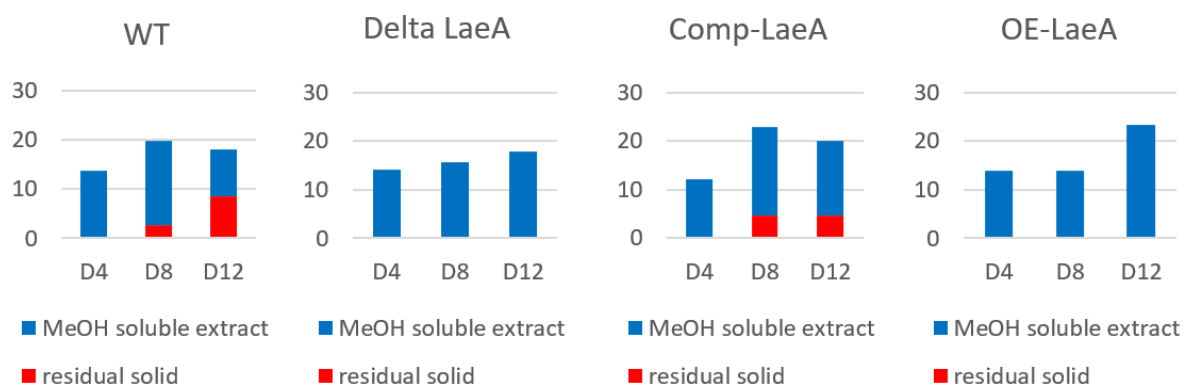


Figure 4.12. The quality of the extracted metabolites for each strain. X-axis (days): time. Y-axis (mg): mass.

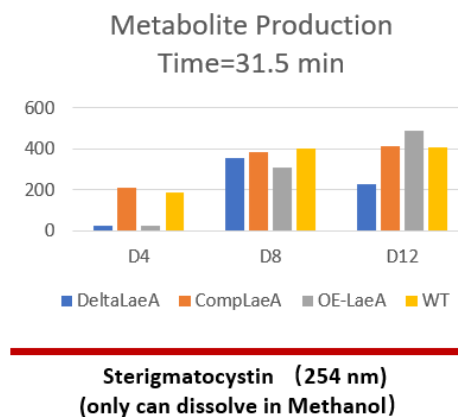
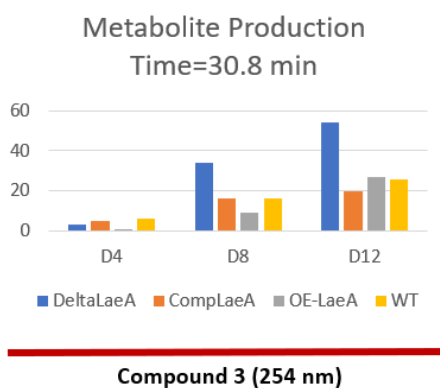
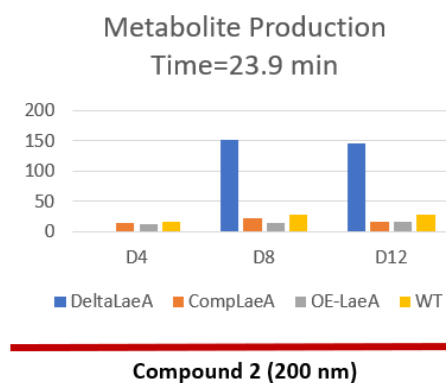
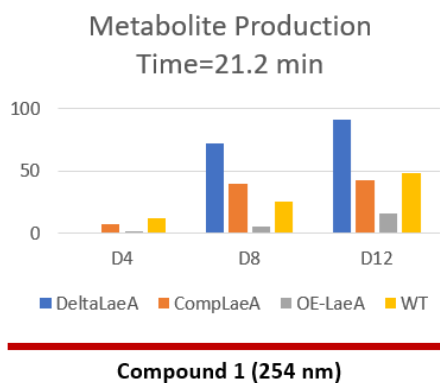


Figure 4.13. HPLC-UV-MS analysis. Analytical HPLC analysis were conducted on a C18 column on a LC-UV-MS Thermo U3000 apparatus. Liquid M2 stationary cultures of *P. anserina* strains were incubated at 27°C for 4 days, 8 days, 12 days. X-axis (days): time. Y-axis: mass.

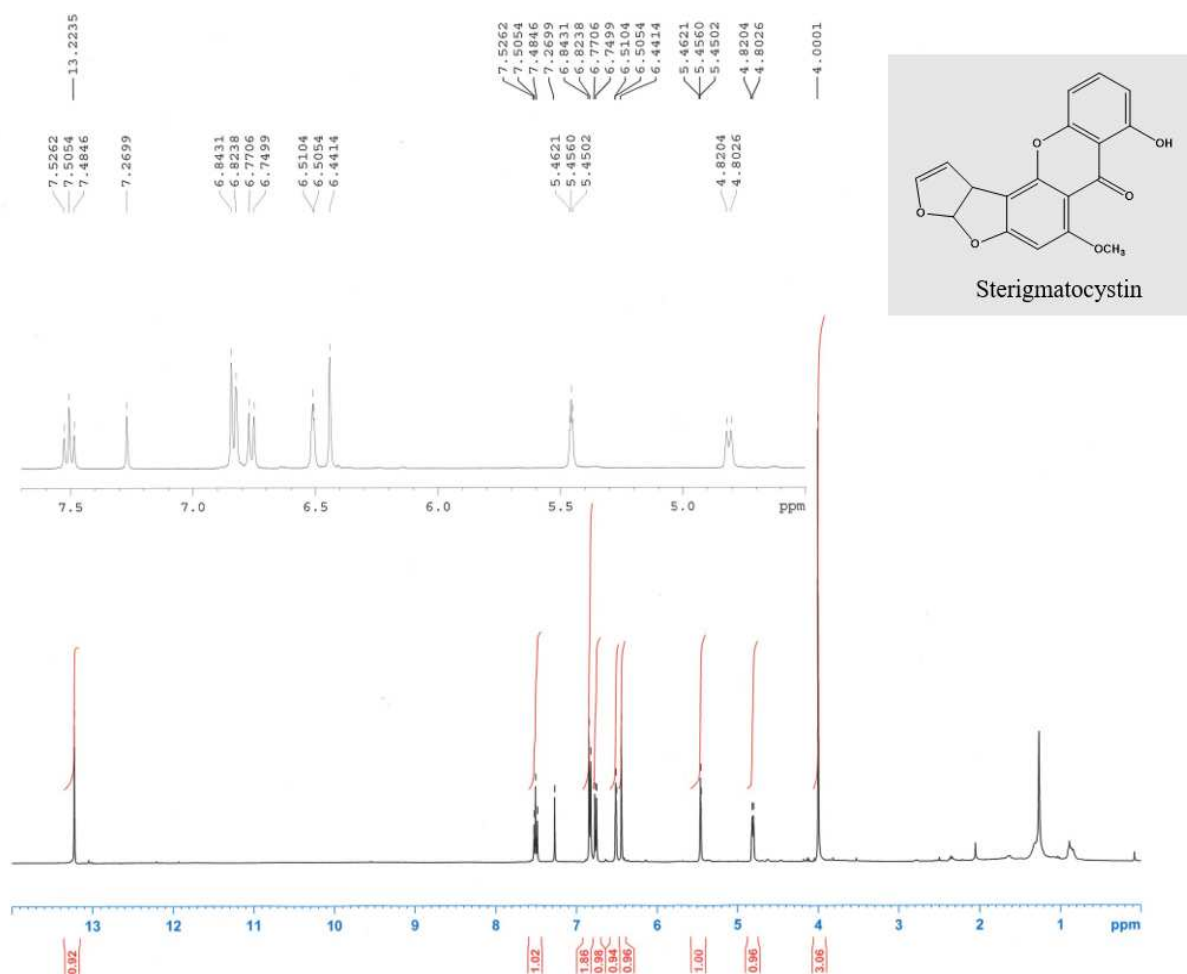


Figure 4.14. NMR analysis. ¹H spectra, of the isolated product corresponding to the peak at 31.5 min (solvent CDCl₃).

Next, we conducted the extensive 12-day cultivation of the *ΔPaLaeA* strain and proceeded with metabolite extraction. Through HPLC separation experiments, I made diligent efforts to isolate each compound and obtain compounds with relatively high purity (**Figure 4.15**). Purification was performed using a preparative HPLC and allow us to isolate six different compounds (A-F peaks).

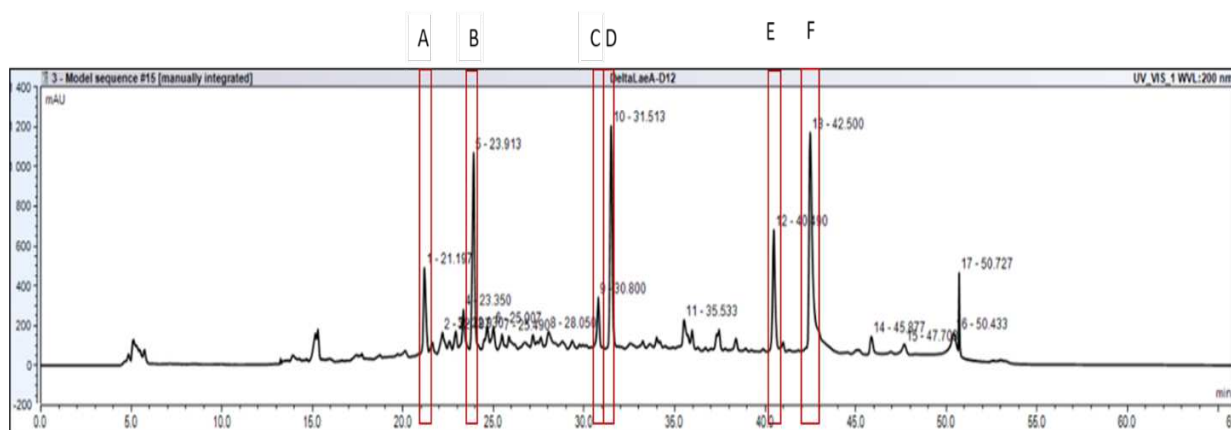


Figure 4.15. Separative HPLC for *ΔPaLaeA* (12 days). Six major peaks were identified: A (21.2 min), B (23.9 min), C (30.8 min), D (31.5 min), E (40.3 min), F (42.3 min).

With a standard, D compound was identified as ST.

4.1.2.9. Analyzing A compound (Rt: 21.2 min)

NMR-based direct analysis is ideally suited for high-throughput metabolomics applications, offering the advantage of detecting a wide range of metabolites in a quantitative and unbiased manner. After isolating and identifying the pure compound (Rt: 21.2 min), we proceeded with multiple NMR experimental analyses. By cross-referencing the NMR spectra with the literature, I hypothesized that the compound might be secosterigmatocystin. We found m/z at 363.1075 in accordance with the formula $C_{18}H_{19}O_8$ for $[M+H]^+$. Upon consulting the literature, we determined that the precise molecular mass of secosterigmatocystin is 362.1002, and its molecular structural formula is $C_{18}H_{18}O_8$. To confirm my hypothesis, I submitted 1 mg of the compound for HRMS experimental testing for further verification, employing chloroform and methanol as the chosen solvents. The results (**Figure 4.16**) indicated that the A compound identified was indeed secosterigmatocystin. This compound corresponds to the degradation of ST. However, given the stability of ST, it is unlikely that degradation occurred during extraction.

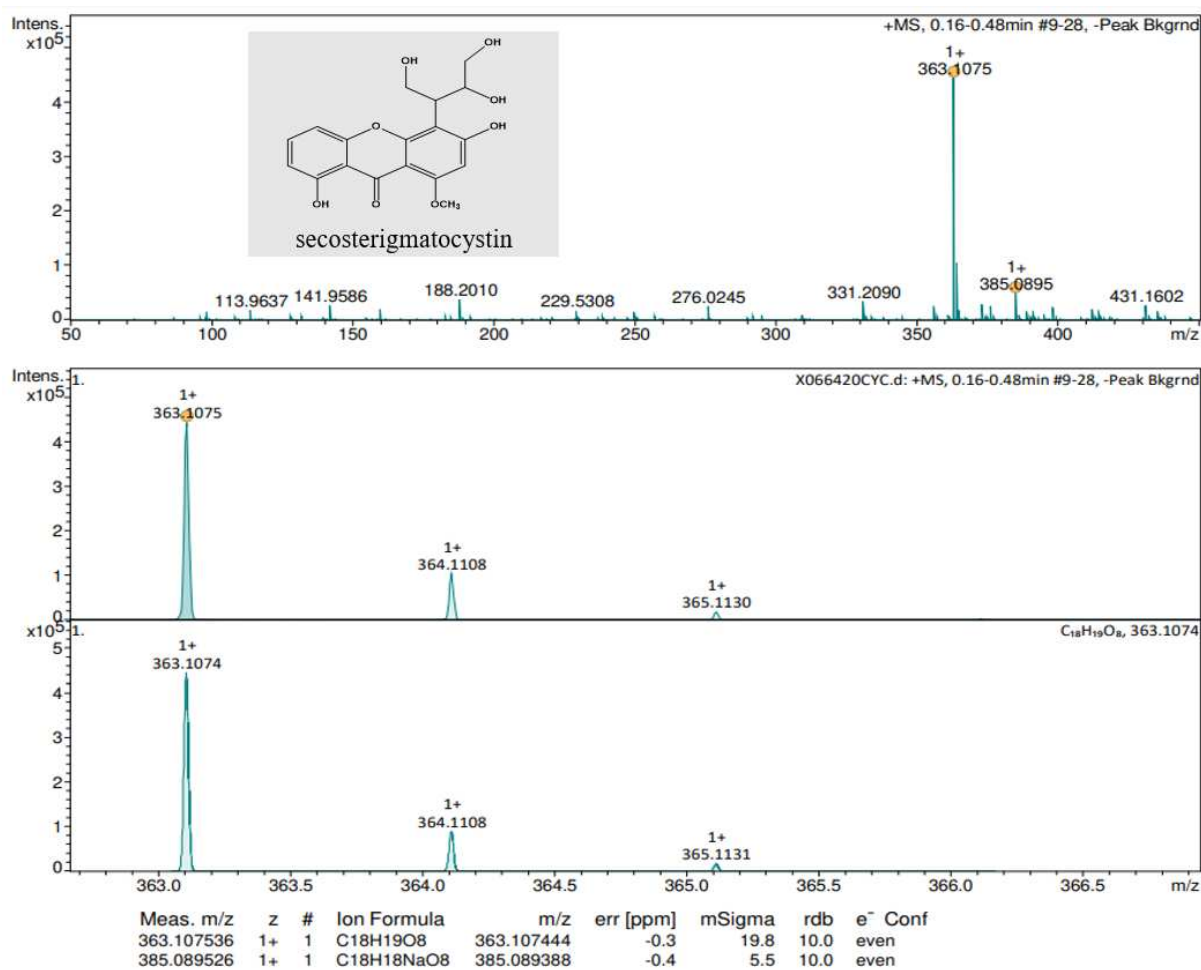


Figure 4.16. HRMS analysis of A compound.

4.1.2.10. Analyzing B compound (Rt: 23.9 min)

To characterize the compound isolated at 23.9 minutes, I performed NMR (300MHz and 600 MHz) experiments to elucidate its chemical structure.

The two-dimensional (2D) NMR heteronuclear shift correlation method allows for clear distinction between resonances due to the separation of correlation peaks by the chemical shifts of the two nuclei. Additionally, 2D-NMR experiments have proven highly effective at identifying novel and known metabolites that correlate with changes in genotype or phenotype, and I conducted the 2D-NMR experiment to further resolve the chemical's structure of compound (Rt:23.9 min) (**Figure 4.17**). The HRMS experimental (**Figure 4.18**) results indicate that the ion of the unknown compound may have four potential molecular formulas: C₁₈H₁₁O₈,

$C_{26}H_{27}O_9$, $C_{26}H_{28}ClO_9$, and $C_{27}H_{28}NaO_{11}$, with the most probable ion formula correspond to $C_{26}H_{27}O_9 [M-H]^-$. Thus the molecular formula could be $C_{26}H_{28}O_9$. After identifying the structural formula, I will combine it with the potential partial structures and functional groups determined from the analysis of 2D-NMR results. Subsequently, I will identify the compound by referring to extensive literature and comparing NMR data obtained under similar conditions to determine its precise name.

HRMS were performed on a Bruker maXis mass spectrometer by the SALSA platform from ICOA laboratory.

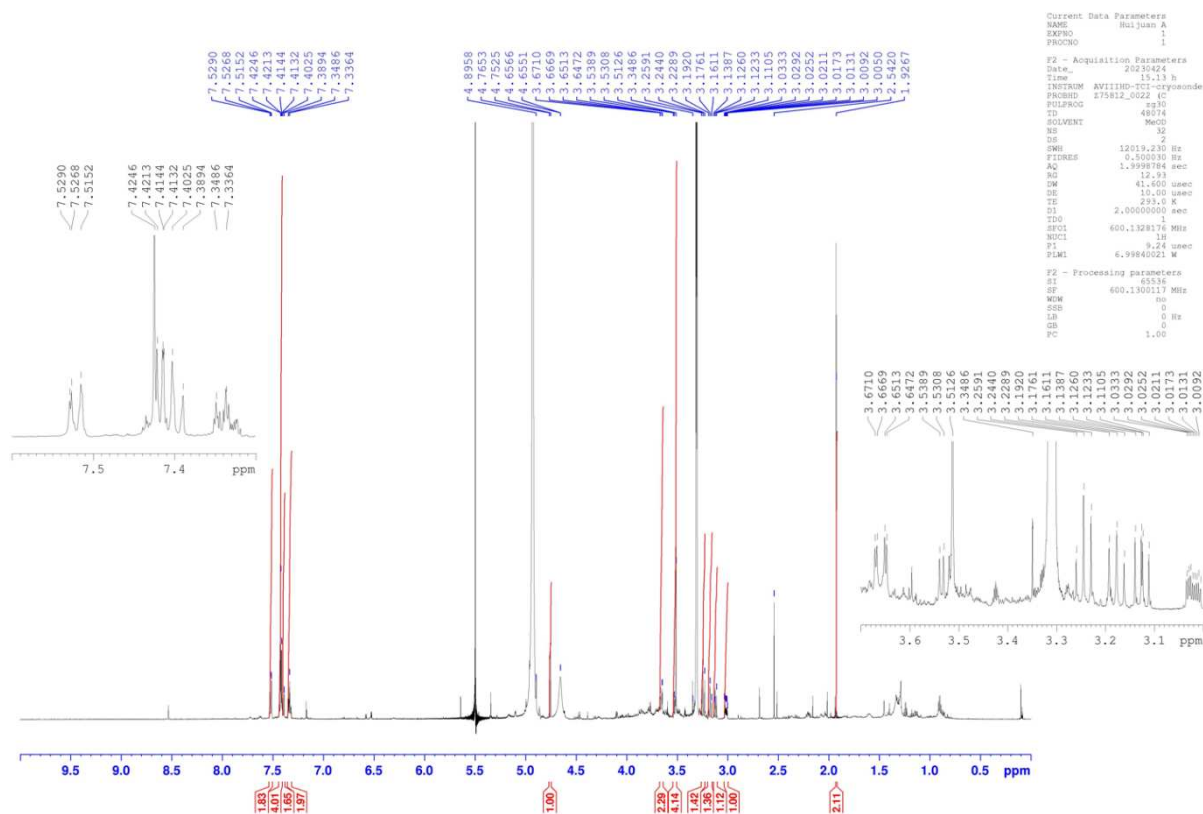


Figure 4.17. 1H NMR spectrum (600 MHz) of B compound.

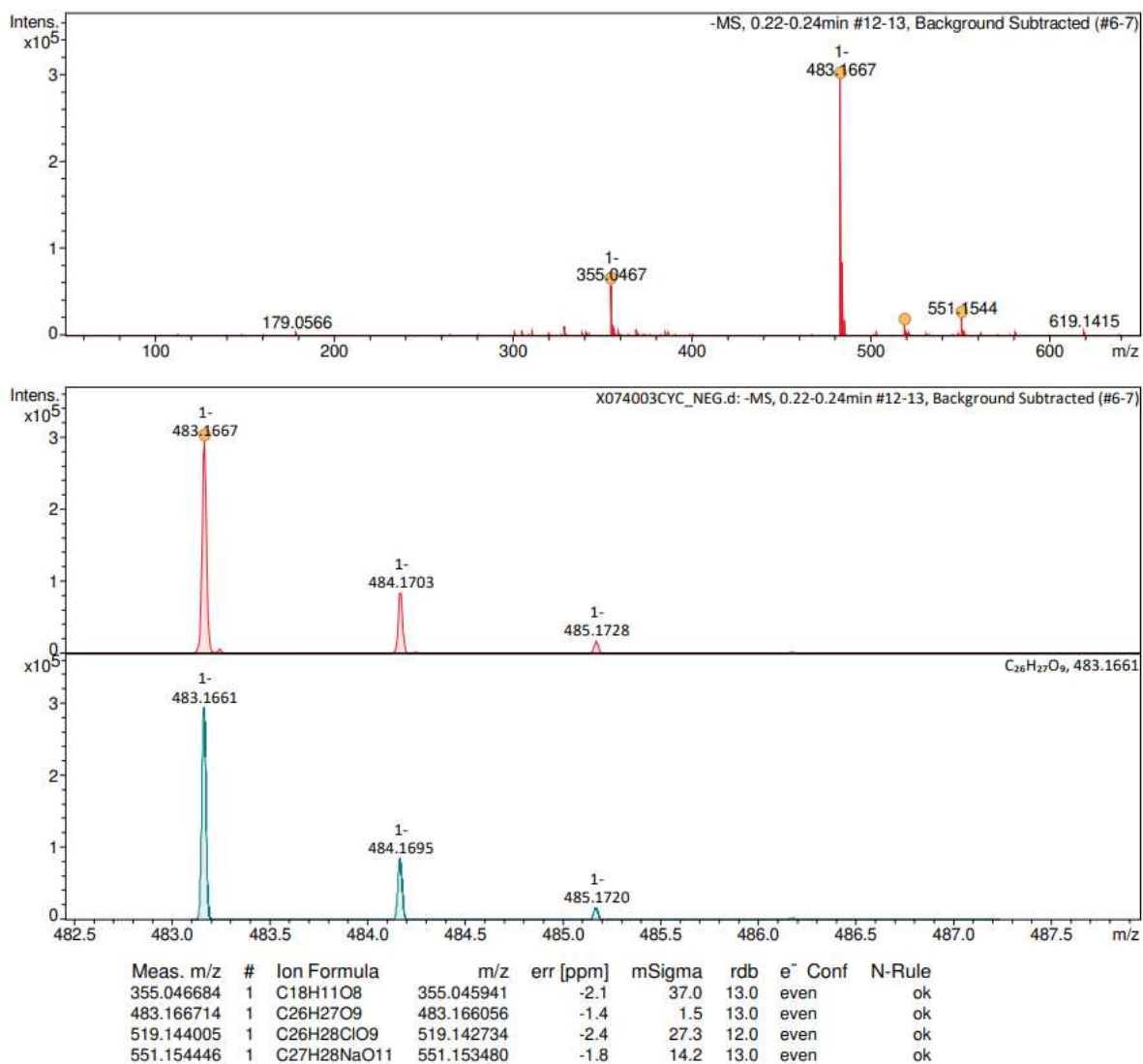


Figure 4.18. HRMS analysis of B compound.

4.1.3. Discussion

Fungal secondary metabolites (SMs) constitute a class of natural compounds crucial for fungal diversification and adaptation to specialized ecological niches. These SMs are of significant interest in various biotechnological and pharmaceutical applications, often serving as starting points for the discovery of novel drugs. Fungal SMs encompass a wide range of compounds that can either benefit or harm human health, exemplified by *Aspergillus*' production of the antibiotic penicillin and the carcinogenic aflatoxin precursor, sterigmatocystin (ST). Biosynthesis of SMs may be tightly regulated. Their expression was often triggered by chemical or environmental stimuli and coordinated with the development and morphogenesis of the producing organism. Secondary metabolic pathways are often tightly correlated with the fungal developmental program and response to external cues including light. *LaeA* is a non-specific gene encoding a global transcription factor, and it is a component of the velvet complex, which orchestrates development and SM production in response to light conditions. *LaeA* has been shown to influence the expression of approximately half of the SM BGCs in other fungi. Consequently, modulating the expression of this protein will inevitably lead to changes in the SM profile.

In this chapter, we focused on the critical role of *LaeA*, a global regulator within the velvet complex, in *P. anserina*. The deletion of *PaLaeA* led to significant changes in colony morphology, a substantial increase in spermatia production, the loss of female fertility, heightened sensitivity to oxidative stress, and the induction of a pink pigment production. These findings indicate that *PaLaeA* plays a crucial role in governing vegetative growth, morphogenesis, sexual reproduction, oxidative stress response, and pigmentation in *P. anserina*.

Filamentous fungi have been recognized for their ability to synthesize a diverse array of natural pigments, including melanins, carotenoids, flavins, phenazines, quinones, and more⁶⁰. Pigmentation is typically regarded as the result of secondary protective mechanisms that help

fungi cope with challenging environmental factors like oxidative stress, light exposure, and unfavorable conditions²³⁶. In numerous fungi, melanization serves as a protective shield against a wide range of environmental stressors, encompassing oxidative stress, UV radiation, and temperature fluctuations. Some fungal pigments are natural products associated with developmental structures, the most common being melanins. Melanins are generally dark brown pigments formed by oxidative polymerization of phenolic compounds and are synthesized during spore formation for deposition in the cell wall. Melanin biosynthesis has been well studied in pathogenic fungi, where the pigment not only contributes to the survival of the fungal spore by protecting against damaging UV light but is also an important virulence factor.

We can speculate that the dark green pigment is linked to melanin biosynthesis; however, additional experiments are required to conclusively identify these pigments. Sterigmatocystin is the penultimate precursor in the aflatoxin biosynthetic pathway. Genes required for the synthesis of aflatoxin and sterigmatocystin are well conserved between aspergilli and are located in large gene clusters. The relative order and transcriptional direction of some of the homologous gene pairs though are not conserved. Thus far, most of the genes in the respective clusters have been shown to encode enzymes required for toxin biosynthesis. At least two genes, *aflR* and *aflJ*, play a regulatory role. Several studies of *aflR* gene have demonstrated it encodes a sterigmatocystin/aflatoxin pathway-specific transcription factor. *AflR* deletion mutants in all three *Aspergillus spp.* do not express biosynthetic genes in the sterigmatocystin-aflatoxin cluster nor do they produce sterigmatocystin or aflatoxin. Loss of *aflR* does not stop spore or sclerotial production but does affect the numbers of spores and sclerotia formed. The function of *aflJ* is not well defined, but disruption of *aflJ* in *A. flavus* results in a failure to produce aflatoxin.

Based on our current knowledge, DHN-melanin plays a central role in giving *P. anserina* its characteristic greenish colony appearance on standard M2 medium²⁶⁷. Under normal conditions, the *P. anserina* WT strain exhibits a greenish hue on M2 medium due to melanin accumulation, with only minimal red-pink pigmentation observed on LB plates. Therefore, we believe that *P. anserina* possesses at least two pigment biosynthetic pathways, one responsible for melanin synthesis and the other for pink pigment production. In our team's research on PaNsdD³⁰⁹, we established that PaNsdD serves as a negative regulator of both the melanin pathway and the pink pigment pathway. We demonstrated the interplay between PaNsdD and the sterigmatocystin biosynthetic pathway. The deletion of *PaNsdD* enhanced red-pink pigment biosynthesis, which only occurred in the presence of the core polyketide synthase (PKS)-encoding gene, *PaStcA*, of the sterigmatocystin pathway, suggesting PaStcA's essential role in this process. These findings align with prior research emphasizing the need for a functional ST pathway for red-pink pigmentation. In this study, we discovered that the pink pigment produced due to the deletion of *LaeA* and the pink pigment generated from *NsdD* deletion are distinct compounds. Consequently, we concluded that LaeA is associated with a different signaling pathway for pink pigment production compared to the pathway in which NsdD participates.

It is widely acknowledged that *P. anserina* lacks asexual reproduction²⁵⁵. Furthermore, the fertilization capacity of ascospores is limited, resulting in an extremely low observed germination rate. Consequently, the abundance of spermatia in the strain does not necessarily indicate a high sexual fertility rate. In this chapter of our research, our data indicate that PaLaeA serves as a positive regulator of sexual development in *P. anserina*. Furthermore, we have demonstrated that PaLaeA functions as a repressor of male gamete production. It is intriguing to note that the findings regarding the role of PaLaeA in the sexual reproduction of *P. anserina* closely mirror our experimental results regarding the function of PaNsdD in the sexual reproduction of *P. anserina*³⁰⁹.

Carbon sources can influence secondary metabolism, as the production of natural products is likely regulated by global regulators, which, in turn, are controlled by the type of carbon source available³¹⁰. Carbon sources have been documented to exert an influence on the production of the trichothecene deoxynivalenol and the polyketide fusarielin H in *F. graminearum*³¹¹. In this study, culturing the *OE-PaLaeA* strain on a medium with an altered carbon source revealed a significantly distinct phenotype compared to M2 medium. In contrast, WT and $\Delta PaLaeA$ strains exhibited nearly identical phenotypes. This suggests that *LaeA* is involved in the carbon source absorption signaling pathway, likely playing a negative feedback regulatory role.

Stress response assays in fungal biology are indispensable, providing insights into environmental adaptation, pathogenicity, antifungal drug development, secondary metabolite production, biotechnological applications, ecological roles, genetic and molecular mechanisms, and evolutionary history. These assays reveal the complex world of fungal stress adaptation and its far-reaching implications. MG primarily arises as a cytotoxic by-product of glycolysis. It has been documented that MG depletes intracellular glutathione, thereby inducing the production of reactive oxygen species. An excess of MG can increase ROS production and cause oxidative stress and thereby is commonly used in phenotypic assays. In this study, the $\Delta PaLaeA$ strain displayed reduced resistance under MG-induced oxidative stress conditions, rendering it more sensitive to this stress than the WT strain. Similarly, when exposed to oxidative stress in the presence of TBH, the $\Delta PaLaeA$ strain also exhibited diminished resistance, rendering it more susceptible than the WT to the oxidative stress provoked by these compounds. Sorbitol-induced osmotic stress inhibited growth but maintained a circular pattern with smooth edges in the WT and *OE-PaLaeA* strains, while the $\Delta PaLaeA$ strain was minimally affected. However, CongoRed introduction caused sluggish growth and inhibited black deposit production in $\Delta PaLaeA$. The $\Delta PaLaeA^{comp}$ strain restored WT levels of tolerance, indicating

that loss of PaLaeA is responsible for this phenotype, thereby indicating that PaLaeA plays an important protective role in regulating the stress response of *P. anserina*.

Confrontation with other fungi can unveil fungal biology, ecological roles, and adaptations. It offers ecological insights into dynamics like nutrient cycling and decomposition, contributes to disease management strategies, optimizes biotechnological processes for enzyme and pharmaceutical production, and is crucial for discovering novel therapeutics, particularly against drug-resistant fungal infections. In this study, we characterized antagonistic interactions between the *P. anserina* WT and mutants by measuring peroxide production and cell death differences. Results indicated that when the *OE-PaLaeA* strain confronted competitors like *A. nidulans*, *P. chrysogenum*, *C. cinerea*, and *T. versicolor*, it displayed increased peroxide levels in the mycelium and confrontation zone. Peroxide experiments revealed the $\Delta PaLaeA$ mutant produced less peroxide than the WT, while the *OE-PaLaeA* strain generated more, suggesting PaLaeA's role in a global competitive response. Yet, the trypan blue method showed no significant differences in cell death between mutants and the WT during confrontations with antagonistic strains.

HPLC-UV-MS experiments demonstrated significantly higher metabolite production in the $\Delta PaLaeA$ strain compared to the WT at specific peaks (21.2, 23.9, and 30.8 minutes) on the 12th day of culture. Hence, identifying the primary compounds in the $\Delta PaLaeA$ strain is essential. The solid that couldn't dissolve in methanol was analyzed using TLC and NMR, confirming it as sterigmatocystin (ST). After isolating the compound (Rt: 21.2 min), NMR analyses were performed, suggesting it might be secosterigmatocystin due to a matching molecular mass (362.1002) and formula ($C_{18}H_{18}O_8$) found in the literature. Through HRMS testing with chloroform and methanol solvents, definitively identifying it as secosterigmatocystin. To characterize the compound isolated at 23.9 minutes, NMR experiments were performed at 300MHz and 600 MHz to elucidate its structure. HRMS results

suggested four potential molecular formulas: $C_{18}H_{11}O_8$, $C_{26}H_{27}O_9$, $C_{26}H_{28}ClO_9$, and $C_{27}H_{28}NaO_{11}$, with the most likely formula being $C_{26}H_{27}O_9$. Upon identifying the structural formula, it will be combined with potential partial structures and functional groups from 2D-NMR analysis. Further identification will involve extensive literature comparison and NMR data analysis under similar conditions to determine its precise name.

By investigating the regulatory mechanisms and the role of the key global regulator LaeA, we've gained insights into fungal adaptation, ecological functions, and survival strategies under diverse environmental conditions. Notably, this research offers practical applications, as the identification of bioactive compounds opens doors to developing new therapeutics for addressing drug-resistant pathogens. It underscores the pivotal role of fungal secondary metabolites in ecology, biotechnology, and healthcare. As we continue our journey into fungal biology, further revelations in the realm of secondary metabolites promise to make lasting contributions to science and society.

4.2. Environmental signal PaHapX and PaCreA

4.2.1. Introduction of PaHapX

Iron is vital for almost all organisms, serving as a cofactor in various cellular processes, including oxidative phosphorylation and oxidative stress detoxification³¹². However, excessive iron is toxic to cells. Hence, iron homeostasis is meticulously regulated to preserve the delicate balance between iron uptake, storage, and utilization³¹³. HapX is the major regulator for adaptation to iron starvation and stimulates the expression of genes involved in iron uptake, including the siderophore system, and represses genes involved in iron consuming pathways, like the tricarboxylic acid (TCA) cycle, respiration or heme biosynthesis.

Iron homeostasis in filamentous fungi, such as *A. nidulans* and *A. fumigatus*, relies on two transcription factors: HapX, a bZip TF, and SreA, a GATA zinc finger TF which are interconnected by a negative regulatory feedback loop³¹⁴⁻³¹⁶. During iron starvation, HapX inhibits SreA-mediated iron usage pathways and activates siderophore production for iron uptake³¹⁶⁻³¹⁸. Conversely, during iron sufficiency, SreA represses HapX and siderophore-driven iron acquisition³¹⁴. During periods of iron abundance, the Cys2-Cys2-type GATA zinc finger transcription factor SreA reduces the expression of HapX and various other genes by attaching itself to a particular motif within the promoter region. SreA inhibits the production of siderophores and the uptake of iron through reductive assimilation to prevent iron overload in iron-rich conditions³¹⁹. In times of iron scarcity, the CCAAT binding complex (CBC)-HapX complex suppresses processes that consume iron, such as heme biosynthesis, the tricarboxylic acid cycle, and respiration, in order to conserve iron. Conversely, it stimulates reductive iron assimilation, siderophore biosynthesis, and siderophore uptake to facilitate iron acquisition^{315,316,320}. HapX also interacts physically with the CCAA T-binding complex and is crucial for iron detoxification by inducing expression of the vacuolar iron importer CccA under

excess iron conditions^{318,321}. In previous studies, genome-wide transcriptional profiling demonstrated that under conditions of iron deficiency, HapX activates genes associated with iron acquisition, such as *mirB* encoding siderophore transporters, while concurrently repressing *CccA*, responsible for encoding the vacuolar iron transporter, along with several other genes involved in iron-consuming processes³¹⁶. It has been reported that iron resistance in *A. fumigatus* is linked to SreA-mediated inhibition of iron uptake and the regulation of vacuolar iron storage through the action of the vacuolar iron importer, CccA³²¹. Furthermore, it has been confirmed that HapX functions as a Janus-type transcription factor, playing a dual role in regulating CccA, and thereby influencing vacuolar iron storage, contingent upon the prevailing iron levels in the environment³¹⁸.

In cells, gene transcription is regulated by various TFs, including the basic leucine zipper (bZIP) family³²². HapX, a bZIP-type TF, interacts with the CCAAT binding complex (CBC) at its N terminus, similar to Hap4's role in *S. cerevisiae*, essential for respiration transcription^{323–325}. The CBC, a heterotrimeric DNA-binding complex, exhibits conservation across all eukaryotic organisms. In *A. nidulans*, the CBC comprises HapB, HapC, and HapE subunits and recruits HapX. Across *Aspergillus* species, the CBC has diverse roles in metabolism and development. In *A. fumigatus*, HapX regulates iron homeostasis, aiding fungal survival under iron stress by repressing iron-consuming pathways and activating siderophore synthesis. Its loss reduces *A. fumigatus* virulence in host environments^{316,318,326}. HapX's roles are also seen in other fungal species, including *A. nidulans*³²³, *Arthroderma benhamia*³²⁷, *A. terreus*³²⁸, *Verticillium dahliae*³²⁹, *Candida albicans*³³⁰, *Fusarium oxysporum*³¹⁷, and *Cryptococcus neoformans*³³¹. These roles encompass adaptation to iron starvation and resistance, except for *C. albicans*. In *A. fumigatus* and *C. albicans*, HapX directly represses iron-consuming genes during iron starvation, verified by chromatin immunoprecipitation^{318,330}. HapX is absent in

humans, and a genome wide analysis revealed the presence of 5,000 to 15,000 CBC-binding sites, with the exact number depending on the type of human cell under investigation³³².

Understanding how fungi regulate iron uptake and utilization can provide insights into potential antifungal therapies. Due to its importance in fungal physiology and potential implications for human health, HapX has been the subject of research in various fungal species. Scientists study its function to better understand fungal pathogenesis and potentially identify targets for antifungal drug development. Remarkably, until now, the mechanism and role of *HapX* gene in regulating iron in *P. anserina* have not been elucidated.

4.2.2. Introduction of PaCreA

The *CreA* (catabolite responsive element) gene, alternatively named *Cre1* in certain fungal species, is crucial in governing carbon catabolite repression (CCR) in fungi. CreA significantly influences the transcriptional regulation of transport, fungal carbon/nitrogen metabolism, and its loss triggers the activation of host defense mechanisms. CCR is a regulatory mechanism in fungi that prioritizes the utilization of easily metabolizable carbon sources like glucose when multiple carbon sources are available. This ensures efficient carbon utilization and energy production while inhibiting the use of more complex carbon sources such as cellulose or lignin. When the preferred carbon source (e.g., glucose) becomes scarce or is absent, CreA's repressive action is lifted.

Transcriptional control tightly manages cellulolytic enzyme production in filamentous fungi, primarily via CCR in the presence of preferred sugars like glucose³³³. In cellulolytic fungi such as *Trichoderma*, *Neurospora*, *Aspergillus*, and *Penicillium*, CCR hinges on the transcription factor CreA. CreA, a C2H2 zinc finger protein, binds to promoters, notably those repressed by glucose or xylose³³⁴⁻³³⁶, like the 5'-SYGGRG-3' motif in *A. nidulans* and *T. reesei*. These interactions inhibit genes encoding xylanases (e.g., *xlnA*, *xlnB*, *xlnD*) and cellulases (e.g.,

cbh1/cel7A, *cbh2/cel6A*, *eg2/cel5A*)^{333,337–339}. Cre1/CreA's subcellular location adjusts according to glucose levels and phosphorylation, crucially affecting its regulatory role^{340–342}.

Cellulolytic gene induction relies on escaping CCR. Deleting, truncating, or using multisite-directed mutagenesis on the *CreA* gene can alleviate CCR, enhancing the expression of key cellulolytic genes across diverse carbon sources, including glucose, lactose, sorbose, cellulose, or a mix of plant polymers^{343–346}. For instance, *CreA* deletion or truncation in *T. reesei* WT strain QM6a leads to cellulase and hemicellulose overproduction when cultivated in glucose-containing media³⁴⁷. The hyper cellulolytic *T. reesei* strain RutC30, capable of cellulase and hemicellulose production in glucose-based medium, possesses a truncated TrCRE1³⁴⁸. Similarly, *Penicillium oxalicum* JU-A10-T, another cellulolytic enzyme hyperproducer, exhibits a frameshift mutation at the C-terminus of *PoCreA*, negatively influencing cellulolytic gene expression under both repressed (glucose) and induced (cellulose) conditions³³⁴. In *A. nidulans*, truncating *CreA* led to the *CreA*^{d30} strain, resulting in a substantial decrease in intracellular trehalose levels³⁴⁹. A similar impact on cellular trehalose concentrations was also observed in the *Aspergillus fumigatus* Δ *CreA* strain³⁵⁰. Moreover, CreA homologs play a role in regulating glycogen metabolism in fungi. Deleting nonessential *CreA* gene in these fungi causes glycogen to accumulate excessively^{350–352}. In *A. fumigatus*, an initial *in silico* analysis identified a potential CreA binding site in the 5' untranslated region (UTR) of the glycogen debranching enzyme-encoding *gdbA* gene, which exhibited significantly reduced expression in the Δ *CreA* strain^{341,350}. Additionally, in *A. fumigatus*, increased intracellular glycogen accumulation is regulated by the protein kinase A (PKA) pathway, suggesting that PKA may be involved in the direct or indirect regulation of CreA³⁵⁰.

Moreover, CreA plays a pivotal role in various fungal processes, encompassing SM production, asexual development, fungal virulence, glycogen metabolism, and circadian rhythms^{352–355}.

CreA regulation involves (i) carbon source-dependent nuclear localization, (ii) protein-protein interactions, and (iii) posttranslational modifications. Notably, CreA's correct cellular localization alone is insufficient for effective repression^{356–358}, suggesting that posttranslational modifications and interactions with corepressors such as RcoA and SsnF likely play crucial roles in achieving its full functionality^{359–361}.

The CreA-mediated CCR pathway plays a pivotal role in fungal growth, adaptation, and survival across diverse environments, allowing fungi to fine-tune their metabolism in response to varying carbon availability. This understanding of *CreA* gene regulation and the CCR pathway has significant implications for biotechnological applications. Researchers can utilize this knowledge to enhance the efficiency of fungal processes, such as biofuel production and industrial enzyme synthesis, by directing carbon utilization toward specific desired products.

4.2.3. Results

4.2.3.1. Generation of deletion and overexpression mutants of *PaHapX*

Generation of primary transformations for $\Delta PaHapX$

Targeted gene deletion was performed as previously described³⁶². Two fusion amplicons containing phleomycin cassette were transformed into protoplasts of *Amus51::nour* strain. In total, 2 transformants were screened on M2 medium containing phleomycin. As shown in **Table 4.2**, 2 of 2 transformants were confirmed by junction PCR verification (**Figure 4.19**), indicating the correct occurrence of homologous recombination. And then 2 independent primary transformants were selected to cross with WT strain, in order to outcross *mus51::nour* marker and obtain homokaryotic phleomycin-resistant progeny. Forty ascospores were fetched from each progeny and then were screened by corresponding antibiotics. As shown in **Table 4.3**, five candidate strains with ideal resistance (Nour^SPhleo^R) were screened out in the progeny of T1 and T2 respectively. Single-core *mat+* and *mat-* mutant strains of T1 and T2 were subsequently

selected through hybridization experiments with the WT. Subsequently, Southern blot experiments were conducted to confirm the T1 and T2 transformants. The results can be found in Appendix 2 (Figure S1).

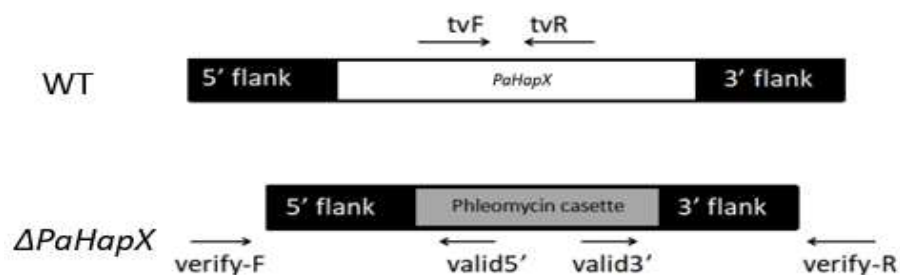


Figure 4.19. Schematic representation of the construction and verification of the deletion of *PaHapX* gene, along with the indicated primers used for confirming the transformants.

Table 4.2. Junction verification for primary transformants

Primers	Transformants	
	T1	T2
vF+valid5'	Yes	Yes
valid3'+vR	Yes	Yes

Yes: successful PCR amplification; No: no PCR amplification.

Isolation of homokaryon

Table 4.3. Resistance selection of targeted ascospores.

Number of spores	Transformants	
	T1	T2
Picked-up spores	40	40
Germinated spores	38	35
Resistance selection		
N ^{RPS} and N ^{SPS}	29	23
N ^{RPR}	4	7
N ^{SPR} (target)	5	5

N: nourseothricin; P: phleomycin; R: resistant; S: sensitive; M2N: M2 medium containing nourseothricin; M2P: M2 medium containing phleomycin.

Construction of overexpressed mutant *OE-PaHapX*

Despite numerous attempts, to construct the *OE-PaHapX* mutant, as described in Chapter 2, it was not successful. We dedicated almost two and a half years to constructing the *OE-PaHapX* mutant, encountering various challenges along the way. Different approaches were exhaustively explored, including primer redesign, PCR condition optimization, promoter variations, polymerase brands, DNA purification and concentration enhancement, multiple protoplast updates for transformation, reagent reconfiguration, stringent environmental controls, and experimenting with various transformation methods by thoroughly reviewing the literature. Changes in the amplification and cloning strategy are being studied to obtain this overexpression.

4.2.3.2. Generation of deletion and overexpression mutants of *PaCreA*

Generation of primary transformations for $\Delta PaCreA$

Two fusion amplicons containing the geneticin cassette were introduced into the protoplasts of the $\Delta mus51::nour$ strain (**Figure 4.20**). A total of 7 transformants were selected on M2 medium supplemented with geneticin. According to **Table 4.4**, junction PCR verification confirmed that 2 out of the 7 transformants had undergone correct homologous recombination. Subsequently, 2 independent primary transformants, T4 and T6, were chosen to mate with the WT strain to segregate the *mus51::nour* marker and generate geneticin-resistant homokaryotic progeny. From each progeny, 140 ascospores were collected and screened with the appropriate antibiotics. Unfortunately, as indicated in **Table 4.5**, none of the candidates from the T4 and T6 progeny exhibited the desired resistance phenotype (Nour^SGent^R). Despite repeating the experiment multiple times, we consistently obtained the same results. In summary, I was unable to obtain a *PaCreA* mutant strain. Therefore, it can be inferred that the absence of PaCreA is

likely lethal in *P. anserina*. Moreover, we have found reports in published articles suggesting that *CreA* can also be essential in other species, such as *A. nidulans* and *P. chrysogenum*^{335,363}.

To confirm the lethality of the *PaCreA* gene deletion, a new transformation experiment using a strain carrying an extra ectopic copy can be carried out. This will enable us to verify that this is indeed an essential gene, by analyzing the genotype of the offspring thus obtained.

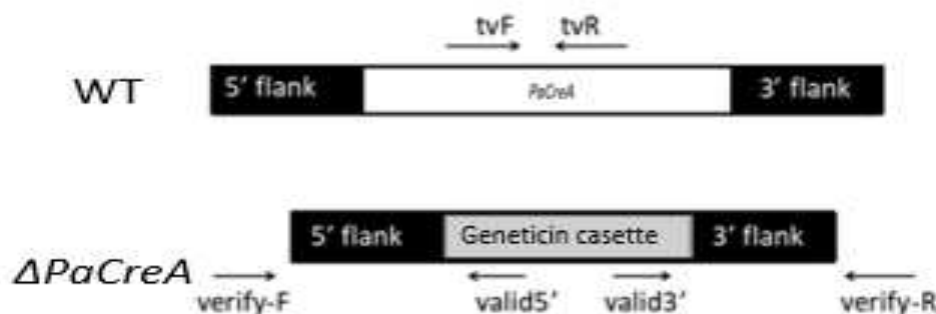


Figure 4.20. Schematic representation of the construction and verification of the deletion of *PaCreA* gene, along with the indicated primers used for confirming the transformants.

Table 4.4. Junction verification for primary transformants.

Isolation of homokaryon

Primers	Transformants						
	T1	T2	T3	T4	T5	T6	T7
vF+valid5'	No	No	No	Yes	No	Yes	Yes
valid3'+vR	No	No	No	Yes	Yes	Yes	No

Table 4.5. Resistance selection of targeted ascospores.

Number of spores	Transformants	
	T4	T6
Picked-up spores	140	140
Germinated spores	101	113
	Resistance selection	

$N^R G^S$ and $N^S G^S$	101	113
$N^R G^R$	0	0
$N^S G^R$ (target)	0	0

N: nourseothricin; G: geneticin; R: resistant; S: sensitive.

Construction of overexpression mutant *OE-PaCreA*

According to the overexpression plasmid construction method described in Chapter 2, we successfully obtained the *OE-PaCreA* plasmid. Subsequently, it was transformed into *P. anserina* protoplasts, and upon verification, the correctly expressed *OE-PaCreA* transformant was obtained (Figure 4.21).

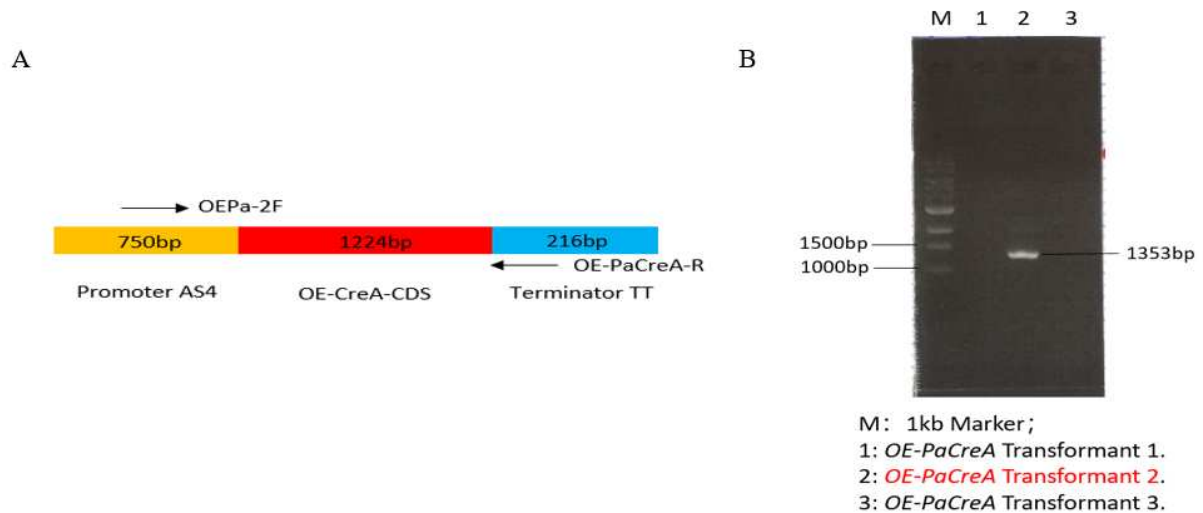


Figure 4.21. Construction of *OE-PaCreA* mutant. (A) Schematic overexpression plasmid construction principle. (B) Verification of overexpression plasmid transformants by PCR.

4.2.3.3. Impacts of PaHapX and PaCreA on vegetative growth and colony morphology

To evaluate the impact of PaHapX and PaCreA on vegetative growth, we performed a phenotypic analysis of both the WT and mutant strains. This analysis entailed incubating the

strains on both solid and liquid M2 media, all at the optimal growth temperature of 27°C. After 8 days of incubation, we compared and analyzed differences in colony size, morphology, and hyphal pigmentation among the $\Delta PaHapX$, *OE-PaCreA*, and WT strains. In our observations, we noted that when cultivated in M2 liquid medium, *OE-PaCreA* produced fewer hyphae than the WT strain (**Figure 4.22**). Furthermore, the WT exhibited a darker green hue in its hyphae, whereas *OE-PaCreA* displayed lighter green hyphae. However, no pronounced phenotypic distinctions were evident in the $\Delta PaHapX$ strain.

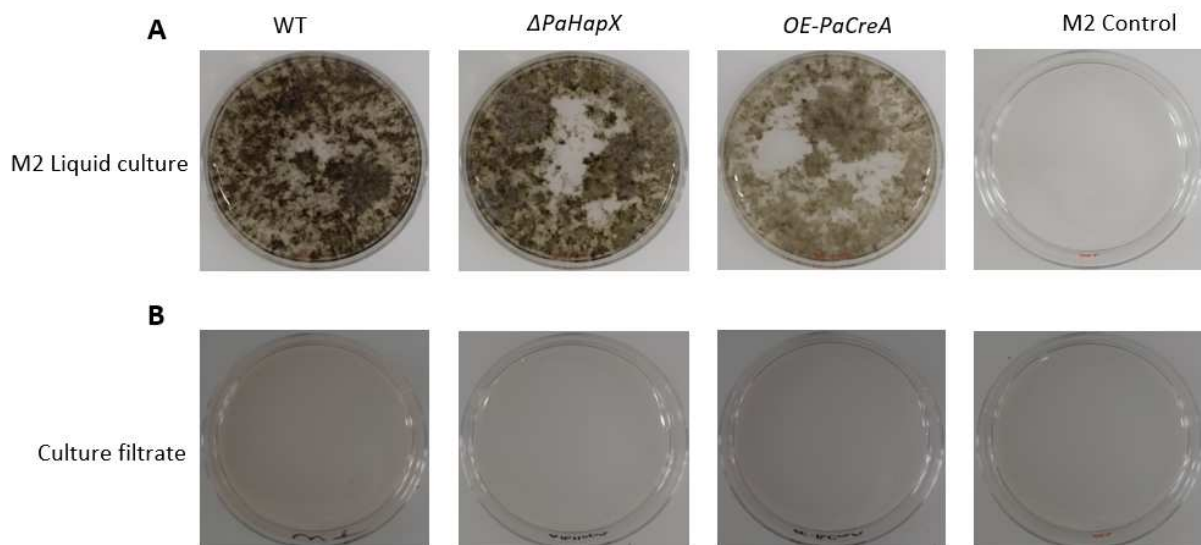


Figure 4.22. Vegetative growth of wild type and mutants. Growth of *P. anserina* strains on M2 liquid medium (A), Culture filtrate without mycelium (B). Pictures were taken after 8-days incubation at 27°C. Photograph the flat panel from a different angle to contrasting color differences. Pictures were taken after 8-days incubation at 27°C. WT: wild-type strain.

4.2.3.4. Role of PaHapX and PaCreA in sexual reproduction

To explore the potential influence of our targeted gene studies on *HapX* and *CreA* on the sexual development of *P. anserina*, we conducted fertility assays. These assays involved using a mixture of fragmented *mat+* and *mat-* mycelia from both wild-type and mutant strains, which were then inoculated on solid M2 medium. Through genetic sexual crosses, we aimed to determine whether PaHapX and PaCreA play a role in the sexual reproduction of *P. anserina*.

As depicted in Figure 4.23, the mutants displayed no discernible phenotype differences in comparison to the WT.

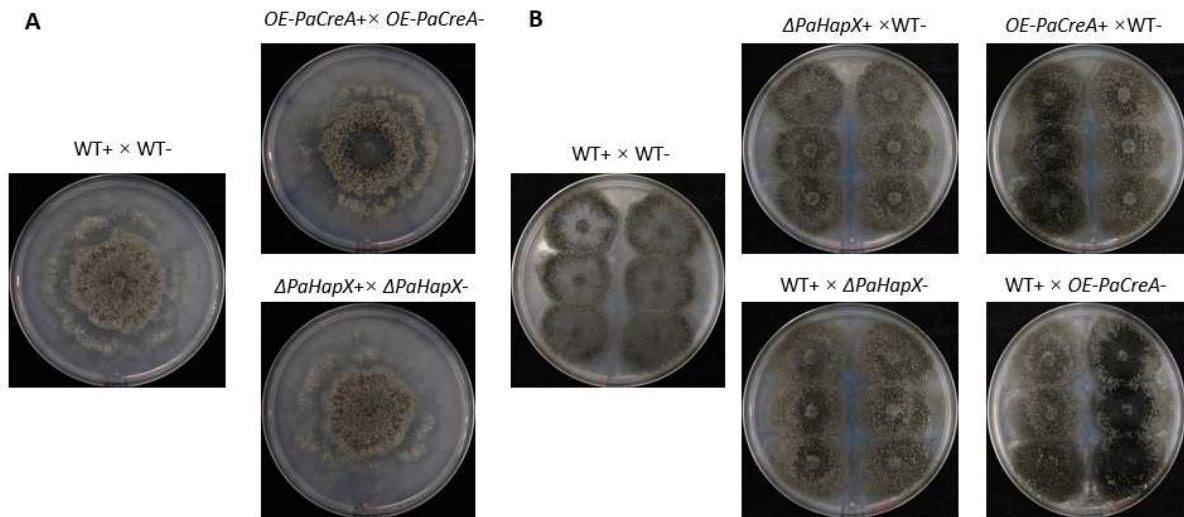


Figure 4.23. Sexual development of wild-type and mutant strains. (A) Heterokaryotic crosses assays. Fragmentated mycelia from *mat+* and *mat-* strains were mixed and then inoculated in the center of plate under constant light illumination. Photographs were taken after 8 days of incubation. (B) Watered confrontation crosses assays. Crosses were made by inoculating the strains 1.5 cm apart. After 3 days of growth, 1.5 ml of water was added and spread all over the plate. The pictures were taken 5 days after fertilization, at which time fully mature perithecia expel ascospores. Mature perithecia became apparent as small dots upon the completion of fertilization.

4.2.3.5. Effect of different carbon sources

As previously noted, altering the composition of the growth medium can trigger previously inactive SM pathways. To investigate this phenomenon, we used M0 medium (similar to M2 but lacking dextrin) and added different carbon sources to examine their impact on the growth and development of both mutant and WT strains. All experiments were conducted in triplicate. As depicted in Figure 4.24, the $\Delta PaHapX$ strain exhibited a modestly reduced growth rate on MAE and M0+Glycerol media relative to the WT strain. Conversely, the *OE-PaCreA* strain displayed a deeper green pigmentation on M3 medium and produced fewer

hyphae when cultured on M0+Whatman medium. Hence, it can be inferred that both HapX and CreA exert influence on the signaling pathway regulating *P. anserina's* carbon source absorption and utilization.

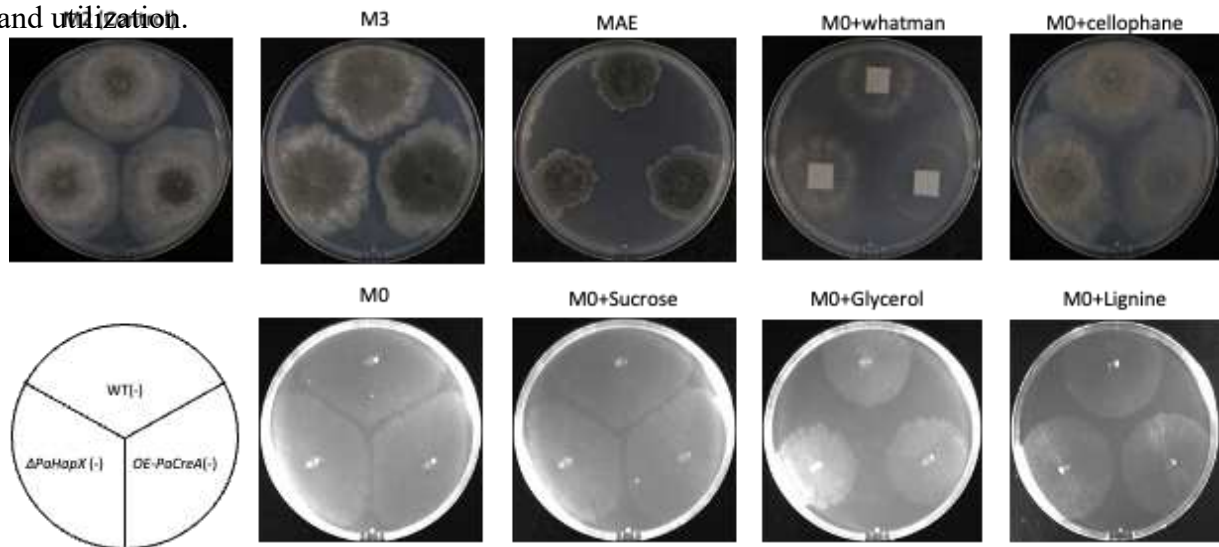


Figure 4.24. Colony morphology WT and various mutants. Some pictures were taken with a camera, and others were taken under gel imaging instrument. M0 is the base medium; M2 Medium=M0 medium + dextrin; M3 Medium=M0 medium + 5.5g/L glucose. Pictures were taken after 4-days incubation at 27°C.

4.2.3.6. Function of PaHapX and PaCreA in oxidative stress responses

Culturing the $\Delta PaHapX$ and *OE-PaCreA* strains on M2 medium supplemented with oxidative stressors (H_2O_2 , MG) revealed that the growth of the $\Delta PaHapX$ strain on M2+MG medium was significantly inhibited compared to the WT (**Figure 4.25**). In contrast, the growth phenotype of the *OE-PaCreA* strain closely resembled that of the WT. It seems that the $\Delta PaHapX$ strain appears to be more sensitive to oxidative stress. HapX could therefore be involved in the signaling pathways of this type of stress.

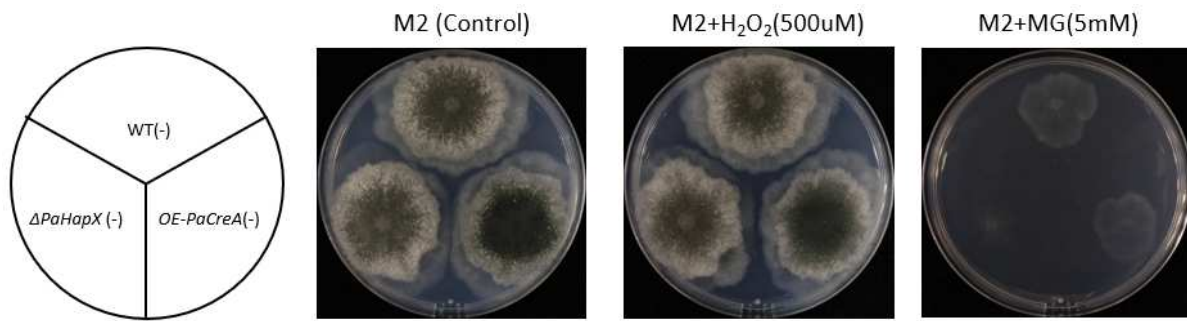


Figure 4.25. Growth of *P. anserina* strains on M2 plates exposed to oxidative stress (H_2O_2 , and methylglyoxal (MG)). Pictures were taken after 4-days incubation at 27°C.

4.2.3.7. Function of PaHapX and PaCreA in osmotic stress and wall-binding compounds responses

Cultivating the $\Delta PaHapX$ and *OE-PaCreA* strains in M2 medium supplemented with osmotic stressors (NaCl, KCl, sorbitol) and cell wall-binding compounds (CFW, Congo red) yielded distinct results compared to the WT. The *OE-PaCreA* strain displayed reduced hyphal production when cultured on M2+NaCl medium. The same thing is observed on the M2+KCl medium, but slightly attenuated. On the M2+Sorbitol medium, the diameter of the thallus is slightly larger, but there are fewer aerial hyphae. So, it seems that CreA is implicated in response to the osmotic stresses but that the answer still depends on the exact nature of the stress, ionic or metabolic. In the presence of calcofluor, the growth of both strains seems unaffected. On M2+Congo medium, the growth of $\Delta PaHapX$ seems slightly reduced compare to WT. The thallus diameter of *OE-PaCreA* is similar to that of WT, but there are fewer aerial hyphae. (**Figure 4.26**).

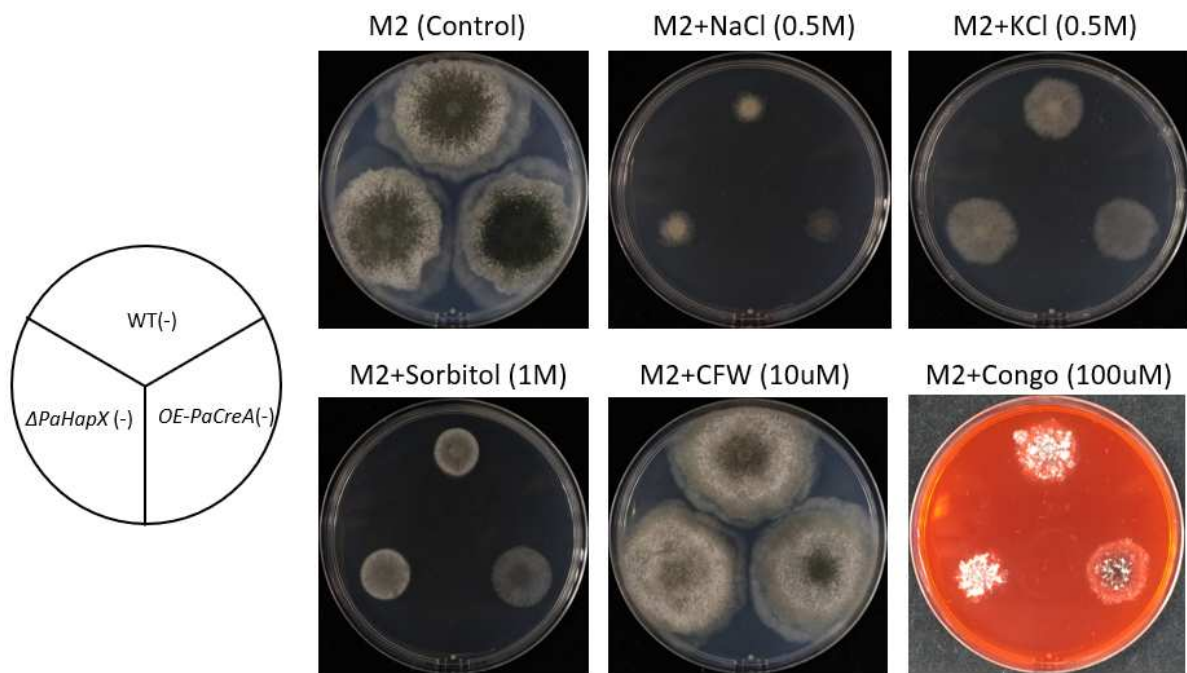


Figure 4.26. Growth of *P. anserina* strains on M2 plates exposed to osmotic stress (NaCl, KCl, Sorbitol) and cell wall-binding compounds (calcofluor white (CFW), CongoRed). Pictures were taken after 4-days incubation at 27°C.

4.2.3.8. Contribution of PaHapX and PaCreA in interspecific confrontations

In the confrontation experiments involving $\Delta PaHapX$ and *OE-PaCreA* strains with other fungal strains, our peroxide detection and cell death tests revealed distinctive outcomes. When *OE-PaCreA* confronted *P. chrysogenum*, *A. fumigatus*, *M. circinelloides*, and *T. versicolor*, it exhibited a marginal increase in peroxide production. In contrast, $\Delta PaHapX$ consistently maintained a phenotypic profile indistinguishable from that of the WT (**Figure 4.27**). Similarly, our cell death test experiments demonstrated that the phenotypes of $\Delta PaHapX$ and *OE-PaCreA* closely resembled those of the WT. Under the conditions tested, HapX do not appear to be involved in hyphal interference, conversely, there may be a slight involvement of CreA in the face of certain competitors.

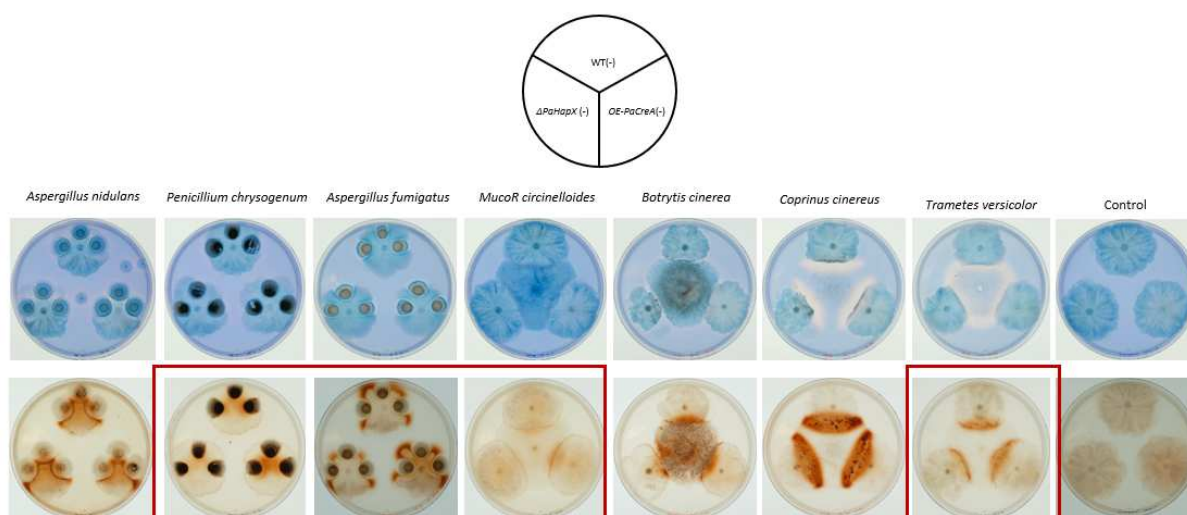


Figure 4.27. Confrontation of *P. anserina* strains other fungal species. Cell death assay and peroxide detection with Diaminobenzidine (DAB) were conducted as previously described.

4.2.3.8. Metabolomic analysis of the different *PaHapX* and *PaCreA* mutants

As in the case of *LaeA* mutants, metabolomic analyses are essential to fully characterize the involvement of HapX and CreA in MSs biosynthesis in *P. anserina*. They will be carried out as soon as all the mutants have been obtained.

4.2.4. Discussion

A connection between AreA and the global carbon catabolite repressor, CreA, has been identified in *A. nidulans*, particularly in relation to arginine catabolism³⁶⁴. Additionally, lactose has been employed as a substrate for penicillin production in *P. chrysogenum*³⁶⁵. Research on *HapX* and *CreA* genes in *P. anserina* has multifaceted importance. These genes provide critical insights into fungal physiology, growth, and adaptation to changing environments. They are closely tied to the regulation of secondary metabolites, offering the potential for discovering bioactive compounds with applications in various fields.

In this party study, we have set out to investigate the function of the HapX and CreA transcription factor in *P. anserina*. We successfully acquired the $\Delta PaHapX$ and *OE-PaCreA* mutants and confirmed that the deletion of *CreA* could potentially lead to lethality in *P. anserina*. During the phenotypic analysis experiment, we observed significant differences in the phenotypes of the $\Delta PaHapX$ and *OE-PaCreA* mutants when compared to the WT. Other experiments are still needed to complete the characterization of the mutants obtained, such as metabolomic analyses.

Chapter 5

Conclusions and perspectives

In this research background paper, we delve into the core of fungal SM in *P. anserina*. Like other coprophilous fungi, *P. anserina* has the potential to produce bioactive SMs due to its highly competitive habitat in dung. In my thesis, I emphasize an interdisciplinary approach to characterizing SM clusters and discovering natural products in the filamentous fungus *P. anserina*. The study's main goal is to unlock the secrets of fungal metabolism and its complex regulations, offering insights into fungal physiology and the discovery of new compounds with potential applications in healthcare. My thesis work involved the study of general cellular factors, NsdD, LaeA, CreA, and HapX, looking at the impact of their modulation on *P. anserina* physiology and MS biosynthesis.

In the **initial chapter**, a comprehensive overview of fungal SMs is provided, setting the foundational framework for this study. The **second chapter** meticulously outlines the experimental methodologies that have been applied in the course of this investigation. The **third chapter** delves deeply into my active involvement in research topics centered around PaNsdD and also encompasses the presentation of published research findings in this context. **Chapter 4** represents the crux of my research effort, offering a detailed examination of SMs in *P. anserina*, particularly under the influence of three carefully selected environmental signal regulators (LaeA, HapX, CreA), a pivotal aspect of my project.

In terms of our research outlook, our future investigations will be twofold. Initially, we will persist in our literature review endeavors, aiming to procure more comprehensive insights into the compounds exhibiting heightened expression in $\Delta PaLaeA$ mutants (Rt: 23.9 min).

Secondly, our ongoing focus will be on the construction of *OE-PaHapX* mutant, followed by extensive phenotypic analyses encompassing aspects such as vegetative growth, colony morphology, sexual reproduction, responses to various carbon sources, performance under multiple stress conditions, and contributions in interspecific confrontations. Simultaneously, we will conduct in-depth metabolite analyses to further enrich our understanding of these mutants.

By delving into the intricate world of secondary metabolites (SMs) within the fungus *P. anserina*, this research not only contributes to our understanding of fungal physiology but also opens doors to the discovery of novel bioactive compounds. The study offers insights into the roles of SMs and their impact on fungal adaptation and survival, shedding light on the broader realm of fungal biology. Moreover, the potential isolation of new bioactive metabolites has significant implications for healthcare, with the possibility of identifying compounds with therapeutic properties. As the paper unfolds, it is expected to provide valuable knowledge and catalyze further research, making it an important contribution to the fields of mycology, biotechnology, and medicine.

In conclusion, this research background paper lays a strong foundation for the study of fungal secondary metabolites, particularly in the context of *P. anserina*. It highlights the importance of understanding the global regulation of secondary metabolites and the role of key regulators. The exploration of these areas is anticipated to contribute to our knowledge of fungal biology, ecosystem dynamics, and potential applications in healthcare and other fields.

Appendix

Appendix 1: List of clusters in *P. anserina*

Table S1: List of clusters identified by antiSMASH in *P. anserina* December 2023 update.

Chromosome	activity	nt start	nt end	Cluster size (Kb)	Pa start	Pa end	Key gene	Cluster numbered	Compound
1	NRPS-like	1795428	1839095	44	1_5028	1_5190	1_5110	34	
	NRPS-T1PKS	1826608	1878895	52	1_5150	1_5300	1_5210	35	
	T1PKS	4090203	4138115	48	1_11790	1_11940	1_11870	26	
	RIPP-like	4859943	4920633	61	1_13290	1_13120	1_13200	43	
	terpene	5380548	5402453	22	1_15230	1_15260	1_15240	25	
2	T1PKS	492901	540969	48	2_498	2_570	2_510	29	melanin (PKS1)
	terpene	536100	558200	22	2_560	2_630	2_610	30	
	T1PKS	2766271	2815007	49	2_6780	2_6910	2_6850	31	
	T1PKS	2909500	2966754	57	2_7220	2_7450	2_7410	32	Sterigmatocystin
	NRPS	3069481	3119984	51	2_7785	2_7950	2_7870	33	
3	T1PKS	1298663	1347323	49	3_1930	3_2050	3_1990	27	
	NRPS	4459405	4505454	46	3_11150	3_11240	3_11200	1	metachelin ?
	Terpene	4589391	4613055	24	3_10830	3_10890		2	
4	T1PKS	31000	66500	36	4_10	4_130	4_40	3	
	Fungal-RIPP-like	159350	220315	61	4_440	4_595	4_560	44	Sordarin ?
	indole	408462	430090	23	4_1090	4_1140	4_1090	36	
	NRPS-like	603177	646191	43	4_1540	4_1700	4_1610	4	
	terpene	737000	760745	24	4_1990	4_2040	4_2030	5	
	T1PKS	1547737	1595038	47	4_3770	4_3890	4_3840	19	Solanapyrone A ?
	T3 pks	1991773	2033038	41	4_9230	4_9340	4_9290	20	
	NRPS-like	3566710	3609367	43	4_4950	4_5090	4_5030	21	
	NRPS	3677258	3740000	63	4_4560	4_4710	4_4640	22	
	terpene / RIPP-like	3739700	3762200	23	4_4492	4_4550	4_4520	37	
NRPS	3755500	3811633	56	4_4350	4_4500	4_4440	23		
5	indole	267234	288427	21	5_2300	5_2370	5_2350	6	
	T1PKS	328011	374589	47	5_2030	5_2140	5_2090	7	9-methylstreptimidone
	indole	432133	452975	21	5_1810	5_1830	5_1815	38	
	T1PKS	472478	514928	42	5_1620	5_1740	5_1690	8	FR901512
	NRPS	700624	746147	46	5_1010	5_1140	5_1070	9	
	T1PKS	936500	987000	51	5_335	5_450	5_380	10	
	NRPS-T1PKS	1567782	1609959	42	5_3665	5_3810	5_3740 and 5_3750	14	
	fungal-RIPP-like	1777051	1837275	60	5_4230	5_4330		45	
	terpene	1996173	2017408	21	5_4910	5_4980	5_4960	17	Squalestatin S1
	other	2811876	2855652	44	5_12390	5_12510	5_12450	11	
	T1PKS	2886701	2923241	37	5_7440	5_7595	5_7530	16	
	NRPS-T1PKS	3094551	3145574	51	5_6760	5_6900	5_6830	15	phyllostictine A / phyllostictine B
	T1PKS	3333081	3384344	51	5_7670	5_7780	5_7750	13	
	NRPS like/T1PKS	4551558	4600976	49	5_10930	5_11090	5_11000 and 5_11010	18	ustethylin A
6	T1PKS	14818	53938	39	6_11200	6_11290	6_11230	24	
	T3PKS-T1PKS	2922000	2976000	54	6_7270	6_7390	6_7330 and 6_7340	28	equisetine / leucinstatin A / Leucinstatin B
	NRPS-T1PKS	3218977	3275256	56	0_160	6_8060	0_240	12	wortmanamide A / wortmanamide B
	other / NRPS-like	3850825	3893332	43	6_9685	6_9830	6_9760	39	
	NRPS	3956247	4001565	45	6_9970	6_10160	6_10100	40	
T1PKS	4021096	4068088	47	6_10260	6_10400	6_10330	41	NG-391 / Solanapyrone A	
7	lantipeptide	312918	346298	33	7_4370	7_4250		42	
	fungal-RIPP-like	3806245	3867741	61	7_755	7_910	7_830	46	

Appendix 2: List of primers

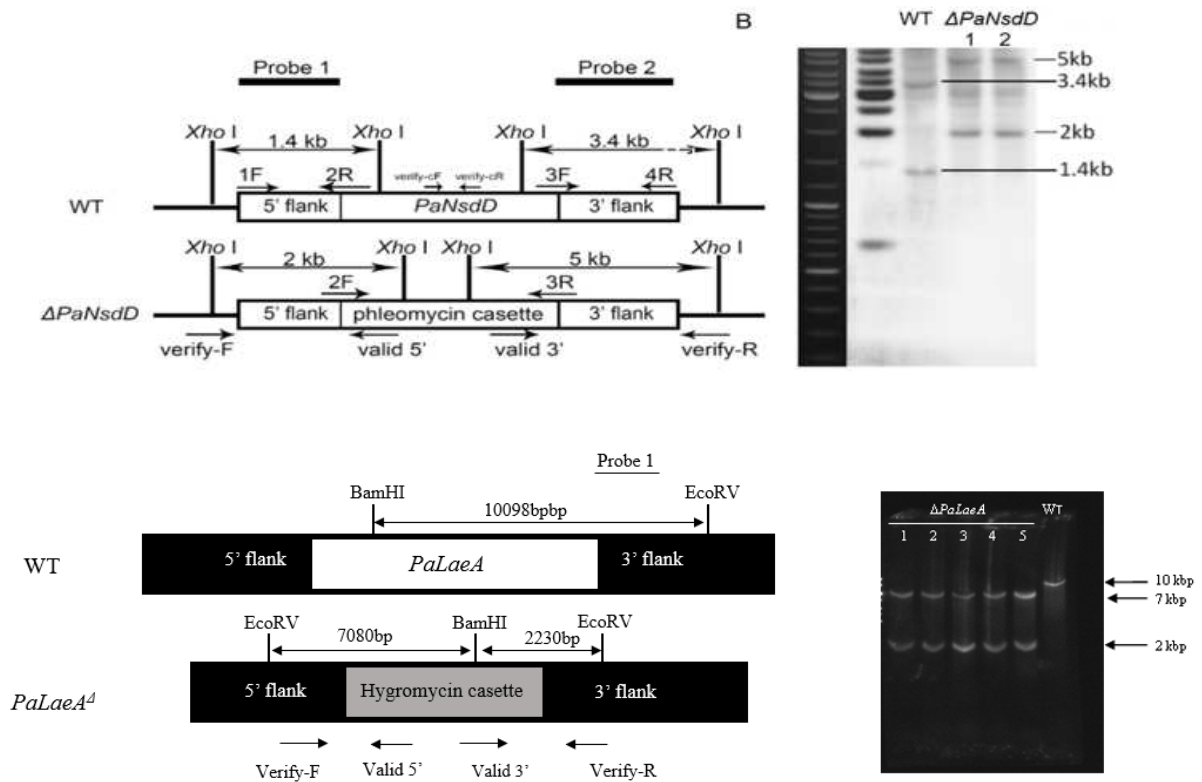
Table S2. Primers used in this study.

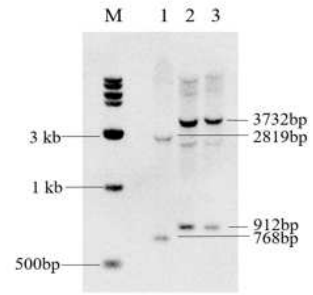
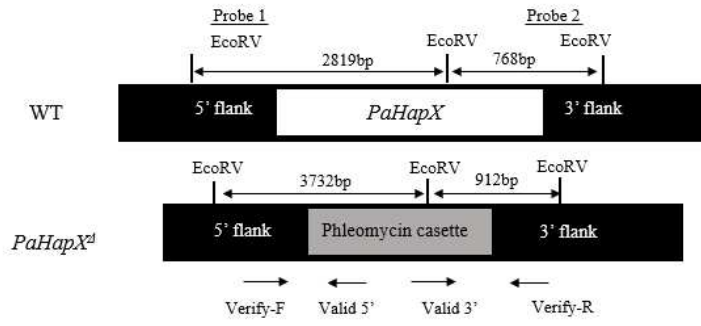
Primers	Sequence (5'–3')	Application
LaeA_A	cagtgcgcatctcgtaggtattctc	
LaeA_B	CTATTTAACGACCCTGCCCTGAACCGtgctggattagctgaactttatcg	
LaeA_mk_E	CGATAAAGTTCAGCTAATCCAGCCAcggttcagggcagggctgtaaatag	Construction of the <i>LaeA</i> deletion cassette
LaeA_mk_F	CAACTCCCATCAATGTCAAGCTCAGcatcgaactggatctcaacagcggttaag	
LaeA_C	CTTACCGCTGTTGAGATCCAGTTCGATGctgagcttgacattgatgggagttg	
LaeA_D	aaagccgtgtcctatcatgaattgc	
LaeA_verif5	ccgcaagaggggctactt	PCR validation for <i>LaeA</i>
LaeA_verif3	gtgtggggacgaagatgc	
LaeAc-F	taccgggccccccctcgaggtegacatgcttggcgatgtcactc	Complementation
LaeAc-R	AGTGGATCCCCCGGGCTGCAGGAATTCcccctgtgttccatcctc	
pBC-reV	cgegcaattaaccctcac	
AS-PaLaeA-R	GGCTGGGACTCCATCGCCATTTTGACGGTTTTGCGATC	Construction and verification of the <i>OE-PaLaeA</i>
OE-PaLaeA-F	GATCGCAAAACCGTCAAAATGGCGATGGAGTCCCAGCC	
OE-PaLaeA-R	GTCGTGCATACTCAAACACCG	
PaCreA-1F	CTATCTCACAACACCCACG	
PaCreA-2R	CTATTTAACGACCCTGCCCTGAACCGAGAAAAGTGTGGTCAACGC	
PaCreA-2F	GCGTTGACCGACACTTTTCTcggttcagggcagggctgtaaatag	Construction of the <i>CreA</i> deletion cassette
PaCreA-3R	CCGTGACCTTTCCTGTTCCAcatecgaactggatctcaacagcggttaag	
PaCreA-3F	CTTACCGCTGTTGAGATCCAGTTCGATGTGGAACAGGAAAAGGTCACGG	
PaCreA-4R	TGTGGTAGTGCTGCTTATCC	
PaCreA-vF	AGAGAGTTGGCGTCTTGTGC	PCR validation for <i>CreA</i>

PaCreA-vR	ACTTTGTTGACTGTCCCGAG	
pBC-reV	cgcgcaattaaccctcac	
AS-PaCreA -R	GCAGATTGCGTTCGTTGCATTTTGACGGTTTTGCGATC	
OE-PaCreA-F	GATCGCAAAACCGTCAAAATGCAACGAACGCAATCTGC	Construction and verification of the <i>OE-PaCreA</i>
OE-PaCreA-R	GACGATATGGCGCGCTTAGTTACAAGAAATCTCCAGCAAC	
TT-PaCreA-F	GTTGCTGGAGATTTCTTGTAATAAGCGCGCCATATCGTC	
pBC-For	cgcgcgtaatacgaactca	
PaHapX-1F	GCGGTGACCCTCTCTCAGCT	
PaHapX-2R	CTATTTAACGACCCCTGCCCTGAACCGGGAGTTCTCTCGGTATCCG	
PaHapX-2F	CGGATACCGAGGAGAACTCCcggttcaggcagggtcgtaaatag	Construction of the <i>HapX</i> deletion cassette
PaHapX-3R	CCTCCCTCCACCAGCACATCcatcgaactggatctcaacagcggaag	
PaHapX-3F	CTTACCGCTGTTGAGATCCAGTTCGATGGATGTGCTGGTGGAGGGAGG	
PaHapX-4R	CAGGTACAAAGCATCCATGG	
PaHapX-vF	CGGCTCGACAAATCGCCAAG	PCR validation for <i>HapX</i>
PaHapX-VR	AGGGTAGCAATTATCGATAT	
pBC-reV	cgcgcaattaaccctcac	
AS-PaHapX-R	GCAGCGGCAGCGGCAGCCATTTTGACGGTTTTGCGATC	Construction and verification of the <i>OE-PaHapX</i>
OE-PaHapX-F	GATCGCAAAACCGTCAAAATGGCTGCCGCTGCCGCTGC	
PaHapX_4R	CAGGTACAAAGCATCCATGG	
Pro-AS4-For	gctatgacatgattacgcc	
AS-PaHapX-R2	GCAGCGGCAGCGGCAGCCATccaattcctgacggttttgcg	Construction and verification of the <i>OE-PaHapX</i>
OE-PaHapX-F2	cgcaaaaccgtcaggaattggATGGCTGCCGCTGCCGCTGC	
PaHapX_4R	CAGGTACAAAGCATCCATGG	

Appendix 3: Southern Blot

Figure S1. illustrates the Southern blot validation of deletion mutants. Genomic DNA was extracted from both the wild type (WT) and two distinct transformants, followed by digestion with specified restriction enzymes. The blots were subsequently probed with a sequence encompassing the pertinent flanking region. The figures provide a restriction map for both the WT and mutant loci, indicating the expected fragment sizes, which are also labeled in proximity to the corresponding fragments on the Southern blot. Additionally, primers used for junction verification are clearly indicated.





M: 1 kb Marker, 1: WT, 2 and 3: *PaHapX*^Δ

Appendix 4: Résumé substantiel en français

Les champignons jouent un rôle vital en tant que décomposeurs, parasites et partenaires symbiotiques des écosystèmes. Alors que plus de 97 000 espèces de champignons ont été décrites, on estime que leur diversité réelle dépasse 1,5 million d'espèces.

Chez les champignons, les métabolites secondaires (MS) jouent un rôle crucial sur le plan écologique. Ils participent à la défense et à la survie dans l'environnement extérieur. Ils facilitent la communication chimique au sein des espèces et entre elles, offrent une protection contre les prédateurs et les agents pathogènes et jouent un rôle dans le développement et la survie des champignons. En outre, les SM sont impliqués dans les relations symbiotiques entre les champignons et les plantes, les insectes et les bactéries, ce qui illustre la diversité de leurs rôles écologiques. Ces produits naturels fongiques représentent un immense potentiel pour produire un large éventail de composés bioactifs ayant des applications en médecine et dans l'industrie. Ainsi, ils interviennent dans la formulation innovante de médicaments et diverses applications pharmaceutiques, notamment les antibiotiques, les antioxydants, les médicaments anticancéreux et antidiabétiques.

Les MS fongiques présentent des structures moléculaires complexes avec une grande diversité chimique, réparties en quatre catégories principales : les polykétides, les peptides non ribosomiques, les terpènes et les dérivés prénylés du tryptophane. Ces composés sont synthétisés par différentes voies de biosynthèse, chacune étant initiée par des enzymes clés telles que les polykétides synthases, les peptides non ribosomiques synthétases et les terpènes synthases. Il existe de plus des MS hybrides. Contrairement aux gènes codant des métabolites primaires dispersés dans le génome, les gènes responsables de la biosynthèse des métabolites secondaires ont tendance à être organisés en groupes de gènes contigus, les clusters de gènes de biosynthèse (CGB). Ces CGB se composent de gènes clés qui synthétisent les molécules précurseurs et sont associés à des enzymes d'adaptation, ainsi que, souvent, à des gènes codant des transporteurs et/ ou à des gènes codant des facteurs de transcription impliqués dans la régulation du cluster.

La compréhension de ces fonctions à multiples facettes des SM fongiques est cruciale pour divers domaines, de la médecine à l'agriculture, et pour mieux comprendre les interactions complexes dans le monde naturel. Avec le séquençage des génomes fongiques, il est devenu évident que le nombre de BGC dépasse largement celui des SM connus, ce qui révèle un potentiel caché important pour des SM bioactifs nouveaux et utiles. Cependant, la plupart des clusters de MS sont silencieux, il est donc important de trouver les moyens de les activer.

La régulation de la production de SM chez les champignons est un processus dynamique et complexe impliquant plusieurs mécanismes de contrôle qui permettent aux champignons de s'adapter à leur environnement et d'interagir avec d'autres organismes par le biais de la production de composés bioactifs. Les CBG fongiques peuvent être régulés en réponse à divers stimuli environnementaux. Ces stimuli peuvent activer ou réprimer des groupes de gènes spécifiques et varient d'une espèce fongique à l'autre. La régulation transcriptionnelle des CBG fongiques est régie par des facteurs de transcription qui peuvent être spécifiques au cluster ou globaux. Ces derniers coordonnent de multiples processus métaboliques en réponse à des signaux environnementaux. Il est essentiel de comprendre ces régulateurs pour optimiser la production de SM.

L'objectif de ma thèse était de participer à l'exploration de la diversité des MS produits par le champignon filamenteux modèle *Podospora anserina*. En plus d'être un organisme modèle qui se manipule facilement au laboratoire, ce champignon présente plusieurs avantages pour cette étude. D'une part, c'est un champignon coprophile se développant en milieu naturel sur les excréments d'herbivore qui est un milieu hautement compétitif où de nombreuses espèces sont présentes (champignons, bactéries, larves d'insectes...). D'autre part, ce champignon filamenteux possède plus de 40 BGC annotés dont très peu ont été caractérisés. *P. anserina* est un champignon filamenteux appartenant à l'ordre des Sordariales, utilisé depuis longtemps comme organisme modèle dans l'étude de plusieurs processus biologiques tels que le développement sexué, la vieillesse, la mort cellulaire, la dégradation de la biomasse végétale, les interactions avec d'autres champignons et le métabolisme secondaire. Afin de mieux comprendre la production des MS chez ce champignon, j'ai participé ou réalisé l'étude de quatre régulateurs globaux, NsdD, LaeA, CreA et HapX.

Le gène NsdD (never undergo sexual development) code pour un facteur de transcription fongique conservé de type GATA à doigt de zinc, essentiel à la régulation du développement sexuel chez les champignons. Ce domaine de liaison à l'ADN est hautement conservé parmi les champignons filamenteux, mais pas chez les plantes ou les animaux. NsdD a été reconnu pour la première fois comme un promoteur central de la reproduction sexuée chez *A. nidulans* (Eurotiales, Ascomycota) grâce à l'observation d'une infertilité complète chez le mutant correspondant. NsdD régit les gènes associés aux structures et processus sexuels, initiant le passage du développement asexué au développement sexué en réponse à des indices environnementaux, avec des fonctions et des cibles qui diffèrent selon les espèces fongiques. J'ai participé à l'étude fonctionnelle de ce régulateur global chez *P. anserina*. Par rapport à la souche de type sauvage, la croissance végétative, les processus de vieillissement, la

reproduction sexuée, la tolérance au stress et les confrontations interspécifiques chez le mutant ont été fortement altérés, en raison de la perte de la fonction de PaNsdD. En outre, la production de 3-acétyl-4-méthylpyrrole, un nouveau métabolite identifié chez *P. anserina*, a été significativement inhibée dans le mutant *DPaNsdd*. Nous avons également démontré l'interaction de PaNsdD avec la voie de biosynthèse de la stérigmatocystine (ST). La délétion du gène *PaNsdd* a également montré l'accumulation d'un pigment rouge-rose qui ne se produit qu'en présence du gène codant pour la polykétide synthase, *PaStcA*, de la voie de biosynthèse de la ST. L'ensemble de ces résultats contribue à une meilleure compréhension de la régulation globale médiée par le gène *PaStcA* chez *P. anserina*, notamment en ce qui concerne son implication inattendue dans le processus de vieillissement fongique et son interaction avec la voie de la ST.

J'ai également réalisé l'exploration approfondie du gène *PaLaeA* chez *P. anserina*, un gène ayant des implications potentielles sur la croissance, le développement, le métabolisme secondaire et les réponses aux facteurs de stress environnementaux. Des mutants de délétion et de surexpression ont été générés ainsi que la complémentation de *DPaLaeA*. La caractérisation phénotypique des différentes souches a été réalisée, suivie d'une analyse de leur impact sur divers processus biologiques. Ces études démontrent que PaLaeA influence la croissance végétative, la morphologie des colonies et la production de pigments chez *P. anserina*, avec un effet notable sur la production d'un pigment rose qui se révèle différent de celui observé lors de l'étude sur PaNsdD. En outre, PaLaeA joue un rôle crucial dans la reproduction sexuée, en agissant comme régulateur négatif de la différenciation des spermaties. PaLaeA affecte également la réponse à différentes sources de carbone, ce qui indique son rôle dans l'utilisation des sources de carbone. De plus, PaLaeA est impliqué dans la médiation de diverses réponses au stress, le mutant $\Delta PaLaeA$ présentant notamment une sensibilité accrue au stress oxydatif. PaLaeA semble également influencer la réponse lors de confrontations interspécifiques, l'accumulation de peroxyde étant affectée dans les souches mutantes. L'analyse des métabolites a révélé une augmentation significative de la production de ST dans la souche $\Delta PaLaeA$, soulignant son rôle dans le MS. Un autre composé, susceptible d'être la sécostérigmatocystine, a été identifié à un temps de rétention de 21,2 minutes. Une caractérisation chimique plus poussée d'un composé au temps de rétention de 23,9 minutes a été réalisée par RMN et analyse HRMS. Cette recherche contribue à une compréhension globale des rôles multiples du gène *PaLaeA* chez *P. anserina*, en mettant en lumière son impact sur la croissance, le développement, les réponses au stress et le métabolisme secondaire, avec des implications potentielles pour la biologie fongique et la production de métabolites secondaires.

Enfin, j'ai initié le travail sur deux autres facteurs de transcription globaux, PaHapX et PaCreA toujours chez le champignon filamenteux *P. anserina*. HapX est le principal régulateur de l'adaptation à la privation de fer, stimule l'expression des gènes impliqués dans l'absorption du fer, y compris le système des sidérophores et réprime les gènes impliqués dans les voies de consommation du fer, comme le cycle de l'acide tricarboxylique (TCA), la respiration ou la biosynthèse de l'hème. C'est un facteur de transcription de la famille des Basic Leucine Zipper (bZIP). Il interagit avec le complexe de liaison CCAAT (CBC) à son extrémité N terminale. Le CBC, complexe hétérotrimérique de liaison à l'ADN, est conservé chez tous les organismes eucaryotes. Chez *A. nidulans*, le CBC comprend les sous-unités HapB, HapC et HapE et recrute HapX. Chez les espèces d'*Aspergillus*, le CBC joue divers rôles dans le métabolisme et le développement. HapX a fait l'objet de recherches dans diverses espèces fongiques afin de mieux comprendre la pathogénèse fongique et d'identifier des cibles potentielles pour le développement de médicaments antifongiques. Le gène *CreA* (catabolite responsive element) joue quant à lui un rôle crucial dans la répression catabolique du carbone (CCR) chez les champignons. CreA influence de manière significative la régulation transcriptionnelle du transport, le métabolisme fongique du carbone et de l'azote, et sa perte déclenche l'activation des mécanismes de défense de l'hôte. Le CCR est un mécanisme de régulation chez les champignons qui donne la priorité à l'utilisation de sources de carbone facilement métabolisables, comme le glucose, lorsque plusieurs sources de carbone sont disponibles. Cela garantit une utilisation efficace du carbone et la production d'énergie tout en inhibant l'utilisation de sources de carbone plus complexes telles que la cellulose ou la lignine. Lorsque la source de carbone préférée (par exemple, le glucose) se raréfie ou est absente, l'action répressive de CreA est levée. Jusqu'à présent, les rôles de HapX et de CreA dans la physiologie et la production de MS chez *P. anserina* n'avaient pas encore été étudiés. J'ai réalisé la construction du mutant de délétion du gène *HapX*, $\Delta PaHapX$, ainsi que la surexpression du gène *CreA*, *OE-PaCreA*, mais les tentatives pour obtenir la délétion de ce gène ont révélé qu'il était probablement essentiel chez *P. anserina*. La surexpression du gène *HapX* n'a pas encore été obtenue, pour des problèmes techniques non encore résolus. La caractérisation phénotypique des mutants obtenus est en cours. Les résultats préliminaires montrent plusieurs différences phénotypiques au niveau de la croissance, de la morphologie des colonies, de la réponse au stress et de l'utilisation des sources de carbone par rapport à la souche sauvage. Par exemple la souche $\Delta PaHapX$ semble plus sensible au stress oxydatif en présence de Méthylglyoxal ainsi qu'une légère sensibilité au stress osmotique.

Les résultats sur l'étude de ces facteurs de transcription globaux devraient permettre de mieux comprendre les rôles de ces régulateurs chez *P. anserina* et leurs implications potentielles dans la physiologie, l'adaptation des champignons et surtout leur implication dans la production de MS. Cela pourrait conduire à la découverte de nouveaux composés intéressants.

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